Contribution to the study of mesenchymal stromal / stem cells heterogeneity, focus on surface markers and senescence

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Contribution to the study of mesenchymal stromal / stem cells heterogeneity, focus on surface markers and senescence

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8th International Symposium China-Europe Stem Cells and Regenerative Medicine, June 19-21 2018, Wuhan, China

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Comprendre l'hétérogénéité des CSM pour des produits de thérapie cellulaire personnalisés

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Mesenchymal stem cell interacted with PLCL braided scaffold coated with poly-l-lysine/hyaluronic acid for ligament tissue engineering
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Charif N, Li Y, Targa L, Zhang L, Ye J, Li Y, Stoltz J, Han H, De Isla N
Aging of bone marrow mesenchymal stromal / stem cells: Implications on autologous regenerative medicine
Bio-Medical Materials and Engineering (2017) 28(s1)

List of abbreviations

ADA	adenosine deaminase
AF	AlexaFluor
AMP	adenosine monophosphate
APC	allophycocyanin
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
ATP	adenosine triphosphate
BCL2	B-cell CLL/lymphoma 2
bFGF	basic fibroblast growth factor
BM MSC	bone marrow mesenchymal stromal / stem cell
BMP2	bone morphogenetic protein 2
BSA	bovine serum albumin
BV421	Brilliant-Violet 421
CCR	CC-chemokine receptor
CD	cluster of differentiation
CDK	cyclin-dependent kinases
CFE	colony forming efficiency
CFU-F	colony forming unit - fibroblast
CIP	CDK interacting protein
COT	chirurgie orthopédique et traumatologie
CSF1	macrophage colony-stimulating factor
CXCL12	chemokine (C-X-C motif) ligand (other name of SDF1)
CXCR	CXC-chemokine receptor
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GMCSF	granulocyte macrophage colony stimulating factor
GMP	good manufacturing practice
GRO	growth related oncogene
GvHD	graft versus host disease
HGF	hepatocyte growth factor
HLA	human leucocyte antigen
HO1	heme oxygenase 1
HPCV	half-peak coefficient of variation
hPL	human platelet lysate

HSP	heat shock proteins
ICAM	intercellular adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon gamma
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
IIS	insulin/IGF1
IL	interleukin
INK4	inhibitor of CDK4
iNOS	inducible nitric oxide synthase
ISCT	International Society for Cellular Therapy
kb	kilobase
LAMP	lysosomal-associated membrane protein
LG	low glucose
LIF	leukemia inhibitory factor
M1	pro-inflammatory macrophages
M2	alternatively activated macrophages
MAC	membrane attack complex
MCP	methyl-accepting chemotaxis protein
MDC	macrophage-derived chemokine
MEM	minimum essential media
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metallopeptidases (or metalloproteinases)
MSC	mesenchymal stromal / stem cells
mtDNA	mitochondrial deoxyribonucleic acid
NK	natural killer
NO	nitric oxide
NRF2	nuclear erythroid-related factor 2
OPG	osteoprotegerin
OSM	oncostatin M
PCA	principal component analysis
PD-1/PD-L1	programmed cell death protein 1/programmed cell death-ligand 1
PDGF	platelet derived growth factor
PE	phycoerythrin
PerCP-Cy5.5	peridinin-chlorophyll-protein-cyanine5.5
PGE2	prostaglandin E2
PTEN	phosphatase and tensin homolog protein
RANKL	receptor activator of nuclear factor kappa-B ligand

RANTES	regulated on activation, normal T cell expressed and secreted
Raf	rapidly accelerated fibrosarcoma protein
Rb	retinoblastoma protein
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
SAA1	serum amyloid A1
SDF1	stromal cell-derived factor 1
SOD	superoxide dismutase
SOX	SRY-related HMG box
SPP1	secreted phosphoprotein 1 (osteopontin)
SSC	side scatter
TGFβ	transforming growth factor beta
TH	T helper lymphocyte
TNFα	tumor necrosis factor alpha
TLR	toll like receptor
TSG6	TNF-stimulated gene 6 protein
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-4	very late antigen - 4
WJ MSC	Wharton jelly mesenchymal stromal / stem cells

Glossary

ADA deficiency

Genetic disorder caused by mutations of ADA gene causing severe immunodeficiency.

Allogenic and autologous

In the context of the transplantation of biological material, allogenic means the recipient is genetically distinct from the donor, but of the same species. Autologous means the transplanted biological material is taken from the recipient itself.

Apoptosis

A kind of cell death precisely organized to avoid to cause tissue damage and inflammation, allowing optimal recycling of cell compounds by phagocytic cells. Apoptosis is often opposed to necrosis that corresponds to a traumatic cell death, where the intracellular compounds like enzymes and oxidants disperse in the tissue, possibly causing damage and inflammation.

Autocrine and Paracrine

A cell can secrete various molecules that will have an action on the neighboring cells, in this case it is called paracrine signaling. The secretions can also act on the secreting cell itself, in this case it is called autocrine signaling.

Cumulative population doublings

It is the estimated number of times the cells are considered to have doubled since their primary isolation *in vitro*. It is an indicator of their *in vitro* proliferation past.

Efferocytosis

Process of a dying cell being removed by phagocytic cells.

Epigenetics

Heritable chemical modifications of nucleotides of DNA and histones that can affect chromatin form and gene expression.

Granularity

Parameter that reflects the cell content, notably in various vesicles. It can be estimated using flow cytometry, by measuring the intensity of the side scatter fluorescence.

GvHD (graft versus host disease)

In case of cancer and blood diseases, a graft of hematopoietic stem cells can be necessary to restore the patient immune system and to fight the malignant cells. Before the graft can be made, the patient must undertake a conditioning treatment to manage the disease and to prepare the graft. This process often causes damage and inflammation in the patient organism. When a graft of allogenic hematopoietic stem cells is then made, mature cells of the donor can sometimes strongly react against the recipient inflamed epithelial tissues. The organs mainly affected are skin, liver and gut. The outcomes can be severe, with the development of infections, hepatic failure, and it can even lead to death.

Heterotopic implantation

When a graft is said to be heterotopic, that means the place of implantation is not in the same tissue than the originating tissue of the graft.

Homeostasis

A dynamic state around a balance point to maintain the conditions allowing organisms to function.

Homing

The process making some kind of cells able to follow gradients of chemoattractants molecules and migrate in specific tissues.

Knock-out

Total inactivation of a gene by genetic engineering.

Mitogens / Oncogenes

These terms commonly refer to proteins that will activate signaling pathways encouraging cell proliferation such as Ras, Raf, PTEN. They can undergo mutations that make them

persistently overactivated, potentially causing uncontrolled proliferation and formation of tumors.

p16^{Ink4a}

INK4 is the name of a family of proteins that are inhibitors of CDK4 and CDK6. CDK are proteins activated by other proteins named cyclins and are necessary for cell cycle progression. p16 is an important member of INK4 family strongly implicated in senescence pathways but also in several other functions.

p21^{Cip1}

CIP is a family of proteins that can bind to both CDK and the cyclins activating CDK, allowing them to play a role in cell cycle regulation. p21 is usually associated to the inhibition of cell cycle progression, but can also be implicated in other functions.

Paracrine and autocrine

A cell can secrete various molecules that will have an action on the neighboring cells, in this case it is called paracrine signaling. The secretions can also act on the secreting cell itself, in this case it is called autocrine signaling.

Passage

After cells have been let grow in a culture vessel for some time, the passage is when they are retrieved and split in other culture vessels to have the space to continue to proliferate or to start a new experiment. The number of passages corresponds to the number of time cells were subcultured this way.

Phenotype

The phenotype of an organism is the result of the expression of its genes in association with the effect of various environmental factors. The phenotype is often placed in opposition with the genotype, the first corresponding to what is effectively produced and observable, while the second mostly refers to the specific variants of different genes an individual can have.

Proteomics

Technique used to study proteins at a large scale, without having to determine beforehand the identity of the searched proteins, but highly dependent on the purification technique used to collect the proteins.

Retrotransposons

They are DNA sequences in the genome that can be transcribed to RNA and copied back into DNA so they can then be inserted in other target sites in the genome.

Systemic lupus erythematosus

Autoimmune disease that can cause joints problems, skin rash, pain, fatigue among many other possible symptoms.

Telomere shortening and telomerase

The region at the ends of each chromosome is called telomeres. For humans, it is composed of double-stranded DNA with the repetition of the sequence TTAGGG around 2500 times. This forms a special structure stabilized by proteins that fix telomeric DNA. This structure has the function to protect chromosomes ends from fusion, degradation and avoids recognition by the detection system for DNA damage.

During each cell division, the last nucleotides of telomeres cannot be replicated, causing a shortening of around 100 base pairs. When they become too abraded, the cell becomes at risk to undergo accidents during division and to transform. The DNA damage is detected and a protective mechanism then activates and proscribes the cell to divide, leading to the state called replicative senescence.

To allow the cells to divide continuously, an important enzyme must be at work: it is called telomerase. When it is fully active, like in pluripotent stem cells or cancer cells, it can reconstitute telomere ends and completely avoid their progressive shortening. For all other kinds of human cells, notably in normal adult cells, telomerase activity is restricted or completely absent, leading to progressive telomeres shortening with age. This process may be one of the causes of the increase of senescent cells with age.

With genetic engineering, the expression of telomerase can be induced in primary cells and allows to avoid replicative senescence *in vitro*, leading to the obtention of immortalized cell lines. It can be said that they are "telomerized".

Transcriptomics

The global and large-scale study of the RNA transcripts in a sample.

Trilineage potential

The possibility for a given stem cells to go toward three different kinds of differentiation pathways.

Tumor suppressor

Tumor suppressor refers to genes or proteins that protect the cells from uncontrolled proliferation, which usually occurs when they are altered or lost.

Summary in French

Les Cellules Stromales / Souches Mésenchymateuses (CSM) ont un grand potentiel pour de nombreuses applications cliniques. Elles sont actuellement les cellules les plus utilisées pour les essais cliniques développant de nouvelles thérapies cellulaires. Cependant, les résultats qui en découlent restent inconsistants. La qualité et l'efficacité thérapeutique des CSM sont influencées par leur amplification *in vitro* et d'autres facteurs tels que les paramètres liés au donneur de cellules.

Des études préliminaires effectuées en laboratoire et l'analyse de la littérature ont révélé que les échantillons de CSM étaient hétérogènes. Des cellules de différents sous-types, à différents stades de différenciation, et des cellules sénescentes cohabitent. Cette hétérogénéité rend difficile la prédiction du comportement de l'échantillon dans son ensemble et pourrait être, en partie, responsable de la variabilité des résultats cliniques.

Le manque de procédure satisfaisante pour prédire et gérer le comportement des échantillons de CSM a poussé l'équipe vers la recherche de nouveaux moyens pour caractériser et contrôler la qualité des cellules.

Le manque d'efficacité des CSM en clinique pouvant provenir d'un mélange non optimal de différents sous-types et de la présence de cellules sénescentes, des marqueurs spécifiques à ces différents types de cellules ont donc fait l'objet de nos recherches.

Différents sous-types de CSM ayant des fonctionnalités spécifiques peuvent exister initialement chez le donneur et leur proportion peut varier en fonction des paramètres du donneur et de la façon dont les cellules sont prélevées. Ces sous-types présents initialement peuvent ensuite évoluer différemment et leurs proportions peuvent changer avec l'avancée de la culture. Toutes les cellules évoluent vers la sénescence avec l'avancée en âge des donneurs et une culture *in vitro* prolongée. De nombreux paramètres à différents niveaux se combinent pour aboutir à l'hétérogénéité globale observée dans et entre les échantillons. Les conditions de culture influencent également fortement l'état des cellules. La possibilité

que différents sous-types réagissent différemment à des conditions de culture données est aussi à considérer.

Afin d'améliorer l'utilisation des CSM dans la recherche et pour des applications thérapeutiques, cette étude s'est donc penchée sur l'hétérogénéité des CSM du niveau inter-individuel au niveau intra-échantillon. L'objectif global de ce travail était de caractériser l'hétérogénéité des CSM en recherchant des marqueurs de surface spécifiques des sous-types de CSM et des CSM sénescentes. Ce travail a ciblé l'étude de marqueurs de surface qui permettent de mutualiser la caractérisation des cellules et la possibilité de trier des cellules vivantes. L'objectif final était de définir des biomarqueurs de fonctionnalité, de trier les CSM pour concentrer un sous-type particulier et d'étudier la fonctionnalité des cellules triées.

Les questions suivantes ont été soulevées :

- Une utilisation plus poussée de la cytométrie de flux pourrait-elle aider à déchiffrer la composition des sous-types de CSM ?

- Existe-t-il un lien entre l'expression d'un marqueur de surface et la fonctionnalité cellulaire *in vitro* ?

- Le tri des cellules est-il faisable pour séparer des sous-types de CSM en culture avec des propriétés spécifiques ? Peut-il améliorer la qualité des cellules ?

- Comment le phénotype des CSM sénescentes change-t-il ? Certains marqueurs de surface spécifiques pourraient-ils être définis et utilisés pour éliminer les cellules sénescentes ? Et à plus long terme, si les cellules sénescentes sont retirées, comment la fonctionnalité de l'échantillon changera-t-elle ?

Pour répondre à ces questions, les objectifs suivants ont été définis :

- Le premier objectif était de décrire la variabilité initiale des CSM, au niveau des marqueurs de surface, dans des conditions de culture classiques, et d'étudier les associations éventuelles entre le phénotype, l'âge du donneur et la capacité de prolifération des CSM.

- Le deuxième objectif était de développer et de tester une stratégie de tri pour séparer les sous-types de CSM selon l'expression de CD146 afin de comparer les propriétés des cellules triées.

- Le troisième objectif était de rechercher des marqueurs de surface spécifiques des CSM sénescentes.

Les CSM étudiées dans ces différents contextes ont été caractérisées par différents essais *in vitro* pour explorer les associations possibles entre leurs potentialités et leur phénotype. Une analyse approfondie de la littérature et des résultats préliminaires obtenus au laboratoire ont permis de choisir un ensemble de marqueurs qui pourraient être associés à la fonctionnalité et aux sous-types de CSM.

L'expression des marqueurs de surface classiques de CSM et des marqueurs de sous-types a d'abord été suivie par cytométrie en flux sur un ensemble d'échantillons de CSM de moelle osseuse provenant de donneurs d'âges différents. Les résultats des niveaux d'expression obtenus par cytométrie en flux étaient très sensibles aux conditions de culture. De plus, chacun des marqueurs de surface suivis a montré une dynamique d'expression spécifique lorsque l'on considère à la fois le pourcentage d'expression et le niveau d'intensité de fluorescence rapporté en médiane normalisée par rapport à un contrôle isotypique.

Malgré la variabilité observée des résultats d'expression des marqueurs de surface, certaines associations ont pu être mise en évidence. Un groupe de marqueurs de surface a été associé à l'âge des donneurs : CD146, CD71, CD105, CD44 et SSC (reflétant la taille des cellules). L'expression de CD146, CD140b et CD71 étaient également corrélés au taux de prolifération. L'expression de CD146 avait de plus la particularité d'être relativement stable en culture. L'expression de CD146 s'est aussi avérée être la plus hétérogène au sein des échantillons. Ces résultats d'expression du CD146, combinés aux liens mis en évidence avec l'âge du donneur et la prolifération des CSM, mais aussi les données de la littérature, ont validé ce marqueur comme l'un des meilleurs candidats pour un tri cellulaire. La deuxième partie de ce travail s'est donc concentrée sur l'optimisation d'une méthode de tri basée sur l'expression de CD146 par les CSM.

Avec les différents échantillons utilisés, il est ressorti de cette étude qu'il était possible d'enrichir un échantillon en cellules exprimant fortement CD146 par une méthode de tri immunomagnétique. Ceci a été faisable même lorsque la présence de cellules exprimant le CD146 était initialement faible. D'un autre côté, une fraction déplétées des cellules exprimant le plus fortement CD146 au sein de l'échantillon a aussi été récupérée pour pouvoir comparer les propriétés de cellules CD146^{high} d'un côté et CD146^{low} de l'autre.

Il a été réalisé qu'un grand nombre de cellules était nécessaire pour obtenir une quantité suffisante de cellules après tri. D'un autre côté, l'expression de CD146 a été maintenue pendant au moins 2 passages après tri. Cela laisse le temps d'amplifier les cellules après tri et de les préparer pour une éventuelle application.

Au niveau des propriétés des cellules triées, il n'est pas ressorti que les cellules exprimant fortement CD146 aient de meilleure capacité de prolifération ou à former des colonies. Par contre, les résultats obtenus avec les tests de différenciation ostéogénique et adipogénique ont montré que les cellules triées exprimant fortement CD146 ont une meilleure capacité à produire des cellules différenciées que les cellules CD146^{low}. La quantification des cellules sénescentes dans les fractions obtenues après tri suggère que les populations de cellules CD146^{low} contiennent plus de cellules sénescentes. Différents profils de sécrétions ont été identifiés entre les cellules CD146^{high} et CD146^{low}. IGFBP3, CHI3L1 et MIF sont apparues comme des cibles intéressantes pour des analyses plus approfondies. Les cellules CD146^{high} ont aussi montré une capacité accrue à migrer en réponse à un signal chimioattractant. Enfin, une analyse multivariée par composantes principales a permis d'inclure tous les paramètres utilisés pour mesurer l'expression du CD146 et les rassembler avec les données de prolifération, de sénescence et de capacité à former des colonies de tous les échantillons triés. Cette approche a mis en exergue notamment les liens entre sénescence, expression du CD146 et taille des cellules. L'intérêt d'utiliser le pourcentage pour suivre l'expression de CD146 et la lier à d'autres variables a aussi été souligné.

Les différences observées entre les fractions triées sont restées cependant moins marquées que ce qui pouvait être observé entre différents échantillons. D'autres paramètres

influençant les capacités globales des échantillons de CSM peuvent donc avoir plus d'impact que l'expression du CD146.

Les recherches ont ensuite été concentrées vers l'étude de la sénescence des CSM, supposée avoir un impact important sur la qualité des échantillons. Les marqueurs de surface sont apparus ici aussi comme les cibles les plus intéressantes, permettant de combiner les approches fondamentales et appliquées. Pour cette étude, un modèle de sénescence réplicative par culture prolongée jusqu'à l'arrêt de la prolifération des cellules a été choisi. Contrairement à la sénescence induite par stress, la sénescence réplicative est plus proche des phénomènes observés lors de la culture classique des CSM et avec des CSM obtenues chez des donneurs plus âgés. L'objectif était de comparer des CSM à passage précoce contenant une petite portion de cellules sénescentes avec des cellules amenées jusqu'à un niveau de doublement de population nul ou négatif, contenant une majorité de cellules sénescentes. Pour cela, les CSM de 3 jeunes donneurs ont été utilisées et étudiées par protéomique et cytométrie de flux. Les cellules ont également été caractérisées pour suivre les changements fonctionnels se produisant au niveau des CSM en sénescence réplicative.

Des échantillons en sénescence réplicative ont été obtenus après 10 à 14 passages. Les cellules à passage tardif ont montré une prolifération fortement réduite et des niveaux accrus d'activité de β-Galactosidase. Par microscopie de fluorescence, le relargage de HMGB1 hors du noyau de l'ensemble des cellules à passage tardif a pu être mis en évidence. Il a pu aussi être observé que les CSM à passage tardif présentaient une taille et une expression de SOD accrues. Ceci a confirmé que les échantillons obtenus étaient bien enrichis en cellule sénescentes.

De manière surprenante, l'analyse fonctionnelle des échantillons sénescents a mis en évidence des capacités intéressantes de migration et d'immunosuppression.

Le suivi des marqueurs de surface le long de la culture des CSM jusqu'à sénescence réplicative a permis de montrer de fortes fluctuations dans les niveaux d'expression des marqueurs de surface, même pour les marqueurs de surface considérés comme étant

constamment et fortement exprimés. Les fluctuations étaient également très dépendantes de l'échantillon.

Une étude protéomique a mis en évidence une augmentation de CD157 dans les échantillons de CSM en sénescence réplicative, tandis que les autres protéines de surface détectées avaient tendance à diminuer.

L'étude de CD157 par cytométrie en flux a ensuite révélé de fortes fluctuations de son expression au cours des passages successifs. L'évolution de l'expression de CD157 ne suivait pas l'augmentation progressive des autres marqueurs de sénescence. Même si le lien avec la sénescence s'est avéré plus nuancé, il reste intéressant d'étudier le lien entre l'expression de CD157 et la fonctionnalité des CSM. Une étude de la littérature a notamment souligné son rôle potentiel pour la migration.

Les importantes fluctuations d'expression des protéines de surface mises en évidence par les analyses approfondies de niveau d'expression à différents passages et dans différentes conditions ont souligné la difficulté associée à l'utilisation de ces marqueurs. Cependant, les marqueurs SSC, CD146, CD71, CD140b et CD157 méritent d'être suivis pour étudier plus en détails leur lien potentiel avec la fonctionnalité des CSM. Des critères associés à leur niveau d'expression pourraient par la suite être inclus dans le contrôle qualité des échantillons afin de sélectionner ceux qui ont le meilleur potentiel pour une application donnée. D'un autre côté, les stratégies de tri cellulaire seront à réserver aux applications avec administration locale ne nécessitant pas un grand nombre de cellules. Les différentes fractions de CSM triées semblent également plus proches entre elles qu'avec les cellules des autres échantillons, ce qui limite le potentiel de ce type de sélection. Elles sont par contre d'un grand intérêt en recherche pour mieux comprendre le comportement des échantillons.

General introduction

Heterogeneity between individuals and cells is often masked and, most of the time, only the average function of the samples is considered.

To understand how organisms can grow and regenerate, how tissues renew and repair, and also the underlying mechanisms of aging, stem cells appeared as key players implicated in all of these fundamental biological processes. Contrary to other kind of cells, stem cells have the ability to produce many progenies that can differentiate into different tissues. The study of stem cell heterogeneity is a very promising field. Such research has the advantage of combining access to fundamental processes for organism physiology while also being implicated in many pathologies with therapeutic application opportunities.

The use of stem cells in regenerative medicine offers new alternatives for numerous clinical applications with unmet medical needs. As the use of stem cells in therapy multiply, their heterogeneous nature is seen as a current drawback for their manufacture and pose new challenges in the pharmaceutical field. Many investigations are undertaken to obtain more homogeneous samples. The identification of the most effective subsets and their isolation is a major focus of research.

This variability between cells of a sample can be considered as noise, drifting of some cells that decrease the concentration of cells of interest. The difficulty to define the mix of cells and the unpredictable behavior of the sample limit its use. However, a mix of cells can also be seen as better prepared to face a wide range of situations. There may have more chances, in a diverse group, that a kind of cells will have the needed resource and resilience to cope with environmental changes. The most adapted cells could then be selected (1–3). In this case, it would be an optimal set of different cells that should be searched for.

Among the different kinds of stem cells, Mesenchymal Stromal / Stem Cells (MSC) are particularly under the spotlight. MSC can be obtained easily and extracted from human tissues. When cultivated *in vitro*, they are very resistant and responsive cells that can be amplified to give rise to many new cells. They can be cryo-conserved for long time and still

be able to provide viable cells after thawing. MSC were initially obtained from bone marrow. The method of culture was optimized and is now effective to grow many kinds of cells harvested from different tissues. That allowed a wide expansion of the research field on MSC and MSC-like cells, comparing cells with each other and trying to decipher their overlapping and distinguishing features (figure 1). The sinuosities that pave the way of MSC research have resulted in a hybrid name used for this work: mesenchymal stromal / stem cells, and they still have many other names.

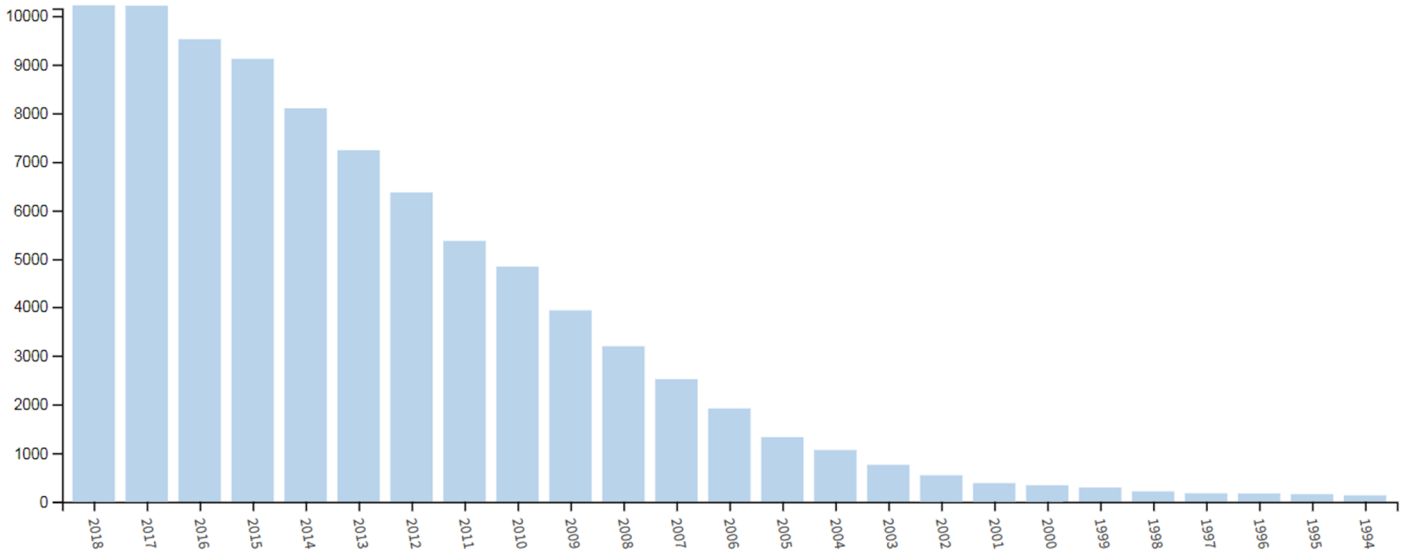


Figure 1: Number of articles on MSC per year.
The abscissa axis is reversed, the most recent year is on the left side.
From Web of Science with the following search:
TOPIC: (Mesenchymal stem cells) OR TOPIC: (Mesenchymal stromal cells)

The use of MSC allowed the undertaking of much more research on human cells close to physiologic conditions; in addition to mouse cells and cancerous cell line that were the most common models. However, contrary to cell lines, MSC display much more heterogeneity.

With their relatively easy harvest and expansion, MSC became a therapeutic tool of major importance, notably thanks to their ability to interact with host cells. They were used in more than thousand clinical trials for a wide set of indications: tissue replacement, immunological disorders, degenerative diseases, among others, all resulting in very variable outcomes. The use of livings cells for therapeutics is delicate, it is difficult to allow their survival, and it adds all their inherent variability to the already numerous parameters to

take into account for any treatment. To ameliorate the success rate of these particular therapies, many studies are more specifically aimed at considering MSC heterogeneity with better cell characterization, selection and preparation.

To access cell-to-cell variability and to deepen the understanding of individual cells, single cells techniques are of major importance. More particularly, **flow cytometry** is an essential tool that gives the possibility to measure multiple parameters on single cells in suspension at high speed. After several decades since its invention, modern instruments provide now large possibilities, that are often hardly understood and thus, underused. Despite the technical complexity, many progresses could be done. The most common application of flow cytometry is to study cells from the immune system in patients. Additionally, its sensitivity allows now to detect rare malignant cells and is used for diagnosis and monitoring diseases in clinical practice. This technique however, as it requires looking at more parameters, is more time consuming than those regrouping all cells.

In addition to extensive cell characterization, **cell sorting** can bring an interesting solution to concentrate the population of interest with a specific functionality. Cell separation can be made according to simple parameters such as cell size with filters. There are more possibilities with antibody labelling associated with magnetic beads. The most complete technique remains with flow cytometry assisted cell sorting, where all the recorded parameters can be used to select more precisely cell populations to isolate from one another. The mastering of this complex technique is expected to become a very powerful tool for research and therapy. For now, there are several limits to overcome. The first is to define the parameters to use for cell separation. Then, the sorting technique must be refined to allow its feasibility and the best quality of the isolates obtained.

This work is integrated in the flourishing field of MSC research. With the help of single cells techniques, it is aimed at improving the understanding of MSC diversity to go toward a better use of these cells in research and therapeutic applications. To put together cell function characterization and ways to act when facing cell heterogeneity, this work was oriented toward the study of surface markers. Indeed, they can be monitored on living cells, and can serve to sort them. The samples obtained are better characterized and allow

to progress in the understanding of the potential and specific contribution of different subtypes. To address these different aspects, a selection of surface markers potentially associated to MSC functionality were studied by flow cytometry on a panel of donors of different ages. Following the work of Yueying Li (4), a previous PhD student, a complementary protocol was proposed to enhance the sensitivity of flow cytometry experiments. These investigations led to the choice of a specific marker to try to separate different MSC subtypes within samples. Cultivated MSC from bone marrow samples were sorted and compared according to their expression of CD146, a multifunctional adhesion molecule. The possible differences in some properties between the sorted fractions were investigated. Working with cells from donors of different ages and after *in vitro* amplification, the consideration of MSC impairment and senescence was also unavoidable. It has appeared as an additional and central cause of MSC variable behavior. For this, a part of the work was oriented toward the research of senescent MSC surface phenotype. MSC were brought to replicative senescence to be compared with younger cells. Proteomic analyses could conduct to relative quantification of MSC surface markers. The results were then confirmed by flow cytometry.

This work is the fruition of collaborations between multiple platforms and clinical centers available in the Nancy area, notably the Cell Therapy Unit, the maternity and the Orthopaedic Surgery and Traumatology Unit of Nancy University Hospital, and the Proteomics and Cytometry core facilities of UMS2008 IBSLor (Université de Lorraine - CNRS - INSERM - http://www.umsibslor.univ-lorraine.fr/). The relationships with the local clinical centers opened the possibility to recover bone marrow and umbilical cord samples and develop MSC research.

One of the purposes of this manuscript is to question some basis and highlight some paradoxes to try to set a framework where future works will be able to use the most of previous experiences. For this, it was needed to reconsider routine practices and start from little to be able to progress further then. To extract the most information from the studies undertaken and clarify the paths for future research, an effort was made to provide details that are often omitted even though can be particularly of use to newcomers in the field.

Outline

In a first chapter, the **introduction** intends to define the general objects of this study and to give necessary knowledge to grasp the situation of MSC in a more global context.

- The first part of the introduction addresses the stem cell concept with the attempt to distinguish cell entities over parameters that are then important for cell use for therapy. Some elements are still at the hypothesis state that remains to be confirmed, but it allows to put a basic framework where it is possible to make links.

- The second part of the introduction focuses on MSC. Their nature is discussed to go deeper in the understanding of these cells and their usages.

- The third part of the introduction is about the use of MSC in different kinds of therapeutic applications, and the potential mechanisms that these experiences could help to identify.

- The fourth part then addresses MSC heterogeneity, at the heart of the work made during this thesis. Different levels of heterogeneity that must be considered together are reviewed.

- The fifth part position itself in a more applied context with the use of surface markers to identify and sort MSC and specific subtypes of cells.

- The sixth part is about senescence to propose a closer look at senescence, as it revealed to be an important process to consider when dealing with MSC and their heterogeneity.

- The last part of the introduction reviews the aspects to take into account to make MSC therapy work, by knowing their limits and the promising strategies for their future use.

After the bibliographic introduction, the next chapter describes the approach that has led us to undertake this work, and provides an overview of the context and objectives.

This is followed by the chapter about methods. It includes detailed description of the techniques used, notably flow cytometry. This chapter also shows the results of protocols optimization made during the PhD work.

The chapter "Results and discussion" then presents a selection of results and their interpretation in the light of scientific literature. It is separated in three parts:

- Part I is about the results obtained when studying surface markers on various MSC samples. The baseline variability of the technique is first mentioned. Then, the links between donor parameters, MSC proliferation and surface marker expression are explored. A special attention is given to the diversity of expression levels that can be found within each sample and that can define different subpopulations.

- Part II reports the feasibility to sort BM MSC at different steps of culture and the functional and phenotypic comparison of $CD146^{high}$ and $CD146^{low}$ sorted cells.

- Part III deals with the study made on replicative senescent MSC. Senescent cells phenotype and functionality were characterized. Results of the relative quantification of young and senescent MSC surface proteins by proteomics are then presented. These results were then further developed by an analysis with flow cytometry.

A chapter of general discussion then gathers the most important findings. It makes the links between the results obtained in the different parts and with the literature.

The last chapter concerns the conclusion and foreseen perspectives for future research on MSC but also for the research approach itself and reproducible science.

At the end of the manuscript is presented the list of bibliographic references used for writing.

Introduction

I. The path to understand "MSC" research

Stem cell existence is difficult to grasp. These cells are very discreet, difficult to find, to describe and thus, to study. Many confusions persist even now and this part intends to decipher the quantities of information that can be found to give clear prerequisite to apprehend MSC research. As understanding of their properties grew, new insights for therapeutic applications are also revealed. They bear a lot of promises but time is still needed to increase the knowledge about stem cells and use them efficiently.

An extended literature exists on animal models, especially murine cells, but this work is oriented toward human biology, adult stem and progenitor cells, and it will place it in a larger context. When there is no specific mention, the cells described in this work are from human origin. This introduction will also try to give the necessary basics to understand the concepts underlying the work problematic and to highlight the different ways to consider it.

A. Cell concepts

1. Cells as the unit of life

a. Cell theory

With the advance of microscopy, the first cells could be observed in the 17th century. The stem cell concept is closely related to the cell concept itself. The cell level is now considered the most basic unit of life. The cell theory, officially formulated in 1839, says that a living being is composed of at least one cell, and each cell comes from the division of a preexisting cell. Cells are thus considered as functional, structural and reproductive units. They are the smallest living units that can be dissociated from tissues of multicellular organisms. Isolated cells can stay alive some time with adequate nutriments and environment. They are autonomous entities. This is what allows to undergo cell extraction from tissues for different kinds of use, from fundamental studies to cellular engineering applications.

b. Cells by the numbers

In every fields of research, it can be useful to have an idea of the orders of magnitude around the observations that can be made. It can give interesting insights that can guide investigations and allow to have critical thought about the results obtained.

First, inside a cell, it is important to have in mind that the space is filled with many molecules, very dense, bringing it to a state called molecular crowding **(figure 2)**.

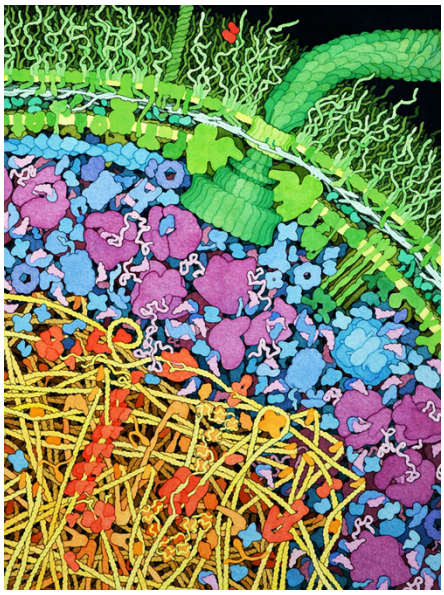


Figure 2: Cell representation made by David S. Goodsell showing macromolecular crowding.

A cell protein content averages 8×10^9 molecules per cell, or 700 pg. Researchers estimated that each cell may contain more than 100 000 different sorts of proteins (5). However, except for some proteins that are present in very big amounts, like structural proteins, it can be considered that there are, at one moment, only few copies of each protein in individual cells. Their diffusion is difficult, and they cannot be considered as always disposable. The position of factors at one moment will then determine the possibilities of the cell. This causes a kind of variability in cells' behavior, that can be called stochasticity. That occurs especially with gene expression needing to gather many factors, or for cell division with the separation of two compartments that do not contain the same material. This stochasticity is regulated by retro controls, especially negative feedbacks, and some genes redundancy notably (3).

At the scale of the human organism, it contains around 3.0×10^{13} human cells. Red blood cells are very small cells and represent the vast majority in cell number (84%). In terms of cell mass however, the main contribution comes from adipocytes and muscle cells (75% of total cell mass with both). They are large cells that represents only a minor part in number (figure 3). A human body contains also around 3.8×10^{13} bacteria (6), the same order of magnitude than the number of human cells, but they only have a minor contribution in total cell mass.

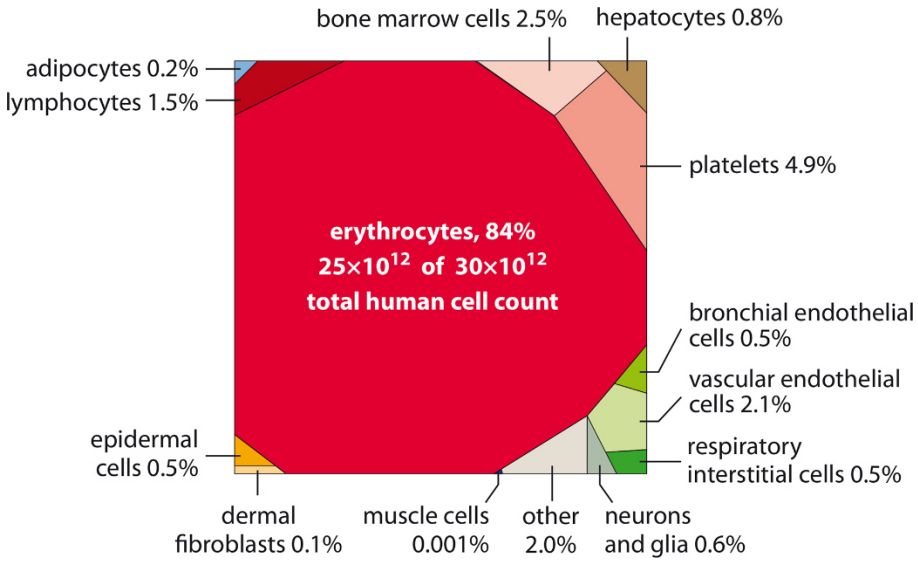


Figure 3: Proteomap chart representing repartition of different cell types in a human body (6).

Each cell type has a specific lifespan in the human body: specialized intestinal cells live around 5 days, retina cells 10 days, erythrocytes 120 days and some hepatocytes up to 400 days for example. Contrary to what was previously thought, all organs renew their cells, even brain and heart, but with some limits and at different rates. These differences are linked to many parameters associated with each cell type environment, functions and capacities. To simplify, it can be said that specialized cells under great stress have a shorter lifespan. That can be the case of cells of the intestinal epithelium or of the epidermis. Cells that reside in more protected areas, in more profound tissues are usually more stable and remain for longer time. That is the case for example of muscle cells or nerve cells.

It is difficult to say how many cell types are found in the human body in total (7). That leads us toward the question of the description and origin of these different cell types.

2. How to define cell types

All the different cell types in an organism are genetically identical, they contain the same genome. It is thanks to gene expression regulation that different cell phenotypes, that are effectively expressed and the resulting cells observed, can be obtained with the same genome. These mechanisms are guided by the action of transcription factors and with the contribution of persistent epigenetic modifications that influence genes accessibility for transcription.

Where is the threshold that allows to distinguish one cell type from another? The question may seem easy for specialized cells like muscle cells or neurons. However, it is trickier when trying to describe different cell types from the same lineage, at different differentiation steps. Cells undergo continuous changes along their life and successive divisions. It may be difficult to say if what is observed are different cell types, or different individuals due to the variability between cells of the same type, or even just different cell states. Group of similar cells can also be described as sub-population or subsets of the same cell type.

The Open Biomedical Ontology Foundry established in 2005 a standard reference nomenclature to define cell types called "CL". Cell types are defined in it by three essential components:

- a minimum set of necessary and sufficient marker genes,
- a parent cell class defined in the CL,
- a specimen source description.

New techniques to gather precise and big amount of data on the single-cell level have allowed new ways to define and create cell types. That concerns particularly high-content cytometry, measuring multiple spectrally and spatio-temporally resolved signals, and single-cell gene expression profiling, but also other high-throughput approaches. These developments are progressively included to improve the CL ontology. They are associated with complex statistical methods and machine learning approaches that allowed to identify numerous new cell types in recent years (8).

The question of cell types definition is linked with major challenges in cell biology. Large scale projects like the Human Cell Atlas Project try to undertake the enormous task to pilot and gather studies in order to map all human cell types and their behavior. The Cell Atlas aims to improve understanding of diseases to the cell level that could be described for large population of individuals. A better understanding of normal and pathological cell state can help to improve diagnostics and to define new therapeutic targets. To realize their aim, this initiative first intends to define a framework to define and distinguish cell types according to their molecular content, estimated by single-cell transcriptomics. These studies will have to be completed by cells function analysis to be able to assign a cell to a particular type. In this project, cell types are conceptualized more as plastic dynamic states in a continuum rather than fixed units (9). Indeed, even for specialized cells, there are some facts about their plasticity and ability to dedifferentiate (10).

Contrary to the usual way to define cell types according to their morphology, molecular characteristics and function, they can also be defined by an evolutionary manner. Cell types diversity are the result of an evolutionary process. They can be defined as groups of cells more closely related between them than with other cells, evolving together and partially independent from other cells that implies independent control of gene expression. Cells of the same type share the use of some genomic information that is not used by other cells. That is possible with the unique cell type **regulatory signature** they share. These regulation mechanisms involve small sets of transcription factors genes that control most of cell type specific effector genes. These factors are also affected by protein interactions. This definition of cell type has the peculiarity that it is the regulatory signature that defines cell types and not cell phenotype, which is more flexible (11).

Now, within the same cell type, it may be considered they are very homogeneous and similar to each other, but it is important to keep in mind that no cell is equal to another. Each has a specific history that will make it different from its neighbor, sometimes at surprising levels of heterogeneity (1).

Thinking of cell types, it is usually associated to their initial function in the organism. However other kinds of classification can also be used, for example by grouping

them according to their cell cycle stage (growing, quiescent, differentiating / engaged, senescent, dying).

How these different cell types are generated? Even the most complex individual emerges from a simple cell at first: the egg.

B. Stem cells

It is when biologists found that cells in the body had limited lifespan and had to be renewed that stem cells were theoretically imagined at the end of the 19th century (12). Mature cells can die for different reasons like programmed cell death, elimination by the immune system after cell exhaustion, or they can also be destroyed throughout injury. To maintain tissues integrity with cells of shorter lifespan than the organism's, a homeostatic mechanism must take place.

1. Actual definition of stem cells

Stem cells are classically defined as cells that display two properties: self-renewal and differentiation capacity. Self-renewal is defined as the capacity to generate a daughter cell identical to the mother cell, also called a clone. Differentiation is a gradual process where cells stop to proliferate to put their energy to develop a specialized function for a specific environment, and to keep this function until cell death.

When a stem cell divides, symmetric or asymmetric division can happen. The cells that can still be called stem cells are the ones that keep the initial capacities of the mother cell.

Four cases can theoretically happen. A stem cell can divide into:

- two daughter cells identical to the mother cell, it is symmetric renewal;

- two daughter cells committed to differentiate toward the same lineage, it is symmetric differentiation;

- one daughter cell committed to differentiate and the other with stem cell potential, it can be called asymmetric renewal;

- two daughter cells that will differentiate toward different lineages, it is asymmetric differentiation (13).

Asymmetric division can also occur when a daughter cell is exposed to different environmental conditions.

It is important to note that a stem cell cannot be defined satisfactorily only by self-renewal and differentiation potential that are not completely specific to stem cells. For example, some mature cells seem to be able to dedifferentiate (10), and it is also the case of cancer cells. A proposed way to really distinguish stem cells from other kind of cells is to define them as at the origin of a lineage. Stem cells can assume life-long renewing of tissues, they can divide without differentiating to allow self-renewal and are the sources of progenitors. Progenitors for their part, are usually generated by asymmetric divisions of stem cells. They are still undifferentiated and show intense proliferative activity to produce a big number of committed cells that will all undergo differentiation toward specialized cells **(figure 4)**. These progenitors remain shorter times and along the differentiation process, the self-renewing capacity is lost. When the differentiation steps of some lineages are described, the terms "precursor" or "blast" cells are often found and refer to cells starting to differentiate. Cells with the suffix "-cyte" refers to those that achieve maturation toward terminal differentiation. Along the differentiation process, the cell cycle is slowed and it can go until cell division arrest called quiescence. A differentiated cell, that can also be called a mature or specialized cell, will display a specific morphology, structural and functional polarization, specific molecular and membrane content and will lose what is not necessary to its terminal function. Cell commitment design the reversible state when the cell starts to differentiate toward a lineage. Cell determination is the term used when the process becomes irreversible in classical conditions.

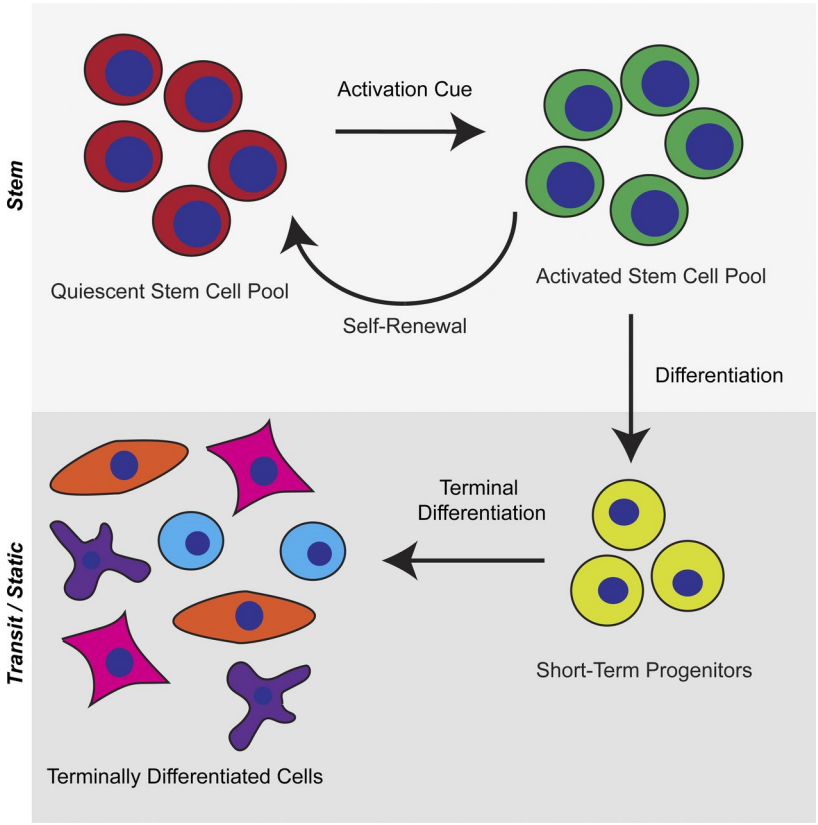


Figure 4: Adult stem cells functioning (14).

To define more precisely stemness, a group of properties can be associated to the stem cell concept, but the definition remains dynamic and context dependent, so it is not necessary to have all the properties to be called a stem cell. This kind of definition can be called a "homeostatic property cluster" (15):

- undifferentiated state,

- capacity to self-renew during a long period of time (throughout the life span),

- ability to give rise to differentiated cell progeny,

- localization in specific niches, cell environment being essential to maintain stemness,

- specific gene expression pattern,

- low division frequency, they are not engaged in the cell cycle most of the time, but it is adaptable. Stem cells usually remain in a quiescent state with reduced metabolic activity to preserve the information and the potential needed to produce other cells.

In the adult body, stem cells are very rare, so the functions they can have other than to produce cells are also difficult to observe and to study. However, they can also have a specific function at their undifferentiated stage, without relying on their progeny (for an example, see part II. "MSC concept" – "MSC physiological role").

2. Different types of stem cells with different "stemness"

There are different kind of stem cells defined by specific levels of self-renewal and differentiation capacities.

It is only during the first steps of embryo development, the four first divisions until morula stage, that cells are called **totipotent** and give rise to an entire individual if implanted *in vivo* in the adequate environment. However, these cells can only give rise to a limited number of cells like themselves and it is not yet possible to amplify cells at this state. Thus, these totipotent cells do not really display strictly speaking self-renewal.

During the next step of embryo development, embryonic stem cells (ESC) can be extracted from the blastocyst and cultivated *in vitro* with extensive proliferation capacity, theoretically indefinitely without oncogenic transformation, while keeping their self-renewing potential. ESC can also differentiate in every cell types of the organism but are not able to generate an entire individual if implanted because they cannot generate the extraembryonic tissues like the placenta. These ESC have the highest *in vitro* self-renewal capacity associated to extended differentiation potential **(figure 5)**. They are called **pluripotent** stem cells. These cells do not persist physiologically after embryo development, even if some confusions can still be found, their presence in the human body is only transient and restricted to the blastocyst step.

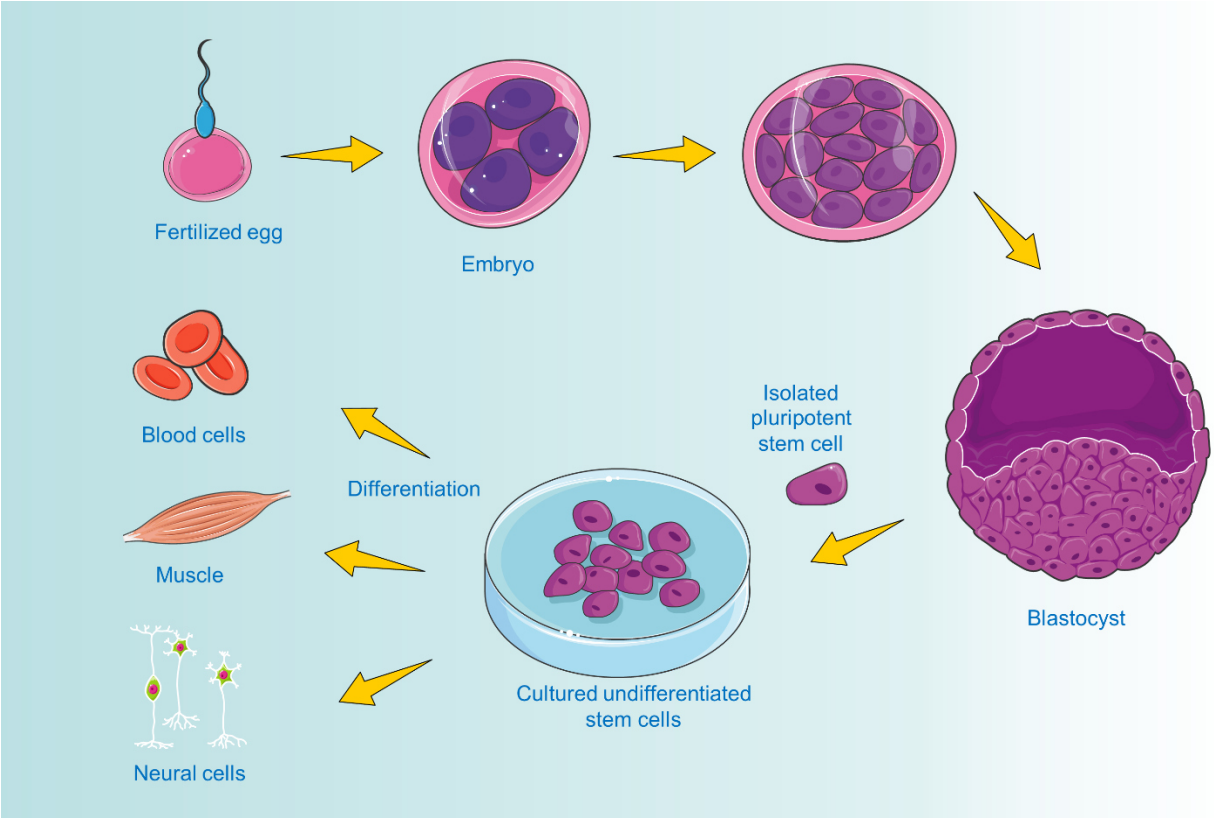


Figure 5: Schematic view of the first phases of embryo development.
After 5 to 6 days, embryo is at the blastocyst stage with around 30 cells in the inner cell mass containing being pluripotent embryonic stem cells that can be extracted and cultivated in vitro and are able to generate 200 different cell types.
(From Servier Medical Art).

However, the stemness term can still be used for a cell that allows to maintain a tissue renewal along lifetime, replacing lost cells and regenerating damaged tissues. These cells remain in the adult body and will not display "unlimited" renewal capacity, they can keep their initial capacities for a time but they will cumulate damages. At one moment they can also undergo senescence and stop dividing or die and the stem cell pool will decrease. Stem cells called **multipotent** will be able to produce differentiated cells from several lineages but with the same embryonic origin. Hematopoietic Stem Cells (HSC), and Mesenchymal Stem Cells (MSC) can be placed in this category. Finally, stem cells called **unipotent** will generate only one cell lineage, that is the case for example of Germline Stem Cells (GSC) producing gametes (16) or intestinal stem cells.

Stem cells present in the adult body during lifetime can be called somatic stem cells (in contrast to germ cells), adult stem cells or tissue resident stem cells (in contrast to embryonic and fetal cells).

In 2006, a new technique opened the possibility to use adult and specialized cells to generate pluripotent stem cells without relying on nuclear transfer or fusion with ESC. These cells were called "induced pluripotent stem cells" or iPSC. The technique implies to reprogram mature cells with the introduction of four transcription factors that were known to play important role for ESC maintenance, Oct3/4, Sox2, c-Myc, and Klf4, with viral vectors. Now, many variants of this technique were developed. These findings have major implications for the stem cells field. Associated to other findings about mature cells plasticity *in vivo*, it also allows to define the stem cell state as something that may be transient and reversible for a cell, even after differentiation.

It is also important to make the distinction between stem cells that are physiologically present during development and in the adult body from stem cells that can be obtained only by lab manipulation like ESC or iPSC.

Stem cells classification has strong implications for research orientation, therapeutic choices and regulation of stem cell use, so it may be of use to question theses definitions, their relevance and how new results may make them evolve (17).

3. General methods to identify stem cells

Stem cells have an elusive nature. They usually do not have specific morphological features, no specific surface markers, and even sometimes no specific transcripts. That makes them difficult to observe, to isolate and to characterize. Extended molecular and functional characterization is needed to be able to grasp them. Thus, it is necessary to harvest them and to undertake functional tests to show their renewal and differentiation capacities. The difficulty is also that the experiments needed to prove their existence also often result in their loss, for example to prove their differentiation capacity, or just being outside their niche that make them lose their stemness. Another limitation is that stem cells are very few compared to other cells in the body, especially compared to progenitors and

quick dividing cells they generate. With *in vitro* culture, the cells isolated from biological samples usually contain only a minority of stem cells. Caution must be taken when these cell mixes are considered. All the cells inside them are often called stem cells but that does not reflect the sample heterogeneity. In the end, it is very difficult to be sure if a sample still contains stem cells. By sacrificing a part of the sample of interest, it is possible to determine the estimated proportion of stem cells in the rest of the sample. The methods generally used depend on the stem cell type that is researched and usually combine functional tests with phenotype characteristics and genetic expression features.

a. Totipotency

To show the totipotency of stem cells, they must be implanted *in utero* and be able to generate a complete organism.

b. Pluripotency

To reveal the pluripotency of stem cells, several experiments must be done:

- Cells implanted in the early embryo must contribute to the generation of all kinds of cells of the three embryonic germ layers that are at the origin of the mature organism. These layers include endoderm, mesoderm and ectoderm and each give rise to specific tissues. A marker, usually a fluorescence gene transfected in a stable way, allows to see which tissues were derived from the implanted cells after development. This test is usually referred as chimera formation.

- Cells implanted in an adult animal must develop a teratoma, a tumor composed of differentiated tissue of the three germ layers.

- Cells must be able to generate functional differentiated cells of the three germ layers *in vitro*.

- The combination of several marker genes must be expressed: Oct4, DNMT 3B, TDGF, GABRB3 and GDF3. The typical surface marker profile for pluripotent cells include the presence of SSEA-3, SSEA-4, TRA-1-60, and TRA- 1-81 and the absence or very low presence of SSEA-1 (18). Nanog is also an important protein that allow the pluripotency maintenance.

Telomerase activity is also used to characterize pluripotent cells. Its activity is fully maintained and provides persistent proliferative potential in pluripotent cells whereas it is less active in adult stem cells (for more details about telomeres and telomerase, see part VI. "Senescence" – "Telomeres shortening and telomerase" and in the glossary).

It can also be noted that cells can also display different levels of pluripotency. The most potent can differentiate in all the types of cells of the organism, that is the case of ESC. Those with less potency must form at least one type of cells of each germ layer to be called pluripotent.

c. Multipotency

To show that a sample contains multipotent cells, the required experiments are less precisely defined.

Historically, the first proofs of adult cells stemness were obtained with graft experiments. This type of experiment showed that bone marrow grafts on mice that are lethally irradiated allow them to survive for the rest of their life. That meant that the sample grafted contained the needed stem cells to replace those that were killed by irradiation. These stem cells could assume the life-long renewal of the mice blood cells. This is made for HSC but it is not easily applicable for other stem cells. This kind of experiment necessitates a technique where the stem cells of interest can be destroyed in the animal and that it leads to short term death without intervention. An alternative to this experiment is to implant stem cells and to follow them during development and life. If the cells are found after long times, that argues for persistence and self-renewal capacity. Their contribution to the generation of differentiated tissues can also be followed to show their potency. These experiments can be done in heterotopic sites where the implanted stem cells form a tissue that would not have developed there otherwise. Serial transplant experiments can also be done to show stemness maintenance.

Their differentiation and self-renewal capacities can also be assessed *in vitro*. Cell differentiation in different cell types is obtained by addition of specific hormones cocktails and with adequate culture conditions. The self-renewal capacity is more difficult to assess, it is not equivalent to proliferation capacity and cannot be tested the same way. Furthermore, the factors required to maintain adult stem cells self-renewal outside their initial micro environment are not yet well known. However, these cells are able to generate progenitors that must be able to proliferate intensively. Thus, it is often considered that,

while being isolated from other cells *in vitro*, stem cells must be able to generate clone daughter cells and form colonies. A way to determine the proportion of stem cells in a sample is to count the percentage of cells able to generate a colony after seeding cells very isolated from each other, with the adequate environment. To be more stringent and to show extensive self-renewal capacity, colony formation can be assessed by maintaining culture more than 2 weeks or after several subcultures. Quiescent primitive stem cells may need longer times in culture to start to divide and generate colonies, while progenitors can generate clones faster but are more rapidly exhausted (19). Another alternative is to combine colony formation with graft experiments. Cells are harvested after implantation, after they could initiate the development of specialized tissues, and are seeded *in vitro* to test if their ability to form colonies remains (20).

Telomerase activity can also be detected in multipotent stem cells when it is activated. However, it is highly regulated and not continuously expressed. Its activity is not sufficient in adult stem cells to maintain telomere length and they undergo telomere shortening during aging (21).

Recent advances in techniques of single-cell genome, epigenome, and transcriptome sequencing can give access to more details on regulatory mechanisms and intrinsic genetic features specific to stem cells (22). Recent studies propose molecular signatures for different stem cell types. These investigations also permitted to reveal the important heterogeneity between cells and led to the determination of new stem cell types that were previously masked with bulk analysis.

4. Stem cells heterogeneity as a fundamental property

The reality of the high variability of cells even with the same genome in the same environment is often not considered and a homogeneity principle is preferred. Indeed, when an entire organ or tissue is considered, even if cells that compose these tissues are not perfectly homogeneous, what matters is that in the end, the ensemble of cells can answer the organism needs, and it can work even if some cells function a little more and some others a little less. Furthermore, **negative retro controls** that are very common in molecular mechanisms allow to narrow cell behavior dispersion (23).

Still, even within the same stem cell type, and for all kind of stem cells, they display an important level of heterogeneity that is often associated to noise while there is more than that. Stem cells heterogeneity allows a diversity of adaptative processes to occur in the human body and is an essential parameter. It is often masked with bulk analyses where single cell variations are inaccessible. Tools permitting to go to the single-cell level can give interesting insights about cell-to-cell variation, even within a seemingly homogeneous population. The level of variation between cells in a population is a parameter of interest by itself and have a functional significance. Variation in gene expression between cells is determined and regulated by different mechanisms like gene promotor accessibility, transcripts degradation rate, gene copy number, or by the structure itself of the regulatory network. Cell diversity and stochastic functioning may be beneficial in several points:

- it allows the population to adapt **quickly** to changes in the environment, with different kind of cells already there and ready to react, each subtype being able to react in a favorable way to distinct contexts,

- population response may be scalable, with different nuances,

- it may allow cells to shift toward a **wider range** of phenotypes, during the differentiation process for example, the most adapted are then selected by the micro-environment, with interactions with other cells and a process of auto-stabilization of their phenotype, thanks to chromatin structure for example,

- it can enhance the information content and information transfer capacity,

- a little subset of cells may have a special ability to sense and respond to specific stimuli and play a coordinator role for the rest of the population by emitting signals (2).

An article highlights this phenomenon with mouse hematopoietic stem cells (1). The variability in some protein expression (Sca-1 protein was used in this case) was not due to noise or lack of device resolution, but rather to distinct cell individualities even within a clonal population in the same environment. Interestingly, when cells displaying opposite expression levels of Sca-1 were separated by cell sorting, right after the sort, they showed different transcriptomes and differentiation potential, but after several days in culture, the distribution of expression level of Sca-1 returned to the same state as before the sort. Even after an efficient sort, the sample regains its initial heterogeneity and seems to return to

what may be an **attractor state**, a kind of steady state equilibrium composed of a given mix of variable individual cells **(figure 6)**.

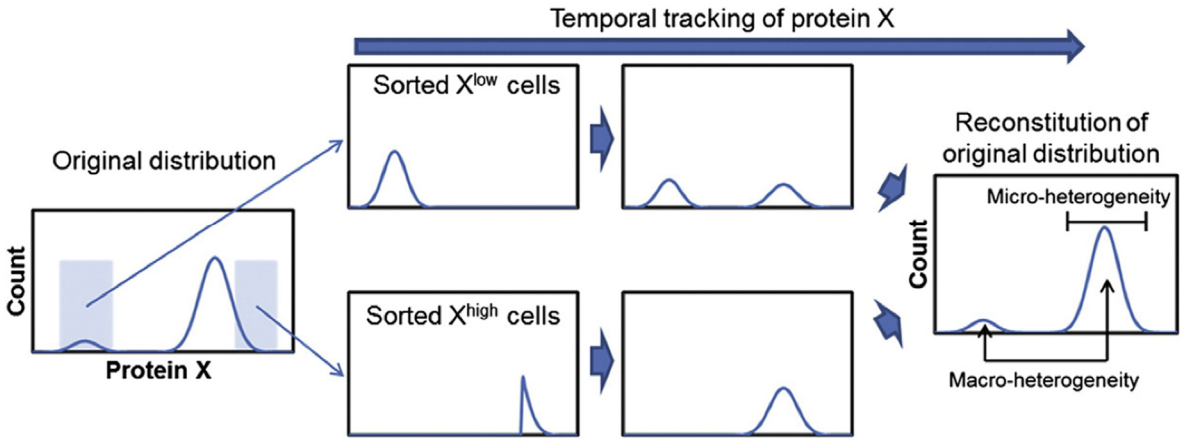


Figure 6: General illustration of the possible robustness of stem cell population heterogeneity.

Protein content is measured via flow cytometry (see the corresponding part for the details of the technique). Protein X displays a bimodal distribution in the initial population. Cells with high expression are separated from cells with low expression by cell sorting. After some time, protein X converges to the original bimodal distribution and heterogeneity. Macro-heterogeneity refers to the existence of distinct peaks whereas the heterogeneity within a subpopulation is called micro-heterogeneity here (24).

These mechanisms may explain the difficulty to culture and obtain cells with homogeneous phenotype. The challenge would then be to be able to take into account this inherent cell heterogeneity without simplifying too much to the average case.

Cell individuality depends on many parameters including micro environment, cell-to-cell interactions, and is intrinsically variable according to the availability and position of the needed factors inside the cell.

5. Stem cells fate determinants

a. Intrinsic factors

Cell fate is in part due to intrinsic possibilities of the cell to be in different states. What can determine these states includes genetic features, especially those that cannot be modified by physiological means, such as DNA condensation level in some regions of the genome. These possible states for the cell may also be affected by stochastic variations, for

example the molecules and organelles that find themselves in the cell and that can vary in abundance following division or to allow gene expression (13).

b. Extrinsic factors

Adult stem cells are rare cells found all over the body in specific **niches** where they can remain at their relatively undifferentiated state and keep their self-renewal potential. Cells immediate environment is of major importance to reveal stem cell potential and for the initiation of phenotypic switches, that will be made according to the specific need of the residing tissue. Adult stem cells have essentially the role to maintain tissue homeostasis by renewing dying cells but they are also active component of the niche, they have a function by themselves as quiescent cells.

Nature of niche composition and function are very similar between different species, it is a widely conserved mechanism. It is composed of diffusible growth factors that can stimulate proliferation and hormones that can promote differentiation. Other small soluble molecule like ions can also be found. Insoluble adhesion molecules and compounds from the extracellular matrix are also present. Extracellular matrix components are not just inert filling, they are important players for cell communication, migration and maintenance. The niche is also composed of other type of cells, often called stromal cells, that can be supportive to stem cells. They are in close relationship, adhere and secrete factors that influence each other, in autocrine and paracrine manners, with feedback regulatory mechanisms. These interactions seem to be needed to maintain stem cell integrity. Other cells in the niche can also transmit signals at some peculiar moments. Spatial localization of each of these components play also a major role to polarize stem cell function. Stem cells are often close to blood vessels that may allow them to have access to the necessary factors for their regulation **(figure 7)**.

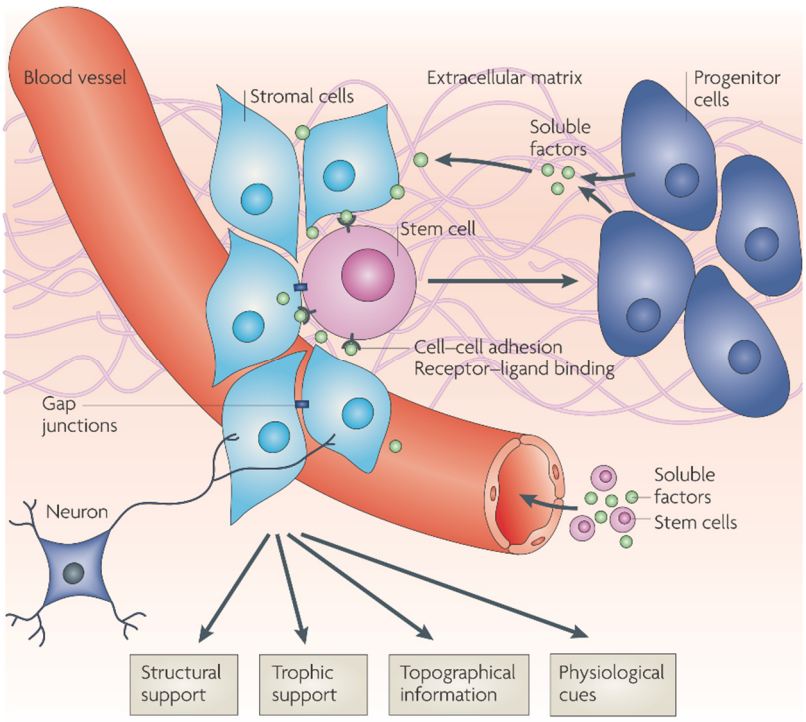


Figure 7: Components and functions of the stem cell niches.
A classical niche may contain, besides stem cells, stromal cells, soluble factors, extracellular matrix, neural inputs, vascular network and cell adhesion molecules (25).

Stem cells also seem to prefer residing in places relatively protected from damage, like the hypoxic niche in the bone marrow for HSC that protect them from reactive oxygen species. Physical factors, temperature and oxygen tension are to take into account. Niches are often thought as restricted areas of specific organs, but it seems stem cells can be found in more various places than previously thought.

The niche environment is thus essential to maintain adult stem cells properties. When they are anchored in the niche, a tight regulation system preserves them from death, exhaustion and excessive proliferation with survival and self-renewal signal. It allows to generate just the needed amount of differentiated cells only when needed. The niche provides cells with appropriate signals depending on the system functioning. When a regular renewal is needed, the niche environment must provide signals that balance self-renewal and differentiation to produce the specialized cells. In other tissues, when stem cells are mobilized more rarely, the niche will provide essentially signals to prevent differentiation but to keep responsiveness when needed, after injury for example. As other cells, stem cells will integrate information from their environment with signal transduction

mechanisms. A cascade of reactions will result in the mobilization of transcription factors that will activate a specific genetic program to make the cell divide, differentiate or remain quiescent. There is also a strong conservation of the type of signaling cascade used by stem cells although they can result in different effects according to cell type. For example, the break of adhesion bindings between cells usually stimulates proliferation. Another example is the signaling pathway with Wnt that can be secreted by surrounding cells or by stem cells themselves (25).

As stem cells properties are highly dependent of their micro environment, it is associated to the major challenges to maintain their capacities after these cells are extracted and cultivated *ex vivo*. More research is also needed to determine the way niches are formed and maintained. These findings could lead to develop better techniques for adult stem cell culture. What is known is that several stem cell types are at the origin of the generation of the differentiated stromal cells that will support them. Growing evidences also tend to point out that stem cell niche dysfunction may be implicated in many pathologies like age associated diseases and tumorigenesis.

The study of stem cells niche is difficult because it requires special techniques that take in account for spatial localization. To label stem cells *in situ*, specific markers are often lacking and it is also a challenge to detect the rare stem cells population.

c. Example of the bone marrow

The term “niche” to describe stem cell micro environment was first employed to describe bone marrow, where reside HSC that produce blood cells. It is found in the center of long and axial bones **(figure 8)**. The inner surface of bone cavities is called the endosteal region. The more central part is called the medullar region. Bone marrow contains also fat cells and is traversed by many arterioles, located near endosteal region, and by sinusoids, distributed more evenly **(figure 9)**. Perivascular cells are present around these blood vessels (26).

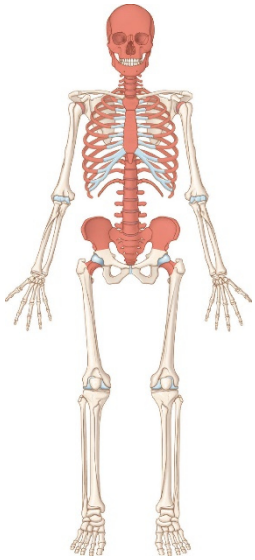


Figure 8: Red bone marrow localization.
It can only be found in the ribs, sternum, vertebrae, skull, pelvis, and upper parts
of both the humerus and femur.
(From Medical Dictionary © 2009 Farlex and Partners.)

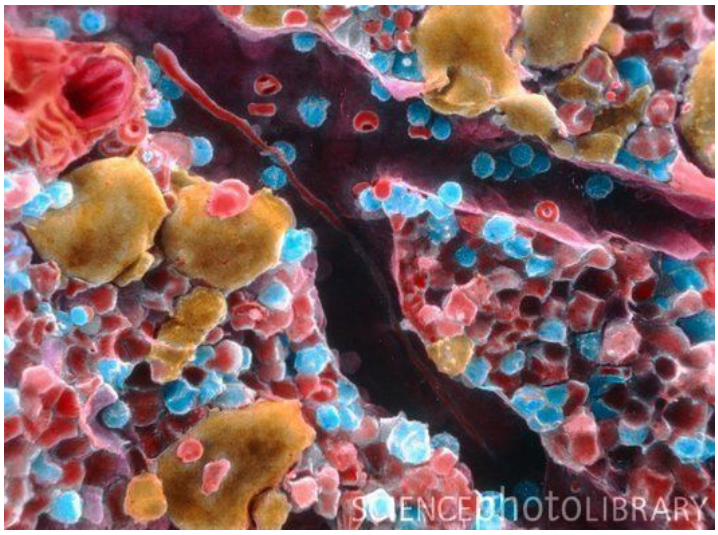


Figure 9: Colored Scanning Electron Micrograph (SEM) of cells making up human bone marrow.
Branching sinusoids (channels, black) are seen in the bone marrow tissue. Bone marrow consists of densely packed red blood cells (erythrocytes, red spheres), white blood cells (granulocytes, blue), and smaller platelets involved in blood clotting (top left, red). Large fat (adipose) cells are colored brown. Bone marrow occupies the spaces in bone and when active, is dominated by production of red blood cell.
Magnification: x660 at 5x7cm size.
(From © Science Photo Library Limited 2019)

Another kind of multipotent stem cell is present in the bone marrow, the mesenchymal stem cells (MSC). They are in close relationship with HSC, each giving mutual support. MSC are important for HSC maintenance and niche homeostasis. They are major actors to develop the niche in the bone marrow (27,28) . They generate osteoblasts and are essential to bone remodeling. They can also differentiate in adipocytes and chondrocytes. Marrow adipose tissue was recently shown to be an active component of the niche with endocrine and paracrine effects that will have important effects in the hematopoietic system and for bone remodeling.

Other kinds of cells are found in the bone marrow and essential to the niche establishment and maintenance. That is the case of some types of macrophages notably and other immune cells like megakaryocytes (28,29). These immune cells are responsible of the development of an immunoregulatory network specific to bone that makes the niche an immune-privileged environment. Macrophages that become cells called osteoclasts, that are

responsible for bone resorption, are implicated in the release of HSC in the circulation, a phenomenon called mobilization. On the other hand, another kind of macrophages called osteomacs supports osteoblast function. Endothelial cells, for their part, contribute to stem cells biology. They can secrete molecules called angiocrine factors that stimulate the surrounding organs repair activities. Endothelial cells also make cell contact with stem cells (27).

Cell contact is important for HSC maintenance. HSC can bind to bone marrow endothelial cells through E-selectin. They also bind to perivascular MSC (27).

The bone marrow is also innervated and under the control of the sympathetic nervous system. It delivers catecholamine signals (a molecule family composed of adrenaline and dopamine for example) that can affect HSC, especially to release them into the circulation, and MSC functions with bone remodeling.

Two types of niches are classically described in the bone marrow. One that is associated to the osteoblasts in the endosteum, and another associated to blood vessels. Endosteum contains regions of significant hypoxia (less than 5% dioxygen). Hypoxia activates Hypoxia-Inducible Factors (HIF) that impact metabolic states of residing cells. In these conditions, quiescent cells rely on a glycolytic metabolism instead of the mitochondrial oxidative phosphorylation to generate ATP. This results in less generation of ATP and reactive oxygen species, both involved in cell differentiation. HIF factors have also the effect in MSC to promote osteogenesis over adipogenesis. Osteoblasts support HSC quiescence also with angiopoietin-1 secretion. The vascular niche, on the other hand, gives access to more nutrients, growth factors and oxygen that promotes proliferation, differentiation and migration of HSC.

Bone marrow niche involves key ligands and receptors proteins that are SDF1-CXCR4, integrin α-4–VCAM1, L-selectin –PSGL-1, hyaluronic acid–CD44, and c-Kit–Kit ligand (30). These factors are implicated in the mechanism that allow stem cells to be attracted to the niche (homing) and mobilization. Ionic calcium concentration is another

factor that can attract HSC (27). SCF is also an essential component in the vascular niche, it can be secreted by MSC and osteoblasts and promotes self-renewal (31).

A more detailed view of the bone marrow niche can be seen **figure 10**.

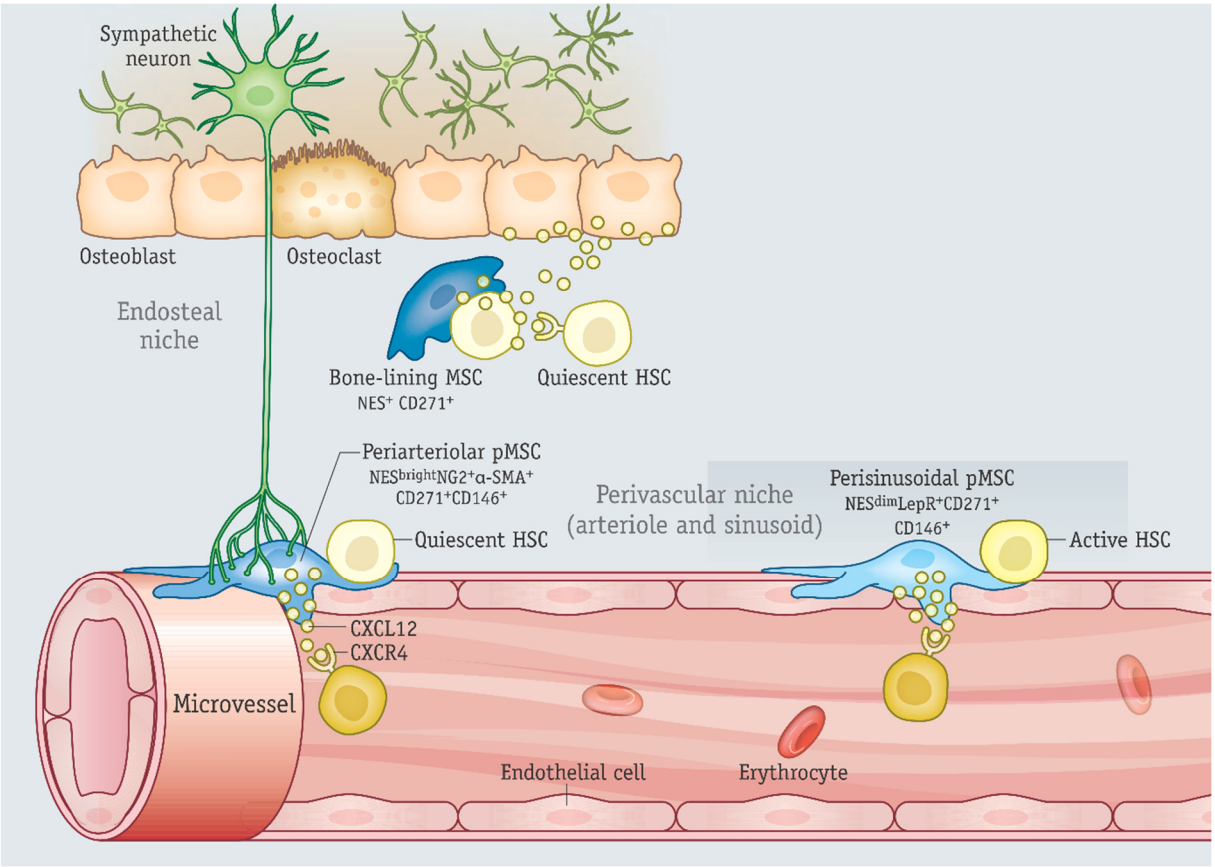


Figure 10: Representation of interactions between different actors of the bone marrow niche.
Different MSC subtypes with different phenotypes, HSC with different activities and all the hematopoietic cells they produce (red and white blood cells), bone cells (osteoblasts and osteoclasts), endothelial cells of the vessels, and neurons.
The different MSC subtypes shown in this figure were highlighted in **mouse** bone marrow (and not in human) as follow: MSC expressing low levels of **Nestin** (NES^{dim}) were found to be a subtype of MSC associated with active HSC while MSC with high levels of Nestin (NES^{bright}) were associated with quiescent HSC.
Other markers could be used to distinguish MSC localization between perisinusoidal, periarteriolar and bone-lining MSC.
MSC can also differentiate into osteochondral lineages.
CXCL12 is another name of SDF1, its secretion is regulated by sympathetic innervation and circadian rhythms. CXCL12 signals cause HSC homing and retention (32).

6. Stem cells aging

A decrease of the maintenance of tissues can be observed with age. It is in part due to the fact that adult stem cells cumulate molecular damages and show functional decline with age. Their self-renewal, proliferative capacities and responsiveness are affected

resulting in a diminution of functional stem cell pool. Differentiation potential is simultaneously impaired. Stem cells can then enter a specific state called senescence. It is a durable state where cells are unable to divide, in the incapacity to answer mitogenic stimuli and with resistance to oncogenic stimuli (33). Cell homeostasis is strongly affected in this state, and senescent cells become a significant source of inflammatory signals. It is an important loss of function for stem cell that should be able to self-renew and produce progenitors (more details about senescence are provided in the last part of the introduction, part VI. "Senescence").

Residual activity of telomerase is found in stem cells but is not sufficient to prevent telomere shortening. By their initial function of self-renewal, stem cells are particularly prone to DNA damage associated to replication. Several studies confirmed the accumulation of genotoxic lesions in stem cells with aging. This is combined with a decrease of repair DNA damage response. Quiescent stem cells usually rely on a particular system to repair DNA that is rapid to avoid cell death and senescence but also prone to errors and may also be in cause in the accumulation of mutations. This DNA repair mechanism also needs a lot of NAD^+, an important cofactor for many reactions. This could lead to NAD^+ reserves depletion leading in alteration of mitochondrial metabolism and of other enzymes needing NAD^+ to work.

Stem cell aging imply metabolic stress despite the important resistance to stress and the fact that most stem cell types rely on glycolytic metabolism that prevents many molecular damages. Metabolic signals in cause are more particularly associated to oxidative stress, mitochondria dysfunction and inflammation. It is then possible to say that aging impacts stem cells for a part and stem cells limitations are also determinant for the aging process. Dysregulations can result in imbalances in the differentiation process. This can lead to tissue defects and degradation or, on the contrary, to the proliferation and over-presence of some cells associated to functional decline, with potential carcinogenesis.

Studies at the genomic and transcriptomic level showed that genes implicated in age associated diseases are generally associated to inflammation pathways and even more to senescence regulation. Levels of inflammatory signals, for example circulating

inflammatory cytokines, increase with age. Systemic factors are particularly important for stem cell functioning and these changes strongly impact them. Among the metabolic signaling that are more specifically altered, there are those of sirtuins, insulin/IGF1, and mTOR pathways. These pathways have particularly important effect associated with aging with their nutrient sensing regulation role. Wnt signaling, which is important for stem cells differentiation and self-renewal, can also be perturbed and associated to senescence and impaired differentiation. Alteration in Wnt signaling can be associated to FoxO signaling that is also an important regulator of stem cells functionality. FoxO transcription factors regulate fundamental processes differently according to cell types. They are classically implicated for the protection from cellular stress, for example by activating the transcription of antioxidant enzymes. FoxO signaling oppose the signaling of mTOR that promotes growth. In case of oxidative stress, a shift favors FoxO signaling and at the same time, the reliance on Wnt pathway decreases. This leads to changes in the cell transcription program implicated in senescence and age-associated diseases. However, FoxOs are also important actors for cell metabolism and are implicated in resistance to metabolic diseases. They can attenuate age-associated diseases, particularly with their effect in the regulation of the immune system and its progenitor pool (34).

Damaged and misfolded proteins accumulate in cells with aging. That is especially the case of proteins implicated in cytoskeletal organization and oxidative stress defense. The lamin A, a protein constituting the nuclear envelope, known to be responsible of the progeria syndrome of premature aging, turned out to be important for adult stem cell and niche maintenance over normal aging too. MSC for example have shown a tendency for lamin A dysfunction that make them more susceptible to stress and may play a role in their decline with age. Facing these kind of damaged proteins, cellular responses that are usually activated to take charge of the altered proteins are reduced at the same time. In this case, specific mechanisms, especially autophagy, are activated to recycle the damaged proteins. Studies with several stem cell types find that autophagy level is more important in stem cells than in differentiated cells. The weakening of this mechanism is also traditionally associated with an increased susceptibility to stress and with aging (34).

Epigenetic modifications that control DNA accessibility are also altered with stem cell aging and are related to the switch of expression program that can be observed. That could be directly linked to the protein management impairment that can affect epigenetic regulators. With aging, the stability and turnover of chromatin modifiers is deteriorated. Persistent inflammation associated with aging could also alter epigenetic regulators of stem cells directly. The inflammatory factors secreted by senescent cells, like interleukin 6, could induce cell plasticity and promote cancer.

The changes that stem cells can undergo with aging are summarized in figure 11.

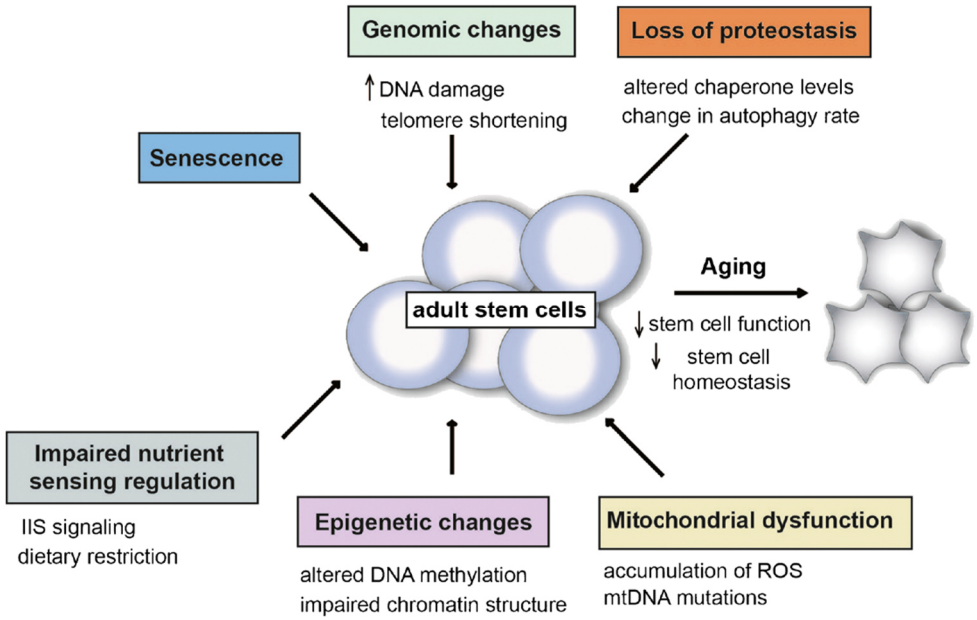


Figure 11: Adult stem cells age-associated changes that result in functionality loss and decline in tissue repair (35).
IIS: Insulin/IGF1.

Micro environment is also an important player leading to stem cell dysfunction. Inflammation can contribute to tissue destruction and niche deterioration. The aging niche sends aberrant signals that can lead to long-lasting epigenetic modifications in the residing stem cells. As stem cells are implicated in the niche remodeling by their secretions and with their production of niche cells progeny, their alteration can in turn lead to aggravation of niche deterioration (34–36).

The cellular composition of the niche is also impacted by aging. The proportion of different subsets of cells, that were constituting functional heterogeneity, change with age.

To continue with the example of bone marrow niche, with increasing age, bone marrow becomes less active, filled with fat cells, and with different proportion of HSC subsets. HSC also show increased heterogeneity between individuals and more variability between cells of the same organism. Some of the altered subsets may earn a selective advantage that make them persist and become predominant. This shift may be more important than aging of individual cells by itself (37).

Aging is also a concern for iPSC, as those derived from aged adults carry more mitochondrial DNA mutations and can have other genetic alterations (38).

The onset of many diseases is associated to advancing age. The understanding of the underlying mechanisms of age associated diseases may help to prevent them and to reduce healthcare costs. An important but difficult part is to distinguish stem cells in the context of aging and stem cells in the context of some specific diseases that are often also associated with aging. Several studies suggest that senescence study *in vitro* is relevant to have interesting insights about *in vivo* aging (34,39). **Figure 12** proposes an overview of the interplay between the main factors implicated in aging. This figure situates stem cell exhaustion as the final event associated to functional decline.

These hallmarks are not always independent, and the occurrence of one can, and often does, impact others. For instance, changes in the epigenetic regulation of genes can result in downstream effects on cellular function, protein stability, cell signaling etc. For this reason, the hallmarks can be categorized into three groups:

- **Primary hallmarks (blue icons)** are those that are the foundational causes of cellular damage
- **Response or compensatory hallmarks (orange icons)**, which are a result of the primary hallmarks
- **Integrative hallmarks (green icons)** that incorporate the first two classes of hallmarks and ultimately lead to the functional decline observed in aging

Figure 12: Hallmarks of aging, separated in 3 categories.
(From merckmillipore.com)

7. Stem cells potential for research and therapy

As stem cells understanding grew, they could progressively be detected, isolated and studied more precisely in the lab. It is now a wide field of research that is often associated to development and aging biology. Stem cells represent an important experimental tool for biomedical research. They can be interesting models of diseases, to screen for new drug discoveries and for cell therapies.

a. Stem cell therapies

Cell therapies consist of using living cells to prevent or treat a pathology. In this part, a focus will be made on cell therapies using stem and progenitor cells specifically. Given the high complexity to develop cell therapies, they are usually considered for applications that could not be solved with single molecules. That is the case for example when large tissue restauration is needed, or when a quick and adaptative answer is necessary.

The fact that the niche is so much implicated for stem cell function makes it an interesting therapeutic aim. It can be used to enhance endogenous stem cell functionality or to facilitate engraftment and efficacy of transplanted cells. Indeed, in the pathological context where stem cell therapies are used, the niche is often damaged and can have negative effect on the implanted cells. Niche consideration can also be of use for stem cells culture, by allowing the cells to grow in an environment where they can keep at best the wanted potentialities for the aimed application. A lot of research effort is placed in biomaterials that could support the cells function. In this case it is possible to rely stem cell field to tissue engineering, where cells are usually seeded in a scaffold before implantation to give mechanical support and sustain tissue repair. Scaffolds can be resorbable and associated with molecules that are progressively released. The different strategies that can be used for stem cells therapies are presented in **figure 13**. Stem cells can also be interesting to use for gene therapy if they persist in the patient and generate daughter cells with the same modification.

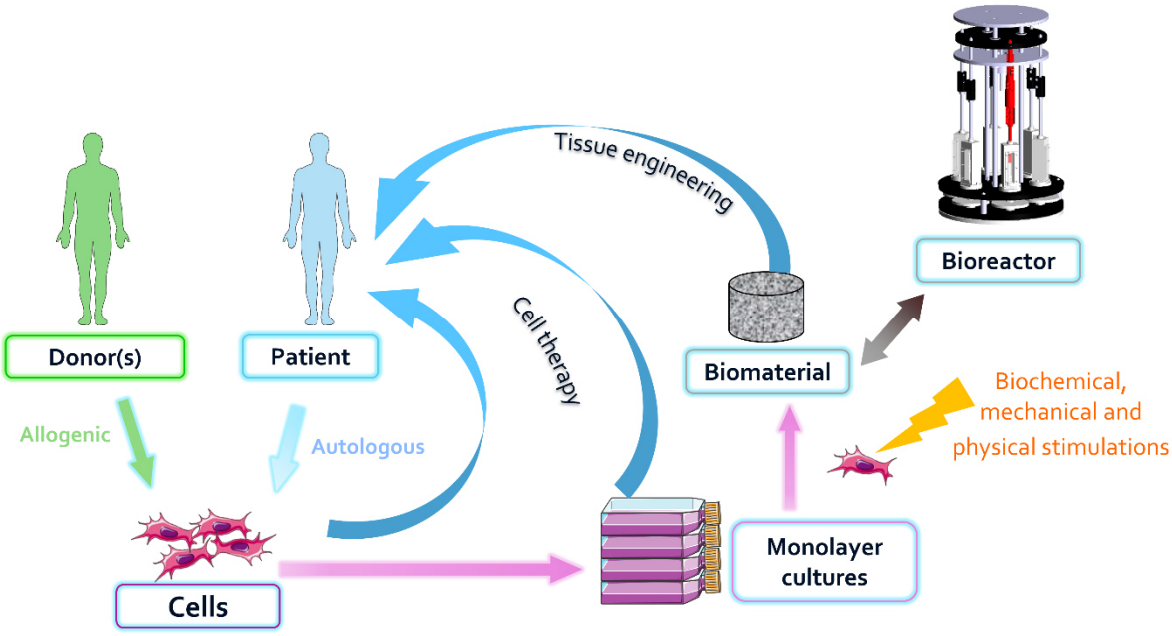


Figure 13: Different possible cases for stem cells use.

An autologous therapy makes use of the patient cells to produce the therapeutic cells. The term allogenic is used to describe the use of cells coming from a donor for another patient. The cells can be used in different ways:

- with separation techniques and minimal manipulation, cells can be extracted and used directly, with no need for expansion or special treatment,
- after monolayer amplification *in vitro* to obtain more cells,
- after culture in a specific environment to direct the cell behavior, for example in special biomaterial and with more sophisticated stimulations.

HSC therapies were the first using stem cells. They are widely used as cell transplant and successful. They can come from bone marrow sampling, from peripheral blood with a specific *ad hoc* treatment, or from cord blood. They don't need to be expanded for actual use. They can be used for autologous transplants but the most common use of HSC is with allogenic transplants for bone marrow failure and hematological malignancies. Allogenic transplants were made possible by the understanding of immune rejection and the development of immunosuppressive drugs that can be used lifelong.

Other actual clinical applications of stem cells exist but they are not so widely available as those based on HSC. Among the most common, there are epidermal and corneal stem cells. These cells need to be expanded *in vitro* to generate autologous grafts in case of skin or cornea severe wounds (40). The products with marketing authorization are presented in table 1.

Table 1: Therapeutic products relying on stem cells with marketing authorization in 2018 (41)

Product name (firm name)	Cells	Indications	Organization - year of marketing authorization
Holoclar (Chiesi)	Expanded autologous human corneal epithelial cells containing stem cells	Severe limbal stem cell deficiency	European Medicines Agency - 2015
Strimvelis (GlaxoSmithKline)	Autologous CD34+ cells transduced with a vector encoding for the ADA cDNA sequence	Severe combined immunodeficiency due to ADA deficiency	European Medicines Agency - 2016
Alofisel (Takeda)	Expanded allogeneic adipose stem cells	Complex perianal fistulas in patients with Crohn's disease	European Medicines Agency - 2018
Prochymal (Mesoblast)	Allogeneic cultured human bone marrow mesenchymal stromal cells	Acute graft versus host disease in pediatric patients	Health Canada – 2015 (authorized but never marketed)
Temcell (JCR)	Allogeneic bone marrow mesenchymal stromal cells	Acute graft versus host disease	Japan Pharmaceuticals and Medical Devices Agency - 2015
Stempeucel (Stempeutics)	Cultured adult allogeneic mesenchymal stromal cells (pooled)	Critical limb ischemia due to thromboangiitis obliterans (Buerger's disease)	Drug Controller General of India - 2016
NEURONATA-R (Corestem)	Autologous bone marrow mesenchymal stromal cells	Amyotrophic lateral sclerosis	Ministry of Food and Drug Safety of South Korea - 2014
Cupistem (Anterogen)	Autologous adipose tissue derived mesenchymal stromal cells	Crohn's fistula	Ministry of Food and Drug Safety of South Korea - 2012
CARTISTEM (Medipost)	Human umbilical cord blood derived mesenchymal stromal cells	Knee articular cartilage defects in patients with osteoarthritis	Ministry of Food and Drug Safety of South Korea - 2012
Cellgram-AMI (Pharmicell)	Autologous bone marrow derived mesenchymal stromal cells	Acute myocardial infarction	Ministry of Food and Drug Safety of South Korea - 2011
Queencell (Anterogen)	Autologous adipose tissue derived adipose cell	Subcutaneous tissue defect	Ministry of Food and Drug Safety of South Korea - 2010

South Korea is the country where the most stem cell derived products are approved. United States registered HSC from cord blood as marketed products while in the other countries it is considered like transplantation. These products were not reviewed here. The cell therapies without stem or progenitor cell implicated are not mentioned here either.

Globally, few stem cell products are currently on the market and their cost is extremely high. They are usually adapted to only a restricted number of patients. Fabrication costs are also in cause, associated to the necessary risk management, and heavy regulations to follow. The maintenance of these therapies is very difficult and many products were withdrawn from the market (41). Within the still young field, clinical trials also often get suspended. Despite the difficulties, and thanks to their unique potential, stem cells therapies continue to be investigated in many clinical trials with more and more different kinds of stem cells. Cliniclatrials.gov is the database gathering the most reports of protocols and results of clinical studies in the world. It is related to the National Institutes of Health in United States. To research for therapies using stem cells in this database, an advanced research tool was used with keywords assigned to the "Intervention/Treatment" field. The study type was selected to be “Interventional studies (Clinical Trials)”. No other filters were used. The last results of the list (less relevant) were checked to avoid some of the false positive results. The last actualization of this search was made on June 8th 2019.

- Biological: Stem Cells: 1774
- Biological: Hematopoietic Stem Cells: 472
- Biological: Mesenchymal Stem Cells: 495
- Biological: Bone Marrow Stem Cells: 403
- Biological: Umbilical Cord Stem Cells: 168
- Biological: Adipose Stem Cells: 93
- Biological: Embryonic Stem Cells derived: 18
- Biological: Induced Pluripotent Stem Cells derived: 3

Progenitor and stromal cells are also included in these numbers, as they are considered as synonyms by the clinicaltrials.gov search tool. Some studies included in the “Stem Cells” number may also concern Cancer Stem Cells. “Bone Marrow Stem Cells”,

"Umbilical Cord Stem Cells", and "Adipose Stem Cells" are other denominations that can be equivalent to the most general term "Mesenchymal Stem Cells". It can also be worth noting that some studies included here are not to treat diseases but only to collect samples.

Despite their large potential, still few clinical trials use pluripotent stem cells and most of stem cell therapies rely on adult stem cells. They can be used undifferentiated or differentiated. HSC remain also investigated to enhance their use and the associated treatments. Among the different stem cell types, MSC are the cells that are the most often chosen for current clinical trials.

When looking at these clinical trials, the indications that are mostly found are blood cancers and bone marrow failure with the use of HSC. HSC transplant can also follow other kinds of cancers when the treatment kills the patient stem cells. BM MSC can be used to enhance HSC engraftment. Graft versus Host Diseases (GvHD) can be a consequence of HSC transplantation and MSC can also be used to try to reduce secondary effects. Stem cells are also tried for even more than the following major indications groups: cardiovascular, immune, inflammatory, bone, brain, lung, kidney, skin, retinal, and liver diseases, infections, osteoarthritis, diabetes and its complications. They are often used to improve wound healing after injury and fibrosis. Also, other original indications like autism are investigated in several countries with stem cells treatments. A surprising clinical trial was also registered in 2018 to try to treat bipolar depression with allogenic BMMSC in Texas.

Using living cells as a treatment comes with huge challenges. It is often difficult to predict implanted cell behavior in humans. Stem cells can behave very differently *in vitro*, outside the organismal context, deprived of many micro environment elements they were used to rely on. They can also show different behavior during normal tissue homeostasis and during tissue injury. Some clinical trial failures could be associated to insufficient preclinical data that reliably supports the cells potential. Even when preclinical data are encouraging, translation to human is often difficult and the conditions for the treatment in the lab are very different from those in a human clinical setup. The industrialization step for cell production can also fail. Stem cells heterogeneity also have major implications for therapies. Passage to the clinical level is motivated to test the beneficial effect of the therapy

but it is also associated with many translational difficulties with an expansion of the parameters that come into play. Combination of different levels of heterogeneity leads to an overall strong variability. However, in a clinical context, the main concerns are still linked to handling, thawing, delivery and dosing instead of sample heterogeneity. To be able to draw more reliable results, more details are often needed, but also more consistence in the critical parameters that are followed in clinical trial protocols. The combination of a more homogeneous and optimized clinical setup and a better understanding and control of cells and donor variability will be needed to improve the success rate of cell therapies.

The collaboration of different fields is a strong necessity to hope to advance in stem cell therapies applications. Mathematical modelling for instance, brought interesting contributions for stem cells comprehension, especially to understand the mechanisms underlying stem cell heterogeneity.

The field is still progressing quickly. A short time after their discovery, ESC and iPSC see their first clinicals approved. Frozen stem cell banks multiply and will open the possibility of a quick delivery of cells and more matching between donors and recipients. The field needs to find solutions to provide treatments with efficacy, safety and affordability, and also to reduce the current considerable costs.

b. Regulation

Stem cell use for research and clinical applications is under the specific regulation of each country. Stem cell regulation is crucial as there is a lot of danger associated with abuse of their use and selling. Patients in need are in high demand for treatment and commercial pressure associated with enthusiastic scientists and ambitious clinicians is a potentially lethal cocktail (40).

To obtain an ESC line for research or clinical applications, it requires embryo destruction. Four types of regulations can be defined in this context.

- Some countries completely ban embryo destruction, it is the case of Austria and Ireland for example.

- Restrictive regulations, in vigor in Germany and Italy notably, don't permit embryo destruction but allow to research with imported ESC.
- Most European countries adopt now permissive regulations with some restrictions. Embryo and ESC research is possible but nuclear transfer and embryo creation is still forbidden.
- Lastly, permissive regulations of other countries such as the United Kingdom, Japan, China, and others, allow almost all techniques except reproductive cloning. USA federal funds are granted with permissive regulations with restrictions, but it is completely permissive for other projects.

For iPSC, there is even more concern about their potential adverse effects. Their use necessitates proofs of their non tumorigenicity before implantation. However, iPSC are made from adult cells, their use is free for research. Fetal and adult stem cells use in research and clinical applications is widely accepted. In this case, informed consent of the donors, or of the mother for the case of fetal stem cells, is necessary.

In Europe, ESC research and applications are not patentable whereas iPSC and adult stem cells can be subject to a patent.

Stem cell therapy products have curative or preventive properties for diseases, or they can act on physiological functions. **In France**, three categories can be distinguished:

- Advanced Therapy Medicinal Products (ATMP): they are considered as biological drugs produced with an industrial process to obtain a standardized product. They are subject to European law.
- Advanced Therapy Medicinal Products prepared punctually: they are prepared for a specific patient. That can be the case for example of autologous stem cell therapies. In this case, this falls within the law of each country.
- Cell therapy preparation: they are not considered as drugs or ATMP. For these products, there is no substantial modification of the initial nature and function of the cells. Only minor manipulations are allowed, and the cells must be used for the

same function in the donor and recipient. These preparations are also under national law.

ATMP must be fabricated with good manufacturing practices, like all medicinal products for human use. In France, this fabrication must take place in a pharmaceutical establishment, or in a public establishment authorized by "*Agence nationale de sécurité du médicament et des produits de santé*" (ANSM). The marketing authorization of ATMP is provided in France by the European Medicines Agency (EMA). It is delivered for five years at first and can then be extended for an unlimited duration after reassessment of the benefits / risks balance. For ATMP prepared punctually, it is ANSM that delivers the authorization in France.

In different countries, many marketing authorizations are under conditional approval or complementary studies to follow the treatment efficacy are often asked for stem cell therapies (42).

Regulations over stem cell research and clinical use can be surprising in some countries and require awareness. There is an example of a clinical trial in India for children with cerebral palsy. They could use undifferentiated ESC between 2007 and 2013 under approval of dubious ethic committees, in spite of the important risk to develop teratoma (43). Dubious private stem cells clinics also proliferate and sell unproven and dangerous treatments, often implying MSC, without following current legislation. One of these clinics, US Stem Cell, was recently defeated in 2019 against FDA in a federal court and asked to stop their activity (44).

8. Misconceptions and controversy in stem cell research

History of stem cell research is young and already full of drawbacks. Their elusive nature is very propitious to lead to fallacy. To this we can add a lot of confusion and lack of rigor to use the adequate vocabulary that mixes up with true controversy and uncertainty. Results published in peer-review journals are not definitive and need to be critically analyzed. Another problem is that true results can often be considered as anomalies or technical errors and it may take a lot of time before a change of paradigm is accepted.

The beginning of cell culture is a typical example. Nobel Laureate Alexis Carrel worked with chicken cells and described cells as immortal in culture when the good culture conditions were found. Afterward, it was found that the apparent immortality of the cells he studied was due to the culture medium used that contained cells unwittingly. Each medium change brought new cells that compensated the death rate of the cells in culture (45). Even now, the field is not guarded against similar errors.

The very existence of some stem cells entities is currently being called very much into question. That is the case of Multipotent Adult Progenitor Cells (MAPC), Very Small Embryonic-Like Stem Cells (VSEL) and Muse Cells that are said to be adult stem cells with pluripotent cells properties. These properties could not be found by other teams and the experiments could not be replicated. Doubts emerged that they could be artifacts due to culture, malignant cell transformation or contamination with other samples. There was also a problem in the vocabulary used, as the cells described initially did not meet all the criteria to be said pluripotent and were still called this way in the articles. Despite criticisms of the scientific community, the teams implicated in these research projects continue to publish and to defend their discovery, clinical trials are even in progress. These concepts and nomenclatures can be found in recent articles and books, as we can see for example in 2018 in Frontiers in Immunology and Stem Cell Research & Therapy (46,47).

A similar case is ongoing with stimulus-triggered acquisition of pluripotency (STAP) cells. This was supposed to be a new and much simpler way to generate pluripotent cells, without genetic transfer. After publication in Nature in 2014, the scientific community failed to replicate the results and an investigation found that some data were falsified. The papers were retracted but there are still ongoing attempts to show the technique could work.

On the other hand, some scientists may fear to use a specific term and the word “stem” is progressively removed from the adult cell nomenclature. Definitions in biology are difficult to manipulate as they often are continuous concepts. It is then useful to take time to precise things and to explain why a term is used instead of another to avoid misconceptions.

Even for HSC research, some scientists try to warn against the possible misconceptions that can take root in the field. For example, HSC are obtained after purification and it is possible that many other interesting stem cells are discarded and thus not represented in the studies. Studies based on highly purified HSC cannot be regarded as representative of the complete marrow stem cell compartment. The appearance of global comprehension in the field, where HSC system seems almost completely characterized, must be considered with care. It must not be a dogma, and it is still far from true comprehensiveness. It is notably with stem cell heterogeneity and fluctuating phenotype that many elements remain to be clarified. Some procedures to obtain the cells that became an unquestionable routine also deserve to be reassessed. That can be the case for example of surface markers that are supposed to be associated to stemness and that are used for cell purification (48). It is still possible that cell functioning conceals new paradigms. Alternative theories are often ignored or quickly disregarded, possibly slowing down scientific progress.

Big differences can also be found in stem cells from different species and can result in serious misconceptions, for example overestimation of the security or danger of the clinical use of a kind of cells. In the case of MSC for clinical applications, it was the MSC coming from mouse that are more prone to malignant transformation.

These examples show us the necessity to take extreme care when considering stem cell research, as misconceptions can happen very easily and can lead to dramatic outcomes.

In this work, most of the studies reviewed were on human cells.

9. Comparison between cell types for research and therapeutic use

The different criteria that can be taken in account to choose a specific kind of stem cells to use for research or therapy are gathered in table 2.

Table 2: Comparison of the advantages and disadvantages of different cell types that can be used for research and therapy (49).

Stem cell type	Advantages and possible applications	Disadvantages
Totipotent cells	- Give rise to an entire individual. Can be used for *in vitro* fertilization, for cloning or to generate transgenic animals.	- Strong ethical concern. - High reactivity needed because totipotency is very quickly lost and does not persist *in vitro*.
Embryonic Stem Cells (ESC)	- Extensive self-renewal. - Can theoretically differentiate in all tissue. - Availability with supernumerary embryos made for *in vitro* fertilization. - Can be implanted to generate transgenic animals. - With genetic modifications, can be used for basic research to understand the role of some genes or generate *in vitro* models for some diseases.	- Risk of teratoma formation when implanted, require precise cell sorting to exclude remaining stem cells that did not differentiate. - Culture conditions are constraining. - Strong ethical concern with embryos destruction. - Immune tolerance problems.
Induced Pluripotent Stem Cells (iPSC)	- Extensive self-renewal. - Can differentiate in all tissues. - Interesting for many basic research fields and can be used to generate models for research on some diseases. - Lower ethical concern than ESC. - Can be used for autologous therapy, from various types of cells easily accessible.	- Risk when implanted, especially of cancer formation. - Culture conditions are constraining. - Difficult production with low yield. Require precise cell sorting to exclude remaining pluripotent cells. - Potential alterations can remain from the cells of origin.
Germline Stem Cells (GSC)	- Can be used to preserve fertility before cancer treatment with autologous transplantation. - Could be used to treat infertility and for therapeutic cloning. - Enhanced safety compared to ESC and iPSC.	- Risk of malignant transformation. - For infertility treatment only. - Ethical issues.
Hematopoietic Stem Cells (HSC)	- Can differentiate in all blood cells. - Remain life-long in the recipient. - Can be used without expansion or precise sorting.	- *In vitro* amplification is difficult. - Compatibility needed. - Engraftment may be difficult. - Many different subsets with complex phenotypes. - For blood and immune diseases only.

Mesenchymal Stem cells (MSC)	- Good expansion capacity. - Can differentiate in mesodermal tissues. - Safety. Do not persist after implantation. - Easy to obtain and to culture. - Immune tolerance for allogenic graft. - Can be used for autologous therapy. - Interesting to make links with donor characteristics.	- Limited capacity to self-renew and differentiate, limited stemness. - Poor persistence after implantation with actual techniques. - Heterogeneity within the samples difficult to follow. - Strongly dependent to donor parameters and diseases. - Big differences in cells obtained with the same procedures.
Fetal stem and progenitor cells	- Expansion capacity is very high. - Less alterations than in aged adults. - Immune tolerance for allogenic use. - Easy access that do not require invasive techniques.	- Require long term banking for autologous use. - Limited potential.
Other adult stem cells	- Accessibility. - Possible autologous therapy. - Stem cell type choice according to the application.	- Lack of unaltered cells in case of disease. - Invasive sampling needed.
Genetically modified adult stem cells	- Ameliorated self-renewal. - Avoid some functionality decrease with culture time. - More applications made possible.	- Secondary effects linked to viral vectors.
Progenitors	- Accessibility. - Fast production of cells. - Easier culture and differentiation with cells already committed.	- Limited self-renewal and rapid exhaustion.
Differentiated cells	- Relatively easy to harvest. - Cells are directly of the desired cell type. - Interesting to make associations with donor characteristics and diseases.	- Require invasive sampling. - Very limited amplification capacity, can be difficult to have enough cells. - Fast function loss. - Immunogenicity and potential dangerous secondary effects.

The type of stem cell used greatly varies according to the application. Researchers that try to find new therapeutics are confronted to the choice between these different kinds of cells, where several types could often be possible to use.

Adult stem cells are often preferred to ESC or iPSC because they can be obtained more quickly, with culture methods that require less interventions. ESC and iPSC are in the end just another mean to obtain cells. If the cells of interest can be obtained by other means, it is not necessary to use these tools. IPSC discovery do not solve all the problems, it is still necessary to determine what kind of cells are to be obtained according to the desired application. They are also confronted to the same challenges as other stem cell therapies with the difficulties for the cells to engraft and take the role of mature cells in a new altered environment. Even for recent iPSC clinical trials, it is still unknown whether iPSC integrate in the tissue or if they just help existing cells. Furthermore, as it takes also a lot of time to generate iPSC and derive differentiated cells, allogenic strategies are currently preferred with the resort of cell banks, despite the immune compatibility problems. It is also related to cost reduction for the firms to have a viable economical model. These difficulties reduce iPSC advantages in practice.

Especially for their safety of use and easy access, with the possibility to amplify them relatively quickly, with limited ethical issues, MSC are the cells that have been used for the highest number of clinical trials. However, failures have been prevalent for MSC therapies in advanced clinical trials whereas there is a large amount of encouraging preclinical results. MSC research is necessary to know more about these cells, their role, their possible alterations, their potential and functions. It is now possible to reap the benefits of twenty years of hindsight on MSC use and to switch to a new version, 2.0 MSC, with enhanced technical implementation, to enable clinical efficiency. This can even benefit many other stem cell clinical applications, including iPSC's, then.

II. MSC concept: From Mesenchymal Stem Cells to Medicinal Signaling Cells

This work will now focus on the cells that were first called mesenchymal stem cells, found in the bone marrow. Similar kind of cells were then identified and called the same way. They all are now widely investigated and considered as promising candidates for stem cell therapy and tissue engineering.

A. MSC initial definitions

The first experiments that could reveal MSC existence were made by Friedenstein in the early 1960's. Bone marrow transplants in mice were shown to give rise to bone and fibrous tissue. Then, bone marrow cells were cultured *in vitro* and it was shown that it contained plastic adherent cells, with fibroblastic shape, that developed in colonies. They were called "colony-forming unit fibroblast" (CFU-F). Further experiments confirmed these results and showed, with heterotopic transplants, that these cells could form miniature bone structures that could be colonized by the host HSC. It was an important step that brought to light the existence of distinct stem cells in the bone marrow. At this time, the cells were called osteogenic stem cells or bone marrow stromal stem cells (50).

The term Mesenchymal Stem Cell was then proposed by Caplan in an article in 1991 (51). This reference explains the existence of plastic adherent cells, that can be obtained and cultured from bone marrow, with extended proliferation capacities. The described cells were also shown to be able to differentiate, with the adequate environment, toward chondrocytes and osteoblasts. They were also thought as theoretically able to generate other lineages like connective tissue cells, adipocytes, ligament cells, dermis cells, and muscle cells. Preliminary animal experiments could also show their potential to migrate to reach damaged tissue and to differentiate *in vivo*. They were thought as stem cells necessary to support tissue renewal. The term mesenchymal is explained to be derived from the Greek "meso" meaning middle and infusion. It initially was used to describe mesenchymal embryonic cells that migrate during early development between ectoderm and endoderm layers. The key characteristics are the migratory and space filling abilities of the

mesenchymal cells, that were associated with wound repair in adult tissues. Adult mesenchymal stem cells potential for therapy was already discussed at this time. In a recent review made by Caplan on MSC, he explains the term mesenchyme refers to cells loosely associated that lack polarity, surrounded by large extracellular matrix. He also points out that he called them "stem cells" provocatively, thanks to their *in vitro* multipotency and clonogenicity, to agitate the then existing dogmas that the only adult stem cells were HSC. Following reports could then confirm by serial transplantations that MSC can support some stem cell definitions (52).

After this, the first studies about MSC suffered from the lack of precision regarding the type of cell that was really studied. Minimal criteria with common surface phenotype and basic properties were defined by the International Society for Cellular therapy (ISCT) to set up things (53). According to this definition, **MSC must adhere to plastic, differentiate in osteoblasts, chondroblasts and adipocytes**. They must express CD105, CD73 and CD90 surface molecules. MSC must not express hematopoietic phenotype with CD45, CD34, CD14 or CD11b and CD79α or CD19. They must also lack HLA-DR. The descriptions of these markers can be found in table 3. It can be interesting to note that the ability of the cells to generate colonies at single cell level were not included in these criteria while it is a standard assay for stem cells to have a kind of evidence about their self-renewal potential. The minimal criteria proposed show impressive constancy in very diverse cases. They cannot be associated to MSC functionality and cannot be used to reflect the underlying cell variability.

Table 3: Description of proteins used to define MSC phenotype.
CD44 and CD166 are additional surface markers commonly assessed to confirm MSC phenotype but they are not included in the ISCT minimal criteria.

Surface markers strongly expressed on MSC	
CD73 (5'NT or NT5E)	Enzyme that converts adenosine monophosphate (AMP) in adenosine.
CD90 (Thy1)	Glycosylated surface protein implicated in many functions like apoptosis, fibrosis, adhesion, tumor suppression, …
CD105 (Endoglin)	TGF-β receptor part implicated in angiogenesis and BMP signaling.
CD166 (ALCAM)	Transmembrane glycoprotein found in many tissues and more often in those involved in growth and migration.
CD44 (HCAM)	Glycoprotein involved in cell-cell and cell-matrix interaction, in adhesion, migration and homing toward bone marrow.
Surface markers absent on MSC	
CD34	Glycoprotein that characterizes hematopoietic progenitor and has a role in adhesion between cells and to the matrix in the bone marrow.
CD45 (PTPRC)	Hematopoietic marker implicated in cell signaling (protein tyrosine phosphatase).
HLA-DR	Molecule of the Major Histocompatibility Complex (MHC) class II that is present on dendritic cells, B and T lymphocytes, monocytes, macrophages. It is involved in exogenous peptides presentation.
CD14	Component of the innate immune system. Acts as a co-receptor for the detection of bacterial lipopolysaccharide. Expressed by macrophages, neutrophils and dendritic cells.
CD11b (ITGAM)	Integrin expressed on the surface of innate immune system cells.
CD79α	Component of the B-cell antigen receptor.
CD19	Transmembrane protein expressed in B lineage cells.

Even with these attempts to standardize and clarify MSC concept, the respect of ISCT criteria is not sufficient to obtain the definition of a homogeneous cell type. The surface markers described here are not specific to MSC and cannot be used for MSC detection or prospective isolation like the surface phenotype that was described for HSC.

MSC definition remains delicate and subject to controversy as their understanding grow and go with new questions. The existence of cells that could really be called stem in MSC samples was questioned. Different names take turns to define the cells more specifically and to reduce misconceptions. More details about MSC tissue sources and their putative physiological roles are necessary to go further.

B. MSC physiological role

Adult MSC were first isolated from bone marrow. Then, cells isolated from other tissues with similar characteristics were also denominated MSC. The tissues concerned in adult organisms are mainly adipose tissues but many others are found to contain similar cells, for example dental pulp, endometrium, and synovial membrane. MSC similar cells could also be extracted from fetal tissues like Wharton jelly from umbilical cord and placenta. Even if they share the same name and some general characteristics, as ISCT criteria, the cells obtained from different tissues also display different functional properties and cannot be considered as equivalent.

The literature on MSC is vast. To select the most relevant information for the thesis work, the following parts will focus on Bone Marrow (BM) and Wharton Jelly (WJ) MSC, even if other kinds of MSC can sometimes be mentioned.

1. MSC niches

Research about *in vivo* MSC is still very incomplete, especially for human MSC. Some studies could still provide interesting insights with tissue section and with the mouse model.

MSC residing in the bone marrow are estimated to represent between 1 in 10 000 in newborns and 1 in 1 million marrow cells in more aged donors (52). It is more than ten times less than HSC (54). The estimated number of bone marrow cells in a human is considered as representing 2.5% of the total number of human cells (2.5×10^{13}). In the approximated 6.25×10^{11} marrow cells, that would make between 625 thousand and 6.25 million MSC residing in adult bone marrow. *In vivo*, these cells allow for the hematopoietic

niche establishment and may be precursor of stromal, bone, cartilage, fat and perivascular cells. To give some orders of magnitude of the renewal rhythms, active osteoblasts lifespan is around 3 months and adipocytes lifespan is around ten years (55).

Different types of MSC could be identified *in situ* in bone marrow thanks to markers such as CD271, Nestin and CD146, that can be varyingly expressed, associated to the lack of hematopoietic and endothelial markers (CD31, CD34, CD45, CD144). These MSC were shown to express CXCL12, angiopoietin-1 and other important factors implicated in hematopoietic progenitor and HSC regulation. In addition to the secretion of growth factors, MSC can secrete extra cellular matrix proteins. HSC were also found to home toward MSC in the bone marrow and to co-localize with them. It turned out that HSC also need cell-cell contact and that their function is impaired without MSC. Several findings come together to suggest MSC have a direct role for hematopoietic support (27,50,56). Other studies made in the mouse have pointed out the role of different subtypes of stromal cells that can induce different HSC functions. These different cells seem to collaborate to provide the necessary cytokine signaling to HSC and progenitors (50). Another interesting study with transgenic mice determined the adult mesenchymal cells of dental pulp, thymus, and bone marrow developmental origins. The cells that were able to generate secondary colonies, estimated as being stem cells, were coming exclusively from the neural crest for dental pulp, entirely from the mesoderm for thymus, and mostly from the neural crest for bone marrow. The cells from these different origins shared similar properties, for example, they relied on Platelet Derived Growth Factor-β to grow colonies *in vitro*, and the dental cell expressed important factors for hematopoiesis (57).

Evidences in human bone marrow demonstrate the localization of MSC in **perivascular regions** (20,58). Perivascular MSC express adhesion molecules mediating interaction with endothelial cells like CD146, and surface receptors implicated in perivascular cells regulation, like CD140b and CD105. MSC are also able to stimulate vessels growth and stabilize new functional blood vessels *in vivo* and *in vitro* (20,59). Some studies complemented this view with the highlighting of another MSC subtype that localize in **endosteal regions**, near bone cells, of the bone marrow. These cells had the specificity to

have a low expression of CD146. They were also found to colocalize and to be associated with HSC, demonstrating the association of distinct BM MSC subsets with different potential HSC niche in the human system. These bone lining cells could display different physiological properties and also answer the important cell replacement demand of the adult skeleton (50,60) (see Figure 10 in the part "Stem cells fate determinants – Example of bone marrow" for the role of different kinds of MSC in the bone marrow niche).

Different kinds of MSC could also be present in the **perivascular region** of other tissues than the bone marrow. That could explain why MSC similar cells can be found in such a wide variety of vascularized tissues. Adipose progenitors in fat tissue for example were shown to reside in the perivasculature (61). From initial researches with adipose tissue, a study suggested the existence of an additional type of perivascular MSC, lacking CD146 expression but expressing CD34. These cells were isolated from adipose tissue by FACS and described as also lacking endothelial markers. They could give rise to MSC cultures that follow ISCT criteria, except concerning CD34 expression. They were found to localize in the tunica adventitia, the outer layer of larger blood vessels. *In situ*, in tissue sections of adipose tissue vessels, they are the only cells, besides similar microvascular perivascular cells as described above, that were found to co-express MSC markers such as CD73, CD90, CD105 and CD44. CD34 expression for MSC-like cells is mostly associated with adipose tissue, even if similar observations could be made in human fetal lung, fetal and adult pancreas, and adult muscle. It seems that this MSC population was not confirmed in adult bone marrow (62). CD34 is also expressed by endothelial cells, fibroblasts and hematopoietic precursors. Verification of its negativity for BM MSC characterization was proposed to verify that the sample was not contaminated with other cell types. An intriguing study tried to select cells from the bone marrow according to their CD34 expression. The majority of MSC still derived from CD34 negative cells but they could show that inside the sorted cells expressing CD34, a small part of the cells could give rise to MSC with classical phenotype, and it was not due to contamination by CD34 negative cells. This could be associated to the existence of these different populations of MSC in adult BM. The expression of CD34 was then lost after plastic adhesion (63).

Progenitors of skeletal muscle and dentin have also been identified as perivascular cells. Different tissues examination could lead to the idea that each tissue specific stem cells are next to their specific perivascular MSC (52). MSC of different tissue are lineage committed, they retain specific transcriptional signatures. In heterotopic transplant experiments, MSC from outside the bone marrow do not develop bone supportive for hematopoiesis. They develop preferentially, in the absence of other cues and material, the types of cells found in their tissue of origin. That would support that MSC share common feature but also display specific functional properties according to their originating environment (64).

Another kind of MSC could be found in umbilical cords, more precisely in the Wharton jelly. It is a connective tissue derived from extra embryonic mesoblast. It protects the blood vessel of the umbilical cord while providing an important flexibility. The matrix is principally composed of mesenchymal and myofibroblasts cells, collagen and hyaluronic acid (figure 14). MSC have been derived from all regions, from the perivascular zones to the subamnion.

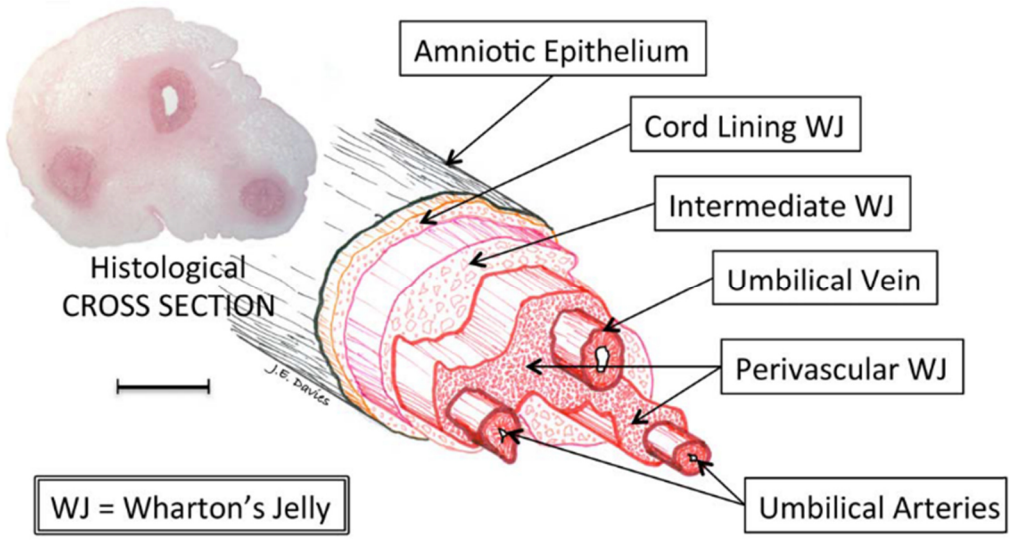


Figure 14: Structure of the human umbilical cord with a three-dimensional exploded diagram.
Scale Bar: 5 mm (86).

2. MSC nature controversy

MSC research is subject to many debates concerning their origins, their self-renewal and differentiation potential, whether this group of cells contains stem cells or not. As we could see in previous part of this work, stemness concept is already subject to debate, and the MSC concept inherits the same type of questioning.

Few articles give the necessary elements to speak about **MSC stemness**. To make a parallel with HSC, their stemness is shown with graft experiments. After elimination of the hematopoietic cells of the mouse by intense conditioning, HSC graft can allow the mouse to survive and maintain hematopoiesis during the remaining normal lifetime. For bone marrow MSC, the exact same type of experiment cannot be done but a variation of grafting experiment was proposed. It consists in serial heterotopic transplantation of murine or human bone marrow MSC in animals (20,56). It was shown the cells could persist and keep their ability to form CFU when retrieved after implantation. The BM MSC transplanted could also produce miniature bone structure, that could host vessels and hematopoietic cells from the recipient. The transplanted MSC were shown to contain **multipotent self-renewing** cells that could differentiate in mesodermal tissue like adipose tissue, bone, cartilage and fibrous tissue (50). The heterotopic implantation aims to reveal the inherent cell abilities to form these structures **independently of the direct cues** that would be applied in the bone. These studies give robust enough evidences for the presence of "true" stem cells in bone marrow MSC. That could lead to the definition of a mesenchymal stem cells as "**the single cells capable of generating a complete heterotopic bone marrow organ (an "ossicle")** ***in vivo*****, including a compartment of perivascular stromal cells with similar phenotype and properties as the originally explanted cell**". The discoveries of these mechanisms were progressively refined. At first, it was with uncultured whole bone marrow that in vivo skeletogenic potential was described. Then, the causing cells were found to be non-hematopoietic cells. It was then refined to a subset of these cells with *in vitro* potential to form colonies. We are now at the step of *in vivo* identified cells with phenotypes associated to a large set of markers (61,65).

In other tissues than bone marrow, studies investigating MSC **stemness** are not as complete. MSC-like cells derived from adipose tissues and selected by FACS either with Stro-1, 3G5 or CD146 surface markers could form bone after heterotopic transplantation in immunodeficient mice, but they could not host local hematopoiesis (66). The aforementioned adventitial MSC expressing CD34 in adipose tissue were tested only *in vitro* but with single cell derived colonies that could demonstrate the *in vitro* trilineage potential to differentiate of these cells (62). For MSC derived from the umbilical cord, experiment undertaken at the clonal level from single cells could demonstrate *in vitro* multipotency and colony forming ability. They were also injected into the femoral marrow cavity of immunodeficient mice and could produce different tissues including bone, cartilage and fibroblasts (67). The MSC with these capacities were obtained from the perivascular zone of the umbilical cord, which is also where the most MSC can be derived.

The extent of **differentiation** potential of different MSC types has been widely investigated *in vitro* and *in vivo* to test their ability to form all kinds of tissues, including non-mesodermal ones, such as neurons or hepatocytes. It was even shown that MSC could dedifferentiate and transdifferentiate between different lineages (68). MSC were even thought to be pluripotent cells in some studies. These results remain inconsistent and MSC phenotype seem mostly to answer to the strong differentiation cues applied exogenously, but in fact they may not be able to give rise to mature cells of these specific lineages. The strong stimulations inflicted to MSC in some studies are considered by some as reprogramming. MSC can be reprogrammed to pluripotency, but natively they are not (64). This is an important debate, associated to many translational purposes, that needs to be addressed with more robust studies (61).

MSC are described by several studies as similar or equal to **pericytes** and **adventitial reticular cells**, or even derived from them. A pericyte common definition is a cell wrapped around endothelial cells in small blood vessels. They are known to be able to regulate blood flow and to communicate directly and stabilize endothelial cells. They are also implicated in the phagocytosis of cellular debris and in vascular permeability. MSC show similar properties with pericytes regarding classical *in vitro* tests (20,59). However, even if MSC

can display strong similarities with pericytes regarding *in vivo* localization, surface phenotype and *in vitro* qualitative assays, they lack the contractility of pericytes and have a different gene expression program (69,70). BM MSC combine expression of genes usually associated to early osteogenic cells (the master gene Runx2 for example) with genes characteristic of pericytes (20,64).

Some studies also asked whether MSC were really different from fibroblasts, as fibroblast cultures were shown to have similar immunomodulation properties *in vitro*. However, it seems fibroblasts culture behave differently. For example, their expansion is favored by high plating density that would make the culture more resistant to oxidative stress, while BM MSC growth is at best at lower densities and is subject to inhibition when the culture becomes too dense (71). Inconsistent data are also reported for fibroblast differentiation. In the end, it seems fibroblasts are more differentiated cells (59). It is however possible that some MSC cultures contain differentiated fibroblasts (50).

To close this list of controversies, some authors also question the adherent nature of MSC. They suggest that a kind of MSC able to form CFU upon serial plating also resides in the nonadherent fraction of bone marrow mononuclear cells. These cells remain to be further studied (72).

Following the debates around MSC, the nomenclature used for these cells has undergone many changes. The ISCT advised in 2005 to use the name “Mesenchymal Stromal Cells”. This nomenclature was proposed to avoid the term “stem” when it is not sure if the cell samples studied contain “true” stem cells. The term stromal is an old term relating to connective tissue. It can be associated to the fibroblastic aspect and osteogenic potential of MSC, but the term could miss an important part of MSC abilities. In a similar manner, some authors underline that the terms stem and stromal cannot be used interchangeably as they currently are, especially for MSC derived from other tissues than bone marrow. For the latter, they prefer the term of “Skeletal Stem Cells” (64). Another common way to avoid the term “stem” is the name “Mesenchymal Progenitors Cells”.

Despite the efforts to define the MSC concept, the associated nomenclature is still often used outside of what was initially defined by already numerous proposals and remains confusing. The different nomenclatures existing make reviews especially difficult in the crowded literature about MSC.

MSC research field is subject to a strong clash of concepts, each team with its specific view on what MSC are and are not, and what they can or can't do. Some teams develop very polarized opinions, with the risk to overlook evidences. However, it can be viewed as a normal step when facing such fascinating and multifaceted cells, that can at the same time produce cells and regulate tissue organization (61).

Before going further in the description of their therapeutic potential, the following part will first describe the general methods to obtain MSC from different tissues.

C. MSC usage and obtention

1. Context of MSC use

MSC can be summarized as multipotent stem cells, able to proliferate in culture and with the capacity to differentiate toward at least three mesodermal lineages: adipocytes, chondrocytes and osteocytes. They can be isolated *in vitro* by plastic adhesion in the adapted culture medium. As they can be easily accessible, MSC represent an interesting source to obtain a big number of cells. They became an interesting model to study the effect of donor conditions like donor age and pathophysiology. They can also be used to study the process of differentiation and cell mechanistic and behavior in response to various stimulations. MSC are also widely investigated for their potential therapeutic activities.

2. Example of MSC obtention from bone marrow

MSC can be obtained from diverse tissues with different techniques.

Figure 15 shows the example of MSC obtention from bone marrow. The cells can be plated directly with the whole marrow suspension. They can also be centrifuged and washed or separated with gradient density. However, it seems the direct method with whole BM can result in the formation of more CFU-F and better cellular growth compared

to cells obtained after gradient separation (73). These gradient procedures did not show global improvement compared to direct plating, but they can limit the presence of contaminating cells during the first days of culture, if important for the application expected from MSC. More specifically, some authors warn that monocytic progenitors (producing monocytes and macrophages) could remain for 2 to 4 weeks in culture (74).

When the tissue source is thick, mechanical and/or enzymatic splitting of the cells can be made. For WJ MSC, the use of enzymes allows to obtain a large amount of cells but

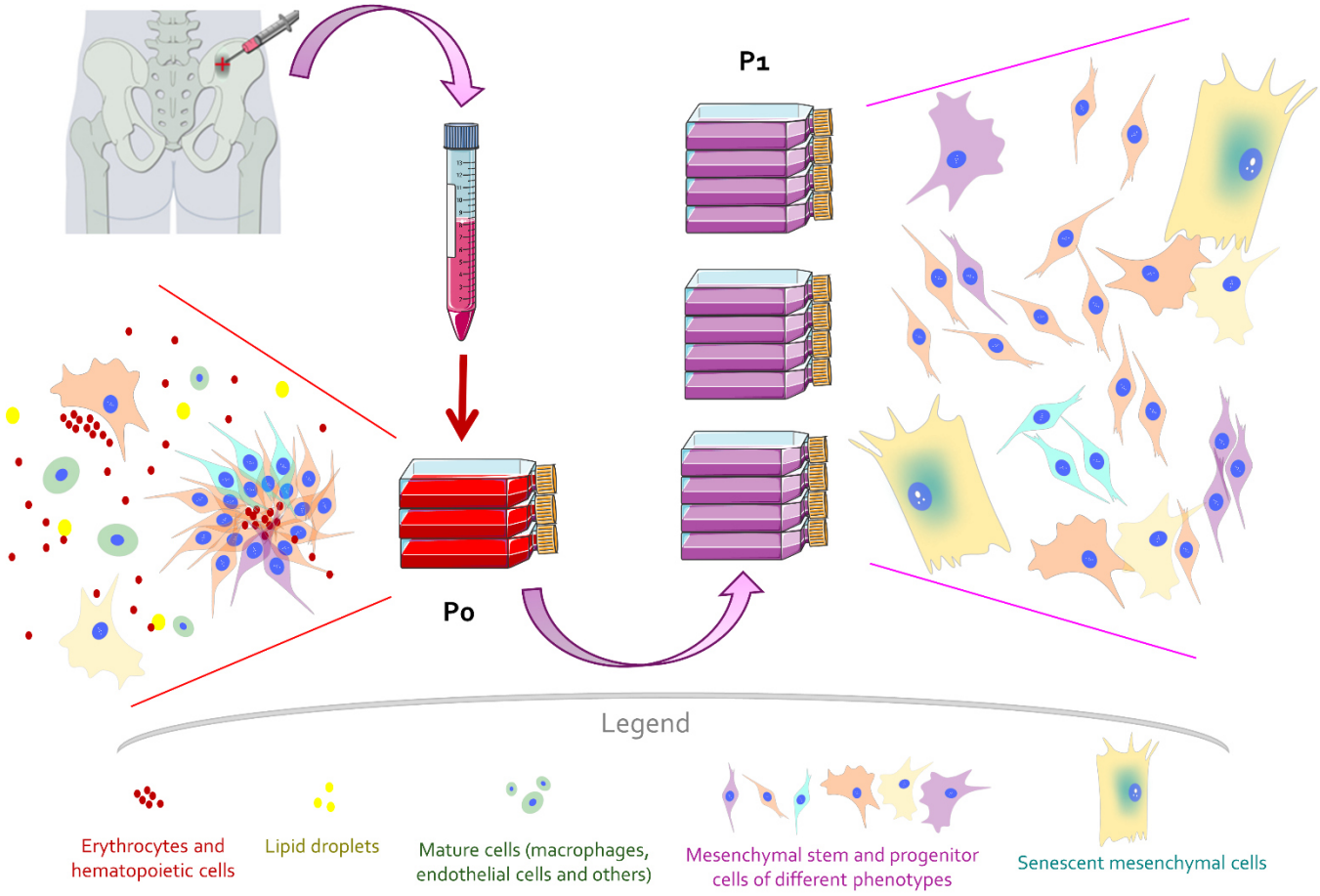


Figure 15: Bone marrow MSC obtention.

Bone marrow aspirates can be directly plated in plastic culture flasks. At this moment, the number of MSC is not known, only the number of mononuclear cells can be estimated and is used to determine the initial plating density, at passage 0 (P0). At this passage, other cells contained in the bone marrow can also be present. They are progressively removed with medium renewal and with their limited survival, while MSC adhere and progressively form dense colonies. Different MSC subtypes are present from the start, and culture conditions can change their proportion and cause the increase of senescent cells. In a general manner, passage 0 is the longest passage. It can be necessary to wait 3 weeks to obtain visible colonies and even more from thicker tissues like Wharton jelly. (They are not represented, but senescent cells can also be present initially at P0).

has the disadvantage to alter surface markers expression and to decrease MSC proliferation (75).

For almost all MSC uses, an *in vitro* step is necessary to isolate MSC and obtain enough cells. It is however possible to use cell separation and gather a cell suspension enriched in MSC that can be used without cultivation.

3. Cell separation techniques

The moment a cell suspension can be collected, it is possible to make a prospective isolation step to obtain a sample enriched in MSC and depleted in contaminating cells. Cell sorting techniques include immunomagnetic separation **(figure 16)** (76) or flow cytometry assisted cell sorting (FACS) **(figure 17)** (77).

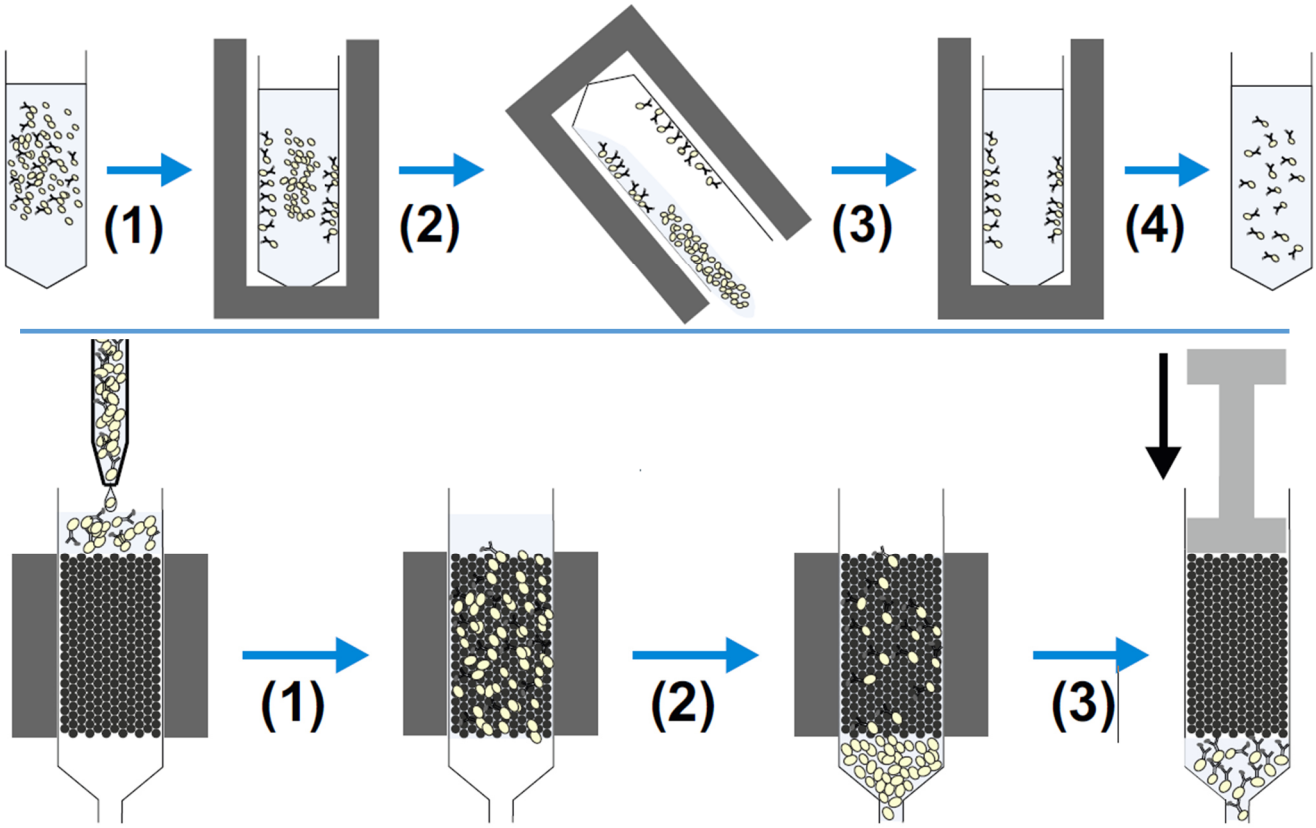


Figure 16: Diagrams showing the common methods used for magnetic cell separation.

In the upper part:

- Step 1: A magnetically labelled cell suspension held in a conical tube is placed in a magnet causing the cells to stick to the sides of the tube towards the magnet.
- Step 2: The tube is then inverted (or aspirated), allowing removal of the non-labelled cells.
- Step 3: The labelled cells are resuspended.
- Step 4: The tube is removed from the magnet to obtain dispersed suspension of labelled target cells.

In the bottom:

- Step 1: A magnetically labelled cell suspension is injected into a column held within a magnet.
- Step 2: Cells then flow through the column and labelled cells are retained, whereas unlabeled cells are washed out.
- Step 3: Following the removal of unlabeled cells, the column is removed from the magnet, and suspension buffer is forced through the column by plunger giving labelled target cells in suspension. (76)

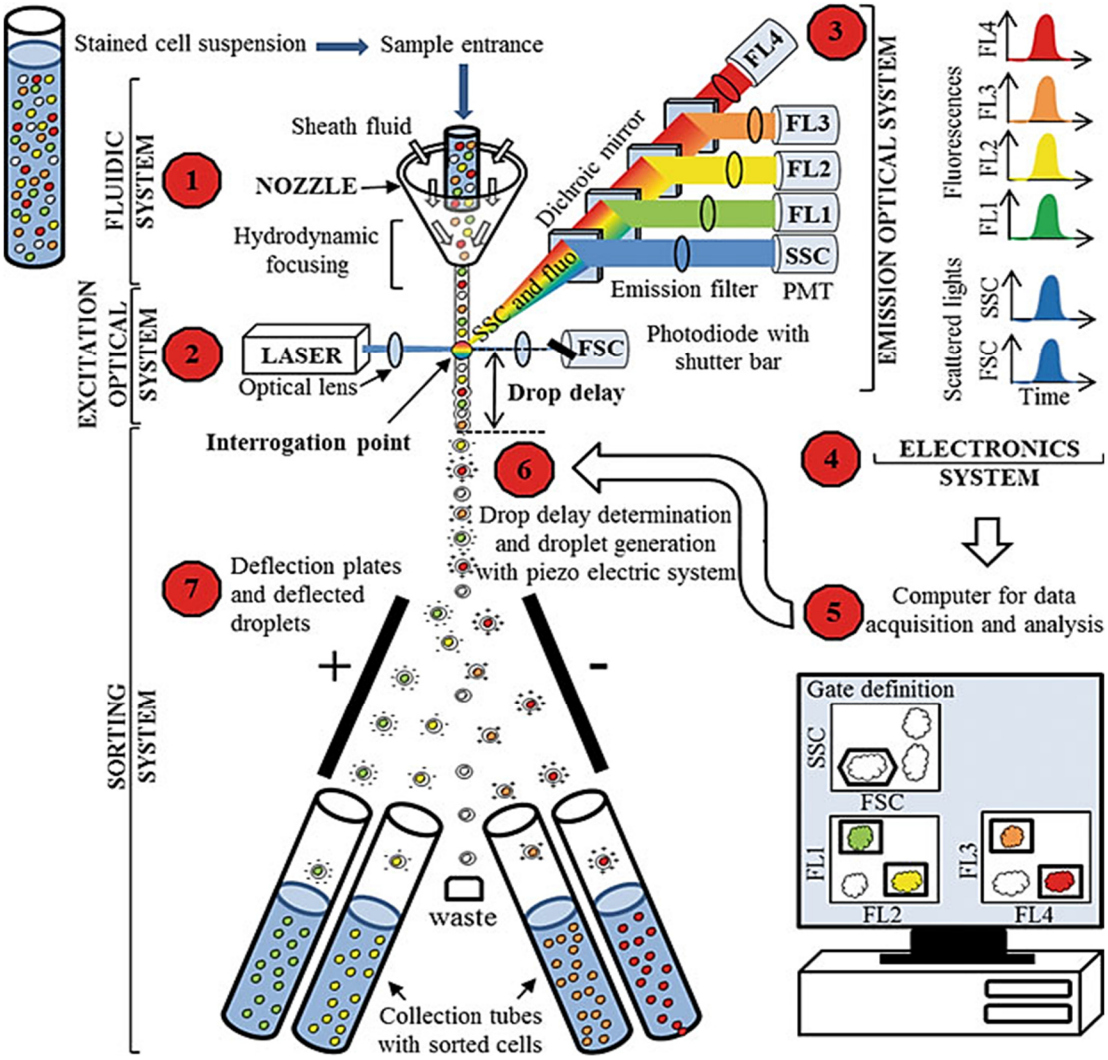


Figure 17: Cell separation by flow cytometry assisted cell sorting (FACS).

Cells are channeled in a continuous stream of individual cells in a sheath fluid. These cells then pass through a light source or laser, and the signature of each cell is detected.

From this detection, the cells will be determined to be above or below a designated threshold value to decide in which tube cells must fall. This is achieved by electrically charging the droplets containing the cells and by passing it through charged deflector plates that deflect the droplets to the appropriate collection tubes.

Fluorescent antibodies can be used to stain and separate living cells according to their surface markers expression. (77)

4. Classical MSC culture

To let MSC grow, they must be placed in incubators and can be exposed to different kinds of physicochemical environments. Once plastic adherent cells are gathered, different kinds of cultures can be made. MSC can be cultured in classical monolayers in relatively dense manner (more than 1000 cells/cm²). They can also be seeded at very low density to obtain clonal conditions where each colony can be harvested independently from the others. MSC are also often cultivated on modified surfaces, in biomaterials or in bioreactors. These alternatives aim to combine the production of numerous cells and conservation of their initial properties of interest.

Some guidelines are proposed and are supposed to be the best compromise between feasibility, the cell quantity and quality that can be obtained (78). MSC culture has the particularity to be more efficient with low densities and poor culture medium. It is recommended that the cells are at the lowest density possible considering the cost, facility, and labor (73). The best culture conditions remain to be determined and depend on the application aimed and the environment cells will have to face.

Fetal Bovine Serum (FBS) batch selection was an important step to allow the generalization of easy MSC culture. Now, to avoid animal products for clinical purposes, human Platelet Lysate (hPL) use is growing. These preparations provide the necessary growth factors for MSC amplification, but they have the disadvantage to be variable in their chemical composition between batches. All the attempts to use medium without these extracts and with precisely defined content gave unsatisfactory results (79).

The culture step can induce some changes in MSC. The challenge is to find the way to obtain enough cells that maintained their functional properties, limiting deviation, deterioration of the cells. To limit these problems, some studies try to use cell sorting to obtain cells enriched in MSC directly, without growing them in culture, but then it is the purity and the number of cells that are limited. MSC expansion in bioreactor or in biomaterial, that could best imitate a physiological environment, may be an interesting alternative currently under study. For example, a study showed that MSC cultivated in a 3D perfusion system better maintained their progenitor properties and displayed reduced inter-donor variability compared to 2D expanded MSC (80).

Whatever the method for MSC obtention, all result in heterogeneous populations of cells, a mix of different committed progenitors and, potentially, some rare stem cells (figure 15 p88). This heterogeneity can be considerable or more restricted with more refined methods associating cell sorting, selective adhesion, and specific culture conditions. In all the cases, the true multipotent and self-renewing stem cells remain certainly a minority of cells in the sample, but they could yield a unique potential that the other progenitors and differentiated cells do not dispose.

Before further exploration inside MSC heterogeneity, the following part will deal with the way they were used in therapeutic application and what it permitted to learn about MSC functioning.

III. Cell therapies and tissue engineering with MSC

A. Overview

Based on MSC properties, new therapeutic strategies were developed. Thanks to their multiple abilities, they were investigated for both the treatment of acute injuries and degenerative disorders, generally associated with inflammation **(figure 18)**.

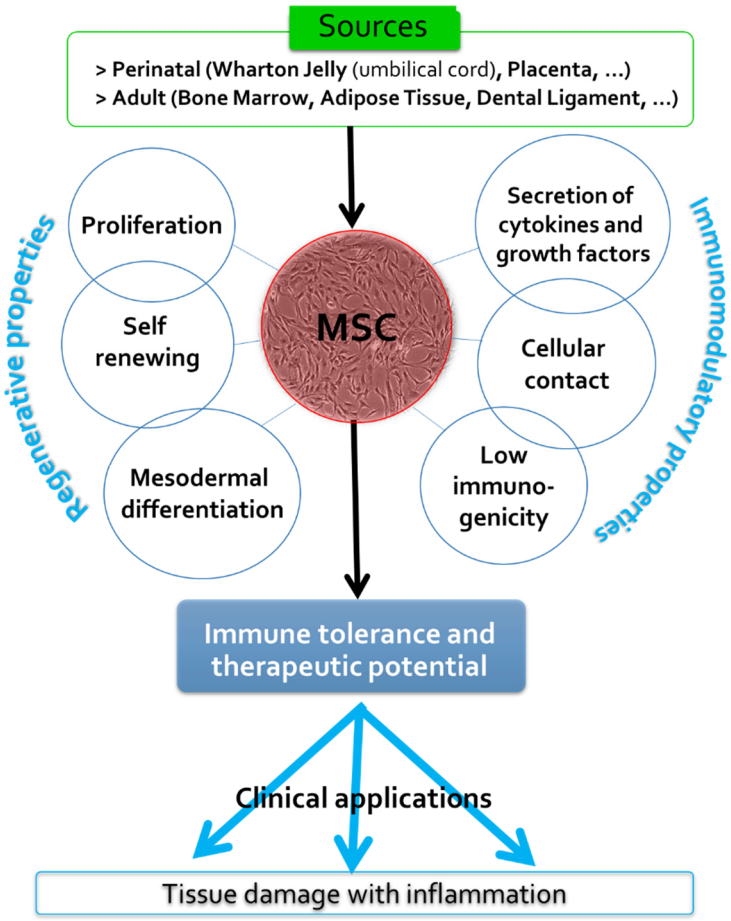


Figure 18: Diagram with MSC commonly reported key properties.

One of their major property, that opened many possible clinical uses, is their ability to be tolerated between different donors. Initial findings showed human MSC could persist in a xenogeneic environment, in sheep (50).

MSC were used in more than 500 clinical trials in different forms (infusion or transplantation in a biomaterial) for applications as vast as bone and cartilage disease, neurological disease, cardiovascular disease, graft versus host disease, liver disease, diabetes, hematological disease, lung disease, kidney disease, Crohn's disease and many others **(figure 19)**.

For ATMP and clinical trials, MSC are mostly obtained from bone marrow, then there is adipose tissue and also umbilical cord and placenta. Other sources remain rare.

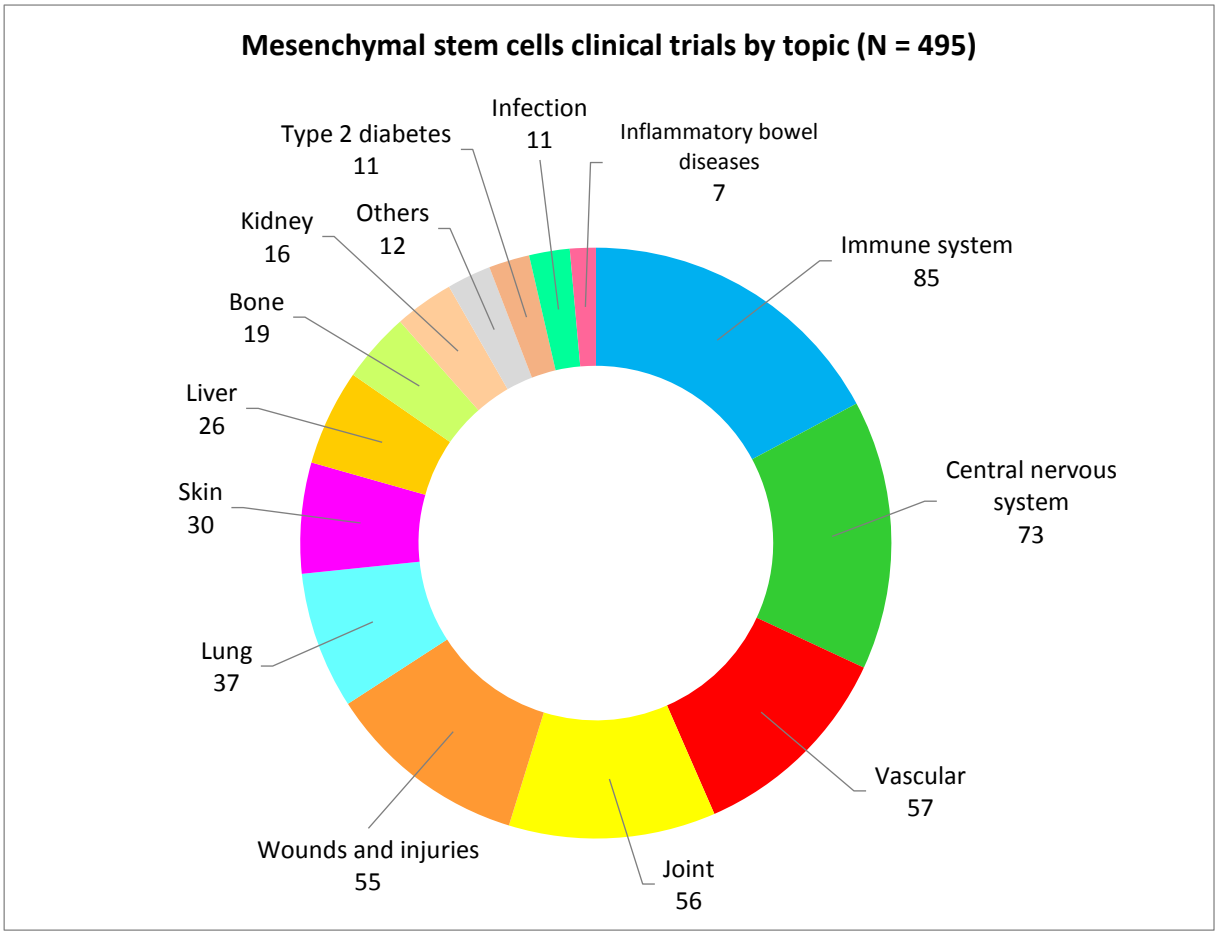


Figure 19: Repartition of the application areas of MSC therapies among clinical trials.
From clinicaltrials.gov (consulted the 09/06/2019) with keyword in the intervention field: "Biological: mesenchymal stem cells".
Immune system diseases include GvHD, bone marrow failure, multiple sclerosis, lupus erythematosus, type 1 diabetes mellitus, arthritis and others. Vascular diseases include ischemia, infarction and stroke notably.
Central nervous system diseases include spinal cord disease, Alzheimer's disease, cerebrovascular disorders, cerebral palsy, amyotrophic lateral sclerosis, and others.

Studies so far showed that MSC administration is well tolerated and some beneficial effect have been observed even if most of the cells seem to be quickly eliminated (81). By their punctual presence, they can communicate *via* cell contact, paracrine and endocrine action to modulate immune system, to promote wound healing and angiogenesis. However the results vary widely, issues in cause may be linked to donor heterogeneity, *ex vivo* expansion, immunogenicity and cryopreservation (82,83). After the early phase clinical trials, precious information could be gathered to precise in which case and how MSC could work at best. Some clinical studies could then come to fruition. As it was described in the previous part about stem cells, MSC are the majority of stem cell products with marketing authorization worldwide.

When looking at all the clinical trials undergoing for very different indications, it could seem that MSC are the new cure-all therapy. More details about the approaches and possible mechanisms underlying this profusion of studies are given in the following parts.

B. MSC for tissue repair by repopulation and differentiation

MSC were initially used with the aim to obtain long term engraftment and to replace damaged tissue thanks to their capacity to produce differentiated cells. However, in almost every clinical trial until now, when benefits could be observed after MSC transplantation, it could not be shown that it was associated with their differentiation and repopulation potential.

1. MSC homing

For the different therapeutic strategies that can be envisaged, as long as entire cells are used, and especially when they are infused, MSC homing abilities are important to consider. It is supposed that MSC display migration capacities to guide them toward inflamed sites, allowing them to exert their therapeutic action **(figure 20)** (84).

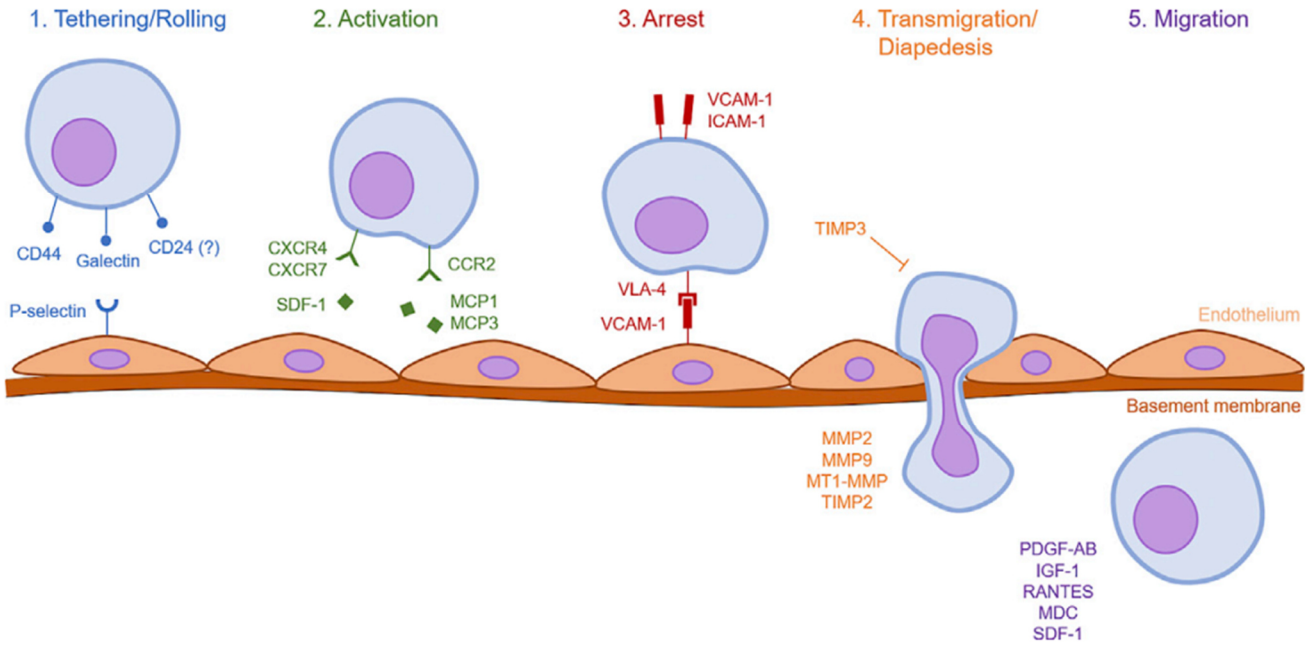


Figure 20: MSC homing mechanisms.

Five steps can be described for MSC homing from the circulation:

- **Tethering**: MSC interact with selectins expressed by endothelial cells *via* different surface receptors;
- **Activation**: inflammatory signals generally mediate this step. SDF1 is secreted by endothelial cells and binds to CXCR4 and/or other receptors that could be expressed by MSC like CXCR7. Secreted MCP could also bind to receptors such as CCR2. This step lead to conformation change and enhanced affinity of integrins.
- **Arrest**: Activated integrins on MSC surface bind to adhesion molecule on endothelial surface, for example VLA-4 (integrin α4β1), bind to VCAM1 (CD106).
- **Transmigration or diapedesis**: MSC go through endothelial layer and basement membrane. This is accompanied by the secretion of matrix remodeler enzymes such as MMP, that can also be induced by inflammatory cytokines that can signal damaged tissues.
- **Migration**: MSC migrate toward various chemotactic signals (PDGFAB, IGF1 notably). (84)

After amplification in culture, MSC display a large size ranging from 16 µm to 53 µm approximatively. The majority of the reports show most MSC are rapidly cleared after intravenous infusion or die after implantation in the pathological site (81). An extensive study even showed that 99% of MSC injected in mice were cleared within 5 minutes. The remaining cells did not persist more than 3 days, but some of them could home to an injured site in the heart during this time (85).

Confusion can often arise from the blur around the mechanisms implicated in MSC therapeutic effect. When a tissue shows a kind of repair, it is often not known whether MSC are inside or not, it can remain obscure and let the idea that the cells are directly responsible

of this outcome, while the studies that tried to follow MSC therapeutic activity could not confirm their presence.

Some authors insist on the fact that MSC do not actually express the necessary molecules involved in migration and homing, at least not the same as other circulating cells. The argument is that MSC are not migrating cells physiologically. They stress the poor engraftment results of MSC given by systemic administration. The most common observation after MSC infusion is they accumulate in the lungs, and can produce an embolism (81). Complement activation may kill most of the cells, and all is cleared within three days. They also note that MSC are very rarely found in peripheral blood, with 1 CFU-F obtained for 10 blood donors (64).

Other authors emphasize that migration of perivascular cells is documented and that they participate in wound healing. They also point out that, even if it is at low frequency, MSC can be found in the circulation. A study reported for example the obtention of 0.5 to 1 million adherent cells from 2 mL of human peripheral blood after two weeks. In vivo, such migration may be enhanced by tissue injury (86).

The possibility for MSC to undertake effective homing and engraftment could also be dependent of the immune context. It seems these mechanisms can be effective only in autologous cases. Several studies with animal models could show that MSC home toward irradiated or injured sites. An irradiated primate model study with autologous GFP-labeled BM MSC associated to HSC transplantation could report MSC persistence after 82 days in several places such as bone marrow, muscle, gut, skin, muscle, and with no cells in the lungs (81). Another study using autologous BM MSC labelled with internalizing quantum dots injected in rabbits with osteonecrosis could show cell implantation and tissue repair with the generation of new osteoblasts by the injected cells. The labels could be detected 24 weeks after treatment (87).

An interesting possibility was also shown with a study on 4 patients affected by advanced cirrhosis that were treated with MSC labelled with a radiotracer. An

accumulation in the lungs was also reported at the beginning, but a shift toward the liver and spleen occurred from the following hours to 10 days after intravenous infusion (81).

2. MSC differentiation

Even *in vitro*, the ability of MSC to differentiate in other lineages than those of the trilineage potential that define them remain unclear. In these conditions, there are still too few evidences to support MSC can be effective to replace missing cells in damaged tissues.

The attempts to use MSC to generate differentiated cells like hepatocytes remain also incomplete. Only a small part of the stimulated MSC undergo partial differentiation. The cells continue to express markers that should be extinguished like CD90, vimentin and fibronectin and they fail to acquire the functionality of hepatocytes (88).

Despite these hurdles, long term engraftment could be obtained in rare studies and with specific kind of cells, for example with immortalized murine MSC (89). This could be due to the fact that the cells are very different from human "natural" MSC. The authors emphasize that it could also be due to administration route and dose. Notably, intra-arterial delivery rather than intravenous delivery was pointed out as an important factor. With this method, the cells could engraft specifically in perivascular locations of radiation injury sites for at least 33 weeks. The intra-arterial route allows MSC to distribute in the body before arriving to the lungs.

These studies remain to be taken with care and are challenged by many others.

Concerning results that can be found in human, it is difficult to extract relevant information from different clinical trials. When looking for example at the reports concerning MSC therapies, the details of preparation protocols and composition are often not adequate. That makes interpretation, reproduction and comparison problematic. A very recent systematic review could only conclude that MSC for orthopedic applications could have an eventual potential but larger and more consistent clinical trials are needed. This review proposes minimum reporting standards to address this issue (90).

3. The case of cartilage and bone repair

a. Cartilage repair with MSC

The efficacy of MSC therapies on cartilage volume is usually limited, but some studies could still report significant clinical improvement. A recent meta-analysis published in 2019 and gathered 33 studies on the effect of autologous bone marrow in knee cartilage repair, including 724 patients. The main finding was that whole bone marrow concentrate transplants had a clinically significant effect for pain and knee function in almost all the studies reviewed. The objective was to preserve the maximal potential of the cells. The results of these procedures seem encouraging with the report of qualitative cartilage matrix regeneration, even for defects larger than 2 cm^2. Cultured allogenic BM MSC were also used and could provide improvement in pain and function in some studies. Many little studies used different kinds of support with the cells (collagen gels, hydroxyapatite ceramic scaffold, fibrin glue), with various outcomes. These procedures are more invasive, but could provide in some cases cartilage and bone regeneration and clinical improvement, but the produced matrix was altered (91). Some studies warned against the possible adverse effect of cell based therapies that could trigger synovial inflammation and lessen cartilage repair (92).

Even if the evidences remain limited for cartilage repair, a systematic review published in 2018, gathering 17 studies, points out that intra articular administration of MSC could improve pain, function and cartilage state (93). Other meta-analyses also point in the same direction, even after 2 years of follow-up. However, these studies remain at high risk of bias and no high level evidence could be extracted yet (94).

The possible mechanism underlying MSC effect are still not elucidated, but several hypothesizes can be made. After injection, MSC usually die massively and they cannot be found after 50 days. During their short passage, they could have direct interaction with other cells and secrete bioactive factors. These activities could stimulate articular chondrocytes and the host stem and progenitor cells to produce new chondrocytes. Some reports could show that MSC secretion could act on synovium and chondrocytes to regulate

their anabolic and catabolic activities. They could also induce the expression of anti-inflammatory factors (94).

b. Bone repair with MSC

With their osteogenic potential, MSC use was also attempted for bone repair and gave promising results, even if no controlled trial could bring conclusive answers yet. Previous clinical experiences concluded that MSC were more interesting to use than whole marrow, as the efficiency seemed to be associated with progenitor content.

Pioneering work undertaken around twenty years ago used allogenic BM MSC for children with osteogenesis imperfecta. After promising results with whole bone marrow transplant, cultured MSC use was tried in infusion, implicating MSC homing. A low engraftment was reported but MSC could still produce functional osteoblasts. The treated children showed significant improvement. MSC action might have been through the production of normal collagen (81).

For other common bone regeneration application, MSC are usually implanted with calcium phosphate materials. Together, they can allow abundant bone formation and were reported to be able to provide efficient fracture healing in clinical trials. Preclinical researches also reported the potential for healing large size defects. MSC were firstly used with the aim to obtain differentiation, in this case in osteoblasts. Some studies could observe the persistence of some transplanted MSC within the formed bone, but cell engraftment remain either very low or completely absent. However, with some little engraftment or not, MSC could still show significant therapeutic effect. The underlying mechanisms is here also hypothesized to be dependent on MSC stimulation of host cells, especially skeletal stem cells and other cells implicated in bone remodeling. Their immunoregulatory effect could also certainly have a role in the process, as immune response is critical to allow bone repair **(figure 21)** (95).

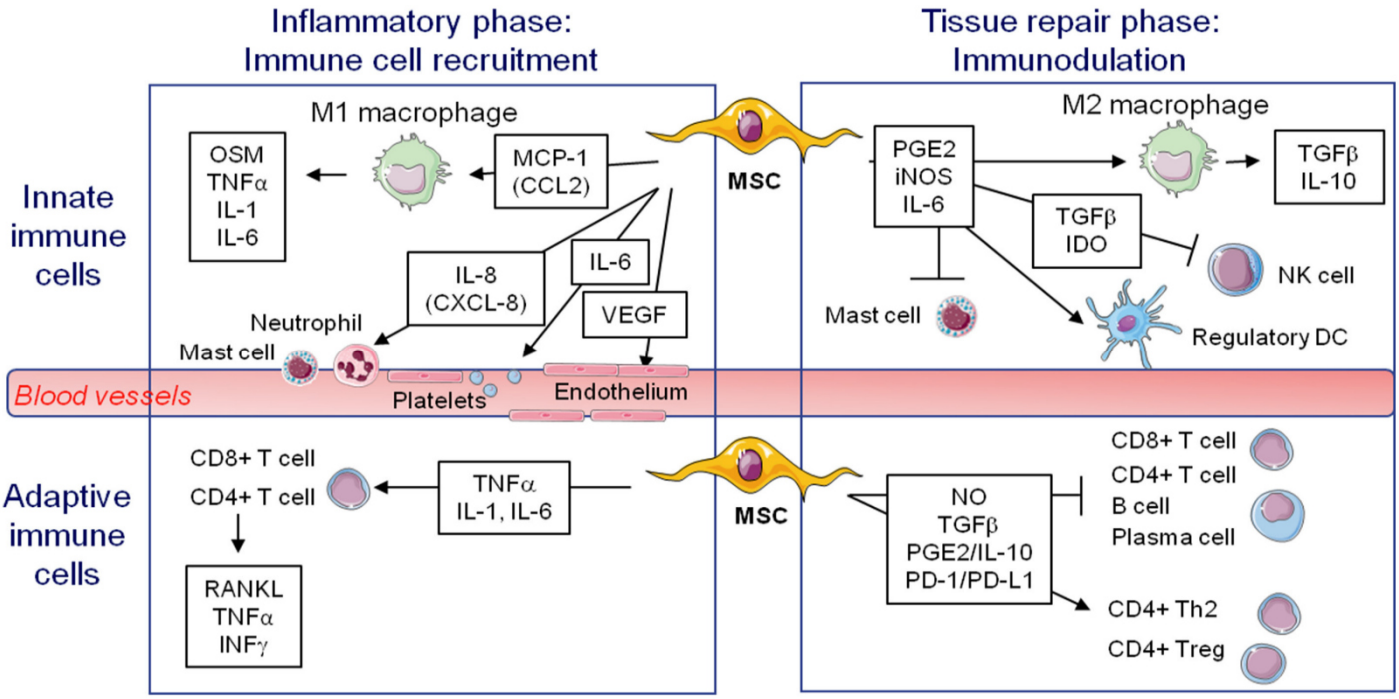


Figure 21: Different MSC immunoregulatory secretion profile favoring or inhibiting various cells potentially involved in bone formation during early inflammation or the later stage of tissue repair.

Resolution of inflammation is needed for tissue repair. MSC have an effect on different kinds of cells. For bone repair, their effect is mediated by macrophages and other immune cells that will stimulate osteoclast with RANKL secretion. MSC are able to improve the efficacy of both inflammatory and repair phases and adopt different phenotypes according to their microenvironment. (95)

In the end, even when working for tissue repair, one of the important reported MSC property was found to be their ability to interact with the immune system and to secrete growth factors.

C. MSC as signaling cells for immunomodulation and tissue repair

1. Possible mechanisms

With the current knowledge and techniques, the effects that were observed after MSC infusion were associated to rapid mechanisms exerted by the cells during their short passage in the organism. The effects could also rely on few remaining cells. After being tested for cell replacement, MSC were then considered as interesting regulating cells. Many studies shifted toward the exploration of their communication capacities. Infused MSC can

home toward site of injury or inflammation, secrete bioactive factors and make cell contacts. They can also secrete factors or act on other cells that are distributed through the circulation and have effect on distant organs. This kind of mechanism of action can be considered independent of a classical stem cell activity.

MSC were extensively studied *in vitro* for their immunoregulatory properties. The majority of what is known derive from cocultures experiments. MSC secretions *in vitro* contains high levels of proteins involved in immune system regulation, extracellular matrix remodelers (metalloproteinase inhibitors, fibronectin, collagen among others) and growth factors and their regulators (VEGF, GMCSF, BMP2, bFGF, IGFBP3, IGFBP4 and IGFBP7). This provides MSC with a strong potential to stimulate endogenous cells for tissue regeneration and to mediate immune cells activity (50). MSC can also display an interesting variety of immunoregulatory function. In case of inflamed environment, MSC can inhibit the immune system activity, while when levels of inflammatory cytokines are too low, they can stimulate immune cells to release activating factors (96). This proinflammatory activity could support the immune response in the first stage of inflammation and may serve to promote defense against pathogens. A feedback then balances MSC effect, as the elevation of proinflammatory signals make them return to their anti-inflammatory phenotype, preventing excessive damage and promoting tissue repair. This mechanism shows an interesting ability of MSC that can adapt their answer to different inflammatory environments **(figure 22)**.

Another important possible mechanism that was also recently highlighted is called efferocytosis. When MSC are administered and accumulate in the lungs, they are predominantly phagocyted by macrophages. MSC can also express apoptotic signals, like phosphatidylserine, that lead to their phagocytosis. MSC engulfment could trigger expression of immune tolerance factors by macrophages such as interleukin-10, TGFβ and IDO. This mechanism is independent of cell viability and could be responsible for an important part of the observed anti-inflammatory effect of administered MSC. That could explain why even dead cells were shown to have some effect (96). These mechanisms are partly summarized **figure 22**.

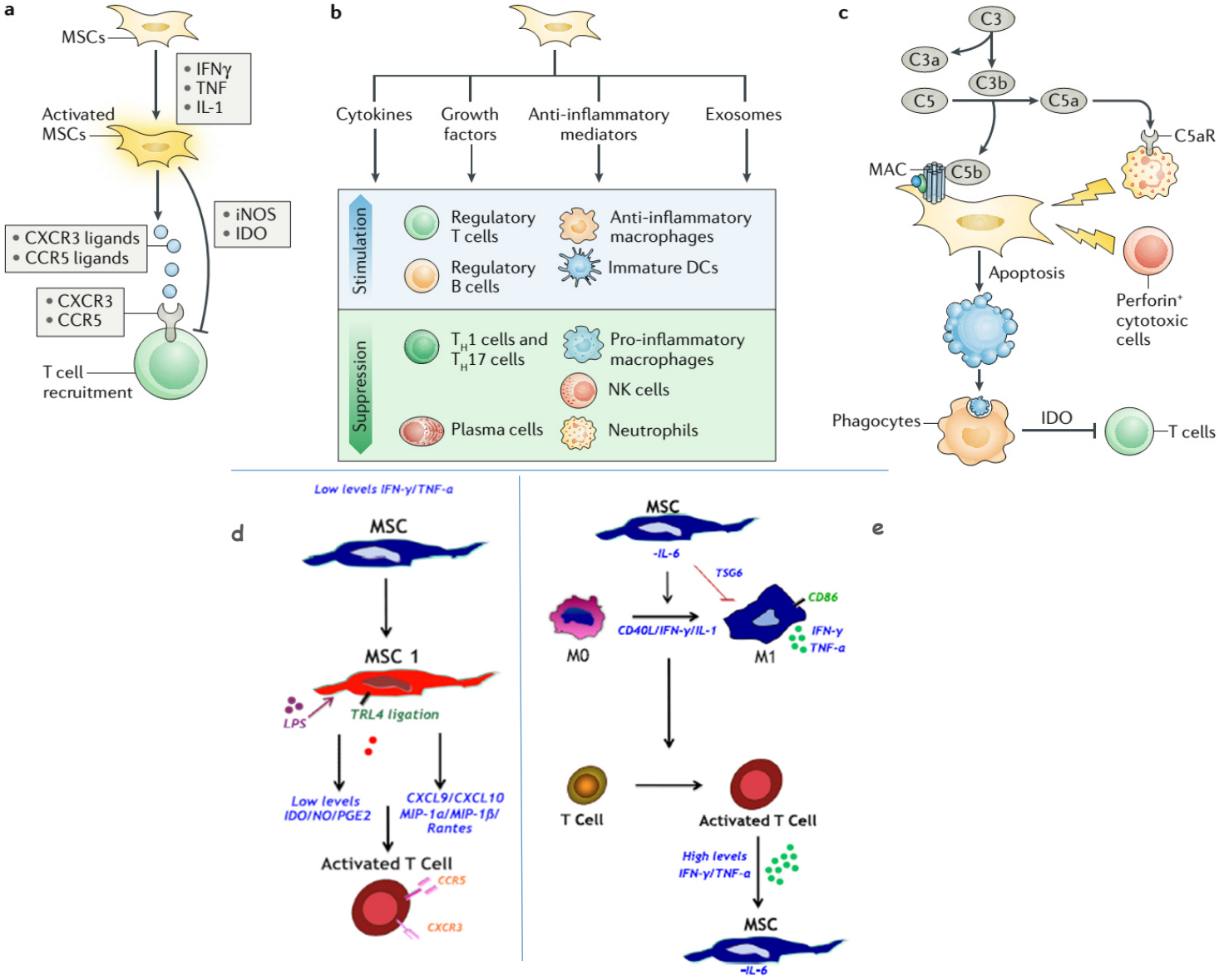


Figure 22: Mechanisms associated to MSC immunomodulation.

a) MSC are activated by IFNγ combined with either TNF or IL1. They produce CCR5 and CXCR3 ligands which recruit T cells. MSC can then suppress T cells activity by expressing iNOS for murine MSC, and IDO for human MSC.

b) The production of cytokines like TGFβ and IL6, growth factors like HGF, LIF, anti-inflammatory mediators such as PGE2, TSG6, HO1 and galectins, and exosomes also mediate MSC immunoregulatory effect. The stimulated anti-inflammatory immune cells can in turn amplify the suppression of pro-inflammatory cells and the emergence of regulatory cells.

c) After administration, MSC can be targeted by the complement system, neutrophils and cytotoxic cells, leading them to undergo apoptosis. MSC can also be apoptotic from the start before administration. These apoptotic MSC can be phagocyted. This induce the phagocytes to express IDO notably. (224)

d) With low levels of inflammatory cytokines, MSC may switch to a phenotype with proinflammatory tendency (MSC1). MSC1 recruit and activate T cells in the site of inflammation through the secretion of several chemokines such as MIP-1a and MIP-1b, RANTES, CXCL9, and CXCL10, that bind to receptors on T cells such as CCR5 and CXCR3. Low levels of lipopolysaccharide can also polarize toward MSC1 phenotype by activation of TLR4.

e) In the absence of IL6, MSC can induce the polarization of macrophages toward a proinflammatory phenotype (M1). This is mediated by the secretion by MSC of IFNγ and IL1 and the expression on their surface of CD40L M1 macrophages in turn secrete proinflammatory cytokines and express costimulatory molecules on their surface that promote T cell activation. A feedback mechanism is then possible, when the proinflammatory cytokines reach a sufficiently high level, the anti-inflammatory phenotype of MSC is activated. (96)

Another interesting aspect associated to MSC ability to interact with immune cells is their expression of molecules implicated in fetal maternal tolerance such as HLA-G and IDO. That could be linked to their capacity to alleviate excessive activation of immune cells. Mechanisms implicated in fetal maternal tolerance may also be related to the effect MSC have after their phagocytosis in the lungs (efferocytosis). Indeed, during pregnancy, some fetal stromal elements are phagocyted daily in maternal lungs and can activate expression of IL10 and IDO (97).

All these complex mechanisms implicating many different effectors are difficult to grasp in common *in vitro* assays. *In vivo* models have brought essential information and could corroborate some of the hypothesis made from the *in vitro* experiments. Several models of autoimmune and inflammatory diseases support MSC capacity to induce regulatory T cells phenotype. Some evidence obtained in a mouse sepsis model could confirm the polarization of macrophages that can prevent excessive tissue damage. The phenomenon was found to be amplified by MSC induction of monocytes mobilization in the circulation. MSC were also found to enhance bacterial clearance by neutrophils, while limiting the oxidative damage they could cause through MSC action on monocytes and macrophages via IL10. In some *in vivo* models, MSC action was shown to be effective even without migration toward the site of injury. Another important element that was confirmed is the effect of the environment on MSC efficacy. In a mouse GvHD model, they were found to be more effective when administered after the inflammatory reaction started. This could be linked to the effect of MSC pretreatment with inflammatory cytokines that could enhance their therapeutic effect in models of GvHD, acute myocardial ischemia and colitis (96). GvHD mouse model was also used to show the importance of MSC fitness. The therapeutic effect of MSC was considerably reduced with apoptotic MSC compared to living cells, despite the importance of the efferocytosis mechanism (97).

It is not yet possible to conclude about the precise mechanisms and the optimal conditions to allow MSC effect. Indeed, existing studies are highly variable, often not comparable and sometimes contradictory. The mechanisms in play are different according to tissue source, culture conditions, senescence level, activation status and analytical

method. A variability is also observed between different donors and between fresh and thawed MSC.

More details on the immunoregulatory mechanisms of MSC and their clinical applications were also described in other thesis more oriented toward the immune properties of MSC (in French) (98–100).

2. Questionings

A part of the researchers implicated in the MSC field are sceptic concerning MSC as immune modulators. They stress that this property now widely associated with MSC has only derived from *in vitro* and clinical studies and that there is no association with MSC physiological functioning. The way common mixed lymphocytes reactions are made is also called into question, some report showing that when the experiments are made with more T cells to MSC ratios, T cell proliferation is promoted instead of being inhibited. In a clinical context, with infusion of 2 million MSC per kg, the maximal ratio would be 1 MSC for 10 000 T cells. Many *in vitro* studies made on the subject of immunomodulation are conflicting and lack relevance because of this kind of inadequacy. Other issues were detected, for example a classical assessment of regulatory T cells is based on a phenotype transiently shared by T cells during their activation (64).

Another fact that is more widely accepted is that despite *in vitro* results supporting the immune suppressive ability of MSC and their immune tolerance, they were found in several *in vivo* studies to be immunogenic, rejected and inhibited in allogeneic conditions. The fact that they express low levels of class II major histocompatibility complex (MHC) molecules (HLA) is not sufficient because of the presence of class I antigens. Furthermore, the expression of MHC class II antigens can be upregulated in an inflammatory environment, for example with the presence of IFNγ (64,97).

A recent systematic review gathered several studies in animal and humans with different kinds of MSC used to treat immune diseases. They could still confirm some immunological mechanisms as the proliferation inhibition of immune cells, except B and T

regulatory cells that were stimulated. The levels of inflammatory cytokines were shown to decrease and the level of regulatory cytokines increased (101).

3. GvHD case

An application that can associate the signaling and potential repopulation functions of MSC is based on their function in the hematopoietic niche. These recognized abilities for BM MSC led to their use in association with HSC transplant to accelerate hematopoietic recovery and to help GvHD management. In an important review published in 2013, it was reported that MSC seemed to be more effective for acute GvHD. It was hypothesized that it was related to the cells' need to be activated by a high enough level of inflammation. Several reports could show positive outcomes after MSC use with important clinical response and significant differences in survival (96). However, these results were not yet confirmed by randomized studies. A recent systematic review and meta-analysis published in 2019 investigated the results obtained so far with MSC for GvHD in 12 completed randomized and controlled clinical trials (879 participants). Contrary to the numerous reports of smaller clinical experiences, this study found no reliable evidence for MSC efficacy for the treatment of acute GvHD in the controlled trials. However, they could report some low level evidence for MSC efficacy to reduce chronic GvHD risk when administered in conjunction with HSC transplant (102). More refinement is still needed for the use of MSC for GvHD, but many precious information could be extracted from these first experiences.

The case of a phase III clinical study for GvHD that could conclude on MSC efficacy is an example of such refinement. MSC treatment was previously found to be more effective in children with gut or liver damage notably, so this trial used these criteria for patient inclusion (103). These results remain to be confirmed, but when the clinical study is made with more specificity, selecting the patients with specific indications, that could reveal in a meaningful way MSC potential for these particular cases.

With current techniques, MSC use remain associated to highly variable clinical response.

IV. MSC diversity

MSC can be subject to the same kind of heterogeneity as the one exposed in the "Stem cell heterogeneity" part of this work. These mechanisms can participate to the observed variability at different levels among MSC. Intrinsic factor and stochastic events can combine with variability associated to environment factors with tissue source, donor parameters, and culture conditions. An overview of the different levels of heterogeneity among MSC is proposed **figure 23**.

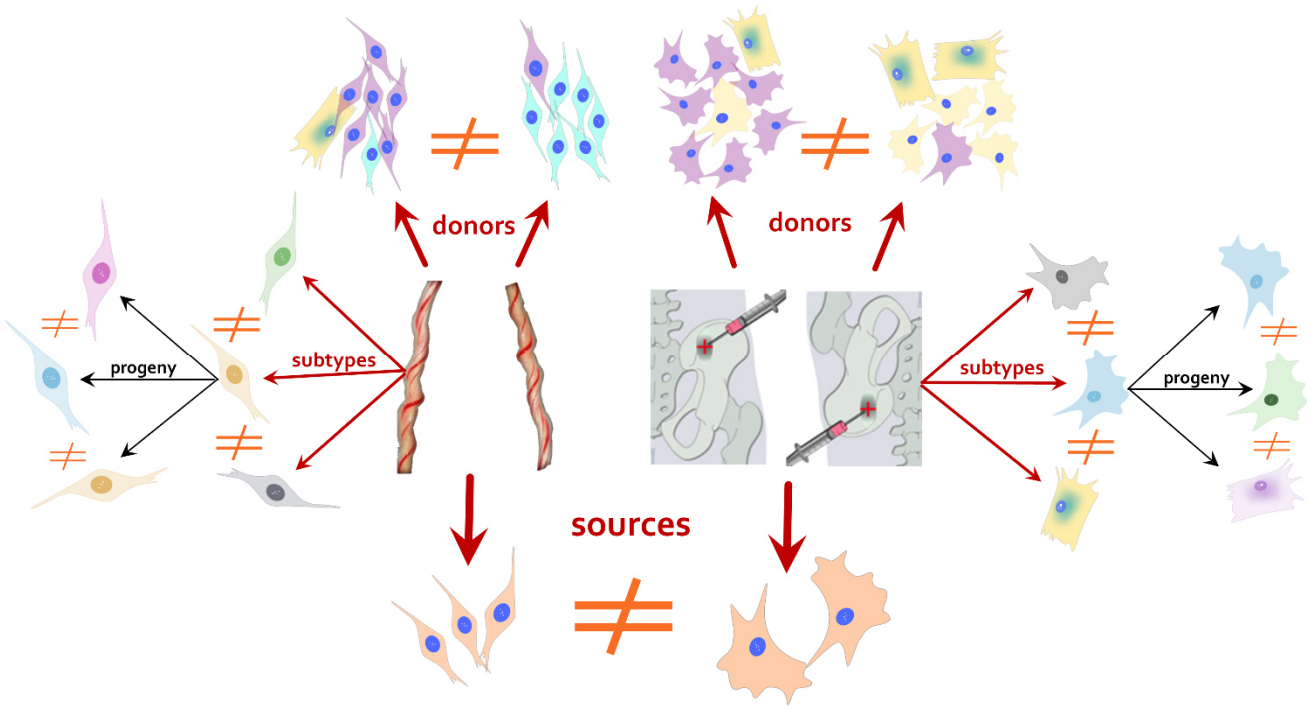


Figure 23: Overview of the different levels of heterogeneity associated to MSC, the example of WJ and BM MSC.

Firstly, MSC obtained from different tissue sources are not equivalent, they will display different morphologies, phenotypes and potentials.
Secondly, MSC populations can vary between different donors even when the same tissue is used.
Thirdly, MSC within a sample are also heterogeneous, and their progeny is also subject to drifting.

A. Apparent homogeneity

In most papers on the use of MSC, when ISCT criteria are verified, there is often an apparent homogeneity assumption, leading to consider that even cells from different tissues or species as equivalent (50,104,105). The common way to interpret and study MSC

summarizes information to an average and often mask their diversity. According to the scale of study and to the application associated, it can be adapted to consider these average parameters. However, it is especially important to deepen the study on variability when trying to understand communication between cells and individual cell function. Because of the baseline stochastic functioning of the cells, it should not be considered as an evidence that different cells within the same cell type or even the same colony will display reproducible mechanisms. **To bolster this homogeneity and reproducibility, other elements are needed, such as the detection of feedback loop mechanisms, high availability of the regulatory molecules implicated, genes redundancy, among others canalization mechanisms**.

In some studies, it is detailed that MSC observable homogeneity in cultured cells constitute a **lineage** homogenous cell population (106). They can be defined by a set of markers that can be found on the whole MSC population and can distinguish their lineage from others such as the hematopoietic lineage. They can also display a relative stable gene expression pattern between different samples from the same tissue source and cultivated in the same conditions. However, cells that are derived from different tissues have more different profiles. Furthermore, the existence of population similarities does not prevent the possibility of heterogeneity within the population. For example, several surface markers were found to be expressed at different levels with a high amplitude (54). In some case, this heterogeneity within the sample can be observed with the various cell morphologies MSC can harbor.

B. MSC variability with tissue source

Bone marrow MSC are the most studied MSC, with the largest diversity of evidences related to their biology. MSC from other tissue still lack this hindsight but could show to harbor some specific features of interest compared to BM MSC. MSC research now tends toward the use of MSC from adipose tissue, Wharton jelly and placenta. This is due more to the accessibility, harvesting and culture ease than to their functional superiority. There are few studies that explored the real stem cell properties of other MSC than BM MSC.

Directed reviews are however common to explain the choice of the type of MSC for a given application.

Even within the same tissue, it is possible to obtain different types of MSC. For examples, despite the fact that Wharton jelly is often associated to the harvest of apparently homogeneous MSC compared to other sources, differences could be observed between cells from different zones of the tissue (86).

Besides the differences, these cells still have the property to display approximatively similar properties *in vitro* with similar culture conditions. However, as MSC from different tissues don't display identical features, it is common that they do not need the same stimulations, for example to be able to differentiate (64). MSC from different tissues can show very different phenotype. Even within the same donor, MSC from different tissues display different gene expression patterns. Their biological features are only partially overlapping, and that can make comparison of the cells difficult. Moreover, different micro environment will induce different cell behaviors.

This part is not an exhaustive review of the subject, only some representative examples were selected, notably of the comparison between fetal and adult MSC. Indeed, neonatal cells were found in several studies to display stronger proliferative capacities and less senescence but they also have different functionalities, especially concerning differentiation potential, migration and cellular mechanistic for immunomodulation (107,108).

BM MSC were found to be more prone to osteogenesis and had a better ability to differentiate toward adipocytes than WJ MSC, but they expressed inferior levels of genes implicated in angiogenesis (98). In a nude mice model, contrary to human BM MSC, ectopic implantation of human WJ MSC did not allow bone formation and did not persist more than 3 weeks where BM MSC persisted without important decrease during the whole month of the study. WJ MSC were instead found to be more angiogenic and induced blood vessels ingrowth *in vivo*. In this study, WJ MSC were found to secrete more pro-inflammatory and chemotactic cytokines than BM MSC. Interestingly, when WJ MSC were

implanted in bone defect (orthotopically), they could promote bone formation as BM MSC. However, bone formation was undertaken by host cells that were stimulated by WJ MSC, whereas bone could be made directly by the transplanted BM MSC (109). This experiment was performed by seeding the cells on ceramic scaffolds embedded in fibrin glue, but the cells were not exposed to specific cytokines or growth factors implicated in differentiation.

WJ MSC are also often presented as more primitive cells because of their fetal nature compared to adult MSC. They were shown to express at higher levels than adult MSC some embryonic markers like Nanog and Sox2, even if these levels remain low. This was hypothesized to be associated to the higher proliferation levels and delayed senescence that can be observed compared to adult MSC. WJ MSC were also shown in a study to be able to overcome the proliferation inhibition that can occur for other MSC types when cells are at high density (110). On the other hand, the studies that report clearly the CFU forming assays with cells harvested after the step of plastic adhesion, and plated at clonal densities, often report a lower capacity of WJ MSC compared to MSC from other sources. They also showed a decline after less time in culture than other MSC derived from menstrual blood and adipose tissue, cultivated in the same conditions (110).

WJ MSC were also reported to be less immunogenic than adult MSC, especially with their strong expression of HLA-G, known to be associated with fetal maternal tolerance. WJ MSC also lack expression of co-stimulation molecules needed for lymphocytes action and express lesser levels of HLA and TLR molecules than BM MSC.

At first glance, WJ MSC seem to display similar immunoregulatory properties and seem to rely on similar mechanisms than BM MSC. However, contrary to BM MSC, they were shown to be able to produce anti-inflammatory IFNα. These two kinds of MSC were also shown to answer differently to inflammatory cues with TNFα and IFNγ, with different levels and kinetics of production of immunoregulatory molecules (98,107). Even without activation, WJ MSC could be more efficient to decrease immune response in a model of acute inflammation (111).

WJ MSC are often chosen as an alternative to BM MSC that can be more difficult to produce. They are presented as equivalent or with higher therapeutic potential. However, they could be more different than what is generally assumed and a less relevant choice. For example, it was shown that WJ MSC migration capacity was lower than that of BM MSC and placenta MSC. This was associated to differential expression of proteins involved in migration (108).

Regarding the phenotype of MSC from different sources, they usually follow the common MSC criteria. However, along successive comparative studies and investigation going up to MSC physiological properties, numerous differences were reported. MSC phenotype is changing according to the initial precise localization of the cells (as it was introduced in the previous section in the part II. "MSC concept" - "MSC physiological role"). Even cells that are in what could seem as the same tissue display different surface phenotype, as it was shown for CD146 in the bone marrow for example according to their localization in perivascular or endosteal region (50,60).

It would be interesting to have clinical studies comparing the effect of different types of MSC sources, but this choice is usually made at the pre-clinical state.

C. MSC variability according to donor parameters

It was shown that donor characteristics can affect MSC functionality, especially donor pathologies and age. It is important to consider it for MSC use in therapeutic application, for donor choice to generate potent allogenic MSC batches and also to study the feasibility to produce enough functional cells for an autologous MSC therapy depending on donor state.

Both adult and fetal MSC characteristics can be affected by donor parameters. Most of the work focuses on adult donor parameter affecting BM MSC.

1. Donor age

a. Technical caution

Association between donor age and BM MSC characteristics were followed in several studies regarding mostly *in vitro* assays. Results are often **controversial** because of differences in donor selection, protocols to collect the samples, culture conditions and results interpretation. An example is when studies about MSC capacity to form colonies are reviewed, they sometime mix different kinds of results comparing the result of plating bone marrow mononuclear cells at passage 0 and other studies using MSC after the first plastic adherence at passage 1 or after. The same terms can be used and lead to some confusions. At passage 0, MSC number is not known and the plating density cannot be precise, it is the total mononuclear cells count that is used. At this step, CFU-F formation reported reflects the proportion of cells that were able to adhere and proliferate as a colony among BM mononuclear cells. After this step, MSC count after detachment is used to plate the cells at a very low density to be the closest to the single cell level. Cells are considered to be isolated and behave independently from one another. The percentage of cells able to divide and produce a big enough colony in these conditions is often referred as the colony forming efficiency (CFE).

An example of different type of analysis that can be found and difficult to compare is that some studies group donors by **class of age** and compare the young and the elderly with statistical tests comparing averages, while other studies search **correlation** between donor age and other parameters without separating groups. The amplitude between the donor ages that were investigated can also be more or less extended. This can result in very different interpretations. A study with a large panel of 61 donors for example could only find correlation between donor age and the expression level of several genes from array assays, but not with proliferation and differentiation capacity, but they did not try grouped comparisons (112).

Other factors that can bring bias in the studies about the effect of age on cells can be the conditions of **sample collection** and the impact of **other donor parameters** like gender, clinical history or actual pathological state. A study made with fibroblasts showed that the

observed differences between cells with donor age were not found any more when donor pathological state and biopsy conditions were taken into account (113).

b. Overview

Despite technical difficulties, a large number of studies were undertaken on this subject and could converge to say that, in a general manner, with chronological aging, MSC have a **higher risk** to display reduced proliferation, colony forming efficiency (from plated single MSC), differentiation potential and altered phenotype, but that is **not systematically the case**. The number of MSC that can be obtained from aged donors is also found to be reduced in several studies, **although not all** (114–118). Moreover, MSC pool was found to be reduced in premature aging disorders such as progeria and Werner syndromes (119). There are few controversies about the decrease of MSC proliferation capacity with the increase of donor age. The most inconsistences reside in differentiation potential of the cells determined with classical *in vitro* assays. For osteogenesis, several studies discussed below tend toward the conclusion that it is the number of MSC able to go toward osteogenic differentiation that is reduced rather than the capacity of the remaining cells (114–118). This could explain some of the discrepancies between studies.

Regarding **gene expression**, upon aging, many homeobox transcription factors, known to be implicated in morphogenesis, differentiation and remodeling, were found to be repressed in MSC. In any case, it is difficult to access to the initiating mechanisms. The downstream effects observed on the molecular level will then vary according to the cell type and environment, with its specific regulatory network (39).

c. Signs of senescence

More consistently, more apoptotic cells and elevated expression of the tumor suppressor p53 and its associated pathway genes were found in MSC from aged donors (120). Markers of **senescent cells** such as β-galactosidase activity and p16 expression were also found to increase (more details about senescence and the associated markers are provided in the last part of the introduction, part VI. "Senescence"). In association to these observations, levels of reactive oxygen species (ROS), reactive nitrogen species (RNS) and

oxidized and glycated proteins were found to increase with donor age. This was combined with a decrease in SOD activity and a lower resistance after exposition to hydrogen peroxide (118,121). Other mechanisms of stress response were also found to be impaired in MSC from aged donor, notably HSP system. More MSC were found to activate HSP in more mature donors, but the system was defective (118). This is combined with the report of moderately shorter telomeres in aged donors, although no sign of telomere dysfunction have been reported (39,122). These different observations are correlated with the decrease in proliferation capacity of MSC from donors at advanced ages (118,121).

A **recent** study on 22 donors compared MSC at early passage (passage 2 to 4) from young and **healthy** aged donors. This study has the advantage to avoid the bias associated to donor pathology. They could confirm the reduced proliferation capacity and colony formation of MSC with donor age. This was associated with an increase of senescence markers with stronger activity of β-galactosidase and higher levels of lipofuscin in the cells. Accumulation of ROS and DNA damages were associated to the activation of DNA damage response and a decrease in the expression level of FOXO4 (implicated in protection against ROS among other pathways). This state was associated to an early senescent stage rather than complete cell cycle arrest. The onset of replicative senescence in MSC culture from aged donor took place from passage 6 to 7, earlier than in younger MSC that could be cultivated until passage 7 without undergoing replicative senescence. This study also reported an increase in the secretion of pro-inflammatory cytokines such as IL6, IL8, IL1α, MCP1, Groβ and CCL4 in older MSC(123).

d. Surface phenotype

Regarding surface **phenotype**, few changes in minimal ISCT criteria markers and additional classical markers such as CD44 and CD166 are generally reported as most studies look only at percentage of positive cells. These markers being strongly expressed, if background fluorescence used to put the threshold of positivity is not too high, the percentage of positive cells can remain at almost 100% while the level of expression of the markers can still change. Despite these limitations, some studies reported a decrease in the presence of CD90 and CD105 on the MSC surface for aged donors compared to young

donors. The protein levels of other receptor such as Stro-1, Notch-1 and receptors involved in bone formation were also found to decrease with age (118). Another more comprehensive study on 52 donors from 13 to 80 years could identify a set of markers with superior levels of expression in BM MSC from young donors (at end of the first passage): CD146, CD106, CD71, CD140b, CD274 (PD-L1), CD166 and CD90. These phenotypic changes were associated with a reduced IL6 level in coculture with T cells (124). Another study on 86 bone marrow donors of different ages could show that, among the CD271 positive cells within mononuclear cells from bone marrow, the proportion of CD146 expressing cells was inversely correlated with donor age. The higher levels of CD146 surface marker were found in fetal and pediatric MSC while the dominant population in adults was negative for CD146 expression (116). These populations of cells were previously shown to be associated with *in vivo* localization, with CD146 positive cells in perivascular regions and CD146 negative cells in endosteal niche (60). The change in the proportion of these different subsets could be associated with changes in the niche composition, and more specifically the increased presence of quiescent HSC, documented in mice, located in endosteal regions (32).

e. Functionality of MSC derived from aged donors

When exposed to aged MSC secretome, young **HSC** were impaired with a reduced clonogenic potential and an increase in genes associated with inflammation. Conjointly, aged MSC were less able to inhibit proliferation of **immune** cells in co-culture experiments. As previous studies, they also observed a global reduction of the expression of transcription factors implicated in osteogenesis in the MSC coming from aged donors. However, they could produce equivalent levels of mineralized matrix compared to MSC from young donors (123).

There are few *in vivo* studies that investigated the effect of donor age on MSC behavior. One of these studies could show that bone formation after implantation in nude mice could be obtained from all donors no matter their age. However, they pointed out that more trials were required with aged donors to obtain it while all the different cultures obtained from younger donors, under age 41, were able to form bone *in vivo*. The success rate of the experiment then decreased progressively until arriving at less than 50% of the

trials after 70 years old. These differences were said to be associated to an inferior proportion of osteogenic cells in the bone marrow of aged donors. A link was also made with other studies that reported a decrease in the number of proliferative cells in bone marrow with increasing age (125).

Another study testing BM MSC in mouse with experimental infarct could show that human BM MSC coming from old donors provided less functional improvement after implantation than MSC from young donors. They displayed less robustness and plasticity (126).

2. Donor pathology

As MSC are investigated to be used for application in autologous therapies, some studies wanted to verify that MSC coming from these pathological environments could show sufficient potential. Furthermore, BM MSC samples used in several research protocols are often taken from patients undergoing operations like hip replacement because of advanced osteoarthritis. Aged donors are also particularly prone to be affected by various pathologies. It is then important to ask what may be the influence of this pathological state on MSC.

Several studies investigated the effect of **rheumatoid arthritis and osteoarthritis** and reported a decrease in MSC proliferation, colony formation efficiency and chondrogenic activity compared to healthy donors (reviewed in (127)). Conversely, other reports indicate similar state and differentiation potential compared to healthy donors (127). A study on rheumatoid arthritis showed in addition that MSC displayed premature telomere length loss. Even with these alterations, it was considered that a sufficient number of chondrogenic MSC can be obtained from these patients and used for therapeutic applications. Another common state that can be found in MSC donors is obesity. This could also have an impact as some studies highlighted a reduced proliferation and differentiation capacity in MSC from obese donors (128).

Another point of interest is the role and state of MSC in **leukemia**. An *in vitro* study used an acute leukemia cell line to study their interplay with MSC. The co-culture of these cells with MSC induced an increase in β-galactosidase activity and activation of p53 gene,

associated to ROS accumulation and cell cycle arrest of MSC. In this state, MSC ability to differentiate was also impaired. The effect of the leukemic cell line was partly dependent on cell contact. Several adhesion molecules such as CD106, CD54 and CD49e were increased in MSC. The leukemic cells, for their part, were strongly adhered to MSC and showed an increased proliferation rate and migration capacity (129).

Patients with **hematologic malignancies** were also studied. BM MSC from these patients showed a reduced capacity for *in vitro* expansion. This was associated to a change in phenotype with inferior levels of the surface proteins CD90 and CD105 compared to healthy donors. MSC from diseased patients also displayed increased angiogenic potential (130). In a similar way, BM MSC collected from patients with **myelodysplastic syndromes**, a class of diseases affecting HSC function, showed all signs of senescence, both in young and aged donors (131). These MSC also showed a tendency to express higher levels of CD184 protein (CXCR4 receptor for SDF1), suggesting an increase in their migration potential (130).

Systemic lupus erythematosus is also a pathology where MSC are involved. A study reported that MSC from both treated and untreated patients were senescent with an increase in p16 expression, and with a decrease in their proliferation, differentiation and immunoregulatory capacities (132).

Another interesting study extracted MSC from vertebral bodies of patients with **idiopathic pulmonary fibrosis** and compared them with MSC from age-matched normal donors. The study showed that BM MSC from the diseased patients were also senescent. They showed sign of mitochondrial dysfunction, accumulation of DNA damage and secreted inflammatory factors that could induce the senescence of fibroblasts. Their functionality was impaired, notably with a decrease in migration capacity. These MSC were also tested *in vivo* and revealed that MSC from patient with pulmonary fibrosis were less able than MSC from healthy donors to prevent fibrotic changes in a model of lung fibrosis induced by bleomycin (an anti-cancer drug). With MSC from diseased patients, the severity of the illness was increased with an important pro-inflammatory state (133). The previous studies hypothesize that MSC may have a role in the onset of these diseases.

3. Obstetric factors

Fetal cells can also be affected by their environment during pregnancy. A study investigated the influence of a large number of obstetric factors on WJ MSC. It showed that cells coming from healthy, **full term** infants, with mother without pre-eclampsia, showed better proliferative and chondrogenic differentiation capacity compared to the others (134). A recent study also showed that WJ MSC can be affected by gestational **diabetes** mellitus, even when the glycemia was controlled. It was notably their proliferation rate, chondrogenic and osteogenic differentiation abilities that were impaired, associated to an increase in adipogenic differentiation, senescence level and oxidative stress (135).

4. Inter-donor general variability

History and state of each patient is complex and may influence MSC state and potential. Donor age and pathology at the time of MSC harvest are important parameters that must be considered for MSC studies, but there are still other factors combining their influence on MSC that would be interesting to investigate. Indeed, even when correcting for age and pathology, a high variability still remains among MSC (112). The maximal number of passages and number of doublings possible for each sample can notably be considerably different. The modifications of the transcription profile along the culture was also found to occur differently between several donors (136).

Interestingly, a study on 53 donors highlighted differences between female and male BM MSC. Female MSC were shown to secrete more IFN-γR1 and IL-6β and displayed a stronger inhibition of T cells in co-cultures experiments. Among BM MSC derived from the younger female donors, cells that displayed a high clonogenicity were more frequent, had a smaller size and higher proliferation rate than the others (124).

D. Intra-sample heterogeneity

A 1999 study already highlighted the heterogeneous nature of MSC samples. They described cells at various stages of committment with distinct potentialities, in particular large variations for growth and differentiation. Interestingly, they reported differences between samples collected from the **same donors** and with the same technique. For some it

was aspirates obtained with six months interval, and for others it was aspirates taken simultaneously from iliac crest on both sides. Fast proliferating and slower proliferating MSC may coexist and are not randomly distributed in bone marrow. MSC heterogeneity from isolation step is inevitable and further propagate during culture (137). As it was shown for HSC, MSC that reside in a tissue are heterogenous and composed of different subtypes of cells. These phenotypic changes are fluctuating and they are amplified with the progress in the cell cycle. These changes occur continuously and in a reversible manner. That means that cells expressing different antigens may still be of the same cell type. The dynamism of BM MSC phenotype may be associated to the constant remodeling of the bone marrow. A large set of markers and the understanding of their variations is then needed to be able to characterize MSC heterogeneity.

1. Colonies

To show true multipotency, the progeny of a single cell must be able to differentiate toward several lineages (61). For this, single cell derived colonies are used. This technique also allows to highlight the variability between the different colonies that can be generated from the same MSC sample. They were shown to differ in size and cell number, differentiation potential and internal composition. They also displayed different progression toward differentiation and senescence (64). More precisely, a study could show that all possible combinations of potential to differentiate toward osteocytes, chondrocytes and adipocytes could be found in different colonies from the same BM MSC sample. No more than 50% of colonies have showed an ability to differentiate toward the 3 lineages. The colonies showing this trilineage potential *in vitro* were found to be more proliferative and contain more clonogenic cells themselves, and they also expressed higher levels of **CD146** than unipotent colonies (138). Also, for WJ MSC, studies with clonal cultures revealed differences in self-renewal and multilineage potential between different colonies from the same sample. A hierarchical structure was proposed where a gradual loss of potential occur progressively along the successive cell divisions. A general observation was that MSC with the highest potential for differentiation were also the rarest cells (67).

The organization for differentiation potential *in vitro* remains unclear and may **not** follow the same structure as the hierarchical HSC model. MSC choice and culture conditions for differentiation are also very different between studies and make it difficult to gather consistent results. Furthermore, the *in vitro* observations may not represent the differentiation sequence of MSC ***in vivo***. A group investigating MSC heterogeneity *in vivo* also showed that cells derived from **different colonies of the same sample** displayed different capacities to form bone and hematopoietic tissue after transplantation in mice. 8 out of 20 colonies were competent in the example of this study (68).

2. Cell individualities

Even within the same colony, cells obtained *in vitro* are not equivalent to the mother cell of the colony. For example, different MSC within the same colony were shown to differ in their osteogenic differentiation potential. That could be due to loss of differentiation potential along with repeated divisions, or to stochastic events. Cell morphology and differentiation commitment were also found to be different between cells from the center and cells from the outer part of the colony. Larger cells were found in the center of the colony while the smaller cells tended to be at the periphery. These differences could disappear if the cells were seeded once more. but important heterogeneity then reappeared in culture within very little time (68,139). An interesting study used **continuous imaging** associated to semi-automated tracking of cell morphology, division and migration to follow the onset of BM MSC heterogeneity in culture. Interestingly, less than 10% of the cells that adhered the first day was found to give rise to the majority of cell progeny while more than half of the cells did not replicate over the 6 days they were followed. Most proliferating cells took around 2 days to divide and were described as smaller cells. This confirmed other studies where smaller cells are reported as more potent and larger cells as more mature and restricted in their potentialities. After each division, there was a probability for the daughter cells to stop division and increase in size. Large cells were also found to display stronger β-galactosidase staining. These large cells arise in a context where the culture density was far from confluence and the growth arrest of the cells was not due to contact inhibition. This implicates that the larger cells observed in culture are not a distinct subtype

of MSC nor an older cell population, but they arise from many different generations of cells that ceased to replicate (140).

These findings were consistent in different culture conditions and with BM MSC from different tissue sources. However, the triggering of the cessation of proliferation and change in cells size may occur at different times. These results have numerous implications and can change the way MSC cultures are considered. For example, with only a little fraction of cells quickly replicating while the majority of the other cells do not divide, that means that common ways to determine population doubling may underestimate the true number of divisions. These proliferative cells have divided more than what could be thought when looking at the number of population doubling of the whole population. It also means that senescent cells accumulate throughout *in vitro* culture (140).

Other studies confirmed that progeny from a single MSC can, in less than 7 days, show important changes in their size and lineage commitment. Functional and biophysical properties of cells derived from single colony can then be considered as highly variable (141).

V. Surface markers to decipher MSC heterogeneity

A. Interest of surface markers

As it could be seen in the previous part about MSC diversity, their heterogeneity is disclosed in a very progressive manner, by comparing different colonies of cells within a sample and with the combination of different markers. To go further, surface markers can be of great interest to distinguish cells that have similar general features but that may harbor different individualities.

The potential contribution of surface markers to improve the way stem cells can be used was demonstrated with HSC and CD34 positive cell selection applications, now widely used to treat blood diseases. Even with the fluctuations of this marker on HSC, this technique has turned out to be very useful. However, for MSC, until now it seems there is no such single marker that can be sufficient to isolate cells with the desired therapeutic property. There is a strong need to determine the combination of markers that could capture their initial biological variability, but also the variability induced by the manufacturing process, and above all their therapeutic potential.

Surface markers are interesting targets to distinguish between different MSC states and subtypes. These markers also have the big advantage that they can be used on living cells. Surface markers can be used for *in situ* identification, cell characterization, prospective isolation without plastic adherence step, or functional selection. With the possibility to isolate different MSC populations, it becomes possible to analyze their specific properties and their interactions. Surface proteins also carry out important biological function such as cell contact, interaction with extracellular matrix, signal transduction, or even molecules transport. A proteomic study could identify around 200 plasma membrane proteins on BM MSC (142). Even with clinical grade manufacturing, a high variability is reported among the MSC produced, and it is reflected by variable expression of surface markers (outside the ones proposed by the ISCT). In a general manner, markers with lower abundance show more variability than markers that are very highly expressed on MSC surface (143).

B. MSC prospective isolation

To reveal the presence of different subtypes of MSC from the initial source of cells, several surface markers were tested in a prospective manner to see if they could allow to extract MSC with cell sorting. These markers were searched on the cells before they were put in culture, when their phenotype is the closest to what it was in the body. The ability of the cells to adhere to plastic and form colonies was notably compared between the cell suspension obtained with different set of markers.

1. Standard or variable surface markers

The classical MSC markers that are strongly and homogeneously expressed on all adherent MSC can be of use in strategies to isolate MSC directly from the tissue source. However, in the initial tissue, MSC are often minority and there are always other cells that will also express MSC classical markers. A more specific combination of markers can be used, but the low number of MSC makes it difficult to be feasible.

Variable surface markers, for their part, can be used to isolate cells with a specific phenotype directly from the tissue source but also after expansion. Isolation directly from a cell suspension obtained from tissue source is particularly interesting to avoid MSC degradation with culture, to keep their maximum potential. Even if the markers are not completely specific, that allows to eliminate a big part of unwanted cells. Isolation techniques allow to enrich the suspension in MSC and give them more possibilities to show their capacities. However, MSC are also rare compared to progenitors, hematopoietic or differentiated cells. This strategy can only be used for applications that do not need too many cells, for local administration for example. When more cells are needed (often for systemic injection), they can be **amplified after selection**, but with the risk of drifting. Cells can also be **selected after amplification** with these peculiar markers, just before their use for the wanted application. The fact that some markers can only be found in uncultured MSC while others only appear with culture adds some constraints to the possible sorting strategies.

2. List of surface protein candidates for MSC selection

An extensive review of the different candidate markers that were associated to MSC and used to isolate them are gathered in **table 4**. In a global manner, the markers proposed in this table enrich the sample in cells with MSC phenotype, and, at varying levels, higher self-renewal and multipotency than unfractionated cells. However, each of them is associated with specific MSC subtype with specific function. The usefulness of a marker can greatly vary according to the context and the aim of the project. All these markers are not associated with the most primitive MSC. Some will select MSC more committed to a kind of differentiation.

These markers can be used alone or in combination in different orders. All the details of what each marker can specifically bring in MSC selection is not detailed here. For many of them, investigations are still ongoing to clarify their reliability, role and interest.

The composition of MSC subtypes is very intricated with overlapping phenotypes and the use of multidimensional parameters is essential to try to obtain a relatively homogeneous and defined sample. Common sorting strategy from initial cell suspension obtained from tissue source can consist on the exclusion of hematopoietic and endothelial markers (CD45, CD31, CD144), followed by positive selection of pericyte markers (CD146, NG2, or CD140b) or of more classical MSC markers (CD44, CD73, CD90, CD105) (144).

Table 4: List of surface markers used, alone or in association, for prospective isolation of human MSC.

Positive selection refers to cell selection when the marker is detected. Negative selection refers to elimination of the cells expressing the marker or differently said, selection of cells that do not express, or at very low levels, the marker. The last column does not detail the expression of markers in cancerous cells. (106,145–162)

Marker used for MSC positive selection	Tissue source	Other cells expressing the marker
CD9 (MRP-1, MIC3)	Synovial membrane	Macrophages, myoblasts, oocytes, variety of hematopoietic and epithelial cells
CD10 (neprilysin, CALLA)	Placenta	B and T cells progenitors, neutrophils, fibroblasts, breast myoepithelial cells, bile canaliculi, brush border of kidney and gut epithelial cells
CD13 (APN, gp150)	Bone marrow	Granulocytes, myeloid progenitors, endothelial cells, epithelial cells, a subset of granular lymphoid cells
CD26 (DPP4)	Placenta	T cells, lymphatic vessels, intestinal cells
CD34 (mucosialin)	Adipose tissue	HSC, endothelial cells, hematopoietic and endothelial precursors
CD44 (HCAM, Pgp-1, ECMR-3, 3G5)	Bone marrow, adipose tissue	Most cell types, epithelial cells, hematopoietic cells, retinal, cardiac and dermal pericytes
CD49a (integrin-α1, VLA1 α)	Bone marrow	Activated T cells, monocytes, NK cells, smooth muscle cells, neuronal cells, fibroblasts
CD49b (integrin-α2, VLA2 α, GPIa)	Bone marrow	Monocytes, platelets, activated T cells, megakaryocytes, neuronal cells, epithelial cells, osteoclasts
CD49e (integrin-α5, VLA-5 α)	Bone marrow	Thymocytes, activated lymphocytes, endothelial cells, osteoblasts
CD56 (NCAM)	Bone marrow	NK, NKT cells, neurons, muscle cells, neuromuscular junction cells, some stem cells
CD63 (MLA1, TSPAN30, LAMP-3)	Bone marrow	Activated platelets, monocytes/macrophages, endothelium, fibroblasts, osteoclasts, smooth muscle cells
CD73 (NT5E)	Bone marrow	Lymphocytes subsets, follicular dendritic cells, endothelial cells, epithelial cells
CD90 (thy-1)	Bone marrow, adipose tissue, synovial membrane, endometrium	Fibroblasts, endothelial cells, smooth muscle cells, nervous system cells, diverse stem cells including HSC
CD105 (endoglin)	Bone marrow, synovial membrane, cartilage, endometrium, Wharton jelly	Endothelial cells, syncytiotrophoblasts of placenta, activated monocytes and tissue macrophages
CD106 (VCAM-1)	Bone marrow, umbilical cord	Activated vascular endothelium, follicular and interfollicular dendritic cells, macrophages, non-vascular cells in joints, kidney, muscle, heart, placenta, and brain
CD117 (C-Kit)	Amniotic fluid	Hematopoietic cells, gastrointestinal system, melanocytes, germ cells
CD130 (gp130)	Bone marrow	All cell types
CD133	Mobilized peripheral blood, umbilical cord blood, bone marrow (controversial)	Hematopoietic stem cells, endothelial progenitor cells, neuronal and glial stem cells
CD140b (PDGFRβ)	Endometrium	Fibroblasts, smooth muscle cells, glial cells, chondrocytes

CD146 (MCAM, MUC18)	Bone marrow, adipose tissue, endometrium	Activated T cells, endothelial progenitors, endothelium, smooth muscle cells
CD166 (ALCAM)	Bone marrow, synovial membrane, cartilage, fetal tissues	Most tissues, activated T cells and monocytes, epithelial cells, fibroblasts, neurons
CD200 (MRC, OX2)	Bone marrow	B cells, a subset of T cells, thymocytes, endothelial cells, neurons
CD271 (LNGFR)	Bone marrow (only from BM MNC), amniotic membrane, chorion, adipose tissue, (not in WJ MSC)	Neurons, Schwann cells, melanocytes, neural crest stem cells, astrocytes, oligodendrocytes, a subset of lymphoid cells
CD309 (flk-1, VEGFR-2)	Bone marrow	Endothelial cells, hematopoietic and endothelial precursors
CD349 (frizzled-9)	Placenta	Fetal and adult brain, neural precursor cells, testis, eye, skeletal muscle, kidney
ALDH	Bone marrow	Epithelium of brain, liver, testis, eye lens and cornea, neurons, hematopoietic stem cells
D7-Fib	Bone marrow	Fibroblasts epithelium, myoepithelium, smooth muscle cells, some leucocytes
GD2 (ganglioside)	Bone marrow, umbilical cord	Cells from the central nervous system, peripheral nerves, skin melanocytes
HSP90β (STRO-4)	Bone marrow	Oligodendrocyte precursor cells, neuronal cells
Integrin αVβ5	Bone marrow	Fibroblasts, endothelial cells, vascular smooth muscle cells
MSCA1 (TNAP)	Bone marrow	Liver, bone and kidney cells, embryonic stem cells
NG2	Bone marrow (only in cultured MSC)	Oligodendrocyte progenitor cells, pericytes, other progenitor cell populations
SSEA-4	Bone marrow	Pluripotent cells, embryonic germ and stem cells, adult spermatogonial stem cells, cardiac tissue, ovarian surface epithelium, pancreas exocrine tissue
STRO-1	Bone marrow, adipose tissue	Nucleated erythroid precursors, endothelial cells
SUSD2 (W5C5)	Bone marrow, endometrium	Endothelial and perivascular cells, lung and kidney tissues, naive human pluripotent stem cells
Marker used for MSC negative selection	**Tissue source**	**Cells expressing the marker**
CD3	Peripheral blood	T lymphocytes
CD14 (LPS receptor)	Peripheral blood	Macrophages, neutrophils, dendritic cells
CD31 (PECAM-1)	Bone marrow, adipose tissue	Monocytes, macrophages, neutrophils, lymphocytes, endothelial cells
CD34 (mucosialin)	Bone marrow, peripheral blood	HSC, endothelial cells, hematopoietic and endothelial precursors, adipose MSC
CD45	Bone marrow, lung, adipose tissue	Lymphocytes, antigen-presenting cells
CD140a (PDGFRα)	Bone marrow	Embryonic tissue, fetal BM MSC
CD144 (VE cadherin)	Adipose tissue	Endothelial cells
CD235a (glycophorin A)	Bone marrow	Erythroid precursors and erythrocytes
Lin-	Bone marrow	Hematopoietic lineage cells

C. MSC surface markers related to functionality

Among the different markers that were used to characterize and select MSC, some were more deeply investigated and could be associated to specific functionalities. Many of these investigations rely on cell sorting but some markers could be highlighted with other means too. Associations could also be made with whole samples or with separate assessment of different colonies of the same initial sample.

Interestingly, in an old study from 2001, associations between MSC phenotype parameters and functionality were already proposed. Filters were used to separate small cells from bigger cells and the small cells displayed better proliferation rate and differentiation potential. Then they compared the surface proteins that were differentially expressed and found that small cells expressed 4 proteins that were not found on bigger or more mature cells: the vascular endothelial growth factor receptor-2 (FLK-1), TRK (an NFG receptor), CD71 (transferrin receptor), and annexin II (lipocortin 2). This study also revealed that cell size was not a sufficient mean to obtain homogeneous MSC, as several of the surface markers studied showed variable staining among the small cells (163).

In a different manner, large scale studies that focused on the gene expression program of MSC to understand the networks controlling MSC functionalities have highlighted two surface markers that may be strongly implicated in MSC proliferation: **CD200** and **CD157** (65). Other studies on gene expression also highlighted a set of surface markers that were overexpressed in cells able to migrate in a chemotactic assay: CD202, CD146, CD73, CD44 and CD106 (mRNA level) (164).

Another study with an interesting panel of samples could reveal correlations between MSC functionalities and the protein expression of CD119, CD146 and HLA ABC (n = 31, except for CD119, n = 25). Many other markers could also be correlated to some MSC functions but these 3 markers were those that were found consistently at different levels. They were all associated with MSC clonogenicity and proliferation rate. CD119 and HLA ABC were also more expressed on smaller cells and CD146 decreased with donor age (124).

Another study highlighted the interest of CD106 that was found on BM MSC with the best clonogenicity, proliferation rate and multipotence of the samples (n=3 for most experiments) (165). These cells were also characterized by better DNA stability and less senescence (165). CD106 was also associated with better cell motility and migration, both with experiments of passage through porous membranes and with *in vivo* administration to follow lung entrapment. **Importantly, the authors highlighted the interest to separate the most potent cells from the others**. Indeed, their data showed that when they are let together, the most rapidly dividing cells were inhibited by the presence of those with low proliferation rate, which were also shown to undergo senescence prematurely. For this, they made coculture experiments, by seeding together rapid expanding cells with cells with low proliferation rate in equal numbers. After 4 weeks of coculture, the proliferation of the rapid cells was greatly reduced compared to what was obtained with the rapid cells alone (165).

There are many studies on different markers of this kind but the functionalities tested remain scarce and there are few studies about *in vivo* efficiency of these markers. To go further in these investigations, the potential "functional markers" would be interesting to add to the minimal criteria proposed by ISCT to make a better MSC characterization and to refine the possible links with specific functionalities. A selection of the markers that were selected for their potential for this thesis work is gathered in **table 5**.

Table 5: Selection of surface markers expressed at varying degrees on MSC depending on their functionality.

Definition of the surface markers with variable expression on MSC		Potential of MSC expressing more the marker
CD146 (MCAM, MUC18)	Adhesion transmembrane glycoprotein found on endothelial cells, pericytes, T and B lymphocytes, smooth muscle cells, and dendritic cells. Its ligand is laminin-α-4. It is implicated in intercellular and matrix adhesion and extravasation. Also implicated in signal transduction, proliferation, senescence, differentiation and migration (166).	*In vivo* self-renewal, multipotency, migration (20,167)
CD200 (OX2)	Glycoprotein involved in myeloid cell regulation and macrophages inhibition.	Immunomodulation, proliferation (65,106,111,168)
CD106 (VCAM-1)	Adhesion protein that binds to integrin-α4β1 (VLA-4). Implicated in leucocytes adhesion, to vascular endothelium and in signal transduction. Overexpressed when in an inflammatory environment.	Immunomodulation, pro-angiogenic, proliferation, clonogenicity , migration / motility (124,169,170)
CD71 (TFRC)	Transferrin receptor. Expressed by all cell types.	Self-renewal, proliferation, multipotence (124,163)
CD140b (PDGFRB)	Beta receptor for PDGF. Implicated in regulation of cell proliferation and differentiation	Precocity (fitter cells) (124)

Among these different markers, one of the most significant is CD146 that has attracted the greatest attention of researchers.

The marker CD146 was particularly interesting as it was the only one to show robust stemness of BM MSC *in vivo* (20) (see part II. "MSC concept" - "MSC physiological role"). Large scale studies that have undertaken transcriptomic profiling of different cell types also highlighted that CD146 expression was the most robust marker to distinguish MSC from other cell types like hematopoietic progenitors and fibroblasts (171).

From bone marrow mononuclear cells (BM MNC), cell sorting with CD146 requires the combination of other markers to facilitate the sort and the obtention of MSC. Within the whole BM MNC sample, CD146 expressing cells may fall under the detectability level by flow cytometry. Starting with the depletion of CD45 expressing cells allows to see as high as around 1.2% of cells expressing CD146 in the sample. When these CD146 cells are sorted, it allows to reduce the presence of contaminating cells (74) and the sorted cells can

then give rise to CFU of which half are self-renewing and multipotent stem cells. This process allowing to obtain such a high proportion of stem cells can be considered as remarkable (20). Some reports also highlight that depletion with Lineage and CD34 could allow to obtain a population of CD146 expressing BM MSC after 3 weeks, at the end of passage 0, while this population is greatly reduced when whole BM MNC are used (74).

An extensive review of cell sorting experiments showing the interest of CD146 high expressing MSC obtained from different tissue sources is presented in **table 6**.

Table 6: Review of the studies showing human MSC functional properties enhancement after CD146^{high} expressing cells selection.

Magnetic sort is made with magnetic beads associated with the corresponding antibody.
FACS sort is performed with a flow cytometer able to detect and sort cells according to fluorescence level detected with the corresponding antibody.
Bone marrow mononuclear cells, stromal cell suspensions and stromal vascular fractions are cells that are sorted before the plastic adhesion step.
Lineage(-) corresponds to the removal of CD3, CD14, CD19, CD38, CD66b, Glycophorin A tetrameric antibody complexes by crosslinking unwanted cells with red blood cells then removed by gradient density.
(+): positive selection (-): negative selection (Ø): No clonogenicity test made

Source of cells for the sort	Sort method	Culture medium (seeding density for single cell colony formation)	Superior functional properties of CD146^{high} cells compared to CD146^{low} cells or unsorted cells
Bone Marrow Mononuclear Cells	FACS sort Stro1(+) and CD146-FITC(+)	α-MEM 20% FBS (Ø)	Cell growth just after sort (172)
Bone Marrow Mononuclear Cells	Magnetic sort CD45(-) followed with FACS sort CD146-PE(+)	α-MEM 20% FBS (2 cells / cm²)	Colony forming efficiency just after sort, ***in vivo*** multipotency and **self-renewal** (implantation after culture expansion) (20)
Bone Marrow Mononuclear Cells	*Lineage(-)* Magnetic sort CD34(-) FACS sort CD146-PE(+)	α-MEM 20% FBS *clonal culture expansion with 1-10 cells / cm²*	Ability to grow in clonal conditions during 12 weeks, functional HSC support with increased colony output in 8 weeks long-term culture with MSC plated just after sort and irradiated (74)
Bone Marrow Mononuclear Cells	FACS sort CD34(-) and CD146-PE(+)	DMEM 10% FBS (Ø)	Endothelial tubular networks maintenance and improved endothelial sprout morphology (pericyte activity) (69)
Bone Marrow Mononuclear Cells	FACS sort Stro1-AF488(+) and CD146-AF546(+) followed by amplification	α-MEM 10% FBS (100 cells / cm² after 2 passages in cultures)	***In vivo*** proteoglycan deposition (173)
	FACS sort CD105-AF-PE/647(+) and CD146-AF488(+) followed by amplification		***In vivo*** collagen matrix production and proteoglycan deposition (173)

Bone Marrow MSC at passage 1	FACS sort CD146-PE(+)	DMEM-LG 20% FBS (Ø)	Chondrogenic differentiation, proliferation (174)
Vertebral BM MSC at passage 1 or 2	FACS sort CD146-PECy7(+)	αMEM 10% FBS + bFGF (Ø)	*In vitro* migration, *in vitro* GAG production upon differentiation, homing toward organ model of intervertebral disc degeneration (cells used just after sort) (164) CD146(-) cells showed better GAG synthesis rate and aggrecan production and better overall promotion of tissue repair **in the organ model**
Telomerized cell line derived from BM MSC	FACS sort CD146-PE(+)	MEM 10% FBS (Ø)	***In vivo*** bone marrow formation, *in vitro* and ***in vivo*** migration (167) CD146(-) sorted cells could produce more bone ***in vivo*** (but less bone marrow)
Wharton Jelly MSC at passage 3	FACS sort CD146-PE(+)	DMEM 10% FBS (Ø)	Multipotency (175)
Wharton Jelly MSC at passages 3-7	Magnetic sort CD146-Biotin(+)	DMEM 10% FBS (Ø)	*In vitro* and ***in vivo*** immunosuppression, ***in vivo*** cartilage protection (arthritis mouse model), persistence ***in vivo*** (in cartilage during 2 weeks) (92)
Umbilical cord blood MSC at passage 6	FACS sort CD146(+) (fluorochrome for sorting not specified)	αMEM 10% FBS (Ø)	Growth rate, telomerase activity, osteogenic and adipogenic differentiation, less senescence
Placenta MSC at early passage	Magnetic sort CD146-PE(+)	GMP DMEM 5% human serum 5% human platelet lysate (Ø)	Osteogenic differentiation, multipotency (176)
Fresh purified endometrial stromal cell suspensions	FACS sort CD146-PE(+)	bicarbonate-buffered DMEM/F-12 10% FBS (50 cells / cm^2 in gelatin coated dish)	Colony forming efficiency (just after FACS sort) (177)
Adipose stromal vascular fractions	Magnetic sort CD45(-) followed with FACS sort CD146-FITC(+)	ND	Hematopoietic stem cell support *ex vivo* (ability to sustain human HSC with multilineage repopulating capacity and self-renewal ability ***in vivo*** after co-culture with irradiated MSC passages 3 to 8) (178)
Periodental ligament MSC at passage 3	FACS sort CD146-FITC(+)	α-MEM 10% FBS (2 to 3 cells / cm^2)	Proliferation, colony forming efficiency, osteogenic differentiation (179)
Periodental ligament MSC at passage 2 or 3	FACS sort CD45-AF405(-), CD44-FITC(+), CD90-APC(+) and CD146-PE(+)	α-MEM 10% FBS (11 cells / cm^2)	Osteogenic and adipogenic differentiation, colony forming efficiency, proliferation (180)

This table allows to have a global view of the different results obtained with CD146 sorted cells. There remains a high variability between these different studies. Even among studies about MSC obtained from the same tissue source, there are often discrepancies. In fact, these studies did not try to answer to the same questions and did not use the same methods. These results can then be viewed as different potentialities that CD146 MSC can yield when certain conditions are fulfilled.

In some cases, it can also be seen that CD146 negative MSC have interesting properties, notably for differentiation. They may be more mature cells, closer to the state where they can secrete extracellular matrix molecules that can promote regeneration (164), while CD146 expressing cells may still be more plastic cells (167).

D. Limits of subtypes consideration

1. Cell number

As mentioned above, cell selection increases the proportion of cells able to adhere and divide but also decreases drastically the total number of cells, especially with sorts with more than one label. A study reported that triple labelling with positive markers could not be feasible for example. With the sorting strategy that could be used until now, MSC samples remain highly heterogeneous (173). Culture also induces phenotypic changes and some of these markers are lost, such as CD271 (116).

2. Combination sequence

The consideration of the subtypes defined and obtained after the use of markers for prospective isolation must also be taken with care. As none of these markers is sufficient alone, the sequence of the different selection procedures is very important and a subtype of cells can be easily missed. That was the case for example of the seminal study with CD146 cells discovered in human bone marrow (20). It could have been thought with this study that all the stem cells were in the CD146 expressing cell fraction. However, a following important study could bring to light another kind of MSC with low CD146 expression. These cells also answered the stem cell criteria after *in vivo* implantation (60). The difference between the two studies was in the selection process. In both studies, they

deplete CD45 expressing cells. In the latter, they add CD271 positive selection before sorting CD146 high and low cells, enabling the discovery of the CD271+CD146^{low} rare subset. With the selection of the first study, this subset was maybe representing a too little portion of cells to be detected. **In the second, the enrichment method made the CD146^{low} population detectable in CFU experiments**.

3. Markers fluctuation

Interestingly, in a similar manner as it was shown for HSC after sorting with a surface marker, MSC can in some cases regenerate the initial expression profile of the marker as in the population before the sort (see part I. "The path to understand MSC research" – "Stem cells heterogeneity as a fundamental property"). It was shown with Sca-1 protein (the same surface marker as in the HSC study) in mouse BM MSC. It can occur within a few days only or longer time after extensive amplification *in vitro*. In this case, the global expression level of Sca-1 is also reduced. This phenomenon was associated with a positive feedback system implicating histone epigenetic modifications regulating promoter and gene transcription. Persistent epigenetic modifications were also shown to affect cells of donor of older age and this regulation system. In this kind of set up, MSC are regulated to undergo state fluctuations and develop a heterogeneous expression of the marker. With these markers, selected MSC should be used directly after sort and not grown in culture, otherwise, the same population as before the sort will be regenerated (181).

Another case that can happen is given by an example of study about CD146 MSC sorting. After seeding the cells negative for the expression of CD146, the cells started to acquire CD146 expression after some time in culture. However, this expression did not allow the cells to acquire the properties of the initially CD146 positive cells, notably for angiogenic function (72). The association between the expression of a marker and cell functionality is then effective only in specific conditions that must be considered in sorting strategies.

To note, differentiation induction also decreases the expression of many of these markers (including CD49b, CD73, CD105, CD146, and CD200) (106).

4. Heterogeneity benefits

It could be thought that an ideal isolation technique of mesenchymal stem cells would allow to obtain a sample with 100% of uniformly multipotent cells able to self-renew. This would be demonstrated with single cells derived cultures assessed *with in vivo* serial transplantations (61). However, even if it was possible, such cells may not be the ideal sample to use for specific applications. Indeed, a mix of stem and progenitor cells at different stages, or cells already committed, could provide a better therapeutic efficiency, as it can be the case for hematopoietic cells transplantation. All of this remain to be tested, and for this, the mix of cells constituting MSC samples needs to be more precisely characterized when these cells are used and, when adequate, with sorting strategies.

For all kinds of MSC, the observations evoked in the previous parts have shown the importance of the phenomenon of senescence. The following part is dedicated to give the necessary elements to better understand senescence and, at the same time, one of the major causes of MSC heterogeneity.

VI. Senescence

The presence of senescent cells in MSC samples represents an additional cause of heterogeneity. This part will come back on generalities about senescence and will give some elements more specific to MSC. The first sections remain more descriptive, centered on senescence, its related mechanisms and markers, while the functional changes of MSC when they become senescent are addressed as a second step.

A. Definitions

As for stemness, senescence cannot be described by a rigid definition. The senescent state is related to signaling, metabolic and cytoskeletal changes and results in a reduction of MSC resistance to stressors. It ultimately leads to an irresolvable disability to maintain chromatin integrity and persistent cell cycle arrest. Senescent phenotype is the result of both intrinsic and external factors, with the contribution of stochastic events. The precise mechanisms however can differ according to the triggers and different types of senescence can occur.

1. Senescence *versus* quiescence

Both senescent and quiescent cells stopped dividing. Both can stay in culture even after several months of growth arrest. They can however be distinguished by several features.

Senescent cells cannot have the specific function they were made for initially and cannot respond to mitogen stimuli anymore. Their behavior changes and they develop a high metabolic activity leading to increased biomass and abundant production of stress granules.

Quiescent cells, for their part, are resting cells with low metabolic activity and reduced protein production compared to active cells. They remain able to enter the cell cycle when stimulated. Quiescence mechanism also rely on different kinds of cell cycle inhibitors ($p27^{KIP1}$) than senescence (33).

2. Different types of senescence

The first type that was described is replicative senescence. It originates with the works of Hayflick with cells in culture that were shown to stop dividing in culture after a limited amount of passages. It was then shown that cells could undergo senescence prematurely, before the threshold of replicative senescence. This can happen when oncogenes are overly activated and then relate to oncogene-induced senescence. Premature senescence can also be the result of excessive stress such as DNA damage or metabolic stress. A more recent type of senescence was also described in normal development and can be called developmental senescence. Indeed, accumulating evidences show that senescence is a normal cell state necessary during development and tissue repair. Senescence is also thought to be a protective mechanism to prevent tumor and cancer development. For this, it can be generalized that when senescent cells are only present transiently, they may have beneficial effects, while when they remain and accumulate in a chronic manner like during aging, they can become harmful and associated to disease **(figure 24)**.

3. Senescent cells interactions

Senescent cells are resistant to apoptosis and have an important metabolic activity. They are able to secrete many active factors that are gathered in the concept of "senescence associated secretory phenotype" (SASP) (182,183). The SASP is notably composed of growth factors (TGF-β, PDGF IGFBP), cytokines (IL6, IL8), matrix-remodeling proteins (MMP), receptor antagonists and decoys. The composition of the SASP can vary according to the cell type and to the trigger of senescence. These factors can act both locally and systemically.

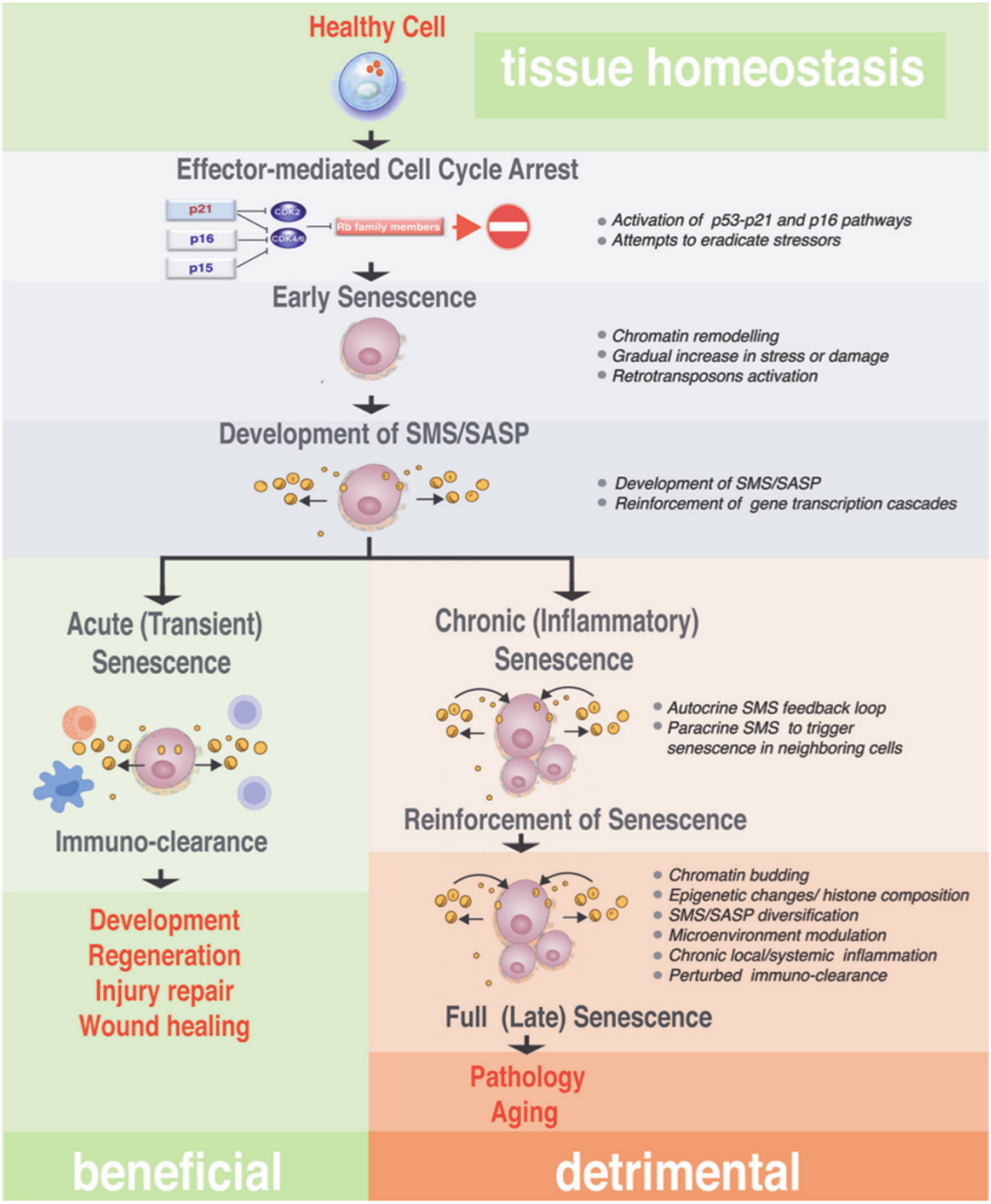


Figure 24: Diagram of the different possible stages of senescence, from early senescence to either acute or chronic senescence.

- Early senescence is mediated by cell cycle inhibitors. At this stage, senescence is still reversible by changes of effectors in the pathway leading to cycle arrest. Early senescent cells appear positive to β-Galactosidase staining but do not all secrete complete senescence messaging secretome (SMS).
- Senescent cells are normally eliminated by the immune system. This acute senescence is presumed beneficial and is necessary for normal development, healing and repair.
- Senescence can become chronic when the immune clearance is impaired, for example with age, and with the accumulation of molecular damages on proteins and DNA that the cell fails to repair. This prolonged arrest leads to an irreversible state and senescence reinforcement driven by epigenetic changes. Cell cycle genes become fully inactivated and inflammation pathways are activated. This causes an increase in the secretion of inflammatory molecules that can act locally or systemically. (184)

On one hand, SASP factors can be of use to promote tissue remodeling and regeneration and to prevent tumorigenesis. These mechanisms are dependent on other cells that are recruited and activated thanks to the senescent cells' communication. The SASP can alert and recruit immune cells that have also the ability to clear senescent cells. The cells implicated in this mechanism were notably NK cells, cytotoxic CD8 T lymphocytes and CD4 T cells that in turn stimulate macrophages to clear senescent cells. While the senescent cells are removed, the secretions they produced can also promote cell plasticity by inducing the expression of stem cells markers in surrounding cells, boosting the replacement of the removed cells. When inflammation remains local and controlled, it is beneficial, favoring tissue regeneration instead of fibrosis after wounding (184).

On another hand, the SASP can also become harmful when persisting longer times and inducing too much inflammation. In this case, it hinders the progress of regeneration and can even potentiate angiogenesis and tumor growth. The factors secreted by senescent cells were also shown to have the potential to reinforce the senescence state of the secreting cell itself and to propagate senescence phenotype to neighboring cells. This effect will vary according to the concentration of the factors, the duration of the exposure and the type of the cells.

Several studies investigated the SASP of MSC. With replicative senescent BM MSC, the secretions of IL6, IL8, ICAM1, OPG, CSF1 and SPP1 were found to increase. On the other hand, the secretion of the factors CXCL12, SAA1, and TGF-β1 were found to greatly decrease with passages. These proteins are implicated in attachment, migration, immune cells function regulation and inflammation. Other studies on MSC SASP used irradiation to induce senescence. A study followed BM MSC brought to senescence by X-ray irradiation. These senescent MSC showed strong increase in the secretion of GRO, IL8, IL12 and MDC (136). Another study used ionizing radiations to induce senescence on BM MSC and report senescent MSC secreted more than ten times the levels found in normal MSC of leptin, TGFα, IL8, eotaxin, IFNγ, VCAM1, IFNβ, IL4, and MCP1. These factors are known to be associated and to stimulate inflammation (185).

The SASP is also dynamic, changing over time and sensible to variations of the environment. Considering the potentialities of SASP factors, the ambivalent properties of the SASP may also be a part of the complex mix of elements responsible for the controversial results obtained with MSC applications. The presence and formation of senescent MSC may indeed play an important and ambivalent role, especially in immunomodulation (184).

B. Inducers of senescence

1. Replicative exhaustion

MSC senescence establishes in a progressive manner until loss of MSC functions. Cells undergoing replicative senescence in culture are characterized by progressive decrease of proliferation rate associated with morphological changes and enlargement. Additionally, there is an increase in cell granularity and lysosomal content. These lysosomes contain increased levels of the β-galactosidase enzyme that is widely used to highlight senescent cells. The expression of tumor suppressors $p16^{Ink4a}$, $p21^{Cip1}$, p53, and/or Rb is also increased and cells lose the ability to synthesize and repair DNA. While in this state, senescent MSC can resist to apoptosis and remain in the sample for a prolonged time (184).

a. Telomeres shortening and telomerase

Telomeres are composed of repetitions of the DNA sequence TTAGGG and form special structures at the end of chromosomes. They protect DNA from degradation and fusion with other chromosomes, but during each division, the last nucleotides of telomeres cannot be replicated. Sensors in the cells, like the protein kinases ATM and ATR, are able to detect DNA damage, in this case when telomeres are too short. This triggers the activation of the DNA damage response pathway. This pathway leads to the activation of repair mechanisms, and when the repair is not possible or fails, it can result in apoptosis or in the exit of the cell cycle mediated by the regulators p53 and $p21^{Cip1}$. The reasons of the orientation between apoptosis and senescence are not yet well known, but it depends on the kind of damage and the cell type.

Telomeres shortening may be one of the factors triggering MSC replicative senescence. Indeed, the decrease in proliferation capacity of BM MSC and the increase in

β-galactosidase staining were found to be associated with shortening of telomeres (184,186). Furthermore, some reports found that growth arrest occurred after telomeres reach a threshold of between 5.8 and 10.5 kb in length, which was also associated to DNA damage to the telomeric region (187). The hypothesis that telomere shortening is the leading cause of MSC senescence *in vitro* is also supported by the fact that it is possible to limit senescence and greatly extend MSC lifespan with the introduction of active telomerase with genetic engineering. This technique provides cells staying in a good state and that can grow to a similar extent as immortalized cell lines, far beyond what is possible with other techniques (except complete reprogramming toward iPSC) (71).

However, it seems also possible that replicative senescence occurs without detectable telomere shortening, on MSC with still long telomere ends (184). Indeed, as in most adult stem cells, several kinds of MSC can still express low levels of telomerase, more specifically in a transient manner during DNA replication. This may be sufficient to avoid excessive shortening and allows MSC to maintain long telomeres (188,189). Telomerase activity could be detected both in early and late passage MSC, even if for most cases, this activity was found to decrease during expansion (156,190). Higher levels were found in WJ MSC, and an increase was even found after the first passage (191). Some studies fail to detect telomerase activity, maybe because of the sensitivity of the technique used or because of other factors, such as the subtype of cells obtained.

Telomerase activity is typically repressed after differentiation, but its initial expression in stem cells, even at low level, was associated to differentiation capacities. A mouse model with telomerase knock-out have shown that murine MSC lacking telomerase lose differentiation capacity, highlighting a potential additional role of the low expression of telomerase for MSC multipotence (190). This process goes along with a strong shortening of telomeres upon differentiation. In BM MSC, this shortening was found to be even greater with cells differentiated after extended amplification (189).

b. Genetic drift and DNA damages outside telomeres

Telomere shortening is not the only mechanism that may be associated to what can still be called "replicative" senescence. DNA damages and errors in replication can accumulate in other locations along successive divisions. When these damages cannot be resolved, they lead to the activation of persistent DNA damage response pathway. This is accompanied by the deterioration of epigenetic regulation and chromatin structure. Endogenous retrotransposons can also be activated and cause more DNA damages (184).

Outside strong DNA damage, replicative senescence may also be caused by changes in genes expression program, that can itself be caused by changes in chromatin state caused by epigenetic regulations.

Interestingly, transcriptomic assessments made on MSC along their culture at several steps until reaching complete replicative senescence showed that the changes in their expression program are nonlinear, **progressive**, and start early, in cultures still looking like young cells. It means the onset of these changes in association with senescence preceded the functional changes (136). In BM MSC, these alterations were even found to start right after the beginning of culture (192). The transition occurred at variable times between different samples. At early passage, some cells can already display important molecular changes in association with senescence (136). In a study on WJ MSC, the first significant expression changes occurred after the fifth passage (with 27 cumulative population doublings), with a rapid accumulation after passage 9 (with 45 cumulative population doublings), senescence being reached variably after passage 10. Genes that are usually found to change with MSC senescence are implicated in cell cycle, replication, cellular movement, DNA repair, differentiation, stemness, inflammatory cytokines and immune response, senescence and apoptosis pathways and more broadly, associated with aging (39,68,136,193,194).

These modifications in expression could be related to DNA-methylation changes in regions associated with histones and lamins that were also reported. These changes also start early in MSC culture, during the exponential phase of amplification, and affect several developmental genes that were also found to be differentially expressed in later passages.

The high reproducibility of the DNA-methylation patterns indicate that replicative senescence and the functional changes underwent by MSC may probably be a regulated process instead of a consequence of genomic instability and random accumulation of cellular damages (195–197).

2. Stress-induced senescence

Cells exposed to high levels of environmental stressors during a prolonged time can undergo premature senescence independent from telomeres length. Common stressors that are reported to be able to cause this kind of senescence are ROS, drugs causing DNA damages, irradiation and hypoxia. The growth arrest can be transient, with the activation of p53 and $p21^{Cip1}$, and it can become irreversible with the activation of $p16^{Ink4a}$ when the stress persists.

In the case of MSC, when they function normally, they rely on glycolytic metabolism and contain an important antioxidant machinery, leading to low levels of ROS. Exposure to low ROS concentration is beneficial in this case and promote DNA stability, cell survival and proliferation. However, the successive divisions of MSC leads to a decrease of MSC defenses while ROS levels and oxidized proteins increase and become deleterious (119). Defenses mechanisms that were found to be affected in senescent MSC were a decrease of the activity of the major antioxidative enzyme SOD and a decrease in MSC responsiveness to heat shock proteins (HSP). HSP are implicated in the trigger of protective mechanisms in case of stress and play a central role for proliferation and differentiation. Additionally, an important transcription factor intervening in response to oxidative stress to activate various antioxidants and promote survival, NRF2, was found to decline with MSC senescence (187).

Several studies have reported that MSC can undergo senescence after exposure to anti-cancer drugs damaging DNA, irradiations and exposure to ROS. In these cases, MSC have been reported to strongly resist to apoptosis and preferentially take the path of stress-induced senescence, sustained by persistent DNA damage response activation. The senescent MSC had different metabolic activities according to the kind of stress they had been exposed to (184). They also undergo important cytoskeletal reorganization (185).

When cells are cultivated *in vitro*, they are subjected to important levels of various stressors that can favor this kind of senescence. Theses stresses include detachment from extracellular matrix and other neighboring cells or exposure to high levels of oxygen, nutrients and growth factors.

3. Oncogene-induced senescence

This type of senescence is induced by the overactivation of oncogenes or when tumor suppressors are inactivated, both risking to cause uncontrolled proliferation. It can occur both *in vivo* and *in vitro*. There are few studies of this type of senescence on MSC. Oncogenes were found to be related to MSC senescence in patients with systemic lupus erythematosus and in other patients affected by a kind of cancer (alveolar soft part sarcoma) (184).

C. Senescence markers

To be able to understand the effects that are commonly reported in research articles, it is important to have an overview of the marker that can be searched to study senescence. A variety of markers can be used to identify senescent cells and sometimes give some indications for the type of senescence. No one marker is specific and it is necessary to combine them to be able to reliably detect senescence **(table 7)**. However, there is no consensus on the required markers (33).

Table 7: List of common senescent markers, their interest and limitations.
(33,123,184,185,198–202).

Markers	Definition	Advantages	Disadvantages
Absence of proliferation	The consequence of stable cell cycle arrest.	Can be assessed easily with different techniques to follow DNA synthesis and cell proliferation (incorporation of nucleotide analogs, absence of the proliferation marker ki67).	Cannot be used alone because it is also found in quiescent or terminally differentiated cells.
Morphological changes	Protein degradation is impaired in senescent cells, causing an increase in content and size and changes in cytoskeleton.	Can be assessed by microscopy and flow cytometry without specific staining.	Not specific and difficult to put the threshold. May appear later than other markers.
β-galactosidase	The increase in β-galactosidase activity in senescent cells reflects the increase in lysosomal content.	Marker that is found easily in many different types of cells and widely used.	Difficulty to determine the threshold of positive staining. Not specific to a type of senescence. Many false positives with confluent or terminally differentiated cells. False negative in some cell types with weak β-galactosidase expression. Not necessary for senescence.
Lipofuscin	Residues of lysosomal digestion containing lipids.	Naturally fluorescent, it causes an increase of the autofluorescence of senescent cells.	Not sensible and specific enough.
$p16^{Ink4a}$	Inhibitor of the cell cycle progression.	Marker that can strongly increase in response to senescence stimuli, and persists and accumulates, in association with senescence maintenance and stabilization.	Not necessarily present according to the kind of senescence pathway and once senescence is established (notably oncogene-induced senescence). Single cells staining problems associated to lack of specificity of commercialized antibodies.
$p21^{Cip1}$	Inhibitor of the cell cycle progression.	Strongly increases in response to senescence stimuli.	Not necessarily found with senescence. The state of the cell can still be reversible. Mainly present during senescence initiation but may not persist.
γH2AX	After double-strand DNA lesion, the histone is phosphorylated.	Specific of a kind of damage and can give indication of the trigger of senescence.	Weak staining that can be difficult to quantify.
Telomeres length	Cells with telomeres critically short can be searched.	Specific of a kind of damage and can give indication of the trigger of senescence. Could be used in a prospective manner.	Many techniques commonly used are not precise enough to accurately determine telomere length. High variability between donors.

Karyotype abnormalities	Reveal genomic abnormalities by looking at chromosomes.	Can detect potential tumorigenic changes.	Small changes cannot be detected. No clear karyotype marker yet for senescence.
HMGB1	Important chromatin protein regulating gene transcription, translocated from the nucleus to the cytoplasm and secreted outside the cell when senescent (no change in transcription or translation).	Specificity of the mechanism, associated to hyperacetylation in the chromatin and inflammation. The relocalization occurs early in senescence development and is easily seen in fluorescence microscopy.	Not specific of senescence, can also be found after injury, after cell activation or in dying cells.
SAHF	Senescence-associated heterochromatin foci are regions of chromatin becoming dense and unattainable for transcription.	Associated to stable senescence and epigenetic drift. Can be seen roughly with DAPI staining or more precisely with specific proteins staining.	When assessing markers associated to DNA content (methylations, histones), it should be kept in mind that some senescent cells may be stopped after DNA replication and contain 2 times more DNA than other senescent cells.
H3meK9	Histone methylation that was found to increase with oncogene-induced senescence in human fibroblasts.	Give some insight about one of the possible mechanisms associated to the kind of senescence studied.	
DNA methylation	Search of a specific epigenetic signature of changes in DNA methylation associated with senescence.	Highly reproducible. May reflect variations between samples and in subpopulations not seen with growth curves.	Very variable between different colonies in the same culture, not synchronously regulated. Validations on the specific cell type must be performed to identify a refined set of genes that are consistently modified before it can be used.
Global gene expression changes	Follow the level of expression of a wide panel of genes associated with senescence.	Fast quantification is possible with microarray techniques. Changes preceding other indicators.	
Lamin A	One of the most important protein for the architecture of the nucleus whose structure changes with senescence.	Functional importance in association to chromatin organization and transcription implicated in aging and senescence.	The changes can be subtle to detect. May not be altered in replicative senescent cells or after DNA damage.
Lamin B	An important mediator for the organization of other lamins, implicated in senescence and proliferation regulation. Its expression decreases with senescence.	Found early in multiple types of senescence before the onset of changes in other lamins, in morphology, in β-galactosidase and SASP.	Lamin B is not found in some cell types.
Apoptosis resistance	Increase in the expression of anti-apoptotic proteins like BCL-2.	Can give an idea of the possible persistence of the senescent cell.	No specificity.
SASP	Activation of the secretion of different pro-inflammatory factors when the cell become senescent.	Give elements about possible effects on neighboring cells.	The onset takes more time than other markers. There are no specific secretions signature defined yet, they are very variable according to the cell type and trigger of senescence.

With MSC as with other cell types, the marker the most commonly used is the increase of β-galactosidase activity. However, this marker was found to be insufficient, for example by not being found while other markers indicated the senescence state of MSC. It can be problematic for studies assessing senescence with just this marker (71). A close alternative to β-galactosidase was proposed in a study to better discriminate the senescent cells from the others, but it was not tried on MSC yet. This marker is the enzyme α-fucosidase. It is upregulated at the mRNA and activity levels in association with the different types of senescence (203).

Senescent MSC were also shown to display the classical increase in $p16^{Ink4a}$ expression, but in some cases with unchanged levels of p53 and $p21^{Cip1}$. To note, a baseline expression of $p16^{Ink4a}$ is present in BM MSC but at a low level. This baseline expression was not detected in WJ MSC, but $p16^{Ink4a}$ still appeared with senescence (71).

D. Functional changes occurring in senescent MSC

MSC undergo senescence with *in vitro* culture but also with chronological age. Several studies have searched the impact of senescence on other MSC functionalities. Senescent MSC were shown to display classical markers of senescence as described above. MSC changes when they become senescent can also be similar to changes that were found on MSC with increased age that were reviewed in a previous part, but they are not exactly the same. It was indeed shown that MSC freshy isolated can contain a high proportion of senescent cells especially when taken from aged donors, but these cells can be impaired for some functions by other means too. In this part, in a complementary way, a focus will be made on studies with senescent MSC observed in vitro (71). However, contrary to some studies addressing the effect of donor age, the number of samples studied in functional assays with replicative senescent cells are often very low (under n = 5). It makes it difficult to precise the level of association (with correlation for example) between parameters and to decipher the variability existing between different samples.

1. Proliferation and clonogenicity

In most studies, MSC of different sources are found to be able to proliferate several months, until an average of 12 passages and a maximum of 50 cumulative population doublings before reaching complete replicative senescence (39,74,136,192). There are very important variations of these parameters between different donors (72). In particular, MSC from old donors exhibited a decreased maximal life span that can reach near half of the cumulative population doublings obtained with the cells from young donors (204).

Studies at the clonal level showed that no more than 1/3 of the colonies obtained at the start of the culture would reach more than 25 cumulative population doublings (65). A study following MSC with individual cell tracking confirmed that a minor proportion of MSC were highly proliferative and produce the majority of progenies, meaning a small number of cells divide more than what could be thought initially. Their proliferation rate decreases progressively and they also finish by becoming senescent in turn. That means that when MSC are extracted and amplified in culture, they are more exposed to damage than *in vivo* and can undergo the equivalent of decades of *in vivo* aging (112,205).

For studies using mathematical modeling for MSC growth, it can seem that the initial presence of senescent cells may not have strong impact as the proliferation of the other cells outgrow their number rapidly and seems to make them negligible. However, other experiments with fibroblasts and epithelial cells could give some evidence that the presence of senescent cells at early stage of culture can limit the growth potential of the whole sample (71).

Concerning clonogenicity, when approaching senescence, it is often let aside because MSC ability is almost completely lost. Some report the capacity of 1% of cells to form little colonies with senescent BM MSC and adipose MSC (136,195).

2. Phenotype

Along *in vitro* culture, MSC undergo progressive phenotypic changes before complete growth arrest. One of the most noticeable change is increase in cell size (68,206). Simultaneously, the expression of most surface markers was found to decrease in several

studies, while autofluorescence and cell "granularity" increase, as assessed by flow cytometry (39,130,192). No appearance of new markers nor complete loss was found to be associated with MSC senescence (71).

More specifically, significant surface markers such as **CD146** and **CD71** were found to be less expressed among MSC undergoing replicative senescence (74). However, in some cases, CD146 expression was found to stay stable between early and late passage (194).

On the other hand, the surface marker **CD264** was found to increase in senescent BM MSC obtained from aged donors (207). For another marker, the leptin receptor (**CD295**), its expression at the RNA level increased in passage 5 cells compared to passage 1 cells and was even inversely correlated with the remaining potential of proliferation before replicative senescence. This increase in expression was also found in cells cultivated with 20% O_2 compared to 3% O_2 and with cells after they reached confluency. The protein, for its part, was highly expressed on a population of dying cells. The authors hypothesized that proliferation decline in aged donors and later passage cells may be caused by the progressive increase in dying cells retrieved from the cell pool, then causing the remaining cells to undergo senescence (188).

In one sample of MSC that may be adipose MSC brought to replicative senescence (the tissue source is not clearly mentioned), the percentage of cells expressing CD106 surface protein was found to decline greatly in late passage cells. CD106 may be important for interaction with endothelial cells and for MSC homing. The authors also found a link between CD106 and hyaluronan synthesis in MSC. Indeed, it seems the production of hyaluronan is also greatly reduced in senescent adipose MSC. Additionally, the authors found that knock-out of hyaluronan synthases in early passage cells caused loss of CD106 expression while supplementation in hyaluronan could restore CD106 protein expression in senescent MSC. This finding can give some ideas of investigations to do when considering MSC migration abilities and culture supplements that can be brought (208).

3. Metabolism

With MSC from different tissue sources and with different culture conditions, the expression of the initial genes implicated in MSC metabolism were robustly found to decrease. This may be one of the causes of the impairment of high passage cells (193). Indeed, it seems that the metabolic phenotype of origin of MSC is glycolytic but it shifts toward more oxidative phosphorylation when cells are placed in culture. This change can contribute to the onset of replicative senescence on MSC (209).

4. Differentiation

In a general manner, MSC ability to differentiate decreases with progress toward senescence (68). However, in some cases, a semblance of chondrogenesis, osteogenesis and generation of adipocyte-like cells could be conserved (39,155,194). Some reports even showed an increase in the potential to take the way of osteogenic differentiation (192). Some discrepancies remain between the currently available reports and may be caused by the limitations of the techniques used. For example, alizarin red staining used to highlight osteogenic differentiation. This can give situations where more cell death gives the false impression of more differentiation (185).

5. Adhesion, migration and survival

With time in culture and senescence drawing near, MSC ability to migrate decreases, in association with the decline of the expression of chemokines and adhesion molecules, and of the ability to react to chemoattractants (155). Some studies also report a decreased adherence to plastic surfaces (71).

A study *in vivo* with murine MSC showed a decline of homing to the bone marrow and spleen with senescence (68). Another *in vivo* study showed that the survival of pre-senescent WJ MSC implanted intramuscularly in mice was also shown to be compromised compared with early passage cells (193).

The decrease in migration capacity of senescent MSC might be related to changes in surface marker expression, such as the decrease in CD106 aforementioned (208).

6. Immunomodulation and hematopoietic support

With the classical assays of mixed lymphocyte reaction, a marked decrease in immunosuppression was reported between fit and replicative senescent BM MSC or close (at least passage 9) (136,210). Cytokine secretions of these senescent MSC were also found to be defective for several important factors important for their immunoregulating function. Notably, IDO and kynurenine, that are very important for MSC effect on lymphocytes proliferation, were downregulated (210). These changes in secretion profile make senescent MSC more at risk to promote inflammation and tumor growth when implanted.

With another type of senescence induced by ionizing radiation, MSC could keep some capacities to limit lymphocytes proliferation and regulate macrophages *in vitro* but had a strong reduction in their migration capacity after inflammatory stimulation, although senescent MSC equally accumulate in the lungs *in vivo* (210). It was additionally shown that the use of MSC brought to senescence by irradiation can abrogate their therapeutic effect in an *in vivo* sepsis model. In this case, the authors incriminated preferentially the loss of migration capacity of the irradiated cells instead of the decline of immunomodulation capacity (211).

In a similar manner, a study using replicative senescent BM MSC this time, showed a decrease in their capacity to support hematopoietic progenitors *in vitro* and HSC engraftment in a mouse model (212).

Reports of clinical trials of MSC for GvHD also point out the possibility that MSC senescence was one important factor implicated in therapeutic failure (83).

7. Overview

The principal changes in functionality undertaken by senescent MSC that can alter their therapeutic potential are gathered **figure 25**.

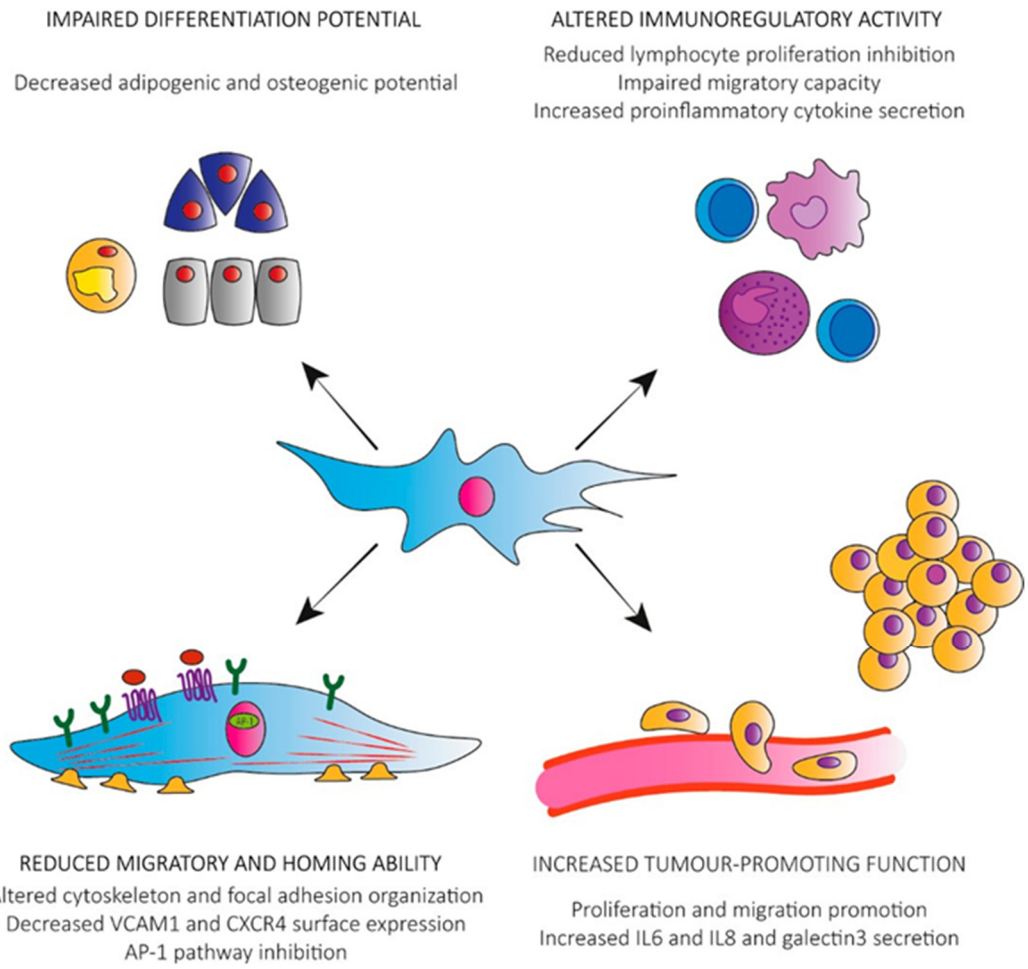


Figure 25: Overview of the main changes of functionality that happens when MSC become senescent (185).

Rather than complete therapeutic failure, the consequence of the presence of senescent MSC may more be responsible of variable clinical outcomes. For specific applications, senescent MSC may even unveil interesting features.

E. Potential of senescent cells

Surprisingly, senescent cells and their secretions can also be interesting for specific therapeutic applications.

1. Tissue repair

One of the most interesting aspect of senescent cells is their secretions. A mouse model with elimination of senescent cells could show that they were necessary for optimal wound healing *via* the action of their secretions and more particularly of PDGF-AA (213).

Another interesting study on this aspect was made on humans with oral submucous fibrosis caused by exposition to areca nut and tobacco. Biopsies could be taken at different stages of the disease and senescence of fibroblast was followed and studied more in depth. They could highlight an accumulation of senescent fibroblasts with the progression of the disease by a mechanism involving ROS. These senescent cells were said to possibly ameliorate the tissue condition thanks to their secretions of MMP before they are cleared by the immune system (214).

2. Immunomodulation

An interesting effect was reported with WJ MSC that were brought to passage 15 with 10 000 cells/cm² for seeding density at each passage (meaning they were let less time in culture than usual passages with smaller seeding densities). At this stage, the proportion of senescent cells had increased a lot, even if all cells of the samples were not yet senescent. These cells surprisingly displayed stronger immunosuppressive activities when compared with passage 3 cells, even if the size of the effect was not very high. In association to this finding, the authors reported a strong increase in IL6 production in the passage 15 cells that may be one important factor influencing MSC immunomodulation (215).

Another study reported an increase in expression of genes with antimicrobial functions in replicative senescent WJ MSC (193).

3. Control after administration

Senescent cells are also viewed as interesting for applications where their action is wanted to be transient, because there is less risk for the cells to proliferate inadequately. This could be of interest for applications where senescent MSC can still display interesting functionalities, like for immunomodulation or to promote tissue repair.

4. Interesting model for *in vivo* aging

The genetic and epigenetic changes showed some similarities between cells brought to replicative senescence *in vitro* and cells observed *in vivo* in aged donors (196). The characteristics of the cells and changes in functionality reviewed in this work also display some similarities. Replicative senescence is above all a mean to obtain a large enough sample enriched in senescent MSC to better observe their specific features.

Senescence is an important mechanism to consider that can have a strong impact on MSC therapies. A better understanding of senescence could even lead to the valorization of the potential beneficial effects that senescent MSC could provide. For this, surface markers that could help to identify and sort senescent MSC would be of great interest.

VII. MSC limits and potential for future therapeutic strategies

In a global manner, MSC have proven to be very plastic cells. Their phenotype and potential are very flexible. Because of this, much attention and meticulousness are required to be able to obtain reliable results. Until now, MSC use in therapy provided limited and short-term benefits in most studies. However, many paths remain to be explored before leaving them aside.

A. MSC limits

1. MSC complex biology

With thousands of publications related to CSM, a lot of information is shared but it can be difficult to extract relevant information. Unfortunately, the quantity is not associated to the quality of the research in the field. An important issue is that teams working on MSC applications can quickly be overwhelmed and do not always have the possibility to dive into in-depth understanding of the cells they use. The easiness to follow the minimal criteria is also tempting to satisfy preclinical requirements while it is far from sufficient. Insufficient consideration of the inherent biological properties of the cells could result in too hasty translation toward unreasonable targets and clinical failure (61). The many hopes associated to stem cell therapies can make lose sight of the reality behind these cells.

MSC **current** possible therapeutic action is not associated to extended engraftment and differentiation in cells that regenerate a tissue. Instead, it seems they can secrete bioactive factor that have a therapeutic potential for immunomodulation or to stimulate the patient cells to regenerate the tissue. For this reason, Caplan proposed to call MSC "Medicine Signaling Cells", especially to avoid patient misconceptions.

Another aspect linked to MSC biology is that it may not be their physiological behavior to have high proliferation rate. That is a critical aspect because MSC rapid and large proliferation wanted for clinical applications. However, it makes it difficult to follow and obtain sufficient quality from MSC amplified too much, too fast, notably in large scale batches and in bioreactors.

2. Experimental models limitation

Animal models are essential, but in MSC field, some important differences between human and other species MSC lead to significant difficulties. Some of the encouraging results associated with MSC in murine models of disease have not yet be translated to equivalent results in phase III clinical trials.

One of these major differences that led to strong doubts about MSC use for therapy is the propensity of murine MSC to transform and to become tumorigenic in normal culture conditions. This instability is now confirmed to be highly improbable in cultured human MSC. The attempts to induce malignant phenotype by long-term expansion did not work. Mouse MSC amplification require a longer time than human MSC and there are some differences in their phenotype. For immunoregulatory mechanisms, murine MSC rely more specifically on iNOS while human MSC depends on IDO. These two molecules are associated with different pathways implicating different inflammatory molecules. Human MSC receptors also do not cross react will all murine cytokines (216). For example, IFNγ is species specific, human MSC cannot be activated by mouse IFNγ (96).

Mouse models also display several differences compared to the clinical context. For many *in vivo* studies, MSC used are fresh and metabolically active, and often in autologous systems. In the clinical set up, the tendency is, for many different applications, to use cryopreserved cells, just after thawing in an allogenic system. With these settings, cell viability and functionality are reduced. That result in most cases in better conditions in mouse models. The doses used for the mouse are often compared to what can be done in humans. It is around 50 million cells per kilogram for mouse and usually no more than 12 million per kilogram for humans (97). Intravenous injections of cells are also especially difficult in mouse because they can easily cause embolism.

It is interesting to see the heterogeneity that is also marked within mouse MSC. Surface epitope of bone marrow MSC were found different between different strains. This was associated with different media requirement for optimal growth (217).

The phenotype stability of the cells is also under question. For example, with experiments that were trying to show that MSC could differentiate and remain in the tissue to repair, the presence of differentiated cells coming from MSC could only be seen transiently.

There is also too much reliance on results obtained *in vitro* that are not associated with the cell functionality *in vivo*.

3. Heterogeneity and standards

The ISCT criteria were made to standardize MSC research with a minimal baseline. However, the verifications proposed could lack some elements to avoid artifacts and to really be able to bring homogeneity between MSC studies. They are very stable and do not correlate with different MSC functionality. However, they can be used as a mean to identify the cells but not as quality validation. In fact, the assays proposed, like the differentiation assay *in vitro*, suffer common artifacts and do not correlate with *in vivo* properties of the cells. Furthermore, the standards for conducting, interpreting and validating these assays were not clearly provided. Furthermore, to show real multipotency, it should be made from single cell derived culture, otherwise the differentiation observed could be the result of several unipotent committed progenitors. Some authors encourage the use of *in vivo* models to study BM MSC properties (64).

Despite standardization efforts, culture conditions remain highly variable between studies and even between runs. The media used to amplify the cells are essentially supplemented with fetal bovine serum or human platelet lysate and their content is not precisely defined, but there is no satisfying alternative. The experimental protocols being very variable, results are often inconsistent.

Even when a standardized protocol is used, a very high variability between samples processed the same way can be found, especially in key functions involved in MSC therapeutic actions. That means the samples were initially different and that can be linked to differences between donors.

To this variability is added the MSC cell population heterogeneity. No method achieves isolation of pure MSC population, and the mix of different subtypes of cells used in different applications remain poorly defined. When MSC are amplified, they can also undergo replicative exhaustion and senescence. This is notably a risk when trying to produce large amounts of cells at the industrial scale. Samples can also harbor a variability according to donor characteristics such as age, genetic traits, and medical history. For the production of MSC products, few donors are used to produce many cells and there are no precise selection criteria. These different levels of heterogeneity sum up and make it difficult to understand the biology and potential of the cells.

When going up to the clinical scale, many other factors crop up. For example, MSC used after cryopreservation will display a further different behavior. They are impaired the day after thawing and can have decreased immunoregulatory capacities. They are cleared more rapidly by immune system after infusion (218). The variety of patients to be treated is also a major part of the heterogeneity that must be dealt with.

These various difficulties make translation from research to clinics tough. MSC use is associated to high level of complexity with much more parameters than classical drugs to take into account.

4. Adverse effects

The overall safety of allogenic and autologous MSC therapy is validated in most of clinical studies, allowing clinical research to go further. However, there is still a limited hindsight on long-term effects. Some acute problems could also be found and should be anticipated.

The example of another kind of stem cell therapy shows that tumorigenesis can occur long time after cell administration. Eight years after olfactory mucosa cell administration in the spinal cord, a patient developed a large mass (128). MSC activities could also promote cancer growth. MSC may indeed favor tumor and metastasis formation by promoting angiogenesis. MSC could also be conditioned by the tumor environment and become tumor resident. Another potential harmful effect would then be associated with

their ability to recruit immune suppressive macrophages. These aspects remain poorly understood (96,219,220). MSC senescence is also important to take into account. With their ability to secrete a large set of bioactive factors (SASP factors), they could also promote cancer cells proliferation or migration (185). An interesting study with adipose MSC exposed to cisplatin, an anti-cancer drug, showed that it caused MSC senescence. These senescent MSC secreted several SASP factors, notably high levels of IL6 and IL8. In this state, they could favor the appearance of cancer stem-like cells and their resistance to cisplatin *in vitro*. In a mouse model, they also caused cisplatin resistance together with increased tumor volume (221).

Another important aspect reported in several cases was MSC immunogenicity. MSC can be lysed by NK cells and could also activate B-cells in specific conditions (222,223). Immunological compatibility of the provided cells and the donor can be essential in some cases, such as engraftment of bone marrow transplantation or for repeated administrations. The formation of antibodies against the transplanted cells (alloimmunization) and against fetal bovine serum used to cultivate MSC were also documented (81,97). These problems can lead to an increased cell clearance and decrease the efficacy of the treatments with multiple administrations.

For some applications, MSC could also disturb normal tissue function, for example when injected in the heart (97).

5. Economic model

It is difficult for the establishments working on cell therapy products to maintain an acceptable economic balance considering the extremely high cost to develop and produce these treatments.

The example of the product Prochymal, allogeneic cultured BM MSC for pediatric acute GvHD, is representative of the difficulties of the field. This product obtained the marketing authorization of Health Canada the first time in 2012 but it was never used in the end. One of the main reasons may be that it was not reimbursed.

MSC correspond to high cost therapies, mostly associated to small markets, ideally personalized therapies, with the necessity of very specialized producers. In this set up, it is hardly possible to correspond to a lucrative business model (97).

B. New paths for future MSC use

What can be done from now on to make MSC therapies more successful?

1. Use MSC in accordance with their functional origin

With the refinement that was made on MSC concept, it could be interesting to put more efforts toward the applications that correspond to the actual MSC biological role identified (61).

MSC can indirectly contribute to stem cell repopulation and differentiation with their ability to **support HSC**. A stronger orientation of MSC use toward applications such as HSC engraftment and recovery, or vascular assembly, appears like an effective path. Another interesting focus would also be toward pathologies where MSC are impaired or associated to MSC genetic dysfunction, like fibrous dysplasia or osteogenesis imperfecta (64). The use of MSC make sense in this case to compensate the lack of efficient endogenous MSC for these patients.

MSC potential to repopulate a tissue may still be an interesting path to explore, but it is more challenging to make it possible. Only few experimental studies support this possibility and the conditions required for effective engraftment are more difficult to gather.

Another strategy is to see MSC as a relatively easily accessible source material that has to be modified to acquire its interest. For example, MSC can be modified for their adhesion receptors to be guided to home toward the desired target (224). This guided migration can also be done through the overexpression of some cytokines. It was made for example with IL10 and resulted in an enhanced anti-inflammatory effect *in vivo.* MSC could then become like delivery vehicles for drugs (96).

2. Improve culture conditions

MSC are very plastic cells that can adapt to different culture conditions and will show different phenotypes depending on them. In the previous parts, it was reviewed that even within one MSC sample, a mix of cells at different stages and with different potentialities is present from the harvest (116,138). This mix of cells is dynamic and will evolve according to the cell's environment. Better controlled and more advanced culture methods can be used to favor different subpopulations and manage MSC heterogeneous composition, or simply to obtain MSC in a better state at the end of amplification.

a. Reduce amplification

With *in vitro* expansion, MSC are maintained in a cycling state and exposed to various stress, like high oxygen. Some markers present in the MSC in the tissue of origin can be lost with culture, and other markers can appear. Along culture, MSC undergo progressive changes toward loss of functionality and senescence (192). A quick loss of homing abilities was observed with decrease of homing receptors like CXCR4 (225). Some groups recommend to use MSC before passage 4 (around 23 cumulative population doubling), before their transcriptome becomes too heavily changed because of culture (193). A clinical trial for GvHD showed a better outcome for patients treated with passage 1 MSC compared to passage 3 MSC (226). Another study for GvHD showed a positive link between early passage MSC with low percentage of senescent cells and the clinical outcome (83). These results encourage the use of MSC with minimal culture time, however that is often not sufficient to obtain the required amount of cells in clinical context, usually at least 1×10^6 cell/kg are necessary for each administration (82).

Even in the case of allogenic context, it can be difficult to obtain enough cells from one donor. Many different culture systems are currently tested to overcome these possible limitations. Some teams explore the possibilities to pool cells from different donors. Pooled MSC are even used in clinical trials (227). MSC production is now oriented toward the industrial scale with cell banking before a patient is identified. Another possible view would be to favor personalized treatments, on a smaller scale, with a production of cells according to the patient needs, but this is not suitable for all applications.

It can be noted that the use of bone marrow mononuclear cells without prospective isolation nor plastic adherence step showed poor regenerative efficacy compared to cultured MSC (155).

b. Improve culture medium

The composition of culture medium strongly influences MSC phenotype. Many studies investigated the effect of different supplements on MSC growth and characteristics.

Essential growth factors

Growth factors are the most critical components. The most common source of growth factors in MSC culture medium remains FBS. Several batches must be tested by research teams to select the solutions that will effectively support MSC growth. The generalization of human platelet lysate (hPL) commercialization, robustly tested to favor MSC growth, opened an efficient new alternative for MSC cultivation, although its cost remains very high. It is particularly interesting for clinical production without animal derivatives. The studies on BM MSC report an increased proliferation together with reduced senescence and changes in morphology for cells cultivated with hPL compared to those with FBS (71,73,228,229). Interestingly, DNA methylations and transcriptome were not affected significantly and changes in proliferation and morphology were reversible by switching from hPL to FBS (228). A systematic review gathered many studies with BM MSC and adipose MSC that related comparison of cell functionality between FBS and hPL culture conditions (230). They concluded that MSC differentiation capacities *in vitro* were similar with both culture medium. The few groups that showed a decrease in differentiation were using hPL with high heparin concentration to avoid hPL clotting, or were using hPL coming from elderly donors. Other functionality tests were globally positive with hPL, notably with immunomodulation tests. Some groups had to deplete fibrinogen from hPL because it could compromise MSC secretion of IDO, one of the mediators of T-cell inhibition. A concentration too high in platelets could also impair MSC ability to inhibit T and NK cells.

Autologous serum was also tested as an alternative to FBS or hPL. This solution is particularly interesting to reduce immunoreactivity risk. It was shown to allow MSC

proliferation at similar level as with FBS. However, the results available until now strongly suggest that MSC capacity to differentiate and other functionalities could be reduced when exposed to autologous serum. A large volume of blood is also needed to obtain sufficient amount of autologous serum (71). Some groups used pooled human serum with WJ MSC. They showed similar characteristics and gene expression with no alteration of cells potential (231).

To better control cell culture conditions and to follow more rigorously good manufacturing practices, the ideal would be to use chemically defined medium, where each molecule is brought with a known concentration. However, until recently, its use was unsuccessful to obtain healthy MSC. More efficient systems are now available but some deleterious effects are still reported, notably a pre-disposition toward osteogenic differentiation, downregulation of surface markers including CD146, and increased cell size and senescence (232).

Optional complements

Many complements to culture medium were also tested to protect MSC from premature senescence. These compounds can be antioxidants (vitamin C, N- acetylcysteine, isothiocyanates), growth factors (FGF, PDGF, EGF), extracellular matrix components and other molecules such as rapamycin (187).

These complements must be handled with care because their effect is not yet well known and could be counterproductive. The addition of FGF for example could lead to a decrease in MSC ability to differentiate (185). Another example is with the use of antioxidants, an increase in premature senescence was reported with high doses commonly used with the aim to protect the cells from oxidative stress (233). The example of resveratrol is also representative. When it was used sparingly, it could improve BM MSC proliferation and osteogenic differentiation, but if MSC were exposed too long, it increased MSC senescence (119).

Cells from elderly patients also seem to answer differently to some stimulations. It was shown that FGF2 could increase MSC growth when they were derived from patients older than 60 years old only. Children and younger adult MSC used in this study did not

benefit of FGF2 addition. These data can be used to adapt culture conditions of BM MSC according to the donor age (65).

Moreover, it seems that coating culture surface is mostly beneficial. The appearance of senescent cells due to in vitro culture can be limited when they are cultivated in soft substrates (234), with extra cellular matrix proteins, for example hyaluronan (235), WJ extract (236).

Pre-treatments

MSC pre-treatments can also be considered just before their use. In particular, stimulation with inflammatory cytokines could help MSC after expansion to retrieve some functionality before administration (225). This kind of treatment was even shown to allow to make senescent cells retrieve their immunomodulatory capacity (210).

c. Adapt enzymatic treatment to detach MSC from culture surface

The method chosen to detach the cells from the culture surface can be important to enable cell therapeutic effect. For example, with pronase instead of trypsin, MSC could better get out from the lungs and reach other tissues after transplantation (81).

d. Reduce cells plating density

Very low seeding densities (less than 3 cells / cm^2) give the best yield with MSC and strongly extend their replicative lifespan (71). However, the amount of material, the incubators capacity storage and the time needed to obtain large numbers of cells make it necessary to find a compromise. This compromise lies usually around 1000 cells / cm^2 (73).

Low density cultures (plating density of 100 cells / cm^2 or less) also possess different metabolic characteristics compared to high density cultures. Cells plated at high density were shown to rely more on oxidative phosphorylation for proliferation and displayed more senescence (209). More energy was produced by oxidative phosphorylation but it was not beneficial for MSC. In low density cultures however, MSC were better able to manage the effects of ROS (209).

Seeding density and confluence can also affect MSC morphology and phenotype. Low density cultured MSC exhibited increased CD146 expression and more clonogenicity. On the other hand, CD200 level of expression increased when increasing plating density and confluence level (168).

To avoid reaching too high density cultures, the ideal would be to passage cells at around 70% confluency to maintain MSC in optimal state (141).

e. Oxygen level

Oxygen level during culture play an important role in the regulation of MSC functions. The classical oxygen level is set at 21% for MSC cultures, it is referred to as normoxia. This level is 4 to 10 times higher than physiological conditions. Many groups tested MSC culture in hypoxia, but results vary a lot depending on the level of hypoxia tested and the MSC tissue source. Most of the time, cells displayed enhanced proliferation capacity and stayed more undifferentiated (237). MSC also produced different secretomes and lipid composition when exposed to hypoxia (238). Some studies could show that hypoxia increased clonogenicity, differentiation potential and sustained immunomodulation (50). The expression of CD146 was also reported to be affected by oxygen level. Its expression was found to increase after exposure to 21% O_2 (60).

3. Storage and cryopreservation

Cryopreservation is an additional parameter that can limit MSC potential. According to the application it would be interesting to use fresh cells, but the most feasible seems to be to let the cells recover in culture a couple of days before they are administrated to the patient (143).

4. Quality controls

During MSC production, many parameters should be controlled and reported. This is necessary to be able to reveal associations between these parameters and clinical outcome. However, the interest of some assays are often debated and many authors write that there is a need of more relevant functional indicators, specific to the desired potential of the cells for the application, and predictive of functional outcome (83,97,239).

a. Proliferation

MSC ability to proliferate under stimulation is a necessary basis when amplification is needed. Researchers also tried to extrapolate other functionalities from the way MSC proliferate. For example, MSC capacity to differentiate with *in vitro* tests was associated with their proliferation rate just before the start of differentiation. Moreover, the proliferation status was proposed as more relevant than donor age to predict MSC potential (240). The other way around, predictive markers were searched to estimate the samples' potential of amplification. Epigenetic analysis of specific CpG sites could predict MSC replicative potential. This signature has been proposed for quality control of MSC (71,241). Some studies also showed links between colony forming efficiency and replicative potential (242), but another group report they did not find such link (243). Furthermore, MSC with poorer proliferation and capacity to form colonies showed a decreased capacity to regenerate infarcted myocardium *in vivo* (126).

b. Senescence

Senescence assessment is still too often let aside. It is of great interest to incorporate it in quality controls (50). Molecular markers linked to senescence were proposed because they could be detected before senescence onset and could be detected before other markers. Despite high variability, 24 genes were found to predict MSC samples with decreased functionality (136). Moreover, in a clinical study, it was showed that clinical efficacy of MSC was associated with expression of genes implicated in senescence regulation. TRCP4 was notably highlighted as a quantitative biomarker to evaluate the potential clinical efficacy of MSC samples (244).

c. Differentiation

Proliferation parameters were proposed to predict MSC trilineage differentiation potential because the success of *in vitro* assays needs cell confluency (240). However, they could not predict *in vivo* bone forming capacity (245). Other markers classically used, such as alkaline phosphatase, osteocalcin, Runx2, collagen type I, and osteopontin, were also questioned by a study on telomerized BM MSC. Indeed, they failed to predict *in vivo* bone

formation capacity of MSC, whereas other markers were found to be significantly correlated (decorin, lysyl oxidase-like 4, natriuretic peptide receptor C, and tetranectin) (167). Contrastingly, another study still validated alkaline phosphatase, measured by several means, as a robust predictor of *in vivo* bone forming capacity of MSC (245).

To note, a study showed that neither proliferation potential nor differentiation potential consistently predict immunomodulatory effect (246).

These latter studies highlight the importance to use relevant methods that can give realistic indication of the cell potential, that do not rely only on a theoretical scenario.

d. Improve methods

Especially in a context of cell production for clinical application, reliable markers are needed to avoid treatment failure. Correlation between different *in vitro* parameters are often made but, for example with immunoregulatory assays, they could not predict failure for GvHD patients (226).

A good example of interesting approach was made in a clinical study that searched links between MSC secretions and clinical outcome in Amyotrophic Lateral Sclerosis patients (247). They found that the levels of VEGF, angiogenin and TGF-β found in MSC culture were good predictors of their effectiveness.

For each application, the relevant assay and analyses to predict their success *in vivo* must be determined and is not generalizable. The definition of predictive biomarkers is challenged by MSC heterogeneity and their high plasticity, even after selection methods (248). However, there is still a lot of potential for improvement with statistical approaches.

Furthermore, in depth cell characterization and reporting would be of great help in defining future quality *criteria*. Details on the patient conditions, and the reporting of clinical events are also needed to be able to give more relevant conclusions about MSC therapeutic and adverse effects. A list of parameters that can influence MSC and their therapeutic effect is given **table 8**. The ideal would be to study and report all of these parameters when MSC are used.

Table 8: Gathering of the parameters that can influence MSC therapy, by category (not exhaustive).

Categories	Parameters
Donor	Age
	Genetic trait
	Pathology
	Procedure of cell collection
Isolation and culture	Isolation procedure
	Thawing precise technique
	Seeding densities at each passage
	Mix of cell initially harvested
	Time in culture
	Passaging technique
	Cell density seeding
	Culture medium and supplements (chemical environment)
	Frequency of culture feeding
	Gas levels
	Type of growth surface and other materials associated
	Mechanical environment
	Storage conditions: precise freezing technique
Cells	Tissue source and localization in the tissue
	Subtype
	Number of population doublings
	Cell cycle state and senescence
	Freezing procedure
	Metabolic profile
	Results to potency assays, stage of committment
	Epigenetic state
	Mutations

Administration	Route
	Liquid or material for administration
	Time after thawing
	Dose
	Timing of administration
	Number of administrations
	Time between administrations
Patient	Pathology state and clinical history
	Inflammation level
	Genetic traits
	Immune compatibility

5. Selection

As high variability is observed between MSC batches, several methods were developed to select the cells with the best potential for the targeted application.

a. Select the tissue

Differences between MSC from different tissues were discussed in a previous part of this introduction (IV. "MSC diversity" – B. "MSC variability with tissue source"). In addition to the parameters discussed, feasibility in practice must also be considered. That is one of the reasons why BM MSC are set aside and that their fetal and adipose counterparts are preferred. BM MSC require more invasive procedure to be obtained, but can be collected during another clinical intervention. WJ MSC are more easily accessible and with a high potential of umbilical cords donations. The tissue is richer in MSC than bone marrow. It allows to obtain more cells, will delayed senescence and strong immunoregulatory properties (193). However, the physiological specificity of MSC according to the tissue of origin should also be taken into account.

b. Select the donor and the sample

The selection can take place before cell sampling, using only donor characteristics, or after MSC isolation, by making a selection between different samples, according to quality criteria.

Factors such as age, gender, health status, genetics, immune identify or other parameters that can impact MSC potential can be considered. For this, there is a strong need to define reliable associations between donor parameters and MSC functionality. The definition of biomarkers that could be tested between donors could lead to appropriate donor screening (97).

Regarding donor age, its predictive potential was debated. Some authors suggest that differences in growth rates were attributed to other variations during sampling for example (72). It could be interesting to deepen the study of MSC between aged donors to understand better why for some of them, MSC potential is better preserved. Depending on the context and application, MSC from aged donors remain an interesting cell source.

With the use of relevant quality control markers, it could allow to decide if another MSC sample must be used. For autologous therapies, it may mean that a new biopsy will have to be performed (137). The results of cell characterization could also lead to adapt the culture conditions (the culture medium with specific supplements for example) to reorient MSC behavior going the wrong way (243).

c. Sort the cells

To have a better understanding and control of MSC action, more refined cell sorting strategies could bring a deep impact. Some authors advise the use of cultures of single cell colonies instead of bulk cultures (64). However, the numbers of cells required for clinical application is too large to be expanded from such cultures. Strategies of large-scale enrichment of MSC subpopulations with FACS or magnetic sorting have a better potential (141,150). Sorting can only be made with markers allowing the cell to stay alive for future use.

For some teams, cell isolation techniques are a mean to bypass cell culture. It allows to concentrate the cells of interest and to avoid loss of potential (155,249). The limits often raised of this strategy is the lack of specific and reliable markers to isolate MSC, and that is can be difficult to obtain a sufficient number of cells from one donor. However, a study highlighted the feasibility of immunomagnetic separation to select MSC with better bone formation, vascularization and chemoattracting potential from different bone marrow sources. They suggest in particular $CD271^{high}$ cells enrichment from reamer irrigator aspirator waste fluids that can be applied as of now by orthopedic surgeons (250). From MSC in culture, cell sorting was also proposed as a way to avoid the most potent cells to be altered by the contact with other cells (165). An interesting article showed the interest to associate different sorted subpopulations (with adipose MSC) to obtain a synergistic effect (251). In a simpler way, cell sorting can also be of use to select cells of smaller size, without necessarily a specific marker. Cell size was consistently associated with more proliferative cells, even if this criterion was not sufficient to obtain homogeneous cells. The advantage of such sorting could also be to prevent the larger cells from clogging and causing adverse effects after injection (206). Cell sorting according to the size can also be made with other types of instruments than FACS that are more simple and rapid to use (252). Another interesting and simple technique to sort cells was proposed with specific coating of culture surface. Fibronectin was used to make fit fibroblasts adhere rapidly (10 minutes), while senescent fibroblasts were unable to attach in such short time. They could harvest both fit and senescent cells easily this way (214). This could work because senescent fibroblasts have a lower density of α5β1 integrin (the fibronectin receptor) on their surface.

When sorting strategies are not feasible, it would be important at least to have samples that are more precisely characterized in their content in different kinds of cells to be able to make association with clinical outcomes.

d. Advantages of heterogeneous MSC cultures

To enhance MSC efficacy, their heterogeneity could be of use and even necessary for some therapeutic applications to be possible. More particularly, it seems important for complex mechanisms such as tissue repair, where different cell populations could

participate simultaneously to regulate inflammation on one hand and limit tissue cell apoptosis on the other hand, for instance. Too rigid population could be counterproductive (68). It remains that it is important to have a better understanding of the constituent populations of MSC complex mixes, and possibly to select or direct them toward the most efficient for the wanted application (138).

e. Select the patient

The patient must also be selected with caution, as current MSC therapy rely essentially on the recipient cells to have a regenerative effect. It is then necessary to research parameters that can be predictive of patient responsiveness. Experiment that combine donor and recipient cells could give precious information, especially interaction with immune cells. An example of the interest of this approach is provided in a recent clinical report with MSC for GvHD. They studied the interaction of MSC and some of the recipient immune cells. It resulted that patient responsiveness was associated to the capacity of the recipient cells to induce MSC apoptosis in this assay (97). This kind of approach could help to select the patient for whom MSC therapy could really be beneficial, or in another way to test MSC samples until they can show favorable features with predictive assay and biomarkers.

6. Administration and dose

Even if initial functionality of MSC is refined with care, the way the last step is made is crucial to allow MSC to express their potential and can spoil the previous efforts. The objective is to allow the cells to access to the sites where they will be able to exert their therapeutic action. Some application would also need to have viable cells that can be sensible to their environment and react accordingly.

Just before the cells are administered, the way they are retrieved is also critical. Especially if the cells had to be cryopreserved, when the application enables it, it could be better to let them recover a few days in culture before administration, notably if cell viability and responsiveness are important (97).

Some authors push that MSC should not be used by infusion but should be locally implanted (64). Indeed, they are the first adherent cells that are examined as a product to be given by infusion. In the *in vivo* models, local implantation works better for MSC survival, persistence and differentiation. Local implantations in heterotopic sites in animals could even attest BM MSC stemness and their ability to organize a hematopoietic environment. The addition of a scaffold can also help to improve MSC survival. For example, in humans, MSC in pellets or in alginate gels could persist more than 3 months in chondral defects where MSC injected freely died. After MSC treatment, the direct comparison between injection and implantation could also show the superiority of implantation (94). For example, in ischemic stroke, the best improvement was obtained after intracerebral administration. The intra-arterial route is also supported for some applications and allow the cells to reach various peripheral tissues before being stuck in the lungs. An increased accumulation of MSC in the affected tissues after intra-arterial injection was found for example for ischemia stroke, kidney and joint inflammation applications (81). However, the route does not always affect the outcome. For example, it was not shown to affect therapy outcome for traumatic brain injury. For some cases, intravenous infusion may even be better, especially for lung applications (84).

It also seems that the use of autologous cells could be the necessary condition to allow cells to migrate, engraft and persist, as shown is the part of this work concerning homing. However, with appropriate administration, the possibility for allogenic MSC to engraft is not yet out of scope. With WJ MSC for example, it was shown they could survive at least 6 weeks after administration in intrafemoral space. These cells could produce bone, cartilage, fibrous stroma, and matrix by themselves, and also stimulated the mouse host cells for tissue repair (67).

Several clinical studies could show improved MSC efficiency with augmented doses (97). Combined with a local application, it allows for a local mass effect (97).

The timing of delivery is also very important to enable MSC effect, according to the inflammatory environment in the patient notably, but also according to the delivery of other drugs.

The treatment that are given in addition to MSC administration must also be considered. (96). The use of immune suppressant drugs for example could decrease MSC regulatory activities on immune cells (224).

Many strategies have been tested in order to enhance MSC homing and engraftment after administration. Cells can be grown to form a sheet that can be applied locally. Scaffolds can also be implanted with the cells and / or can be used to release factors such as SDF1 in the target site to improve MSC homing. An original method in development is the use of pulsed focused ultrasound. These waves could produce mechanical pressure without tissue damage. This leads to the activation of mechanotransduction pathways that upregulate activating and chemoattractant molecules expression. Another possible way is to modify MSC behavior or surface directly. MSC can be exposed to hypoxia and soluble factors, often inflammatory cytokines, to increase their migration toward chemokines. They can be grown on specific coating to enhance the expression of some adhesion molecules. Another strategy developed is to label cells with magnetic particles to drive cells toward the target with an external magnetic field. The surface of MSC can also be directly modified to express adhesion molecules to improve homing. Enzymes can be used to increase the efficiency of surface receptors and adhesion molecules. It was done for example to convert on MSC surface CD44 into HCELL, the functional form that allows MSC to bind to common selectins. The desired ligands or chemokine receptors can also be directly conjugated to MSC surface, for example with streptavidin biotin, or other linker molecules. Similar approach can be made with antibodies. Genetic engineering approaches are also tested (84).

7. Less adverse effects

Considering the various uses of MSC therapy and the hindsight acquired now after more than 20 years of clinical use, the reported safety associated with their administration is encouraging.

To avoid the problems associated to immune reactivity in allogenic context, some authors encourage the reliance on autologous therapies despite the difficulties for production. Large banks of allogeneic cells could also provide the necessary diversity to allow donor close matching with recipient.

To avoid some of the potential adverse effects and to facilitate the therapy process, many groups go toward the use of cell secretions only with no cells implanted. A recent systematic review on animal studies showed similar effects of secretions only and MSC that could improve new cartilage formation (253). A kind of vesicles secreted by MSC, more precisely exosomes, were administered in one patient with severe GvHD, and could improve his state. This field of research experiences strong growth (81,254).

This approach is promising for many MSC current uses, but could not replace the entire cells for some application where cell responsiveness and adaptative phenotype is necessary. It can also be limited if cell contact and presence (for example when being phagocyted) is essential for MSC therapeutic effect.

8. Economic model

An interesting example of a stem cell therapy product is the one of Temcell, allogeneic BM MSC for acute GvHD, that has been successful is in Japan. This success has been allowed because Japanese National Health Insurance authorized the reimbursement of the product, that can go up to $ 170 000 (97).

To sustain an economic model, MSC therapies are currently oriented toward the use of allogeneic MSC that can be produced in large scale and banked. That could be deleterious and decrease the resort to autologous therapy that have unique advantages for some indications and could be the only strategy with practicable engraftment. Government could have a specific politic to promote the development of autologous therapy, with financial support, facilitated regulation and procedures for public establishments.

9. More research on MSC biology

Despite many setbacks with MSC clinical results, a big effort of research is still ongoing and the results of long years of struggle are starting to draw the framework in which MSC use can be of interest.

Progress remains to be done, for some applications, to permit to MSC to stay alive after administration.

More research on MSC basic properties is also necessary to explore the maximum potential of these cells. More particularly, it would be necessary to better understand how MSC exert their regulatory function and are themselves regulated *in vivo*.

There is a need of models that could better represent the mechanisms happening *in vivo*. Even if it leads to heterogeneity between protocols, it is interesting to develop different models and it is even necessary to reveal some of the potential of the cells. The consistence between results originating from different kinds of experiments and studies would be a strong proof but is hardly conceivable in the current stage of MSC research.

MSC heterogeneity may also be their strength. MSC diversity allows to have access to a wide range of different functionalities and many possibilities of action. What is needed is to find the means to make the best use of this complex resource. The definition of biomarkers that could be predictive of MSC functionality and the use of cell selection methods could provide a great advance for MSC therapy.

Among all these challenges, this work has focused on the study of MSC heterogeneity.

Approach

I. Historic background

A. Background of the research team

MSC research in the team started with BM MSC and was principally focused on tissue engineering. It was then redirected towards the use of BM MSC and WJ MSC for cell therapies. With the multiplication of the samples studied, striking differences were observed between them, especially in proliferation capacity, but also in differentiation, and then in immunomodulation properties. The first hypotheses pointed out donors age, but that was not sufficient to explain the high variability. Furthermore, heterogeneity and changing behavior in culture were also observed with WJ MSC. Different culture conditions were tried, with the use of hypoxia for example, to try to enhance MSC functionality (237). Growth curves of different MSC samples were analyzed and markers predictive of cell potency were searched. Different *in vitro* assays were developed for a more complete cell characterization. Importantly, the study of senescence was incorporated in the basic quality control of the cells.

B. Previous PhD student

An important part of the bases used for this work comes from a previous doctoral work undertaken in the lab by Yueying Li called ”Vieillissement des cellules stromales mésenchymateuses de la moelle osseuse : implications en médecine régénérative“. During this previous work, associations could be made between donor age, *in vitro* culture, senescence level, and the decrease in MSC capacities to proliferate, to form colonies and to differentiate (117). MSC coming from aged donors also displayed an overall greater heterogeneity. The importance to consider donor age was highlighted. Two classes of donors could be defined around the threshold of 40 years old, in accordance with the behavior of the samples studied in particular in terms of cells variability and proliferation capacity.

Surface markers also appeared as interesting tools of study to more precisely characterize the cells, but also to improve cell sorting. At the same time, clinical trials reports of MSC therapies accumulated with very variable results, where MSC heterogeneity and senescence were pointed out as one of the potential responsible factors. An extensive literature review allowed to choose a set of markers that could be associated to MSC functionality and subtypes. Interesting correlations were obtained, notably between CD146 expression and donor age and with MSC proliferation capacity. CD146 appeared as an important marker for MSC as it was reported in an important study that identifies stem MSC *in vivo*, the only evidence of this kind in the MSC field (20).

II. Research questions and objectives

Preliminary studies performed in the laboratory and found in the literature review showed that MSC samples were heterogeneous. Different subtypes, different stages of maturation, senescent and young cells coexist. This heterogeneity induces a difficulty to predict the sample behavior and could be responsible, in part, for the variability of clinical outcomes.

The different subtypes that can be found can result of intrinsic cell functioning and initial cell mix present in the donor, but also from changes occurring during culture **(figure 26)**. Cell culture and characterization appeared as an irreducible step for MSC use. The lack of satisfying procedure to predict and manage samples behavior drive the team toward the research of ways to fill the gap. In order to improve MSC use in research and for therapeutic applications, this study address MSC heterogeneity and its consequences. Because MSC lack of potency and impairment could come from non-optimal mix of different subtypes and from the presence of senescent cells, specific markers of these different kind of cells were searched.

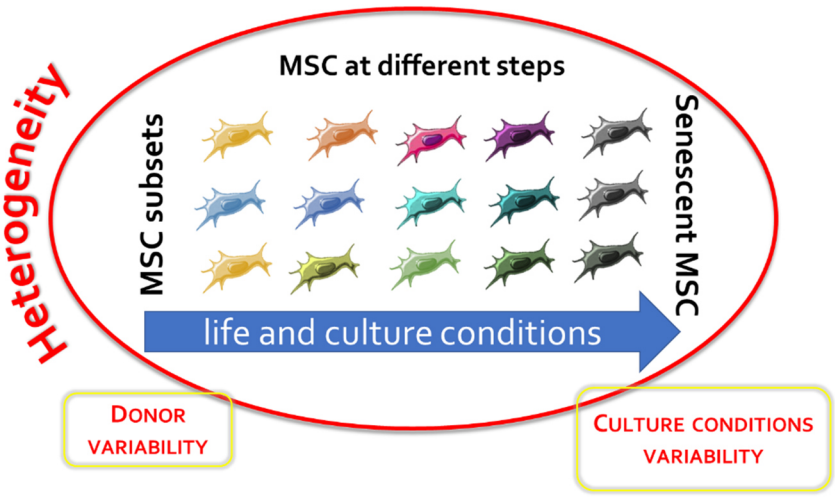


Figure 26: Schematic representation of MSC heterogeneity.
Different subsets with different potential can be existing initially in the donor and their proportion may vary according to donor parameters and the way cells are harvested from the donor. These different subsets can evolve differently and their proportions can change with the progress of culture. They all move towards senescence with advancing age and prolonged *in vitro* culture. The presence of different subtypes and of different proportions of these subtypes also participates to the heterogeneity observed at other levels. Culture conditions also influence the state of the cells, and different subtypes can react differently to given culture conditions.

The global objective of this work was to characterize MSC heterogeneity. For this, specific **surface markers** of MSC subtypes and senescent MSC were searched. The final aim was to define **functionality biomarkers**, sort MSC to concentrate a subtype and study functionality of the sorted cells. A careful attention was also given to **culture conditions** and their influence in MSC heterogeneity and functionality, but the work undertaken during this PhD work could not explore this part of the subject in depth, which is currently taken on by ongoing studies.

In order to improve MSC use in research and for therapeutic applications, this study addressed MSC heterogeneity from inter-individual to intra-sample level, the following questions were raised:

- Could a more refined use of flow cytometry help to decipher the composition of MSC populations?

- Is there a reliable link between some surface marker expression and *in vitro* cell functionality?

- Is cell sorting feasible and reliable to separate distinct MSC subtypes in culture with specific properties? Can it improve cells quality?

- How does the phenotype of senescent MSC change? Could some specific surface markers be defined and used to remove senescent cells? And on longer-term, if senescent cells are removed, how sample functionality will change?

To answer these questions, the following objectives were addressed:

- The first objective was to describe initial MSC variability, at the level of surface markers, between and within different samples in classical culture conditions and study eventual associations between phenotype, donor age and MSC basic properties.

- The second objective was to develop and test a sorting strategy to separate MSC subsets according to CD146 expression to compare the properties of the sorted cells.

- The third objective was to search for specific surface markers of senescent MSC.

The MSC studied in these different contexts were characterized with different *in vitro* assays to uncover possible associations between their potentialities and their phenotype.

Methods

This chapter includes the methods used and some results about methods development and optimization.

I. Isolation and culture of MSC

Human bone marrow samples were obtained from the University Regional Hospital of Nancy-Brabois. Samples come either from femoral bone marrow of patients undergoing hip replacement in the Orthopedic Surgery and Traumatology department (in French: “Chirurgie orthopédique et traumatologie", COT) or from iliac crest of healthy donors selected for allograft managed by the Cell and Tissue Therapy Unit **(table 9)**.

The samples were processed as previously described (117). They were washed with PBS and centrifuged at 300 g for 10 minutes. Mononuclear cells were counted in Thoma chamber after dilution in Leucoplate and seeded at 50 000 cells / cm² in culture flasks (Corning). The samples were cultured with alpha MEM medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine, penicillin and streptomycine at 10 mU/mL and fungizone at 2.5 µg / mL (this medium was also called “complete αMEM” in this work). Cells were grown in an incubator in normoxia at 37°C, 90% humidity, 5% CO_2. MSC were detached with 0.025% trypsin when reaching around 80% confluence or before if it is not reached after a maximal time of 21 days for passage 0 or 14 days for the next passages. After the first passage, MSC were seeded at 1000 cells / cm². The time in culture of BM MSC (after P0) was between 9 and 14 days for each passage, depending on the growth rate of the cells.

Cells were frozen at different passages with cold DMSO-10% FBS solution in cryotubes. Tubes were first placed in CoolCell freezing containers (BioCision) in a -80°C freezer. Then, after at least 24 hours, they were moved in a -150°C freezer. Cells were thawed at least 1 passage before experiments. For this, hot culture medium was used to dissolve the frozen cell suspension and place cells in a large amount of medium rapidly. This was made to avoid high concentration DMSO deleterious effects on cells at ambient temperature.

Table 9: List of the bone marrow samples used with donor information.
The date of retrieval is in the format year/month/day. M: male; F: female; ?: the information could not be retrieved at the time of writing.

Age and denomination	Sex	Provenance	Date of retrieval
9	?	UTCT	16/12/21
15	M	UTCT	13/05/30
18	M	UTCT	14/04/29
20 A	?	UTCT	14/05/28
20 B	?	COT	15/03/27
24 A	F	COT	14/02/19
24 C	F	UTCT	14/09/09
27	F	COT	17/04/14
28	F	COT	17/02/22
30	F	COT	14/07/10
32 A	M	UTCT	12/04/11
32 B	?	COT	15/04/17
33	M	UTCT	16/05/12
52	F	COT	16/08/03
56	M	COT	11/12/02
58	F	COT	16/08/17
59	?	COT	15/03/10
60	M	COT	15/08/17
65	?	COT	15/01/09
68	M	COT	14/11/05
76	M	COT	14/12/19
78 A	F	COT	15/10/21
78 B	F	COT	16/12/21
84	F	COT	13/04/10
85	F	COT	13/09/03
86	?	COT	14/10/28

Cell proliferation rate was estimated at each passage with 2 parameters: Population Doubling (PD) and Doubling Time (DT) as follows:

$$PD = \frac{\log N_x - \log N_i}{\log 2} \qquad DT = \frac{T \times \log 2}{\log N_x - \log N_i}$$

Where:

N_i = Initial number of cells seeded

N_x = Number of cells counted after detaching

T = Number of days between seeding and detaching

Living cells were counted using trypan blue staining in Thoma chamber. The counting was repeated at least 3 times. Population doubling can be cumulated for each passage and represents the number of times MSC population divided. Doubling time represents the speed of MSC division.

II. Quality controls and functional characterization

A. Colony forming efficiency

To evaluate the content of Colony Forming Units (CFU) in the cultured sample, MSC were seeded at less than 2 cells per centimeter square (100 MSC in 57 cm² petri dish, 3 replicates). After 14 days of culture, cells were stained with crystal violet (Sigma). The number and size of CFU were analyzed with ImageJ (National Institutes of Health). This allows to estimate the ability of MSC to generate a progeny with almost no support from other cells. The very low seeding density makes this test different from classical proliferation tests. Only some types of cell including stem and progenitor cells are able to grow under these conditions.

B. Differentiation

MSC were differentiated toward osteogenic and adipogenic lineages with specific commercial media (Lonza) according to the manufacturer protocol. Three to five wells were seeded for each differentiated sample. Control cells (seeded in 3 wells) were cultivated simultaneously in the same plate. All washing steps were made very gently to avoid the detachment of cells and extracellular matrix components. Fixation and staining of differentiated cells were made at ambient temperature.

1. Osteogenic differentiation

Cells were seeded in 24 or 48-well plates at 3100 cells/cm² in complete αMEM. After 24 hours to allow cell adhesion, the medium was changed for a commercial differentiation medium from Lonza containing FBS, L-glutamine dexamethasone, ascorbate, penicillin and streptomycin. Negative controls for osteogenic differentiation were cultivated with classical complete alpha MEM medium. After 21 days, cells were washed with PBS and fixed with 10% formaldehyde, freshly prepared from a stock solution of 37.4% formaldehyde. Cells were then washed with water and stained with Alizarin Red (Sigma) solution at 40 mM. This solution was prepared by mixing the powder with distilled water and was filtered with 0.45 µm filter. The pH of the solution was adjusted to be between 4.1 and 4.3 with HCl when needed. This solution was left 20 minutes on the cells. After this, the cells were washed 4 times and the plate was air dried. At least 5 microscopy images were taken (Leica DMI3000B) of each well to determine the stained surface with ImageJ.

Alizarin Red reveals the calcium deposits that can be found when cells acquire osteogenic phenotype. To note, amorphous calcium deposits are very soluble at the pH used in the experiment and if present, they would be lost during the procedure.

2. Adipogenic differentiation

Cells were seeded in 24 or 48-well plates at 21 000 cells/cm² in complete αMEM. The cells were grown until they reached confluence. At this time, 3 cycles of medium change with alternance of induction (3 days) and maintenance phases (1 to 3 days) are initiated. Commercial induction medium (Lonza) is notably composed of FBS, L-glutamine, dexamethasone, indomethacin, insulin, 3-isobutyl-1-methylxanthine and gentamicin sulfate-amphotericin. Negative controls for adipogenic differentiation were left in maintenance medium regularly renewed. After 3 cycles, all the cells were left 7 additional days in maintenance medium (with medium replacement every 2 to 3 days).

At the end of the differentiation process, cells were stained with Oil-Red-O (Sigma) or Adipored (Lonza) to reveal lipidic vesicles formed in adipocytes.

For Oil-Red-O staining, cells were washed with PBS and fixed with 10% formaldehyde, freshly prepared from a stock solution of 37.4% formaldehyde. Cells were then rinsed with 60% isopropanol and stained with Oil-Red-O solution. This solution was made by mixing 30 mg of Oil-Red-O powder with 6 mL of 100% isopropanol and with 4 mL of distilled water. The solution was also filtered with 0.45 µm filter before it was placed on the cells. The staining solution was left 30 minutes on the cells before washing 3 times with distilled water.

Adipored staining was made on living cells. 400 µL of PBS was placed in each well, and 12 µL of Adipored solution was added over with gentle mixing. The solution was left for 15 minutes.

Right after staining, at least 5 microscopy images were taken of each well with Leica DMI300B. ImageJ was used to measure the stained surface.

C. Senescence assessment

Cells of each sample were seeded in 3 wells of 24-well culture plates (Corning).

After 4 to 7 days, Senescence β-Galactosidase Staining kit (Cell Signaling) and Click-iT Plus EdU Alexa Fluor 594 Imaging Kit (ThermoFisher) were used together on the same cells to assess senescence and proliferating cell rates according to the manufacturers protocols.

EdU is a nucleotide substitute left in MSC culture for 24 hours, it is incorporated **only in the cells undergoing DNA replication**. Then, the cells were fixed with a solution of 2% formaldehyde – 0.2% glutaraldehyde. After this, β-Galactosidase staining was performed by adding a solution containing the substrate named "X-Gal" (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). β-Galactosidase can be found in all cells. This staining is made at **suboptimal pH** (pH 6.0 while the optimum for the enzyme is between 4 and 4.5), so that **only cells that strongly overexpress the enzyme, one of the signs of senescence**, will be revealed by the staining. The action of β-Galactosidase on X-Gal leads to the formation of a blue compound visible in bright field microscopy. The staining

solution is left overnight in an incubator at 37°C without CO_2. Then, to reveal EdU staining, cells were permeabilized with 0.5% Triton X-100 and blocked with 0.5% BSA. After this, a solution containing AlexaFluor594 picolyl azide fluorescent compound, made to fix incorporated EdU, is added. Ultimately, DAPI (Sigma) staining was also performed to stain all nucleus and count the total number of cells.

At least 15 fields were photographed under the microscope (Leica DMI3000B). Images with DAPI fluorescence were used to count the total number of cells. Images with EdU-AF594 fluorescence were used to determine the proportion of proliferating cells. The percentage of cells stained with senescence associated β-Galactosidase was determined with bright field images. Indications of the way the cells were counted are given **figure 27**.

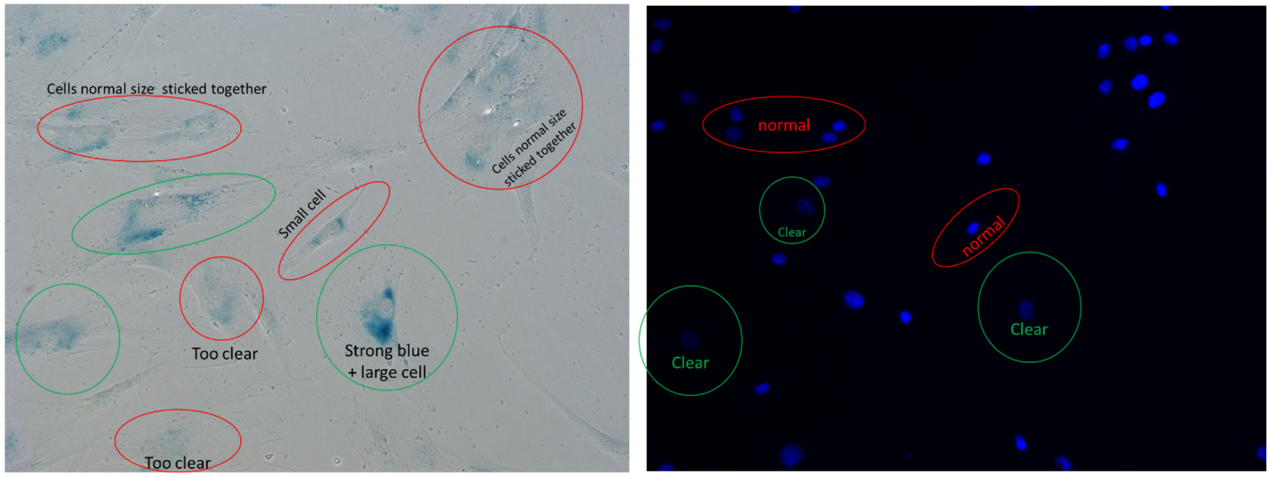


Figure 27: Example of images obtained after senescence associated β-Galactosidase and DAPI staining and precisions about their analysis.
In green are shown the cells that were effectively counted as positive for β-Galactosidase staining. The cells that could falsely be counted as positive are encircled in red.

The cell was considered positive for β-Galactosidase staining if it had clearly visible blue staining in its cytoplasm and at least one of the following characteristics:
- the cell is large,
- the nucleus is large,
- the nucleus is clear (low brightness),
- the staining is strong on a single cell isolated from others.

The cell was not considered positive in the following situations:
- the blue color is too clear,
- the cell is small size and the staining not so strong,
- the cells are normal size and stuck together,
- the cells are dividing (2 round cells near one another),
- the cell is detaching.

The association of EdU staining also allowed to avoid some of the false positives with β-Galactosidase staining. When a nucleus was positive for EdU label (regardless of the brightness of the fluorescence), even if there was blue coloration or other signs that may be associated to senescence, the cell was not counted as positive for β-Galactosidase staining.

For cells said to be in replicative senescence state, they were cultivated until cell exhaustion (more details are provided in the Results chapter, part III called "Senescence and surface phenotype"). This model has the advantage to show some concordance with *in vivo* senescence associated with aging. Notably, methylation changes in MSC were overlapping in long-term culture and aging *in vivo* (39).

D. Migration

Migration capacity of MSC was assessed with the use of porous inserts called transwells or Boyden chambers **(figure 28)**.

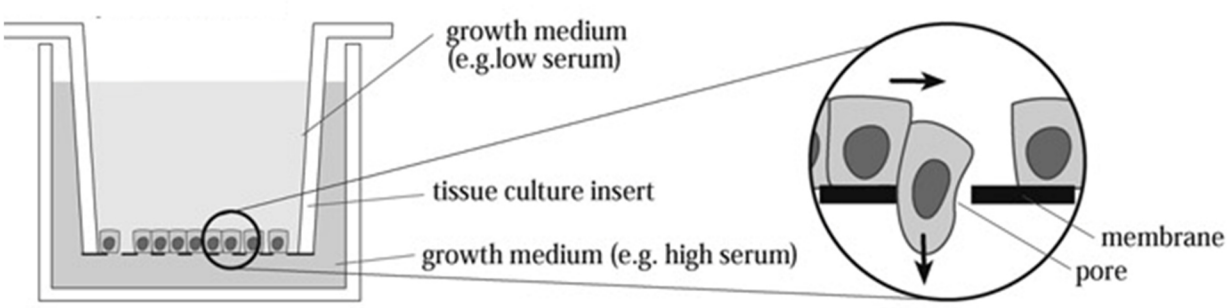


Figure 28: Representation of the migration assay with transwell inserts.

This technique has the advantage to test MSC capability not only to move but also to deform themselves to cross a pore with a defined size. In the studies proposed in this work, it was not used to test different chemoattracting molecules, but to compare MSC potential of different kinds of samples.

The protocol used was inspired by Corning protocol called "Migration of Human Mesenchymal Stem Cells Using Corning FluoroBlok Cell Culture Inserts"
(accessible with the following link:
https://www.corning.com/media/worldwide/cls/documents/CLS-DL-CC-054%20REV1%20DL.pdf).

Fluoroblok inserts (figure 29) permit to follow the migration of living cells on the underside of the insert with fluorescence dyes like Calcein without the need to remove cells from the upper part of the membrane, the light being blocked by the opaque membrane. The removal of cells needed when using transparent membranes was reported to be too random and cause very variable results (255).

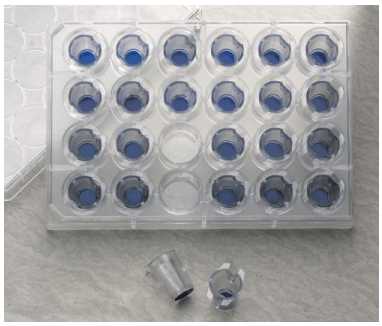


Figure 29: Photography of Fluoroblok inserts to use with 24-well companion plate.

40 000 MSC were seeded in alpha MEM medium without FBS on Fluoroblok inserts with 8 µm pores (Dutscher) in 24-well companion plates. Attraction of cells toward 10 to 30% FBS according to the cases (the details are mentioned in the legend of the figures) was compared to migration through the membrane of cells toward medium without FBS after 15 to 20 hours in the incubator. FBS was chosen because it was reported to induce the migration of MSC the most strongly. Living MSC were stained with Calcein (Invitrogen) and the fluorescence intensity detected by bottom reading using a microplate fluorescence reader (Varioskan Flash, ThermoFisher). Fluorescence microscopy images were also made to visualize cells on the underside of the membranes (Leica DMI3000B).

Different pore sizes (3 µm and 1 µm in addition to 8 µm) were also tested to compare young and senescent MSC capacities.

E. Secretions analysis by cytokine array and ELISA

MSC at 80% confluency were placed 48 hours in medium without FBS to gather cell secretions. Supernatants were frozen at -80°C for subsequent analysis. To have a global view of MSC secretions, Human XL Cytokine Array Kit (R&D Systems) was used according to the manufacturer protocol. This array allowed to assess the relative expression level of 102

human soluble proteins. The plugin “Protein Array Analyze for ImageJ” was used to analyze spots density.

Aliquot of the same medium used for cytokine array were used to confirm the results for CHI3L1 (Bio-Techne) and IGFBP-3 (ThermoFisher) with ELISA technique according to the manufacturer protocol. Four-parameter logistic curve model was used for the interpolation of the concentrations with the software GraphPad Prism.

F. MLR

Blood samples from healthy donors were collected the day of the experiment in EDTA tubes. For CD4 activation, a 96-well plate was prepared with CD3 and CD28 (R&D) coating at 10 µg / mL. The coating solution was left for at least 2 hours at 37°C. Leucosep tubes were used with Lymphoprep density gradient medium (StemCell) to retrieve peripheral blood mononuclear cells (PBMC). After counting PBMC, they were sorted with CD4+ T Cell Isolation Kit (MACS Miltenyi Biotec) according to the manufacturer protocol. CD4 cells are then labelled with CellTrace Violet Cell Proliferation Kit (ThermoFisher) following the instructions of the kit. This label allows to follow cells proliferation, the fluorescence of the label per cell decreasing with cell divisions. After washing the 96-well plate, 50 000 CD4 T cells in 100 µL of RPMI supplemented with 10% FBS were placed in each test well. For the condition in coculture with MSC, 50 000 MSC in 100 µL of RPMI-10% FBS were placed per well together with CD4 cells. All cell suspensions were gently mixed with a pipet. For activated T cells, 2 wells were gathered for one flow cytometry tube. For non-activated T cells, the content of 3 wells were pooled. After this, the cells were left 5 days in an incubator in normoxia at 37°C, 90% humidity, 5% CO2. The plates were then centrifugated at 400 g for 3 minutes with slow braking. Supernatants were removed and cells were labelled with CD4-PerCP-Cy5.5 antibodies, during 30 minutes at 4°C. Cells were then collected in PBS for flow cytometry analysis. At least 50 000 events were analyzed to determine the proliferation status of CD4 positive gated lymphocytes. CD4 T cells alone and without activation were used as control cells. They displayed a population of cells homogeneously labelled with CellTrace with a high fluorescence and were used to

put the threshold corresponding to negligible proliferation of CD4 cells. The percentage of CD4 cells in proliferation was gated with this threshold. The percentage of proliferating cells in activated CD4 cells alone was used as the reference to determine the inhibition of proliferation caused by MSC on CD4 cells in coculture.

III. Immunofluorescence

A. Immunophenotyping analysis by flow cytometry

1. General principles of flow cytometry

Flow cytometry is one of the oldest methods to study single cells. This technique allowed to grasp cell-to-cell variability very early, but it took time before it was really considered.

The fluidic system of the flow cytometer allows cells to pass one at a time through a laser light. After being excited by the laser, cell fluorescence and light scatter can be detected by different detectors. The detected photons are converted in electronic signal **(figure 30)**.

All analyses were made on Gallios flow cytometer from the Cytometry core facility of UMS2008 IBSLor **(figure 31)**. Calibration beads were always used at the start of the instrument.

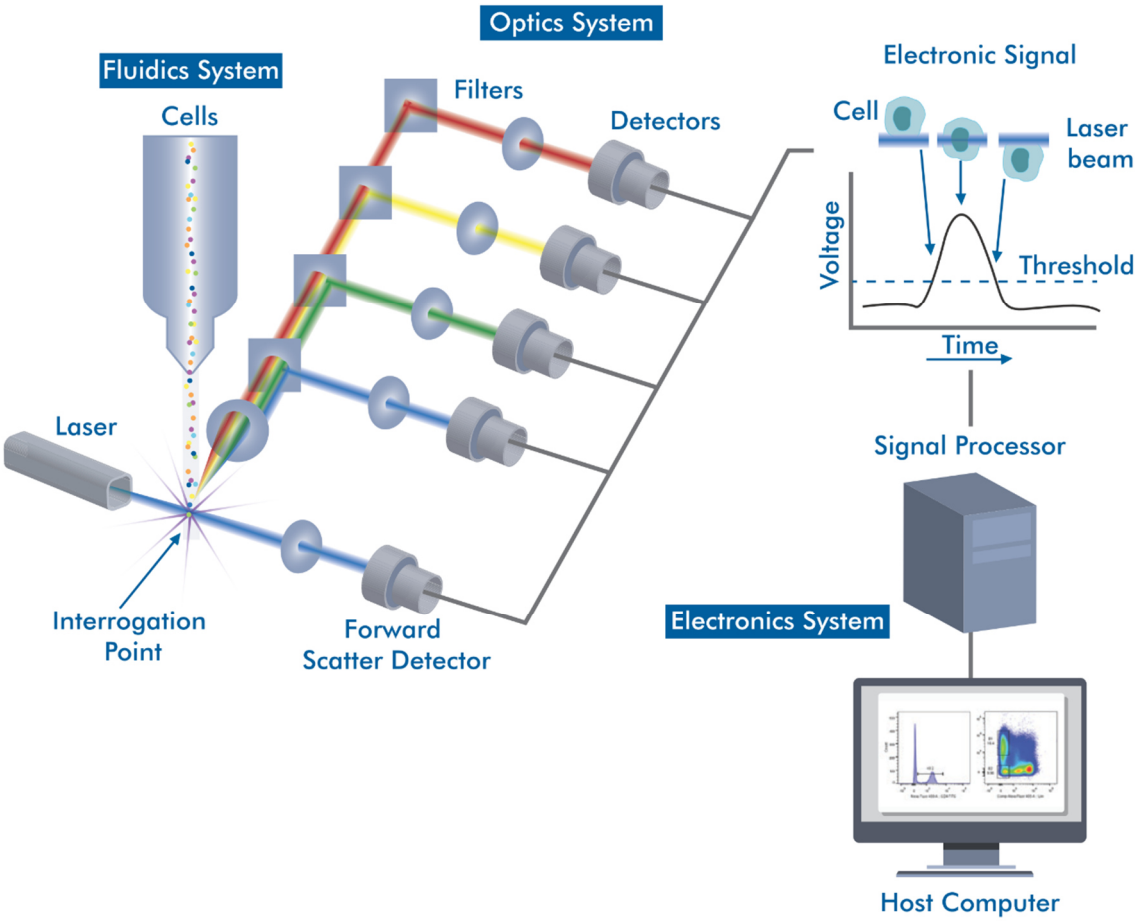


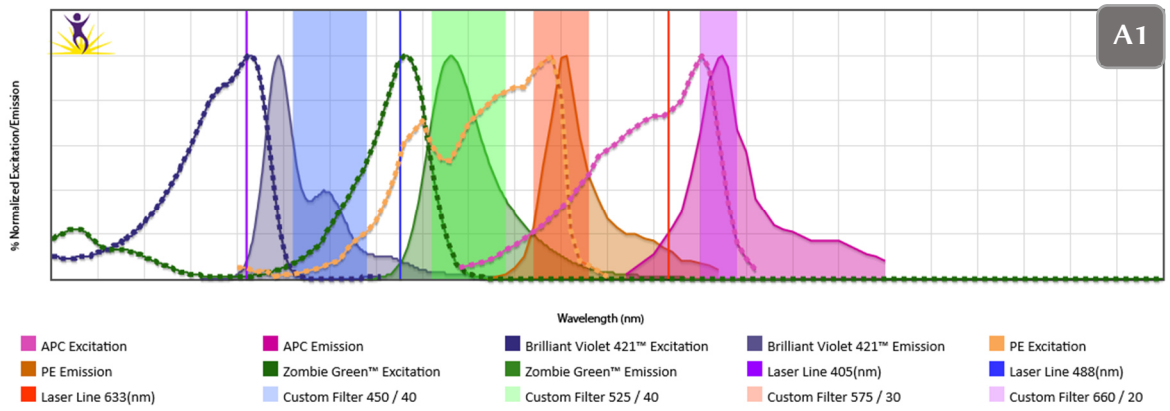
Figure 30: Basic components of a flow cytometer.
From Bio-techne Flow Cytometry eHandbook
(available at https://info.bio-techne.com/flow-cytometry-handbook.html).

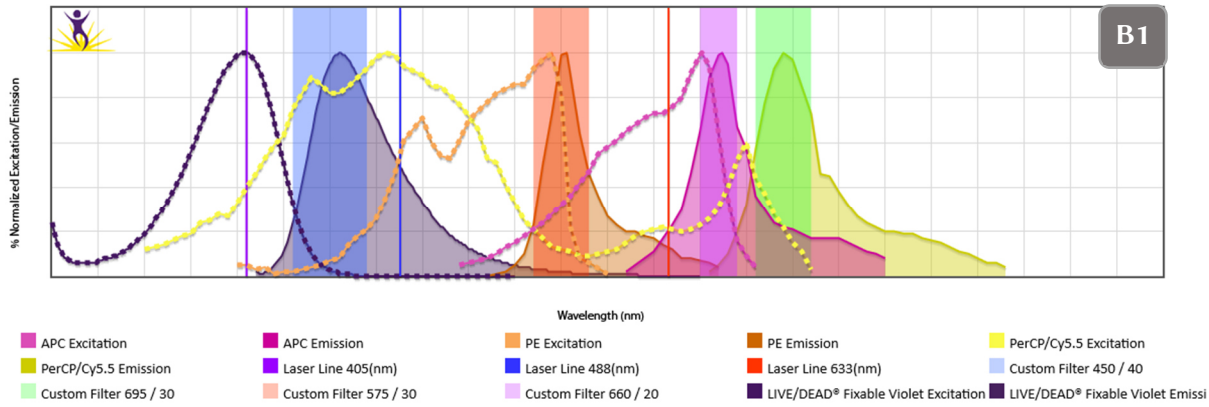
Figure 31: Gallios flow cytometer from Beckman Coulter.

2. Settings and development of flow cytometry panel

New antibody panels were designed to provide a better detection of the level of surface proteins expression, notably to have a better resolution for markers with low expression. The objective of these panel was to allow the detection on the same cells of several surface proteins that could be expressed differently in different MSC subtypes and at the same time to improve detection sensitivity of these markers. For this, the brightest fluorochromes were used for subtypes markers that were weakly expressed. For example, CD146 presence assessed with FITC-conjugated antibodies can be missed because FITC is not bright enough and its fluorescence emission too close to the autofluorescence of compounds present in all cells. Tandem dyes combining several fluorochromes were avoided as much as possible to limit several reported issues, notably the lot-to-lot variability and instability because of their sensitivity to light, storage conditions and handling (256). BV605 fluorochrome associated to CD106 was tested but did not work. The number of labels made on the same cells was limited in the end to avoid to have too much fluorescence spillover, and to be able to detect the studied markers with more sensitivity. To allow the cells to be precisely analyzed and to avoid cell clumps, the flow cytometer speed was always placed the closest to 100 cells per seconds. The associated fluorochrome spectra, voltage of detectors and compensation matrix are displayed **figure 32.**

At first, different photomultiplicator (PMT, detectors) voltages were tried on cells known to have some expression of the tested marker and labelled with only one antibody. Voltages were chosen to allow the best separation between unlabeled and labeled cells and to limit electronic noise contribution in the fluorescence intensity detected (256).





A2

	FS	SS	FL1	FL2	FL4	FL6	FL9
Voltage	100	50	250	279	500	450	510
Gain	1	2	1	1	1	1	1

	FL1	FL2	FL4	FL6	FL9
FL1		1,30	0,00	0,00	0,00
FL2	14,80		0,00	1,60	0,00
FL4	0,00	0,00		0,00	0,00
FL6	5,24	1,10	0,00		0,00
FL9	0,00	1,20	0,00	1,20	

B2

	FS	SS	FL1	FL2	FL4	FL6	FL9
Voltage	100	50	250	400	500	450	350
Gain	1	2	1	1	1	1	1

	FL1	FL2	FL4	FL6	FL9
FL1		0,00	0,00	0,00	0,00
FL2	0,00		0,40	0,00	0,00
FL4	0,00	8,39		1,00	0,00
FL6	0,00	0,10	1,70		5,30
FL9	0,00	0,20	0,70	0,00	

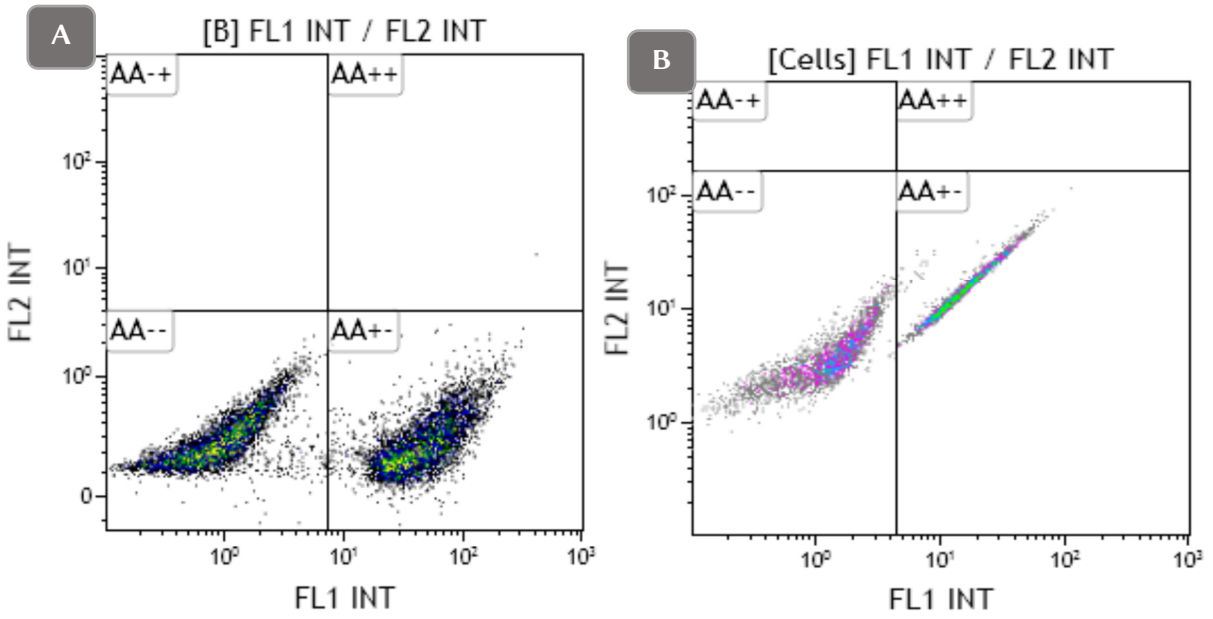
Figure 32: Spectra of fluorochromes used in the antibody panels for flow cytometry and the detail of voltage, gain and compensation matrix values.

Images were obtained with "Spectra Analyzer" tool from Biolegend.com

A1 and A2: the first panel of antibodies designed combined CD146-BV421 (FL9), ZombieGreen (FL1), CD105-PE (FL2) and CD157-APC (FL6) (at first CD157-AF647 was used but it was no longer sold and changed to CD157-APC. AF-647 and APC spectrum are very similar).

B1 and B2: the second panel of antibodies designed combined Livedead Violet (FL9), CD200-PE (FL2), CD71-APC (FL6) and CD140b-PerCP-Cy5.5.

After voltages were defined, the levels of needed compensation were determined. For this, cell samples known to strongly express or fix the marker tested were chosen as follows: WJ MSC passage 5 for ZombieGreen, CD200-PE, CD157-AF647; BM MSC from a 33-year-old donor at passage 1 for CD140b-PerCP-Cy5.5 and Livedead Violet; Hela cells at passage 23 for CD146-BV421 and CD71-APC. These samples were separated in two: a group of unlabeled cells was compared with the labelled cells (one antibody at a time). The compensations in the other channels were determined by adjusting the median fluorescence intensity to be the same as the one obtained with unlabeled cells **(figure 33)**.



Gate	Y-Med
All	0,2
AA--	0,2
AA-+	N/A
AA+-	0,2
AA++	13,6

Gate	Y-Med
All	6,3
AA--	3,3
AA-+	N/A
AA+-	13,4
AA++	N/A

Figure 33: Examples of biparametric plots used to determine compensations.

These examples were made with ZombieGreen viability dye. Its fluorescence emission is normally retrieved in FL1 channel and the leaking in the channel FL2 (used for PE fluorochrome) can be viewed. Unlabeled cells are in the AA- - gate and labeled cells are in the AA+- gate. The median fluorescence intensity of FL2 in these two gates must be equal to avoid the contamination of ZombieGreen fluorescence in the FL2 channel.

A: With FL2 voltage at 279, after asking the software to make compensation up to 14.8% for the contribution of ZombieGreen fluorescence in FL2 channel, the labeled cells were brought back to the same level of median fluorescence intensity as the unlabeled cells.

B: With FL2 voltage at 400, even after asking the software to apply 100% compensations, the labeled cells were still leaking too much in FL2 channel, proscribing this combination.

The use of ZombieGreen as a viability dye was not possible in combination with a high voltage in PE channel (FL2), it caused too much spillover **(figure 33)**. To be able to use a PE-conjugated antibody in this panel, only low voltage could be applied. A marker known to always be highly expressed on MSC was then chosen here. CD105 was one of the most interesting classical MSC marker and a low voltage, compatible with ZombieGreen, was sufficient to detect it.

During the first tests, PE fluorescence seemed to be better detected with less noise contribution and more resolution with a higher voltage (400 instead of 250). However, after the experiments were made with CD200 conjugated with PE in the panel with a voltage of 400, the results were more mitigated. CD200 was better detected when made in the classical setup, alone or just in combination with another FITC-conjugated antibody and with low voltage.

During the panel design, the controls called "fluorescence minus one" (FMO) were made for CD146-BV421 and CD157-APC fluorescence channels (respectively FL9 and FL6) and confirmed that there was no leaking of other fluorescent labels after compensations were applied. The level of fluorescence detected was inferior to what could be seen in the cells labelled with isotype controls, and it was similar to the results obtained with unlabeled cells.

3. Reagents

To determine MSC surface marker expression, cells were suspended in 0.5% Bovine Serum Albumin (BSA) and stained with the antibodies listed **table 10**, associated depending on the experiment.

Table 10: List of antibodies used with their reference and clone.

Antibody	Supplier, reference	Clone
FITC Mouse IgG1 κ Isotype Control	Dako, X0927	DAK-GO1
PE Mouse IgG1 κ Isotype Control	Dako, X0928	DAK-GO1
CD90-FITC	Beckman Coulter, IM1839U	2G5 F15-42-1-5
CD34-PE	BD Pharmingen, 555822	581

CD45-FITC	Dako, F0861	T29/33
CD166-PE	Beckman Coulter, A22361	3A6
CD44-FITC	Beckman Coulter, IM1219U	J.173
CD105-PE	Beckman Coulter, B92442 A07414	TEA3/17.1.1 1G2
HLADR-FITC	Beckman Coulter, IM0463U	B8.12.2
CD73-PE	BD Pharmingen, 550257	AD2
CD200-PE	BD Pharmingen, 552475	MRC OX-104
CD106-PE	BD Pharmingen, 555647	51-10C9
CD146-FITC	BD Pharmingen, 560846	P1H12
CD71-FITC	BD Pharmingen, 555536	M-A712
CD140b-PE	BD Pharmingen, 558821	28D4
BV421 Mouse IgG1, κ Isotype Control	BD Horizon, 562438	X40
CD146-BV421	BD Horizon, 564325	P1H12
AlexaFluor647 Mouse IgG1 κ Isotype Control	BD Pharmingen, 557714	MOPC-21
CD157-AF647	BD Pharmingen, 564870	SY/11B5
APC Mouse IgG2a, κ Isotype Control	BD Pharmingen, 555576	G155-178
CD157-APC	ThermoFisher, 17-1579-42	SY/11B5
CD71-APC	BD Pharmingen, 551374	M-A712
PerCP-Cy5.5 Mouse IgG2a, κ Isotype Control	BD Pharmingen, 550927	G155-178
CD140b-PerCP-Cy5.5	BD Pharmingen, 562714	28D4

MSC viability was checked with Zombie Green (BioLegend), Livedead Violet (ThermoFisher) or Propidium Iodide associated with Annexin V (Life Technologies) for apoptosis depending on the experiment.

Antibodies were always used with large excess concentrations. Isotype controls were used at the same concentrations as the antibodies (concentration provided by the supplier).

4. Gating strategy

Analyses were performed with Beckman Coulter's Gallios flow cytometer and Kaluza analysis software.

The data that could be identified as cell clumps were excluded from the analyses **(figure 34)**.

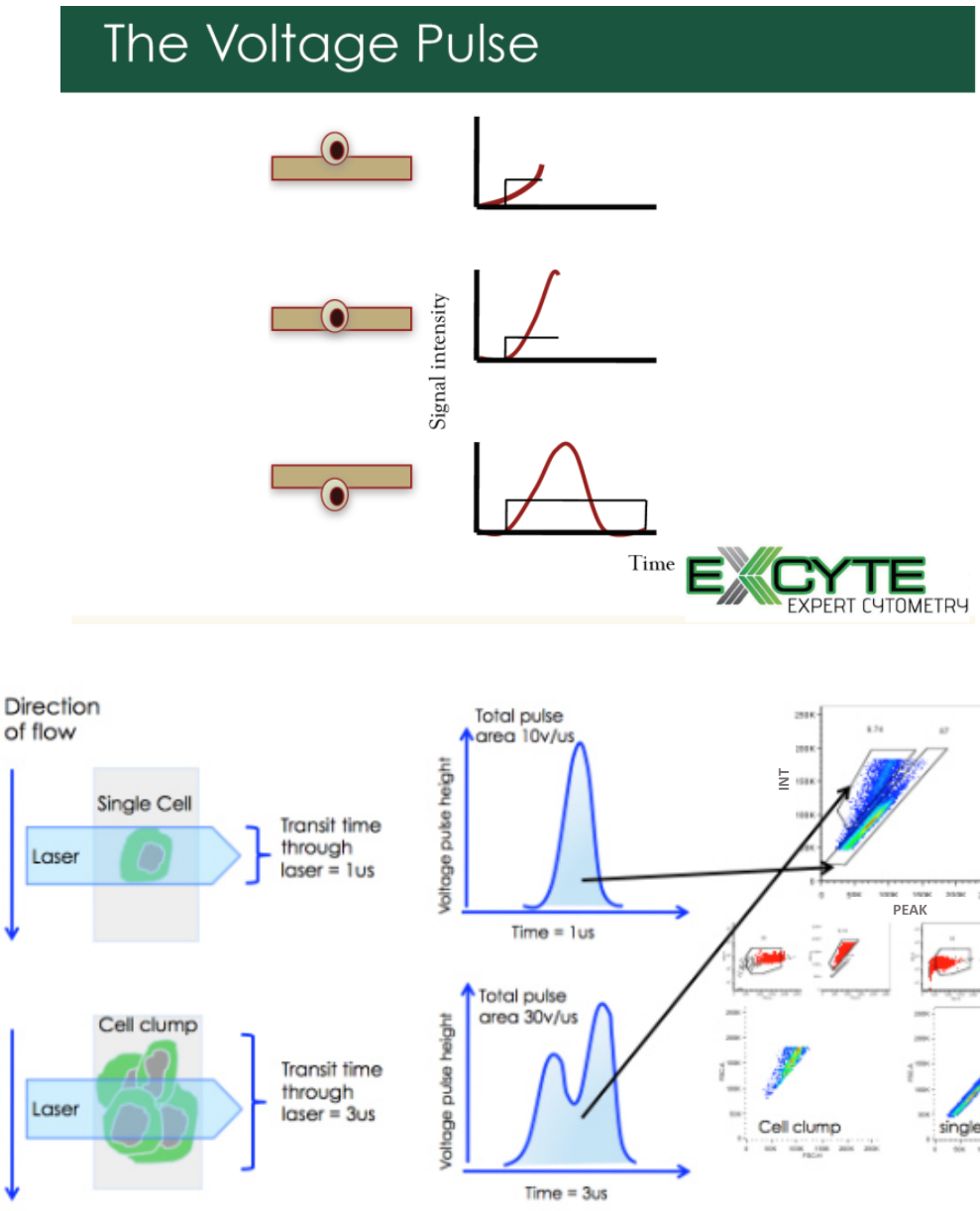


Figure 34: Principle behind the gating strategy to exclude data obtained from cell clumps.

(Figures are from Tim Bushnell and were retrieved from the following links: https://expertcytometry.com/optimize-flow-cytometry-hardware-for-rare-event-analysis/ and https://expertcytometry.com/gating-strategies-get-your-flow-cytometry-data-published/)

Each time a cell cross the light of a laser and fluoresce, a detector converts photons in an electronic voltage pulse. The parameters used in flow cytometry are calculated from these voltage pulse. The abbreviation "INT" that can be seen after the name of a channel (FL1 INT for example) means the integral of the pulse (the area under the curve) was calculated to obtain the represented data. The term "PEAK" means the maximal height of the pulse was used to obtain the represented data.

A cell clump will take more time to cross the light of the laser, resulting in a bigger area under the curve, but for the same maximal height. This can be used to visualize the events corresponding to clumps by making a biparametric plot with one axis with data of pulse height and the other with data of pulse area. Like this, single cells appear in a diagonal and clumps appear with increased area for the same heights.

When viability labels were included, only living cells were analyzed, the gating strategies are shown **figures 35, 36 and 37**.

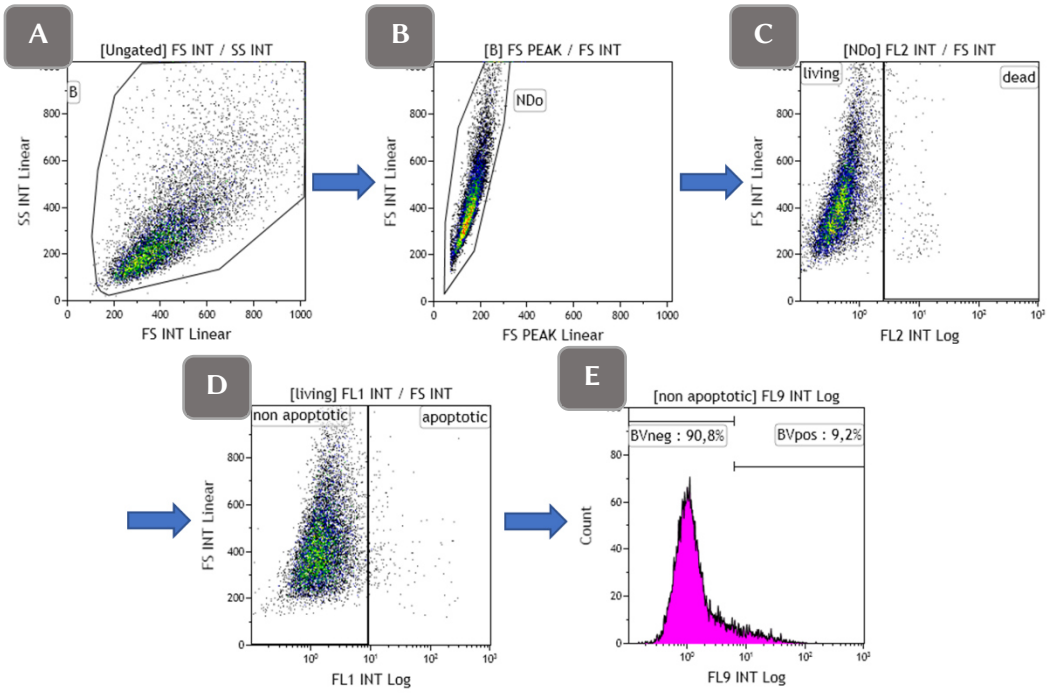


Figure 35: Gating strategy used to analyze CD146-BV421 staining associated to annexinV and PI.

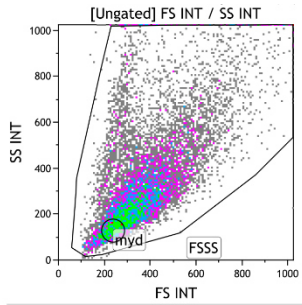
A: 10 000 events are processed and a large population is selected in the FSC (Forward Scatter) / SSC (Side Scatter) chart.

B: Doublets are excluded with the FSC peak / FSC area integral chart. Doublets are revealed because they display a more important area signal for the same peak height.

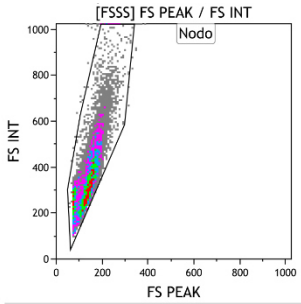
C+D: Selected cells without doublets undergo a last step of selection with annexinV and PI staining to exclude apoptotic and dead cells.

For cells with FITC and PE staining, LIVEADEAD violet is used to exclude dead cells.

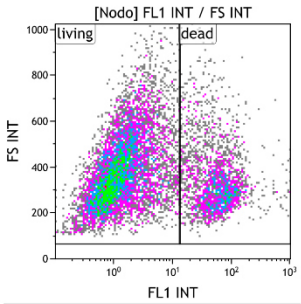
E: The analysis of CD146 expression can then be made on living and non-apoptotic cells.



Gate	Number	%Total	X-Med	Y-Med
All	10 000	100,00	355,89	314,67
FSSS	9 714	97,14	352,72	307,70
myd	933	9,33	239,66	133,26

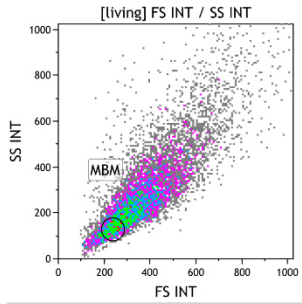


Gate	Number	%Gated	X-Med	Y-Med
All	9 714	100,00	145,74	352,72
Nodo	9 513	97,93	144,66	348,44



Gate	Number	%Gated	X-Med	X-Stdev
All	9 513	100,00	1,93	56,79
dead	2 334	24,53	55,05	95,58
living	7 187	75,55	1,32	2,33

Gate	X-CV	Y-Med	Y-Stdev	Y-CV
All	283,96	348,44	150,63	39,72
dead	127,64	312,79	144,46	41,00
living	107,41	362,54	151,73	39,10



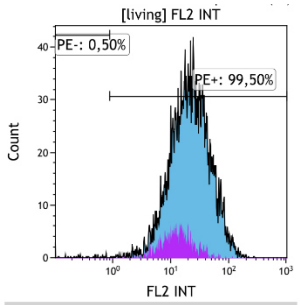
Gate	Number	%Gated	X-Med	X-AMean
All	7 187	100,00	362,54	388,06
MBM	873	12,15	240,31	240,55

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	306,00	151,73	39,10	2,64
MBM	236,00	23,48	9,76	1,26

Gate	X-Min	X-Max	X-GMean	Y-Med
All	100,91	1 012,64	359,18	265,08
MBM	191,47	288,27	238,88	132,45

Gate	Y-AMean	Y-Mode	Y-Stdev	Y-CV
All	307,43	195,00	177,51	57,74
MBM	132,77	124,00	22,55	16,98

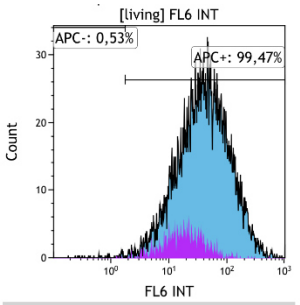
Gate	HP Y-CV	Y-Min	Y-Max	Y-GMean
All	4,57	15,73	1 024,00	260,97
MBM	7,53	81,27	179,73	130,30



Gate	Number	%Gated	X-Med	X-AMean
All	7 187	100,00	21,82	28,57
PE-	36	0,50	0,35	0,33
PE+	7 151	99,50	21,91	28,71

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	24,72	23,74	83,11	24,07
PE-	0,10	0,43	131,81	0,38
PE+	24,72	23,72	82,61	24,07

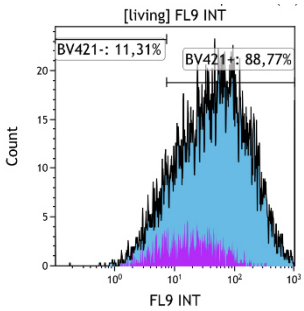
Gate	X-Min	X-Max	X-GMean
All	-1,86	277,95	20,99
PE-	-1,86	0,78	0,32
PE+	0,92	277,95	21,44



Gate	Number	%Gated	X-Med	X-AMean
All	7 187	100,00	40,64	63,24
APC-	38	0,53	0,94	0,93
APC+	7 149	99,47	40,88	63,57

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	52,63	71,91	113,71	5,71
APC-	0,89	0,65	70,56	0,38
APC+	52,63	71,95	113,19	5,71

Gate	X-Min	X-Max	X-GMean
All	-1,69	1 023,73	38,77
APC-	-1,69	1,76	0,79
APC+	1,82	1 023,73	39,58

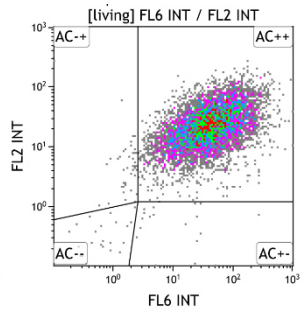


Gate	Number	%Gated	X-Med
All	7 187	100,00	47,02
BV421-	813	11,31	4,60
BV421+	6 380	88,77	57,41

Gate	X-AMean	X-Mode	X-Stdev
All	91,92	98,78	126,65
BV421-	4,50	7,02	1,87
BV421+	102,98	98,78	130,30

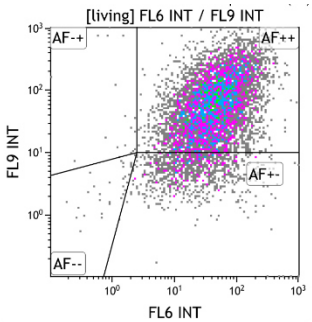
Gate	X-CV	HP X-CV	X-Min
All	137,78	0,77	-7,18
BV421-	41,49	3,11	-7,18
BV421+	126,54	0,77	7,40

Gate	X-Max	X-GMean
All	1 023,99	42,40
BV421-	7,43	4,01
BV421+	1 023,99	57,17



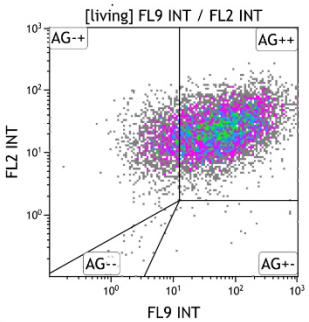
Gate	Number	%Gated	X-Med	X-GMean
All	7 187	100,00	40,64	38,77
AC--	22	0,31	0,67	0,66
AC-+	44	0,61	2,01	1,67
AC+-	20	0,28	15,77	13,29
AC++	7 101	98,80	41,24	40,16

Gate	Y-Med	Y-GMean
All	21,82	20,99
AC--	0,28	0,32
AC-+	4,77	5,09
AC+-	0,67	0,46
AC++	22,02	21,69



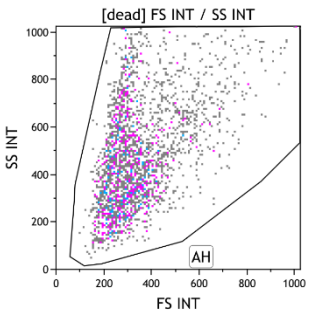
Gate	Number	%Gated	X-Med	X-GMean
All	7 187	100,00	40,64	38,77
AF--	32	0,45	1,39	0,94
AF-+	17	0,24	1,56	1,12
AF+-	1 104	15,36	18,82	20,44
AF++	6 034	83,96	45,91	44,91

Gate	Y-Med	Y-GMean
All	47,02	42,40
AF--	2,91	2,86
AF-+	35,91	56,23
AF+-	5,83	5,08
AF++	62.05	63.36



Gate	Number	%Total	%Gated	X-Med
All	7 187	71,87	100,00	47,02
AG--	31	0,31	0,43	2,21
AG-+	1 371	13,71	19,08	6,82
AG+-	19	0,19	0,26	85,93
AG++	5 766	57,66	80,23	65,98

Gate	X-GMean	Y-Med	Y-GMean
All	42,40	21,82	20,99
AG--	2,24	0,30	0,33
AG-+	5,96	14,80	14,81
AG+-	76,61	1,08	0,78
AG++	68.56	24.01	23.58



Gate	Number	%Gated	X-Med	X-AMean
All	2 334	100,00	312,79	352,36
AH	2 334	100,00	312,79	352,36

Gate	X-Mode	X-Stdev	X-CV	X-GMean
All	296,00	144,46	41,00	327,38
AH	296,00	144,46	41,00	327,38

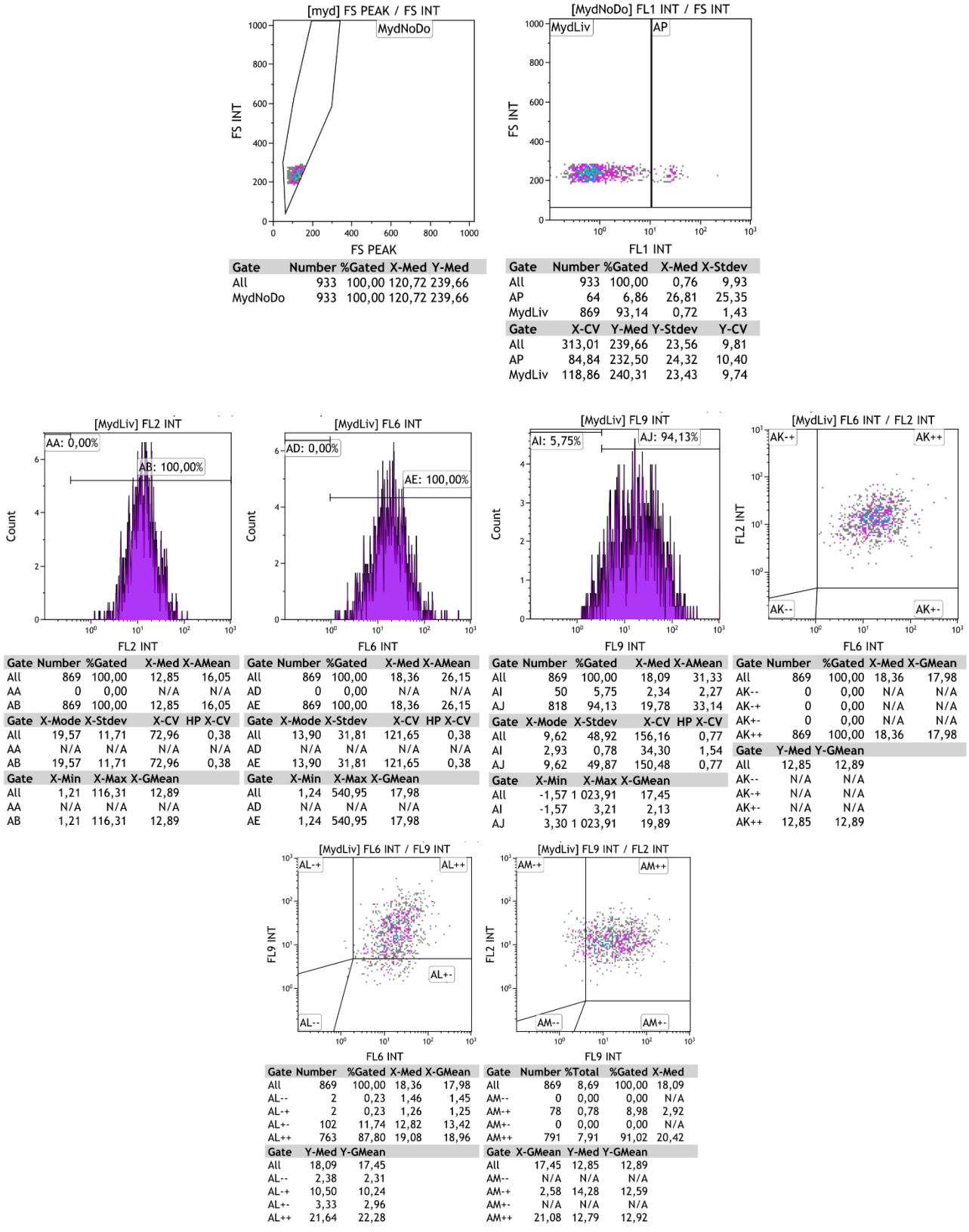
Gate	Y-Med	Y-AMean	Y-Mode	Y-Stdev
All	458,80	486,77	288,00	215,91
AH	458,80	486,77	288,00	215,91

Gate	Y-CV	Y-GMean
All	44,36	435,28
AH	44,36	435,28

Figure 36: Successive gating and data visualization made when using antibody panels combining viability staining and the detection of 3 surface proteins.

This example shows the output of the panel combining ZombieGreen (FL1), CD105-PE (FL2), CD157-APC (FL6) and CD146-BV421 (FL9).

These results were obtained from BM MSC of a 58 years-old donor at passage 1.



Gate	Number	%Gated	X-Med	Y-Med
All	933	100,00	120,72	239,66
MydNoDo	933	100,00	120,72	239,66

Gate	Number	%Gated	X-Med	X-Stdev
All	933	100,00	0,76	9,93
AP	64	6,86	26,81	25,35
MydLiv	869	93,14	0,72	1,43

Gate	X-CV	Y-Med	Y-Stdev	Y-CV
All	313,01	239,66	23,56	9,81
AP	84,84	232,50	24,32	10,40
MydLiv	118,86	240,31	23,43	9,74

Gate	Number	%Gated	X-Med	X-AMean
All	869	100,00	12,85	16,05
AA	0	0,00	N/A	N/A
AB	869	100,00	12,85	16,05

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	19,57	11,71	72,96	0,38
AA	N/A	N/A	N/A	N/A
AB	19,57	11,71	72,96	0,38

Gate	X-Min	X-Max	X-GMean
All	1,21	116,31	12,89
AA	N/A	N/A	N/A
AB	1,21	116,31	12,89

Gate	Number	%Gated	X-Med	X-AMean
All	869	100,00	18,36	26,15
AD	0	0,00	N/A	N/A
AE	869	100,00	18,36	26,15

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	13,90	31,81	121,65	0,38
AD	N/A	N/A	N/A	N/A
AE	13,90	31,81	121,65	0,38

Gate	X-Min	X-Max	X-GMean
All	1,24	540,95	17,98
AD	N/A	N/A	N/A
AE	1,24	540,95	17,98

Gate	Number	%Gated	X-Med	X-AMean
All	869	100,00	18,09	31,33
AI	50	5,75	2,34	2,27
AJ	818	94,13	19,78	33,14

Gate	X-Mode	X-Stdev	X-CV	HP X-CV
All	9,62	48,92	156,16	0,77
AI	2,93	0,78	34,30	1,54
AJ	9,62	49,87	150,48	0,77

Gate	X-Min	X-Max	X-GMean
All	-1,57	1 023,91	17,45
AI	-1,57	3,21	2,13
AJ	3,30	1 023,91	19,89

Gate	Number	%Gated	X-Med	X-GMean
All	869	100,00	18,36	17,98
AK--	0	0,00	N/A	N/A
AK-+	0	0,00	N/A	N/A
AK+-	0	0,00	N/A	N/A
AK++	869	100,00	18,36	17,98

Gate	Y-Med	Y-GMean
All	12,85	12,89
AK--	N/A	N/A
AK-+	N/A	N/A
AK+-	N/A	N/A
AK++	12,85	12,89

Gate	Number	%Gated	X-Med	X-GMean
All	869	100,00	18,36	17,98
AL--	2	0,23	1,46	1,45
AL-+	2	0,23	1,26	1,25
AL+-	102	11,74	12,82	13,42
AL++	763	87,80	19,08	18,96

Gate	Y-Med	Y-GMean
All	18,09	17,45
AL--	2,38	2,31
AL-+	10,50	10,24
AL+-	3,33	2,96
AL++	21,64	22,28

Gate	Number	%Total	%Gated	X-Med
All	869	8,69	100,00	18,09
AM--	0	0,00	0,00	N/A
AM-+	78	0,78	8,98	2,92
AM+-	0	0,00	0,00	N/A
AM++	791	7,91	91,02	20,42

Gate	X-GMean	Y-Med	Y-GMean
All	17,45	12,85	12,89
AM--	N/A	N/A	N/A
AM-+	2,58	14,28	12,59
AM+-	N/A	N/A	N/A
AM++	21,08	12,79	12,92

Figure 37: Following figure 37, output obtained after gating a restricted FSC/SSC area.

To compare marker expression between cells of different samples and avoid the bias that bigger cells may appear brighter but not express more a marker, **a small round gate was created in the FSC/SSC chart and always placed at the same position**. This allowed to compare cells of similar size between different samples. This gate was only used as an additional indication to help interpretation of the results with whole samples. If a marker was found to increase in the whole living population but do not increase for the **FSC / SSC restricted population of cells**, that can mean the increase observed is associated to the presence of bigger cells, and may not show a real increase of expression. That could especially be the case for senescent cells.

To help the interpretation of results obtained from samples with various cell sizes, a gate was created to compare cells of similar size between each sample (figure 37). In this work, cell volume was also approximated by side scatter (SSC, area integral) parameter rather than forward scatter (FSC) parameter. Indeed, with its orientation, SSC channel is usually quieter than other fluorescence collection paths. The detector of SSC is also usually more sensitive, providing better resolution than FSC. A study comparing flow cytometry results with measures of cell volumes also confirmed that SSC was a better parameter than FSC to make approximation of cell size (257).

The cells were gated from a maximum of 10 000 events acquired with the flow cytometer.

Isotype controls (noted ISO) were used for each experiment at the same concentration and with the same combination as the test antibodies. Isotypes were chosen to have the same constant part than the antibodies used for cell labelling. They do not recognize any specific antigen in humans and fix cell surface in a non-specific manner. They are considered in this study as an intern reference obtained within the same cell population. They provide a baseline level of fluorescence obtained with the same settings, on the same cell population and with the same fluorochrome, but not associated to the presence of a precise antigen. These controls also enabled to follow the variations associated to cell autofluorescence, with the instrument or with other environmental factors. However, isotypes should not be considered as good indicators of background fluorescence of the other antibodies used because of the differences in their variable parts and different fluorochrome / antibody ratio notably (258).

The fluorescence of isotype controls was also needed to position to threshold used to determine the percentages of expression of the markers studied. The gate was set to have less than 2% positive cells in the events measured with the isotype. This gate was applied to the cells of the tested samples, from within the same cell population and labelled with the antibodies with the same fluorochrome.

With these measures, two kinds of approach were combined to analyze the different markers studied in this work: percentages and fluorescence intensities.

- Isotype controls were used to normalize the fluorescence intensities obtained with the tested samples. This process allows to obtain more reliable results in several ways: this is more robust to compare experiment undertaken at different times and less sensible to outliers. It also has the advantage to be able to better differentiate the level of expression of a protein between different samples. This parameter reports more precisely the shift in fluorescence intensity of the entire population than percentages (259).

- The approach with the percentages is the most commonly found in the literature but it is usually better adapted for flow cytometry results that allow to distinguish different population of cells expressing differently a marker without the need of isotypes, as it can be the case to distinguish different types of white blood cells for example. For MSC, clear distinctions of cells expressing differently a marker are rare and isotypes were needed to obtain percentage results. These results however, cannot be interpreted as real distinction between positive and negative cells, as the threshold often cut a homogeneous cell distribution in a too arbitrary manner with the 2% method. When all a distribution is translated unaltered toward higher levels of fluorescence in the test sample, but not enough to completely go beyond isotype distribution, that means all cells were labelled but display a weak fluorescence signal, so all cells are positive in a way, but probably with a low expression of the studied marker. In this case, the percentage method with the isotype will falsely separate the distribution in two. Furthermore, percentage is a binary parameter (positive/negative) and does not permit to distinguish between different levels of expression, notably for medium and highly expressed marker, when the fluorescence intensities are very far from the one obtained in the control (all cells find themselves in the positive gate without distinctions). The percentage parameter is also more prone to bias. It is more sensible than normalized fluorescence to the presence of outliers and a very slight move of the gate threshold can also result in a jump in percentage when it crosses the middle of a population distribution.

Percentages remain an indicator that can be interesting in some cases. For some very low expressed markers on MSC, percentage being more sensible to outliers, they can sometimes reveal a change in population shape and position that normalized fluorescence intensity would miss. It is still important to remain prudent, as it was reported in a study with monocytes and macrophages, the isotype control can even display a fluorescence intensity greatly exceeding the fluorescence of some low expressed marker, such as Tie2 (260).

The most representative parameter of the entire cell population is the Median Fluorescence Intensity (MFI). More information about the advantages and disadvantages of different parameters can be found in part VI. "Statistics" of the Methods chapter, in the table in part 4. "Multivariate analysis".

B. Fluorescence microscopy

1. Staining of fixed cells

Cells were seeded at 1000 cells / cm² on coverglasses, previously washed with ethanol and rinsed with PBS, placed in 24-well plates. Cells were grown in the classical conditions described in the chapter Methods, part II: "Isolation and culture of MSC". The cells were left between 7 and 14 days to obtain as much cells as possible without confluence. They were washed with PBS 2 times and fixed during 15 minutes with 1% formaldehyde freshly prepared from 4% formaldehyde diluted with PBS (Gibco). Cells were then washed 2 times with PBS and permeabilized with 0.5% Triton (Sigma) during 45 minutes. Blockage was then made with 0.5% BSA during 20 minutes. Cells were then washed and incubated with primary antibodies during 45 minutes in the dark with dilutions and reagents presented table 11. After 3 washes with PBS, cells were stained with secondary antibodies associated to Goat anti mouse or anti rabbit AlexaFluor488 fluorochrome (ThermoFisher) freshly diluted 1/50. All dilutions were made with a solution of PBS with 0.5% BSA and 0.1% Triton. A final staining with DAPI (Sigma) diluted 1/2500 was then made. All the staining steps were made at room temperature.

Table 11: List of antibodies and dilutions used for fluorescence microscopy.

Antibody	Dilution
Rabbit antibody for HMGB1 (Abcam)	1/500
Rabbit antibody for SOD2 (Abcam)	1/150
Rabbit antibody for H3me3K9 (Abcam)	1/500
Mouse antibody for laminA (Abcam)	1/1000
Mouse antibody for ki67 (Millipore)	1/100
Mouse antibody for p16 (Abcam)	1/200
Phalloidin-AlexaFluor488 (ThermoFisher)	1/50

Images were acquired with Leica DMI3000B microscope with Leica Application Suite (LAS) software. When early and late passage cells were compared, images were taken with the same settings and at the same moment.

Rabbit antibody for laminB antibody was tested on different MSC samples from BM and WJ at different dilutions but it always resulted with no signal.

2. Staining on living cells

Fixation reduces greatly the quality of the staining of surface proteins by altering the cell membrane. To observe CD146 protein on MSC, the staining was made on living cells as follows. Culture medium alpha MEM without FBS and without phenol red, but with 0.5% BSA added is used to block and for the dilution and incubation of antibodies. After 20 minutes of blockage, CD146 primary antibody (Mouse anti-Human CD146, Clone P1H12, BD Pharmingen, 0.1 mg) diluted 1/200 was placed during 45 minutes at room temperature. After washing with PBS, AlexaFluor488 secondary antibody diluted 1/50 associated with DAPI diluted 1/40 were then added during 45 minutes. After washing, images were acquired with Leica DMI3000B microscope using the same settings for the different cells to compare (unsorted, positive and negative fractions).

IV. Cell sorting of CD146^{high} BM-MSC

After detaching and counting, MSC underwent magnetic sorting to separate CD146^{high} expressing cells from CD146^{low} cells **(figures 38 and 39)**. MSC from the same sample were kept unsorted to constitute a base reference.

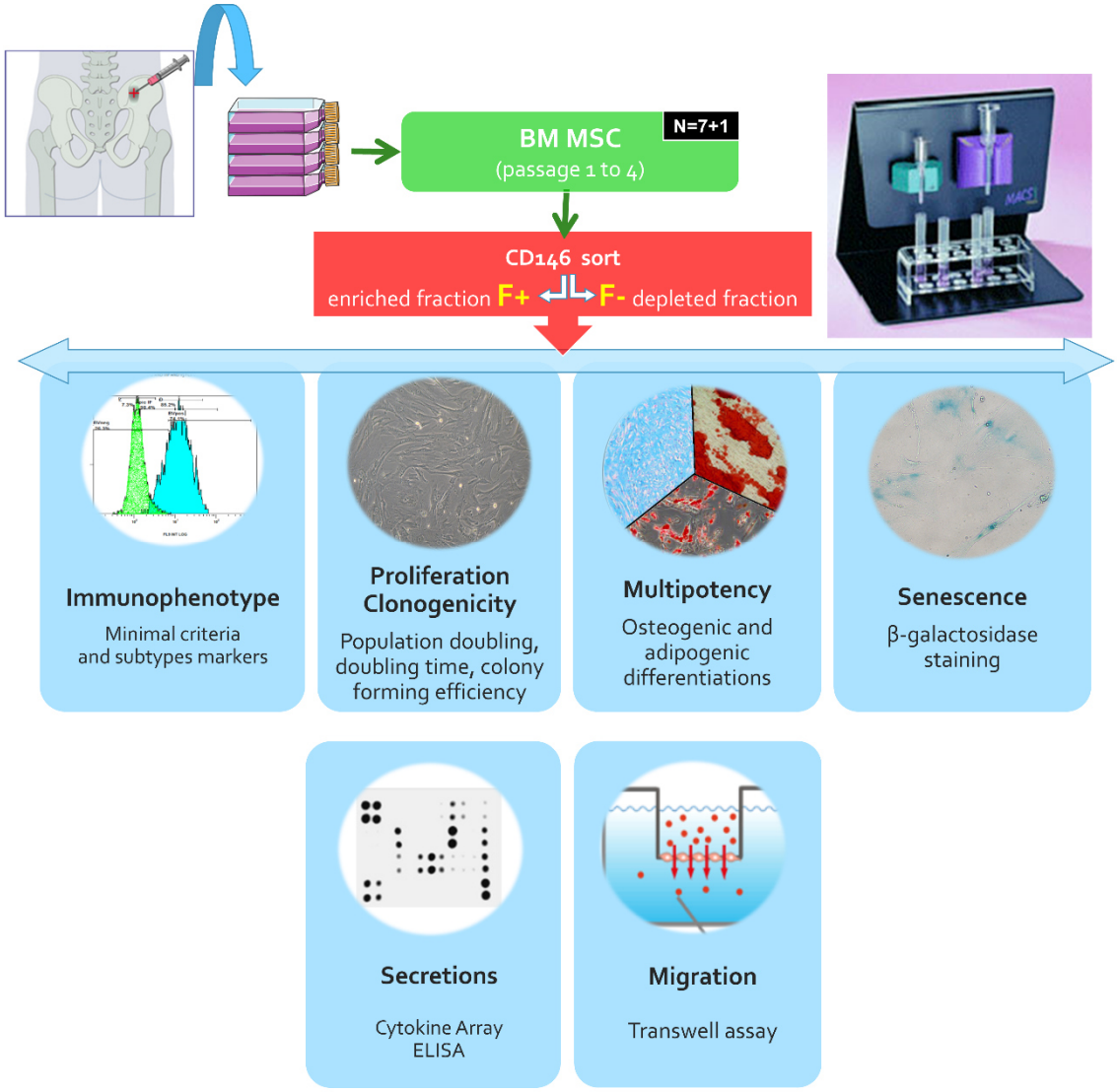


Figure 38: Experimental setup to compare the properties of CD146 high expressing and CD146 low expressing MSC after cell sorting.

Cytokine array and migration were only made on 2 sorted samples.

N=7+1 means a total of 8 samples were sorted, but one was sorted with FACS while the others were sorted with immunomagnetic technique.

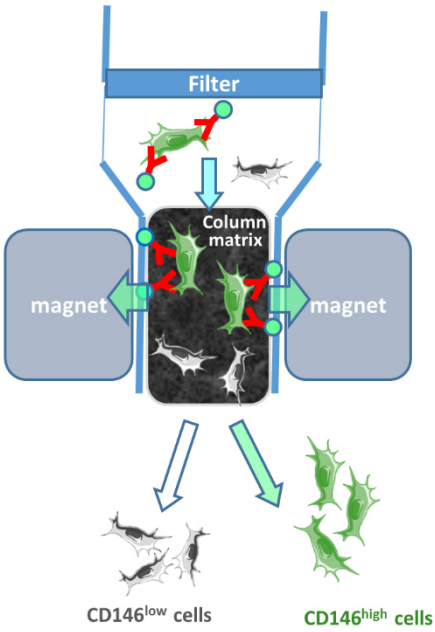


Figure 39: Schematic representation of CD146 magnetic sorting principle.
Magnetic beads are represented as green discs associated to CD146 antibodies represented in red. CD146 expressing cells are covered with antibodies associated to magnetic bead that allow them to stay in the column when placed in a strong magnet while $CD146^{low}$ cells are washed out. Then the column is removed from the magnet to flush $CD146^{high}$ cells out of the column.

MACS Miltenyi Biotec CD146 MicroBeads Kit human was used according to the manufacturer's protocol. All the steps were made at 4°C and volumes are given for 10^7 cells. MSC were filtered and centrifuged, the supernatant was aspirated and 20 µL of FcR blocking reagent were added to the pellet volume for 5 minutes. Then, 20 µL of CD146 microbeads were added for 15 minutes. Afterwards, 1mL of washing buffer made with PSB, 0.5% BSA and 2mM EDTA was added and the cells centrifuged. MSC were resuspended in 500 µL of washing buffer and deposited through a pre-separation filter of 70 µM in a LS column moistured beforehand with 3 mL washing buffer and positioned on a MidiMACS separator (Miltenyi Biotec). The column was washed 3 times with 3 mL washing buffer. The column was removed from the separator and magnetically labeled cells were flushed out with 5 mL washing buffer with the help of a plunger in a second separation LS column to obtain a better enrichment in $CD146^{high}$ cells. This column was also washed 3 times with 3 mL washing buffer and the cells remaining in the column were flushed out with a plunger in 5

mL washing buffer. All unlabeled eluted cells were gathered and constitute $CD146^{low}$ fraction of cells.

Fluorescence activated cell sorting (FACS) was also performed with Beckman Coulter MoFlo Astrios by the Cytometry core facilitiy of UMS2008 IBSLor (Université de Lorraine - CNRS - INSERM). MSC were blocked with 0.5% BSA and stained with 100 µL/mL CD146-BV421 antibody. Around 30% of the cells that express the most CD146 were gathered on one way and 30% of cells that express the less CD146 were gathered on the other way. The cell count on the flow cytometer was used. Some cells after separation were used to analyze CD146 expression.

After this separation step, cells from both fractions were counted and resuspended in culture medium to seed them and further cultured in the same manner as the control group. A part of the cells after separation were used for CD146 analysis in flow cytometry.

More details about the sorting principles are provided in the introduction, in part II. - C. “MSC usage and obtention”.

V. Proteomics

For this proteomics study, bone marrow MSC from 3 young donors were studied. The experimental setup is shown **figure 40**.

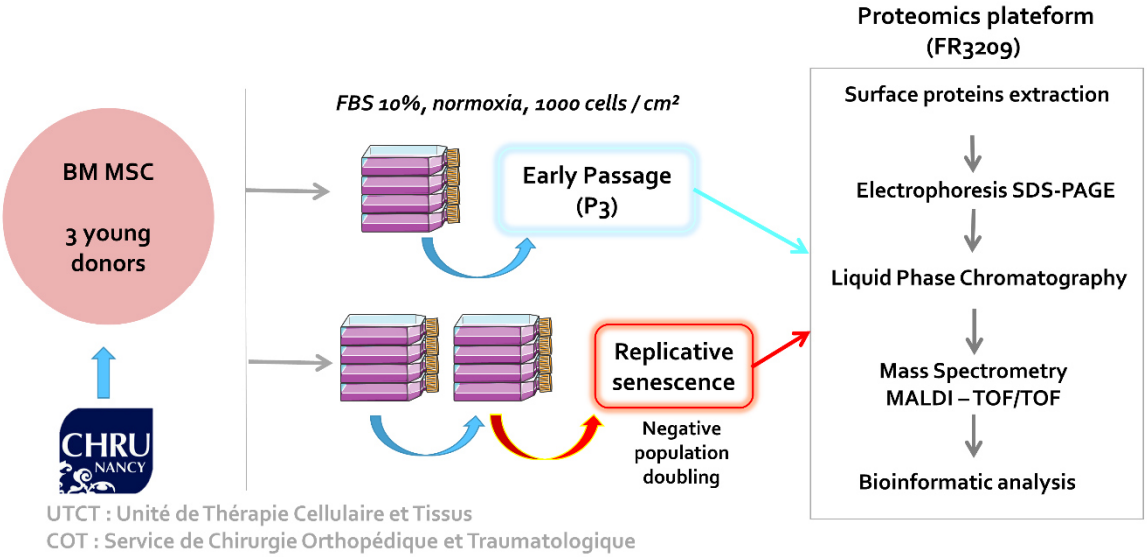


Figure 40: Experimental setup for the comparative proteomic study of replicative senescent and young MSC.

A. Cell surface proteins purification

Proteomics study have the advantage to allow a global view of the proteins in the samples without needing prior knowledge of what is searched. However, quantitative proteomics is particularly challenging for less abundant proteins, such as plasma membrane bound proteins. It was made possible thanks to the help and tenacity of the proteomics core facility of UMS2008 IBSLor.

Surface protein purification was performed on early passage cells (passage 3) and replicative senescent cells seeded in petri dishes at 1000 cells/cm² for passage 3 cells and 2000 cells/cm² for senescent cells. 3 replicates were made for each point.

All the experiments were made at 4°C. MSC in petri dishes were washed 3 times with PBS pH 8.0. Then MSC surface proteins were biotinylated with EZ-Link™ Sulfo-NHS-SS-Biotin (ThermoFisher) 480 µg/mL during 30 minutes. MSC were then washed 3 times with PBS complemented with 25 mM Tris pH 8.0. Cells were then scraped in 700 µL PBS pH 8.0 for

each petri dish. Gathered cells were put in ultrasound bath for 1 minute, vortexed 15 seconds, and centrifuged at 16 000 g 15 minutes at 4°C. The supernatant was eliminated and cells were suspended in 1 mL lysis buffer containing Igepal CA-630 1%, Deoxycholate 0.5%, PBS pH 8.0 and a tablet of cOmplet Mini Protease Inhibitor with EDTA (Roche) in 50 mL. Tubes were left at least 1 hour with agitation. After this, lysates were centrifugated at 16 000 g, 15 minutes at 4°C. Supernatant was collected. 400 µL of Pierce NeutrAvidin UltraLink Resin (ThermoFisher) were pipetted, washed with lysis buffer, and resuspended in 1 mL lysis buffer before use. 250 µL of prepared neutravidin resin was then mixed with the collected supernatant. This mix was passed through empty column. Washing buffer was prepared from lysis buffer with addition of 0.1% triton and 1 M NaCl. Columns were washed 3 times with 5 mL washing buffer. Elution was made with 500 µL DTT 100 mM by passing 5 times through the column. 1.2 mL of absolute ethanol at -20°C were added to the eluent before storing the tubes at – 80°C.

B. Relative quantification

Label-free relative quantification applied to Liquid chromatography (LC) - Matrix Assisted Laser Desorption Ionisation (MALDI) technique was made to compare replicative senescent and early passage surface protein extracts by the proteomics core facility of UMS2008 IBSLor. Dedicated automatic methods were used using WARP-LC software on an Autoflex speed MALDI-TOF/TOF (Time of Flight) mass spectrometer (Bruker). The method was described more precisely in the article presenting the technique (261). The triplicates of the six samples were gathered and analyzed together.

VI. Statistics

1. Univariate analysis

For comparisons of medians and means between different groups of low sample size or when the normality of the distribution of the data could not be confirmed, nonparametric Mann-Whitney tests were used to estimate p values with GraphPad Prism 6.0 (GraphPad Software, USA). The null hypothesis was that the medians or means were equal.

To allow a better visualization of the effect size of differences in migration capacity between groups in part III of the results, ordinary one-way ANOVA followed with Tukey-Kramer's multiple comparisons test were performed with representation of confidence intervals. In this case, it is assumed that the data of the population follows a Gaussian distribution.

When a high number of points were collected for each sample, the results were displayed in boxplots. The body of the box is determined by the first and the third quartile, the distance between the two is called inter-quartile distance. The whiskers represent minimum and maximum values when not mentioned otherwise. In the case of representation in Tukey Boxplot, the length of the upper whisker is stopped at the largest value in the dataset less than the sum of the third quartile plus 1.5 times the inter-quartile distance. For the lower whisker, it is stopped at the lowest value greater than the first quartile minus 1.5 times the inter-quartile distance. Values outside these ranges are plotted as individual points.

2. Bivariate analysis

The degree of linear correlation between two variables was measured with Pearson correlation coefficient, noted “r”. “r” and the associated p values were calculated with XLSTAT. Confidence ellipses shown in the charts are the representation of confidence intervals. The null hypothesis is that the coefficients are not significantly different from 0.

3. P values

P value consideration in this work corresponds to Fisher's significance testing. In this context, p values are determined after the experiments were undertaken, *a posteriori* from the observed data. Fisher p values can be applied to any single experiment, they are more suited for the kind of research made in this work, with series of related experiments and evolving hypotheses. In this case, p values are only an additional measure of evidence to be considered with the rest, no strict threshold dictates the way to interpret results, but different weights can be given according to the order of magnitude of the p value (between 0.045 and 0.055 are considered alike, while 0.0001 have more weight). Results can also be weighted with other arguments considering background knowledge and coherence with other results for examples. This approach is in contrast with the Neyman-Pearson's Hypothesis Testing, with risk error rates α and β determined *a priori*. In this case, a precise threshold is decided to conclude with the p values, and a difference of 0.002 in the p values obtained can lead to opposite conclusions. This is more suited for experiment where a decision must be taken at the end, like for clinical trials.

As a reminder, p values represent the probability of observing similar or more extreme data than the one obtained, when considering the null hypothesis is true. When the effects observed are very marked, very different from what is proposed by the null hypothesis, p values become so low that is can be considered that either the null hypothesis is not true, or an exceptionally rare event has occurred. When the observations obtained are similar or in line with the null hypothesis, p values are high and show there are high probabilities that similar data would be obtained with the null being true. It can be summarized that the smaller the p value, the less plausible it would be that the null hypothesis is true.

To avoid additional heaviness in writing, the classical assessment about "the significance of results" were made, but the meaning behind is detailed here: "Considering the null hypothesis is true, there was only a "p" probability (according to p value) to observe the results obtained (or more extreme results)" (262,263).

4. Multivariate analysis – Principal Component Analysis (PCA)

Combining cell phenotype and functional parameters represented too many parameters to allow a global vision of the possible links when considering only parameters 2 by 2. To serve as a basis for exploration and in order to have a better view of the correlations links that could be revealed from the data, a multivariate statistical analysis was undertaken, in this case Principal Component Analysis using XLSTAT software. The global Pearson correlation matrix allowed to determine possible linear relationships between the numerous variables. Eigenvalues represent quality of the projection of the initial multi-dimensional table. The charts are based on the first two factors (F1 and F2) and must represent the highest percentage of the variability to give the most information. Some information remains hidden in the other factors and care must be taken with interpretation of the maps.

To our knowledge, PCA was not yet used to have insight about data obtained from phenotypic characteristics and cell functionality after cell sorting. It is usually used for genetic expression pattern recognition according to cell type, but have also been used to correlate morphological features with cell functionalities (264,265). Sorting experiment conduces to declination in 3 fractions to compare for each sample and PCA showed the advantage to allow to consider many different parameters at the same time and to reveal potential links that could not be seen otherwise. We also used PCA to compare different type of variables that we can extract from the same raw data set. In the literature, only a few parameters are usually tested, for example percentage of positivity for flow cytometry results. To have the best chance to notice clues that could come out of the results, we chose to add all the relevant parameters that we could access. Each parameter has its advantages and bias and their combination permits to sharpen the possible analyses. The interests and bias associated to each kind of variable are listed in **table 12.**

Table 12: Interest and bias of the different parameters used for PCA.

Category	Parameter	Interest	Bias
Proliferation	**Population Doubling**	Number of times cells divided to allow the population to double during the corresponding passage.	All samples do not remain the same time in culture for one passage because trypsin is made at around 80% confluency, even if it is most of the time close to 14 days for BM-MSC.
	Doubling time	Consideration of the speed of proliferation by taking account of the time in culture for the corresponding passage.	Do not give information about the number of divisions that will depend on the time let in culture for the passage.
	Cumulative population doubling	Related to the total amount of time a cell divided at the time of the measure and gives an idea of which step in culture the cell is.	Will depend on the passage of each sample and vary according to the time let in culture for each passage.
	CFU	Proliferation capacity very isolated from other cells that can be related to stemness.	Beads for cell sorting might reduce MSC adhesion, cells may also need more time or specific medium stimulation to express their stemness.
	Senescence rate	Single cell detection of B-galactosidase overexpression.	Overestimation of percentage of senescence rate can happen because of low specificity of the test and variations with culture conditions (confluence).
Differentiation	**Osteogenic differentiation**	Potential to obtain osteocyte phenotype and to secrete calcified matrix.	Lack of specificity in the measure method. Differences observed qualitatively are not represented in the quantitative estimation with percentage of stained surface.
	Adipogenic differentiation	Potential to become cell from adipogenic lineage and form lipidic vesicles.	Very sensitive to cell culture conditions, especially confluence.

Data	**Raw Data**	Complete information without loss.	High variability between samples. Sensible to instrument variations.
	Ratio between fractions of the same sample run on the same time	Better to compare results obtained at different moments and possibly with some variable conditions.	Smoothing of the data and less detectable differences.
Flow cytometry	**Percentage**	Reflect change in population shape and position compared to isotype. Can be more sensible to the appearance of small populations of cells expressing a marker.	Not precise, cannot be considered as a true percentage of positive cells but remains an indicator. Not sufficient for low to medium expressed markers.
	Median	The most robust parameter to describe a global population.	Differences between samples are more difficult to identify, small population are not considered.
	Geometric Mean	Describe more sharply the population with more possible detection of the presence of small populations. For logarithmic scales (124).	Outliers have a strong impact.
	Arithmetic Mean	For linear scale (FSC and SSC).	Outliers have a strong influence.
	Half-Peak Coefficient of Variation (HPCV)	Reflect dispersion of the population.	Very variable parameter.
Flow cytometry analysis	**Ratio / ISO**	Better to compare data of different experimental runs (259).	Smoothing of the data and less detectable differences.
	Delta / ISO	Results with indicator of unspecific fixation of antibodies subtracted (124).	Isotypes do not represent true unspecific fixation and background staining (266).

Results and discussion

This part combines the description of results with some comments associated to precise elements of method or experiment setup that can help to better understand the results. The discussion related to the specific results is also presented in the last paragraph of each section. A more general discussion is proposed in the next chapter.

I. MSC heterogeneity of surface marker expression

Surface markers were used to address an aspect of MSC heterogeneity and to follow it according to culture, donor and MSC proliferation parameters.

A. Parameters associated to MSC culture

Before considering the variability that can be found between different MSC samples, preliminary studies were made on the same samples analyzed at different times. This allowed to set the conditions needed to compare the cells and to observe the amplitude of variability of the flow cytometry technique.

1. Variability between BM MSC from different runs

After many years of MSC assessment by flow cytometry, some frozen samples were studied several times, sometimes after more than 3 years of cryostorage. The variability observed between the results combines particularly the influence of cell storage, difference in handling, and variations due to the measure instrument. Such results could only be gathered for some of the studied markers **(figure 41)**.

Results showed, for some samples, important variation in nMFI measured between different runs. It is important to know this to better appreciate and weigh the results obtained with this technique. The percentages associated to CD106 expression were the most variable among the observed parameters (figure 41-B). It can be consistent with other reports that highlighted CD106 expression to be sensible to stress induced by culture conditions such as cell density ((267), mRNA level). Two samples out of six showed strong variation in SSC reflecting cell size changes (figure 41-C). One sample out of four showed

important fluctuation on CD90 nMFI, increasing more than twice as much (figure 41-A). CD44 results were also variable but stayed in similar order of magnitude (figure 41-A).

CD146 results were relatively stable (figure 41-D). They stayed within close orders of magnitude, with only one sample out of six showing an important variation in nMFI, variation that was not found in percentage results. For CD146 assessment, both nMFI and percentages can then be relevant to follow.

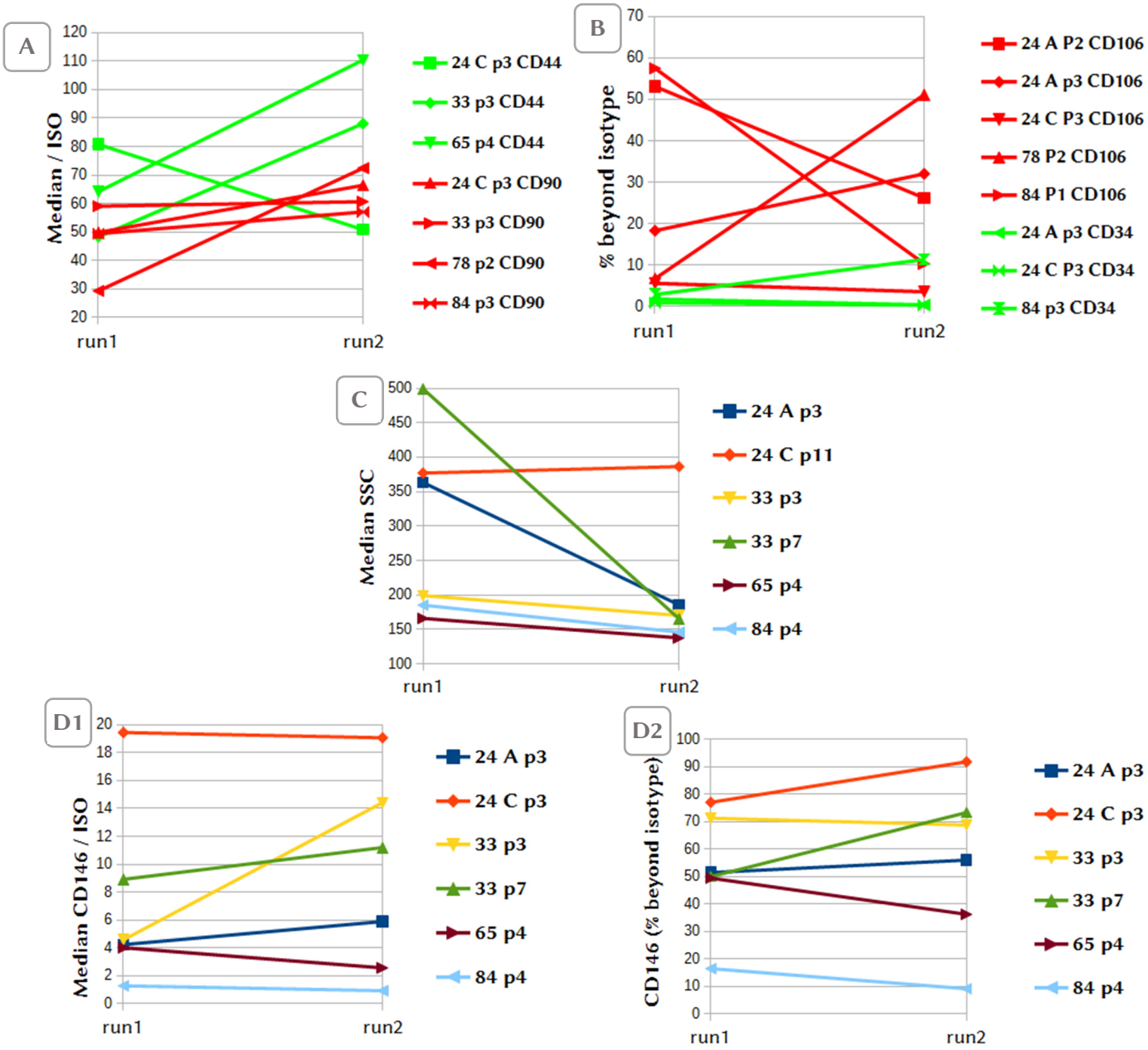


Figure 41: Differences of flow cytometry results between 2 different runs.

A: CD90 and CD44 that are highly expressed markers were presented in normalized MFI.
B: CD106 and CD34 results were displayed in percentage.
C: SSC results were presented with raw medians.
D1 and D2: CD146 results were displayed both in nMFI and in percentage.
Various passages are presented.

2. Fresh and thawed BM MSC

The results obtained from 3 samples allowed to compare the expression of several surface markers on fresh and thawed cells (figure 42). It is similar to the previous approach comparing different runs, but this time with a difference in the protocol. The principal interest was essentially to check if the cryostorage and thaw process could limit the detection of some markers.

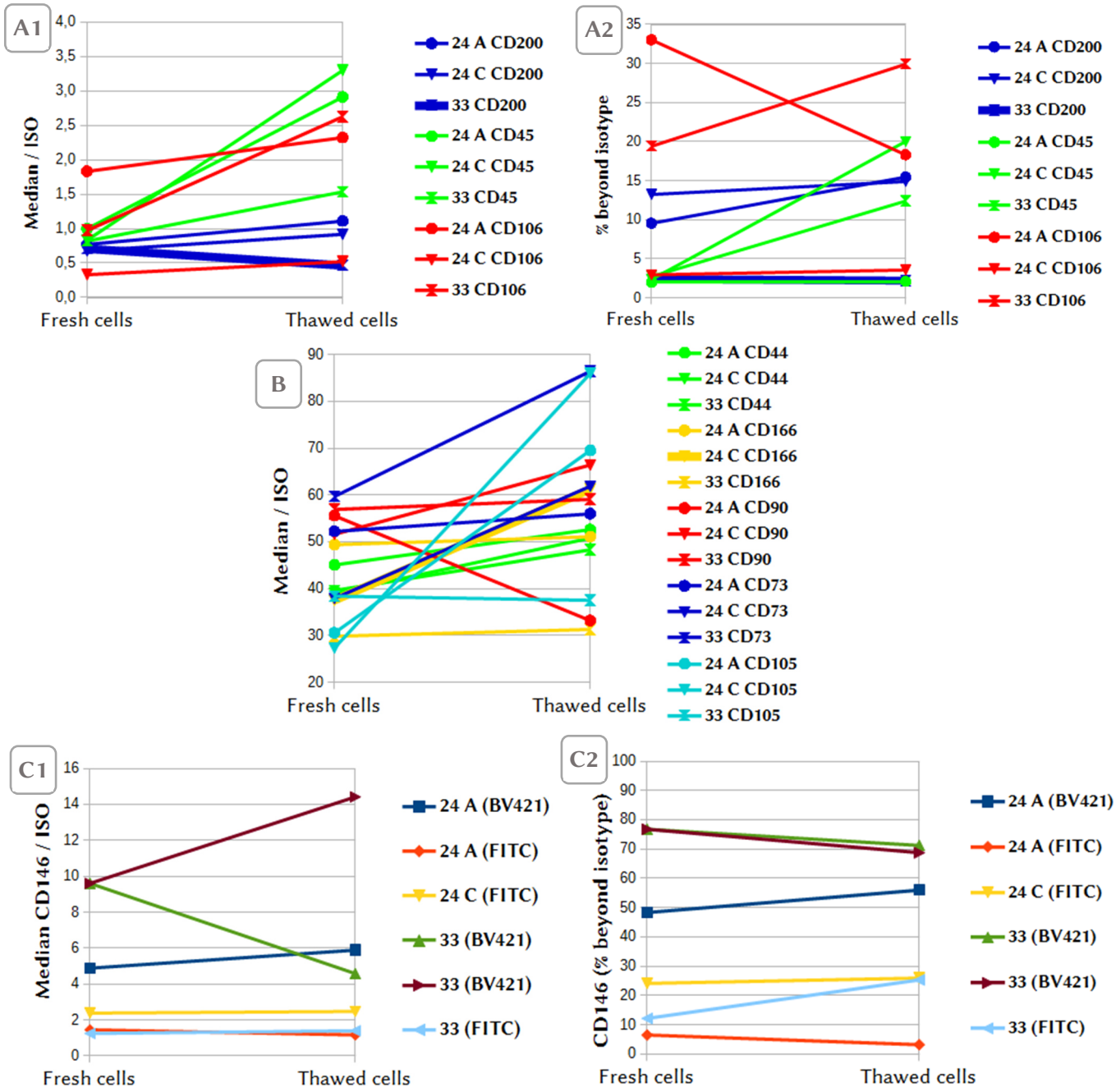


Figure 42: Differences of flow cytometry results between fresh and thawed cells.

The 3 samples were at passage 3. The samples were cultivated a first time and some cells frozen. Then passage 1 frozen cells were placed in culture in a second run. Fresh cells just after trypsinization were analyzed together with thawed cells coming from the same donor at the same passage.

Markers were gathered in one chart according to the scale of values: in A, lowly expressed markers in nMFI and percentages, in B, highly expressed markers, in C, CD146 results in nMFI and percentages.

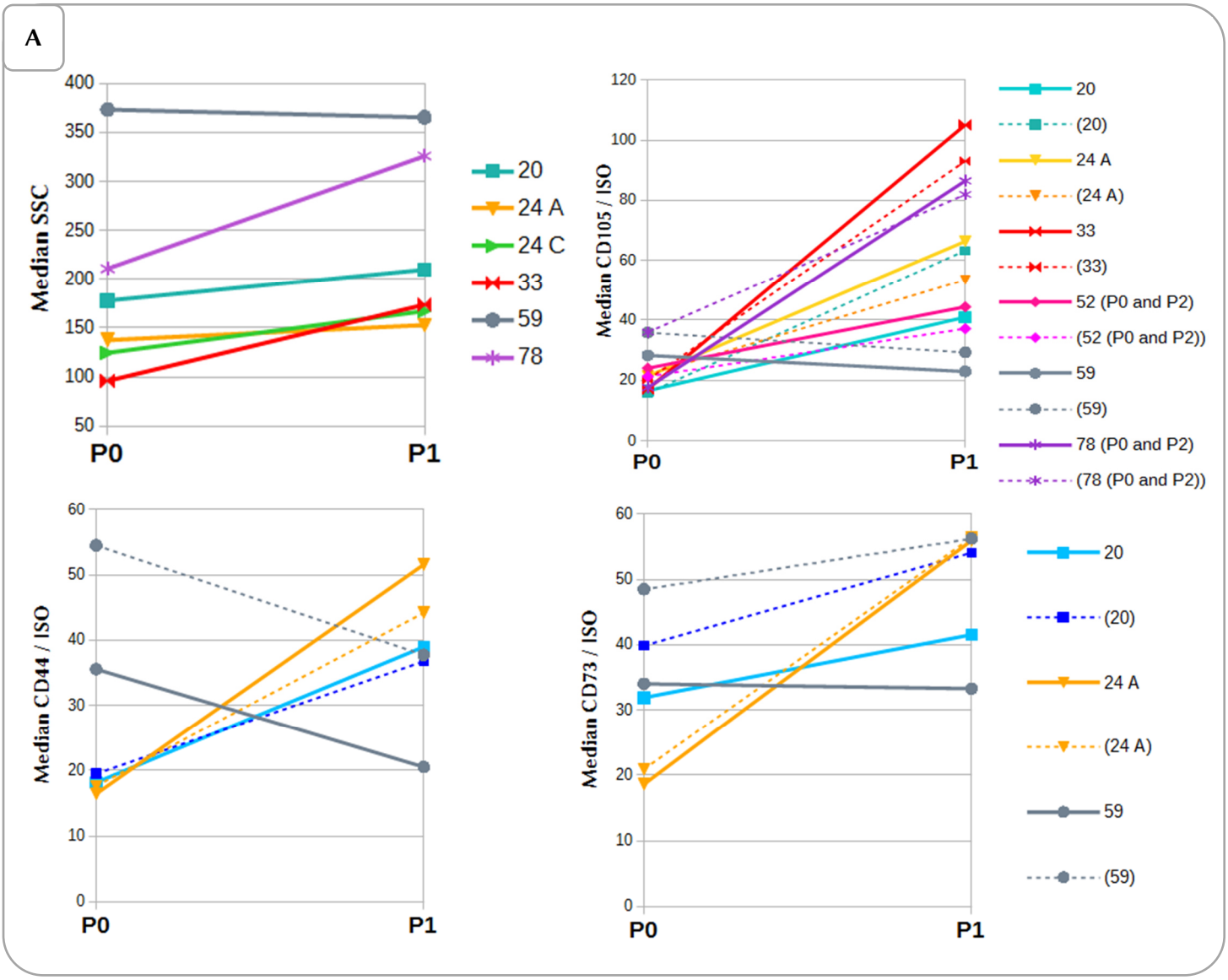
There was no case where a marker could only be detected in fresh cells and not in thawed cells. Some samples showed big variations, like nMFI results of CD105 for “24 A” and “24 C” (figure 42-B), but these variations were of the same type as those between different runs. Interestingly, CD146 expression was also found to be relatively stable, notably percentage results (figure 42-C2). It made it a good candidate to be a biomarker that could be searched to characterize cell state.

This kind of verifications is surely made by numerous research teams, but few report it in papers. There was one group that studied classical markers on BM MSC. They concluded that cryostorage did not affect the expression of these markers (268). One extensive study took also into account subtypes markers, but it was only on adipose-derived MSC. They also did not find any important change between fresh and thawed cells (143).

All the other flow cytometry experiments presented in this manuscript were then made with frozen cells.

3. Evolution between passage 0 and passage 1

P0 is an interesting time point, being the closest to MSC initial state. The evolution between these first passages can be informative about the first impact of culture on MSC phenotype. It can also help to estimate if early passage results can be gathered together or if they are too different to do so. The results of the evolution of MSC surface markers between p0 and p1 are shown **figure 43.**



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B

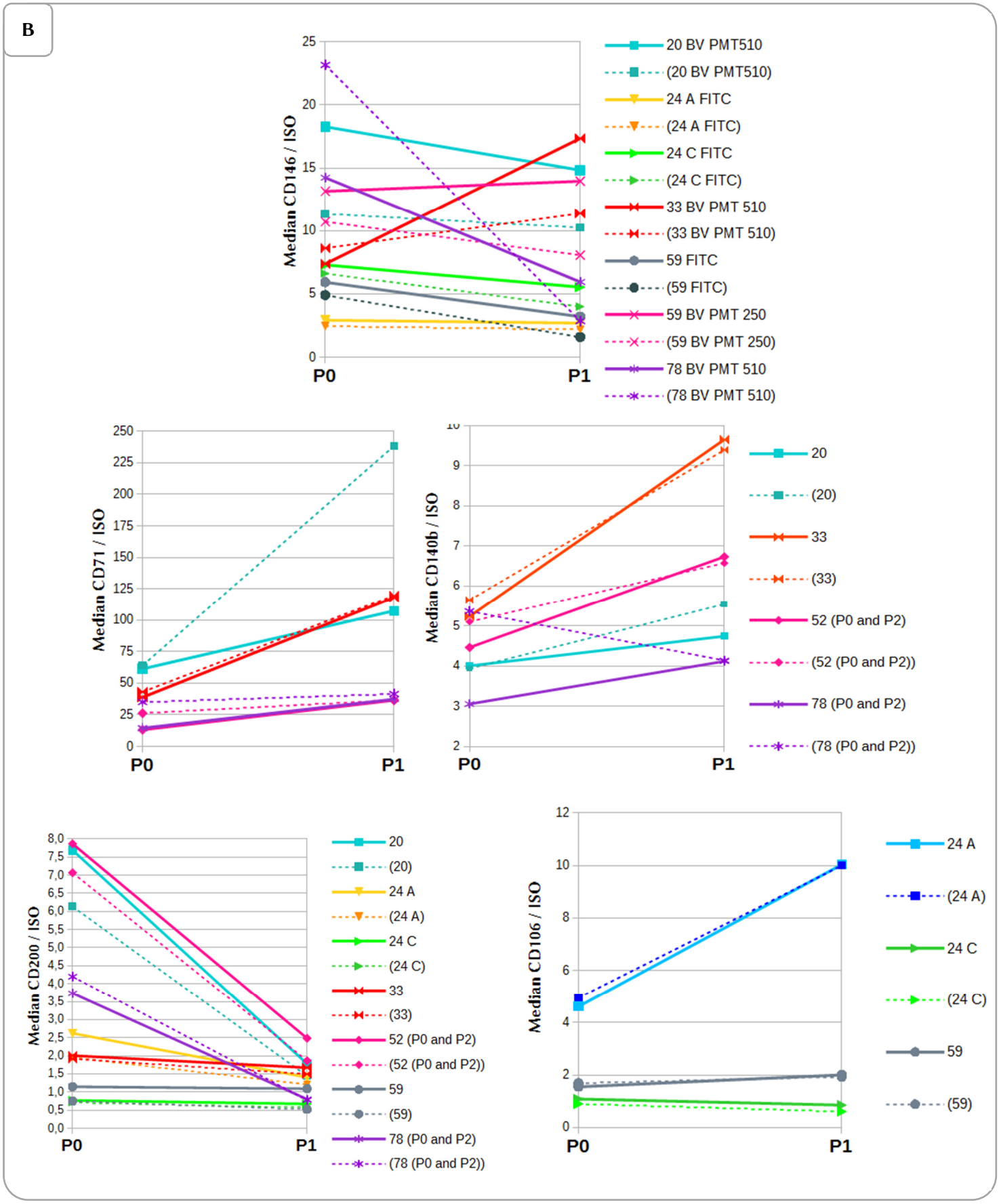


Figure 43: Evolution of MSC surface markers assessed by flow cytometry between p0 and p1.

All results are displayed with normalized median fluorescence intensity parameter.

Between brackets in the legend, and represented by dotted lines: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size and limit the bias that bigger cells can appear brighter.

A: SSC and a set of highly expressed classical MSC markers.

B: MSC subtype markers.

SSC values reflecting cell size showed a slight tendency to increase between p0 and p1 for 5 out of 6 samples studied. In general, the lowest SSC values were found at passage 0 (figure 43-A).

The expression of CD105, CD44, and CD73 showed a tendency to increase between p0 and p1, independently from the increase in cell size, except the sample "59" that behaved differently. This could be explained because the MFI value of isotype control of this sample was particularly high at p1 compared to the p0 value. When looking at the raw MFI values of CD105, CD44 and C73 for "59" sample, they increased like for the other samples between p0 and p1. For these other samples, the MFI values of isotype control were similar between p0 and p1, limiting this kind of bias.

For subtype markers, a similar trend of increase between p0 and p1 was observed for CD71 and CD140b. Interestingly, for CD200, all samples that expressed the marker at p0 lost most of it at p1. Those with no expression from the start stayed at the same state after the first passage. For CD106 and CD146, the results were more variable with no clear trend followed by all the samples.

To avoid additional bias and variability, cells from different donors were compared at the same passage. Another interesting way to do would be to compare cells at the same population doubling level, but this approach limits too much the number of samples that can be gathered.

B. Parameters associated to MSC donor

1. Global variability between donors

To have a global view of surface marker MSC relative expression between BM MSC donors and between the different markers themselves, the flow cytometry data obtained at passage 1 were gathered in boxplots **(figure 44)**. These charts allow to view the orders of magnitude of the levels of expression and variability of all the markers monitored.

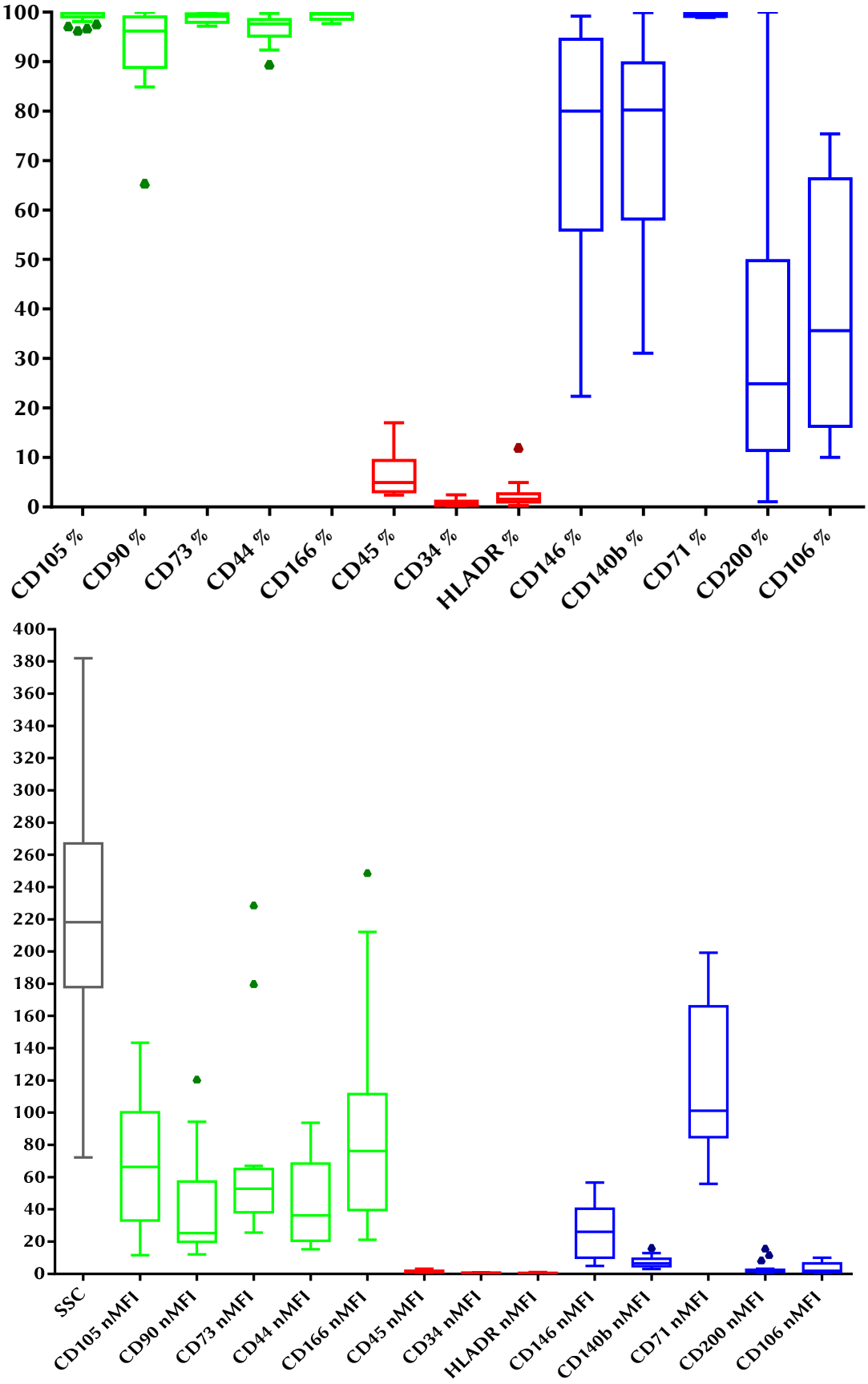


Figure 44: Tukey boxplots representing BM MSC surface markers expression from different donors at p1.

Results were displayed with percentages (at the top)
and with normalized median fluorescence intensity (nMFI) (below).
n ≥ 4 for classical markers (in green and red) and n ≥ 8 for additional surface markers (in blue).

Classical MSC surface markers were the most highly expressed (green boxplots). With percentage parameter, they remain close to 100%. With nMFI, a variability in expression levels was highlighted, notably for CD105 and CD166. SSC parameter was also very variable and covered a wide range of values. CD90, CD73 and CD44 nMFI results were less dispersed.

These results also confirmed that MSC classical negative markers, CD45, CD34 and HLADR, were very lowly expressed.

With this chart where all markers were gathered, the variability of subtype markers with a lower level of expression is less visible with nMFI, but it can be seen with percentage results (blue boxplots). CD200 and CD106 showed similar results, with very low levels of expression but very variable percentages. CD140b level of expression was higher than for CD200 and CD106, but remained at relatively low nMFI values that were poorly dispersed. Percentage of expression results however, showed strong variability. CD71 behaved in a similar way as classical and highly expressed markers that were variable in their level of expression, but near 100% expression. CD146 expression was found to be intermediate between the different subtype markers. Despite optimization of the staining to be as sensitive as possible, the percentages remained variable. The range of expression levels with nMFI was less variable and with smaller values than CD71, but more variable and with higher values than CD140b.

Furthermore, care must be taken in interpreting the data. The relative quantification based on median fluorescence intensities in flow cytometry is very dependent on the instrument settings and staining protocol. For example, for CD140b, it may be possible that the protocol used make it appear at low level of expression and high variability of percentages, but with a more sensible protocol, it could be seen as always near 100% and more variable in nMFI. In this study, subtypes markers were studied for several years and different protocols have been used. The results that are shown in this work are representative of the behaviors that were consistently found with these different protocols for the analysis of surface markers expression.

2. Surface markers expression association with donor age

Results obtained previously in the laboratory showed difference in BM MSC functionality with donor age. In particular, cell proliferation rate was affected **(figure 45)**.

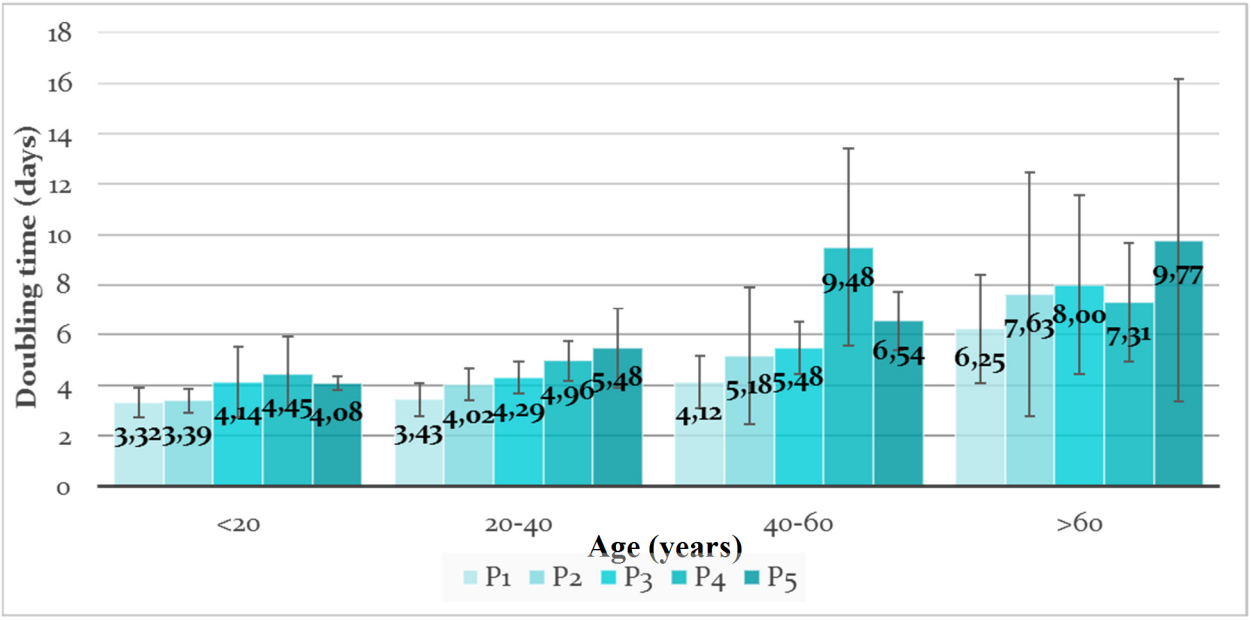


Figure 45: BM MSC doubling time for four different age groups from passage 1 to passage 5.
Results obtained during Yueying Li PhD.

BM MSC obtained from donors below 40 years old were less heterogeneous and had a faster growth compared to BM MSC coming from donors of more than 40 years old. After seeing these results, phenotype of MSC was reviewed more precisely to see if the functional differences observed between samples and according to donor age could also be reflected by surface phenotype changes.

a. Classical MSC markers

The levels of expression of CD105, CD90, CD73, CD44, CD166, usually assessed when studying MSC, were analyzed in several samples coming from donors of different ages **(figure 46)**. Side scattering SSC was also studied as a reflection of cell size. The level of association between expression level of these markers and donor age was determined with Pearson correlation test.

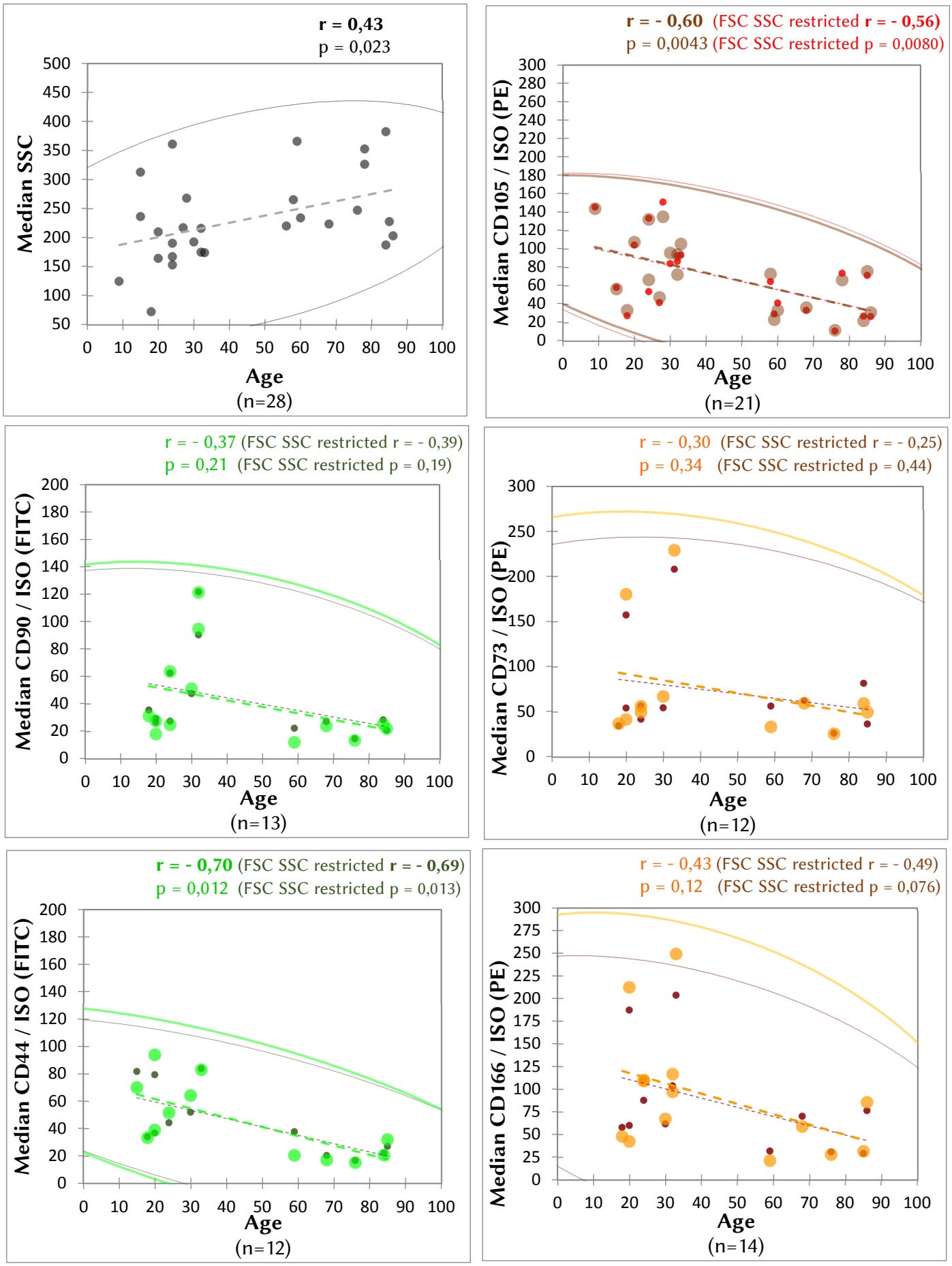


Figure 46: Scatter plots showing MSC classical surface markers expression according to donor age.

Results are displayed with normalized median fluorescence intensity data. All the samples were at passage 1. Smaller and darker points in the charts: results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples, allowing to compare the results obtained on cells of similar size. Pearson correlation tests were made to determine correlation coefficients "r" and p values associated. Confidence ellipses as representation of confidence intervals were displayed.

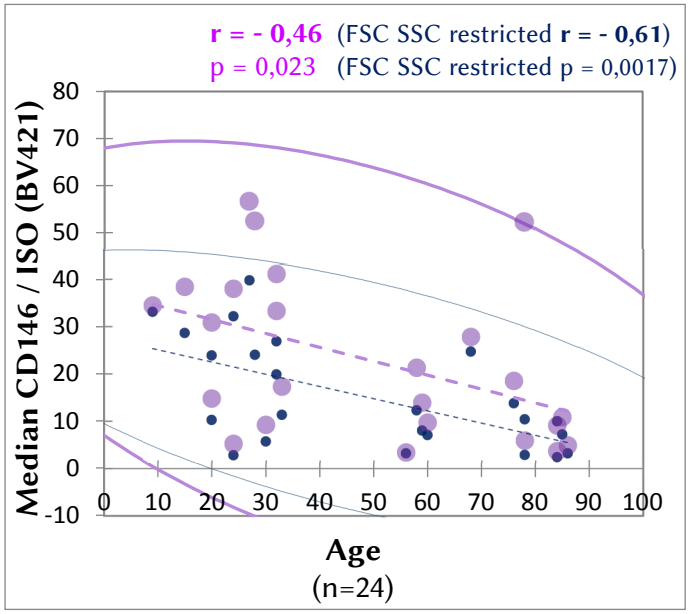
The global tendency observed was that, at passage 1, MSC classical surface markers tended to decrease with advancing donor age. Furthermore, cells size reflected by SSC was positively associated with donor age. The change in expression observed were not associated to change of cell size with age, as they even went in opposite directions. The stronger links between expression level and age were observed with CD44 and CD105.

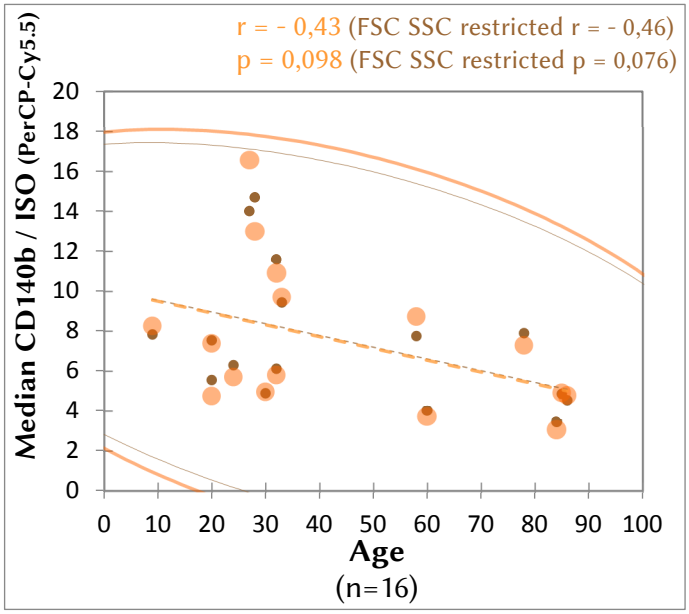
These markers are usually assessed to confirm the cells identity. In most studies, only the percentages are looked at. For these highly expressed markers, percentages obtained were most of the time reported to be between 90 or 100%. It is usually considered that these markers do not reflect a particular cell state, but it may be because the way they were assessed was not precise enough, notably by considering varying levels of expression. Some studies still tried to study these markers more in depth. One of them also tested association between expression level and age on 34 BM MSC samples. For them, the strongest associations were found with CD90 and CD166 that also decreased with age (124). To note, in this latter study, they found no association of donor age with cell size, but they used a CASY cell counter (Roche) to measure cells diameter. In another study, CD90 and CD105 on BM MSC surface were also less expressed in the donors group of more than 40 years old compared to the group of younger donors ($n \geq 3$ in each group) (118). In this study, a slight increase of CD44 in the aged donor group was also identified.

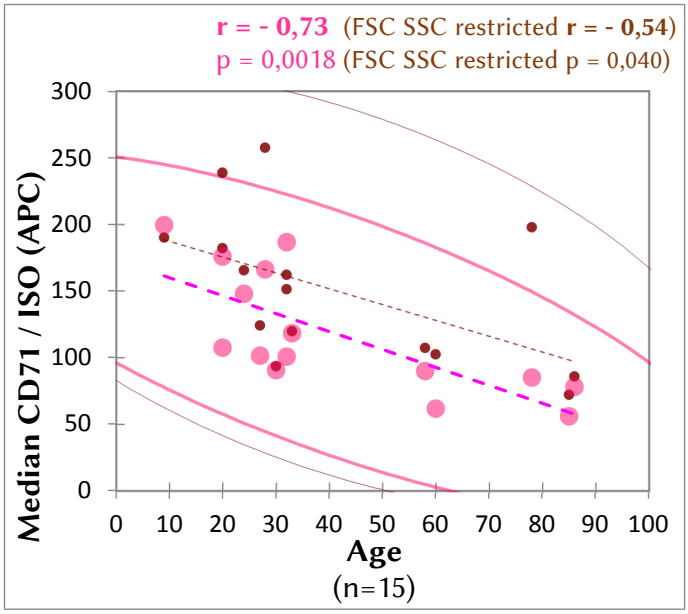
b. Subtype markers

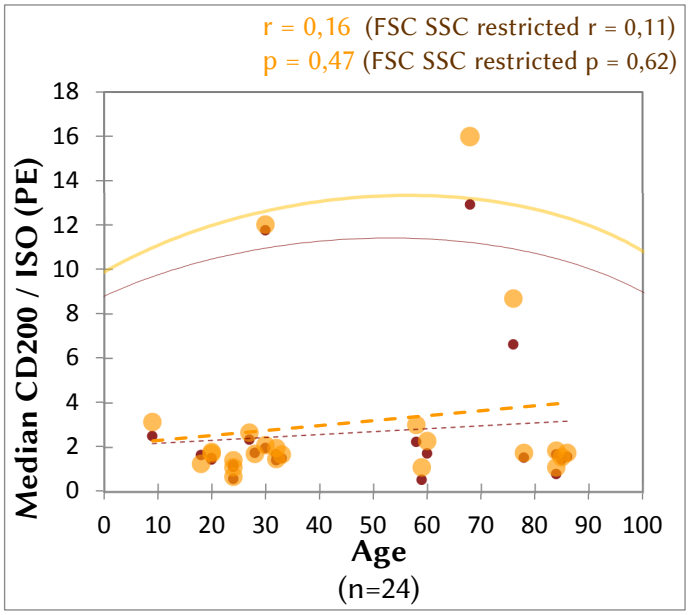
The association between donor age and expression level of surface markers were more particularly tested with subtype markers, that were precisely chosen because of their potential to be linked with MSC state and capacities: CD146, CD140b, CD200, CD71 and CD106. These results are displayed **figure 47**.

The strongest associations were found for CD146 and CD71, that showed a negative correlation with age. This link was more pronounced with CD71, while some samples from the young donors had also CD146 weak expression and high level of heterogeneity remained even with the age consideration.









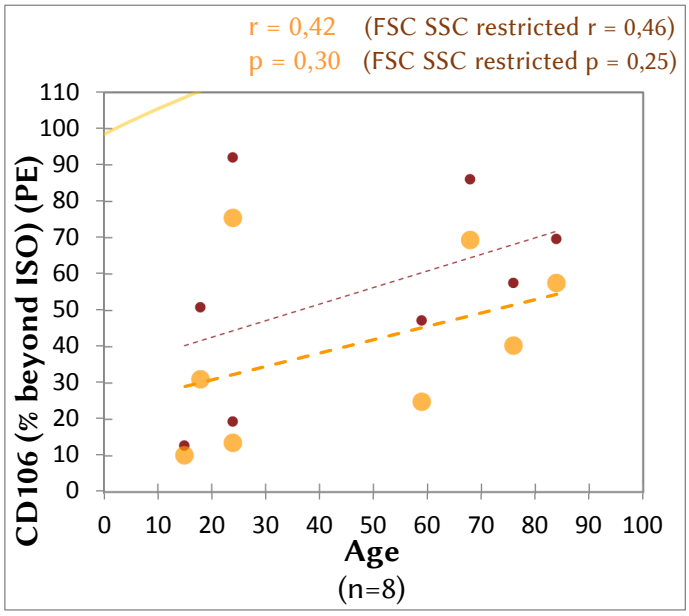


Figure 47: Scatter plots showing MSC subtype markers expression according to donor age.

Results are displayed with normalized median fluorescence intensity data, except for CD106 where percentages were used.

All the samples were at passage 1.

Represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples, allowing to compare the results obtained on cells of similar size. Pearson correlation tests were made to determine correlation coefficients "r" and p values associated. Confidence ellipses, as representation of confidence intervals, were displayed.

CD140b expression also tended to decrease with age, but the link was less marked.

No specific tendency was seen for CD200 expression with age. Interestingly, some rare samples (3 out of 24) stood out with higher level of CD200 expression.

The only marker showing a tendency to increase with age was CD106, althought the association was weak. This trend is consistent with other studies linking CD106 with inflammatory environment (269).

The research team that tested similar associations found a negative correlation of CD71, CD146, CD106 and CD140b expression with donor age (n=34 donors) (124). CD146 expression was assessed in another large study with 86 bone marrow donors and it also decreased with donor age (116).

C. Surface marker and MSC proliferation capacity

Potential associations between surface marker expression and MSC proliferative capacity were explored **(figures 48).**

Classical MSC markers were less associated with MSC proliferation than with donor age. Only slight tendencies were observed. The most marked trend was for CD105 expression with a positive but weak association with population doubling. Cell size, from SSC results, also showed a tendency to be smaller in highly proliferative cells.

Stronger links were highlighted with subtypes markers. The most significant associations with population doubling were obtained with CD71, then CD140b and then CD146 expression. These markers showed increased expression when the cells proliferated more. Interestingly, CD140b was only weakly associated with donor age but was more importantly associated with population doubling. No clear trend was observed for CD106 or CD200.

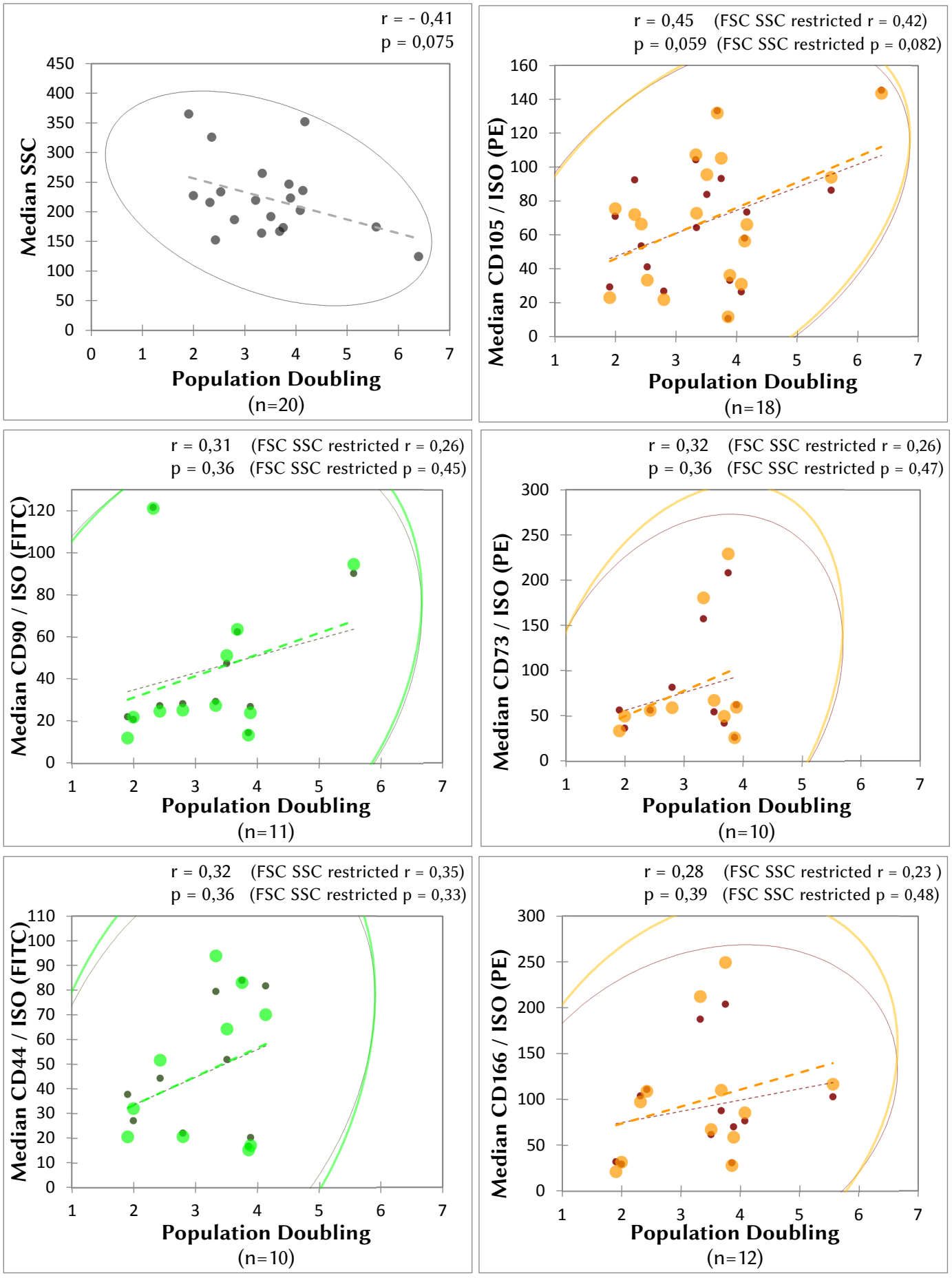


Figure 48-A: Scatter plots with MSC classical surface markers expression and SSC plotted against population doubling.

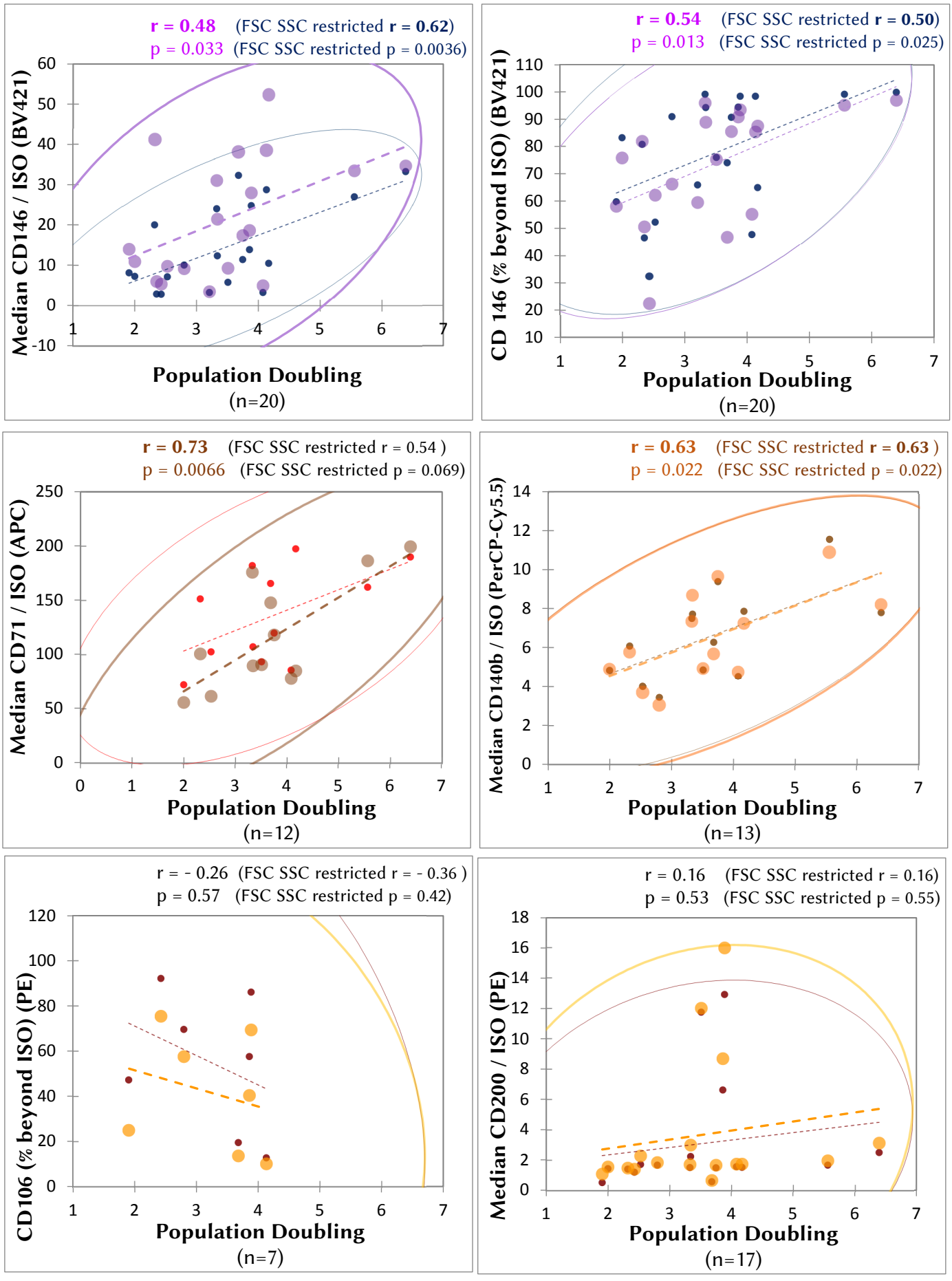


Figure 48-B: Scatter plots with MSC subtype markers expression plotted against population doubling.

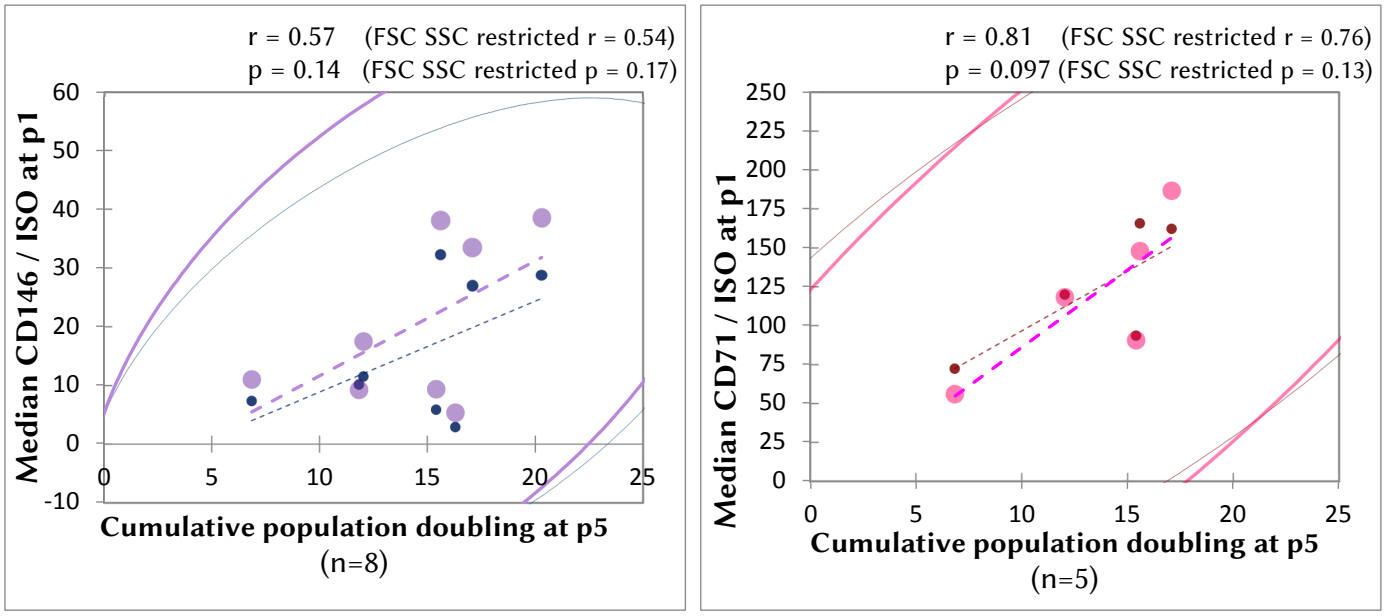


Figure 48-C: Scatter plots with CD146 and C71 expression at p1 plotted against cumulative population doubling at p 5.

Figure 48: Scatter plots with surface markers expression plotted against MSC proliferation parameters.

Figure 49-A: MSC classical surface markers expression results are displayed with normalized median fluorescence intensity data. The population doubling levels shown were calculated at passage 1.

Figure 49-B: Classical MSC marker expression results are displayed with normalized median fluorescence intensity data, except for CD106 where percentages were used. For CD200, the choice was made here to use normalized median fluorescence data that were more informative than percentage in this case. The population doubling levels shown were calculated at passage 1.

Figure 49-C: Normalized median fluorescence intensity data associated to CD146-BV421 and CD71-APC obtained at passage 1 were plotted against the cumulative population doubling obtained at passage 5.

Smaller and darker points in the charts: results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples, allowing to compare the results obtained on cells of similar size.

Pearson correlation tests were made to determine correlation coefficients "r" and p values associated.

Confidence ellipses, as representation of confidence intervals, were displayed.

Some samples included in this study were cultivated until passage 5 and cumulative population doubling (CPD) data at p5 were available. The associations between markers expression at p1 and CPD at p5 (figure 48-C) were checked. This allowed to look at the predictive potential of the different surface markers followed. Among all the surface markers tested, the most significant associations were obtained with CD146 and CD71, the latter showing a strong positive link. However, the results with these 2 markers remained weak at the statistic level. This analysis would require more samples to improve its power, but these trends are already interesting to observe.

Subtypes markers chosen for this study were selected in part because they were reported to be associated with MSC showing interesting functionalities. Among those, their capacity to proliferate was often highlighted (Part V. in the Introduction chapter). However, few reports make the same kinds of association tests, they often group results in two groups to compare. A study, already mentioned in the previous part "Parameters associated to MSC donor age", still made the same type of correlation tests between surface markers and MSC proliferation rate (population doubling time). For them, among the markers tested here, the ones that were correlated with proliferation capacity were CD105 and CD146 (with percentages for the latter) (124). To note, instead of using normalized fluorescence intensity, the authors used geometric mean subtracted by the geometric mean of the control isotype for most of their analyses.

D. MSC subpopulations within a sample

Flow cytometry allows to obtain results at the single cell level, and to see distribution of expression in the sample cell population. With this, MSC heterogeneity within one sample can be grasped. This approach is relevant for markers that are not homogeneously expressed in the whole cell population. The data presented until now using median fluorescence intensity did not take into account the dispersion of results within samples. The assessment of different dispersion parameters such as standard deviation and variation coefficient of flow cytometry data was tried but could not be used efficiently. The most revealing thing remains to show the histograms. Several examples of markers expression distribution are proposed **figure 49 and 50**. WJ MSC samples were also assessed as a reference of more immature cells compared to BM MSC from donors of different ages. MSC classical markers were almost always homogeneously expressed in MSC samples and were not included in these figures.

Among the subtype markers assessed, the highest level of heterogeneity among MSC within a sample was with CD146 expression, with the largest distributions. Intra-sample distributions of CD200 and CD146 expression were more homogeneous on WJ MSC than on BM MSC. CD200 and CD146 expression was also stronger on WJ MSC. CD140b appear to be expressed in a homogeneous way in all the samples, but its level was weaker on WJ MSC.

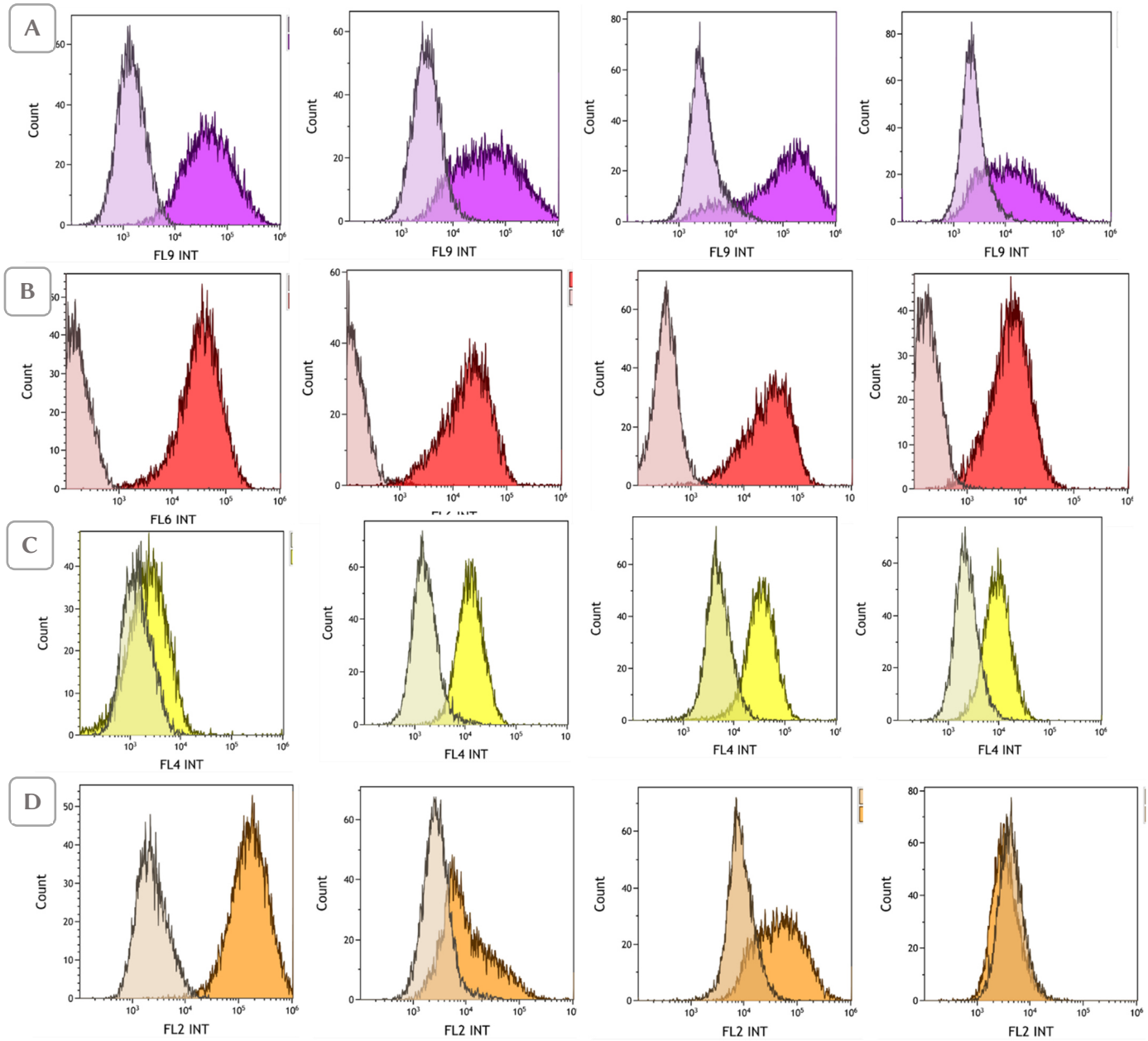


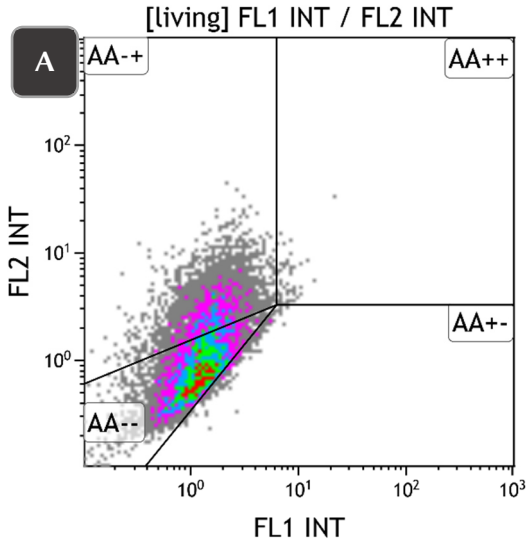
Figure 49: One-parameter flow cytometry histograms of surface markers expression among MSC from different samples.

From left to right:

- **A**: CD146-BV421 staining on WJ-MSC, BM-MSC from 20, 32 and 86 years old donors.
- **B**: CD71-APC staining on WJ-MSC, BM-MSC from 9, 30 and 84 years old donors.
- **C**: CD140b-PerCP-Cy5.5 staining on WJ-MSC, BM-MSC from 9, 30 and 84 years old donors.
- **D**: CD200-PE staining on WJ-MSC, BM-MSC from 9, 30 and 84 years old donors.

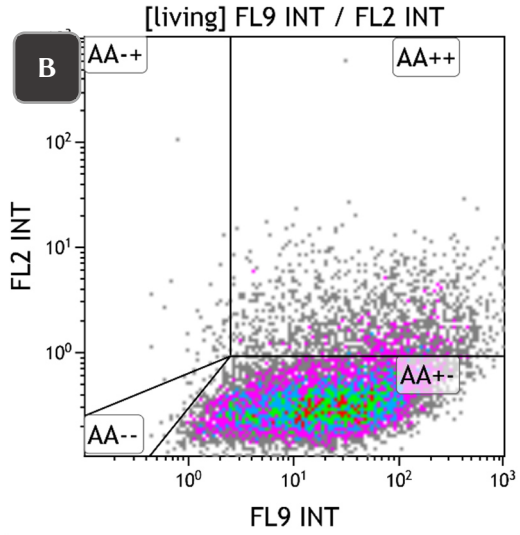
Superposition of control isotype in light color and staining of the surface marker in deep color.

Only one example of WJ-MSC sample was shown because the results were less variable according to donor parameters than with BM-MSC.



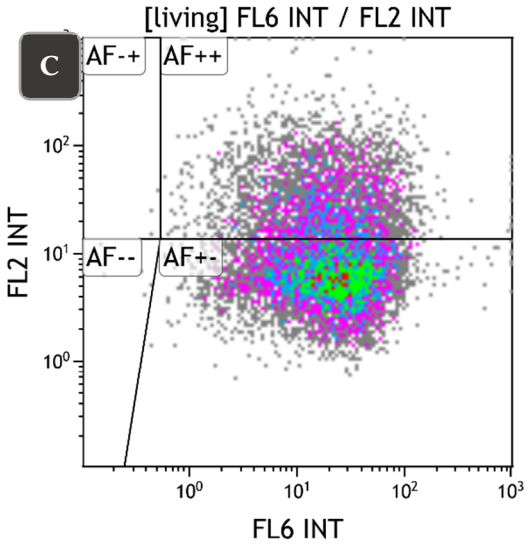
Gate	Number	%Total	%Gated	X-Med
All	9 802	98,02	100,00	1,28
AA--	6 520	65,20	66,52	1,14
AA-+	2 935	29,35	29,94	1,61
AA+-	298	2,98	3,04	2,66
AA++	49	0,49	0,50	7,94

Gate	X-GMean	Y-Med	Y-GMean
All	1,26	1,10	1,21
AA--	1,09	0,79	0,77
AA-+	1,55	3,05	3,32
AA+-	2,77	1,02	1,04
AA++	8,12	4,79	5,72

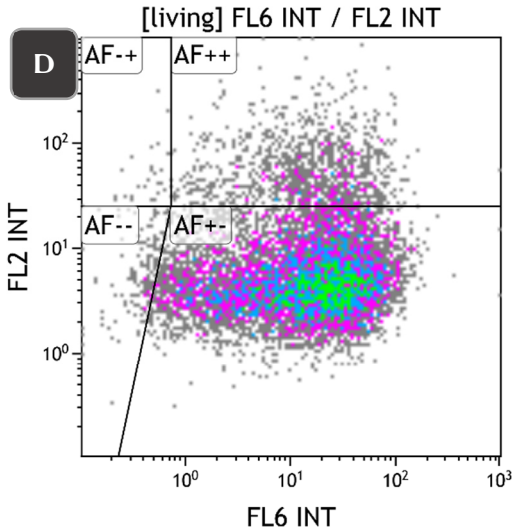


Gate	Number	%Total	%Gated	X-Med
All	9 928	99,28	100,00	22,50
AA--	22	0,22	0,22	1,07
AA-+	44	0,44	0,44	1,78
AA+-	8 279	82,79	83,39	19,22
AA++	1 583	15,83	15,94	70,76

Gate	X-GMean	Y-Med	Y-GMean
All	21,99	0,36	0,44
AA--	0,99	0,42	0,38
AA-+	1,51	1,48	1,77
AA+-	18,50	0,32	0,33
AA++	60,97	1,67	1,97



Gate	Number	%Gated	X-Med	X-GMean	Y-Med	Y-GMean
All	9 332	100,00	19,93	17,67	8,43	10,64
AF--	5	0,05	0,37	0,27	6,56	6,58
AF-+	3	0,03	0,46	0,46	25,74	24,80
AF+-	6 056	64,89	20,81	18,11	5,76	5,50
AF++	3 268	35,02	18,20	17,06	31,96	36,17



Gate	Number	%Gated	X-Med	X-GMean	Y-Med	Y-GMean
All	9 466	100,00	15,43	11,76	5,16	6,67
AF--	164	1,73	0,42	0,37	5,05	5,55
AF-+	31	0,33	0,50	0,42	40,73	53,74
AF+-	8 080	85,36	15,82	12,32	4,55	4,86
AF++	1 191	12,58	17,11	15,00	49,02	56,02

Figure 50: Flow cytometry biparametric histograms showing surface markers expression in heterogeneous samples.

A: Density plot of CD106-PE (FL2), no staining in FL1, from a 52-year-old donor at p3.
B: Density plot of CD146-BV421 (FL9) against CD200-PE (FL2) from a 33-year-old donor at p1.
C: Density plot of CD71-APC (FL6) against CD200-PE (FL2) BM MSC from a 9-year-old donor at p1.
D: Density plot of CD71-APC (FL6) against CD200-PE (FL2) from a 28-year-old donor at p5.

CD200 expression was either absent or low and heterogeneous in BM MSC samples. CD71 expression was always quite high and relatively homogeneous in most of the samples, but it could also be heterogeneous in other cases, as it can notably be seen in **figure 50**. This latter figure shows the examples where the cells are the most dispersed. CD106 was only shown with a density plot with unique staining to be able to better observe the population expressing it.

From flow cytometry histograms, CD146, CD200 and CD71 appeared as relevant candidate for cell sorting experiments using more than one marker. However, such experiment would be difficult to undertake, notably because a very high number of cells is needed.

The shape of distribution in flow cytometry histograms should also be interpreted with caution. Flow cytometry experiment can result in very different outputs according to parameters such as instrument settings, fluorochromes, antibody concentrations, and many others. Previous results obtained by studying CD146 expression with FITC-conjugated antibodies showed that it could fail to detect it or the different levels of expression could not be discriminated (the population appeared more homogeneous). High processing speed on the flow cytometer for example can also result in more dispersion of the results. That would not be related to real differences in expression levels. For the study presented in this manuscript, speed of the flow cytometer was placed closest to 100 cells per seconds.

In the literature, the shape of flow cytometry histograms can more often be found in studies making cell sorting from bone marrow. With MSC already in culture, it is more rarely reported, especially for subtype markers. Some reports could still be found with CD146 distribution in cultivated BM MSC samples. They also obtained large distribution with different levels of expression (174). CD140b expression was also found by others to be very homogeneous (20). For CD200, similar results and shape of distribution were obtained by another team (168). However, they also obtained higher and more homogeneous CD106 expression than what was seen during this thesis work. Intriguingly, they also report a homogeneous and low CD146 expression, while the culture conditions were similar as the study undertaken in this work, and bright PE-antibody was used. It is possible that the

samples shown were those with low CD146 expression. Another hypothesis may be that the samples were retrieved at globally higher levels of confluence. That would be consistent with increased CD106 and decreased CD146 expression. CD71 was assessed on MSC by others but they did not show the distributions (20,124,163).

After seeing CD146 heterogeneous distributions, microscopy imaging was also performed to see how CD146 surface marker was distributed on the cell surface **(figure 51)**.

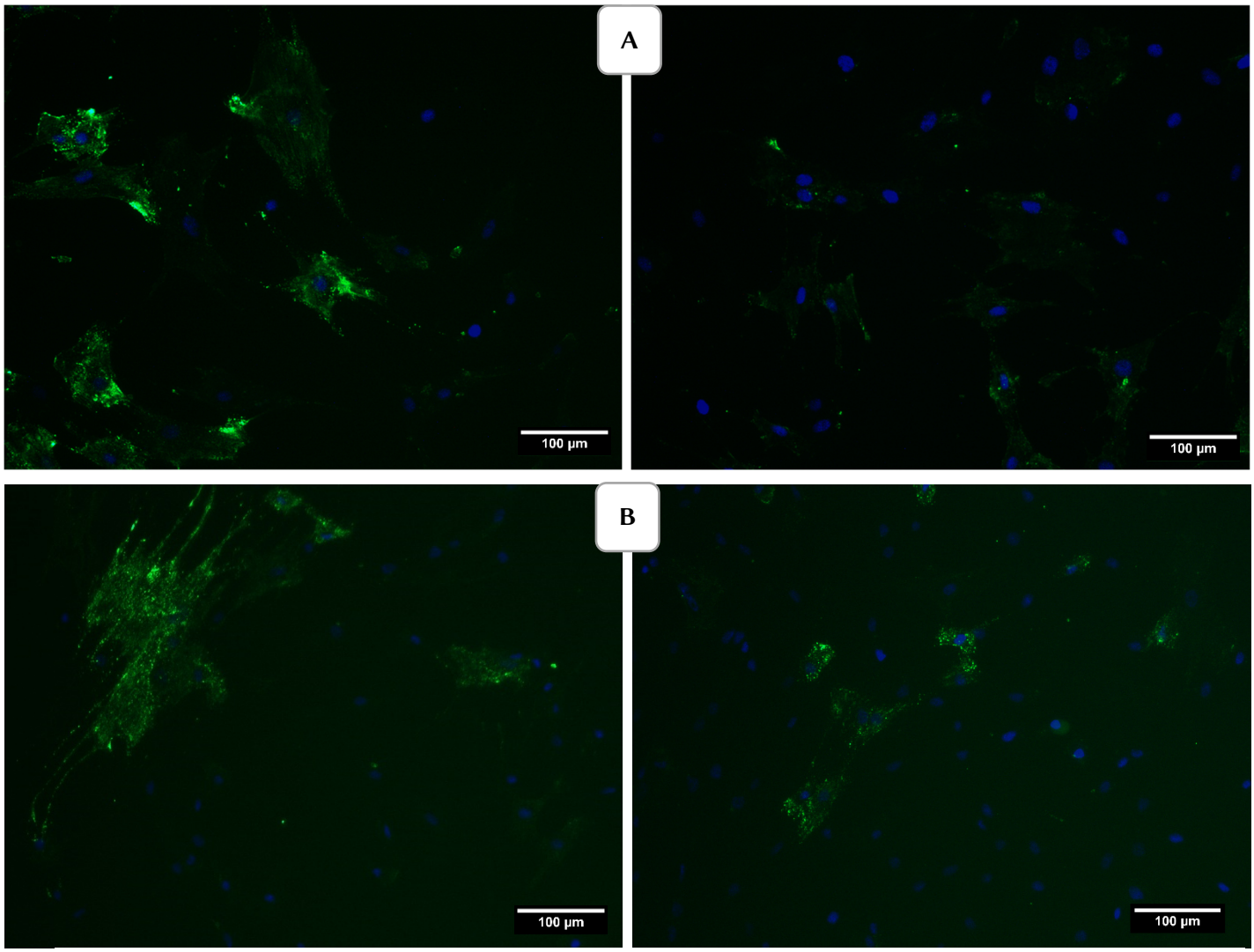


Figure 51: Fluorescence microscopy image of living BM MSC stained with CD146-AlexaFluor488 antibodies (in green) and DAPI (in blue) to stain nucleus.
A: BM MSC from 78 p3 sample.
B: BM MSC from 65 p4 sample.
Microscope used is Leica DMI3000B, scale bar is 100 µm.
Gimp was used to make overlay of DAPI and CD146 fluorescence images, 20% opacity for the DAPI layer, normal mode.

Some brighter cells with many CD146 spots could be distinguished from other cells with fewer spots, and many other cells with no detectable signal. The bright spots were also distributed heterogeneously on the surface of some cells.

Histological images with CD146 staining were seen in the literature (20), but no other images of CD146 on cultivated MSC were found.

E. Main points of part I

- Results of expression levels obtained by flow cytometry were very sensitive to culture conditions. This variability must be taken into account to make reliable comparison between different samples.

- Each of the surface markers monitored showed specific expression dynamics when reported with nMFI and percentage on BM MSC from different donors.

- Some associations were strong enough to stand out from MSC variability. A group of surface markers were found to be associated with **donor age**: **CD146, CD71, CD105, CD44 and SSC** (side scatter reflecting cell size). **CD146, CD140b and CD71** were also correlated with **proliferation rate**.

- CD146 expression had the particularity to be relatively **stable** in culture and turned out to be the **most heterogeneously** expressed when looking at cell population within the samples.

II. Separation of MSC CD146 subtypes within one sample

Preliminary results about subtype surface markers expression on BM MSC showed significant association of CD146 expression with donor age. This marker expression was also the most heterogenous within BM MSC cells populations. Other studies showed different links between CD146 expression and cells functionality, especially with cell multipotency and true self-renewal abilities. Some studies also showed a potential link between CD146 and senescence, for example with CD146 downregulation causing accelerated cell senescence (270).

With all these elements, it was decided to perform a sort experiment on BM MSC according to CD146 expression and to compare *in vitro* cell functionality of $CD146^{high}$ and $CD146^{low}$ expressing cells. BM MSC from donors of different ages were used at different passages to test the possibilities of the cell sorting procedure used.

A. CD146 sorting feasibility

Magnetic sorting experiments were performed successfully on 7 BM-MSC. An eighth sample from a 52-year-old donor was also sorted with FACS. The details about the samples are displayed in **table 13**. Results associated with the sample sorted with FACS are presented at the end of the table.

The samples used were at various states, with different culture time and CD146 expression. In average, 14% (± 5%) of the total number of sorted cells were obtained in $CD146^{high}$ fraction. CD146 associated fluorescence intensity was in average 3 times higher in $CD146^{high}$ fraction than before the sort, with a certain disparity between samples. When using percentage of CD146 expression, the average enrichment factor was 2 ± 1.1. The most important enrichments were achieved with sample “84” at p4 and “78” (at p1). The sample “84” at p4 was also the one with the lowest CD146 expression before sort, and with the smallest proportion of cells obtained in the $CD146^{high}$ fraction (F+, 9%).

Table 13: Overview of the different samples of BM MSC used for CD146 magnetic sorting experiments, their state at the moment of the sort, the percentage of cells obtained in the positive fraction and their respective Median Fluorescence Intensity for CD146 expression.

All the samples were obtained from femoral bone marrow of women who underwent hip replacement.
The cells were at the end of the passage indicated when they were sorted.
The last sample in the table (52 years old) was sorted with FACS while the others were sorted with magnetic beads.
The cells from the donor aged of 84 were sorted at two different passages. The cells were obtained from different runs.
The enrichment factors were calculated by dividing the value in F+ fraction after sorting by the value before sort.

Age	24	30	65	78	84	84	85	52
Passage	1	3	2	1	2	4	2	2
Population Doubling (PD)	2.4	1.8	2.0	2.4	3.0	1.9	0.9	2.8
Doubling Time	6.2	8.2	8.3	5.9	6.4	8.0	16.7	5.1
Cumulative PD	6.9	10.6	6.4	2.4	4.4	9.3	2.8	4.3
CD146 MFI before sort (ratio ISO)	5.3	1.6	3.3	5.9	5.0	1.2	4.0	12.1
CD146 % beyond ISO before sort	38.2	29.4	22.6	50.7	57.2	12.2	42.7	68.4
Proportion of sorted cells retrieved in F+ (%)	18	20	23	12	18	9	12	20.5
CD146 MFI in F+ after sorting (ratio ISO)	10.9	3.3	5.7	30.7	13.575	6.0	13.1	16.7
CD146 % in F+ beyond ISO after sorting	66.5	53.9	30.3	96.2	92.0	56.5	84.5	63.6
Enrichment factor (with nMFI)	2.1	2.1	1.7	5.2	2.7	5.0	3.3	1.4
Enrichment factor (with percentages)	1.7	1.8	1.3	1.9	1.6	4.6	2.0	0.9

Two examples of flow cytometry histograms obtained before and after sorting are displayed in **figure 52**.

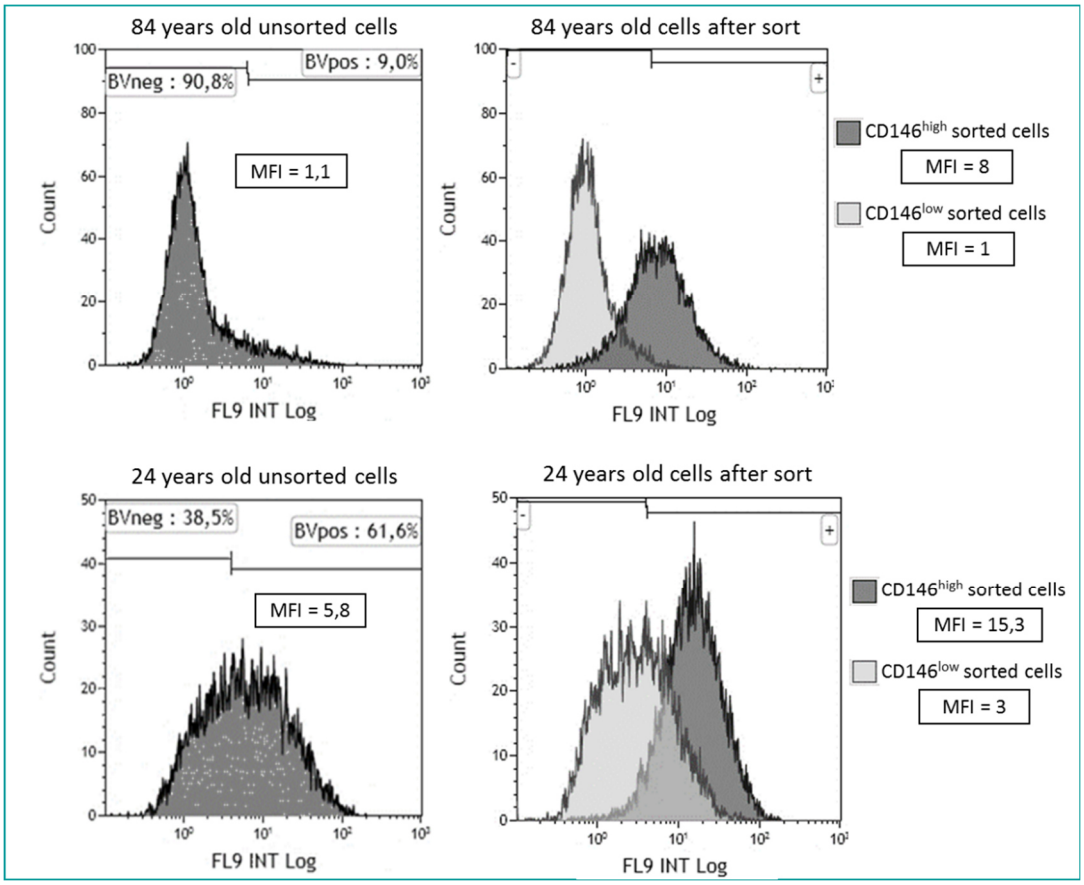


Figure 52: One-parameter flow cytometry histograms of 2 representative BM MSC samples before and after CD146 magnetic sorting.

On the left: unsorted cells. On the right: superposition of $CD146^{low}$ (in clear grey) and $CD146^{high}$ (in dark grey) one parameter histograms.
MFI displayed are raw data (not normalized with MFI from isotype control).

An example of a sample studied by fluorescence microscopy is presented **figure 53**.

This confirms the enrichment of the positive fraction, but the sample still contains some $CD146^{low}$ cells. The negative fraction was depleted of most CD146 expressing cells.

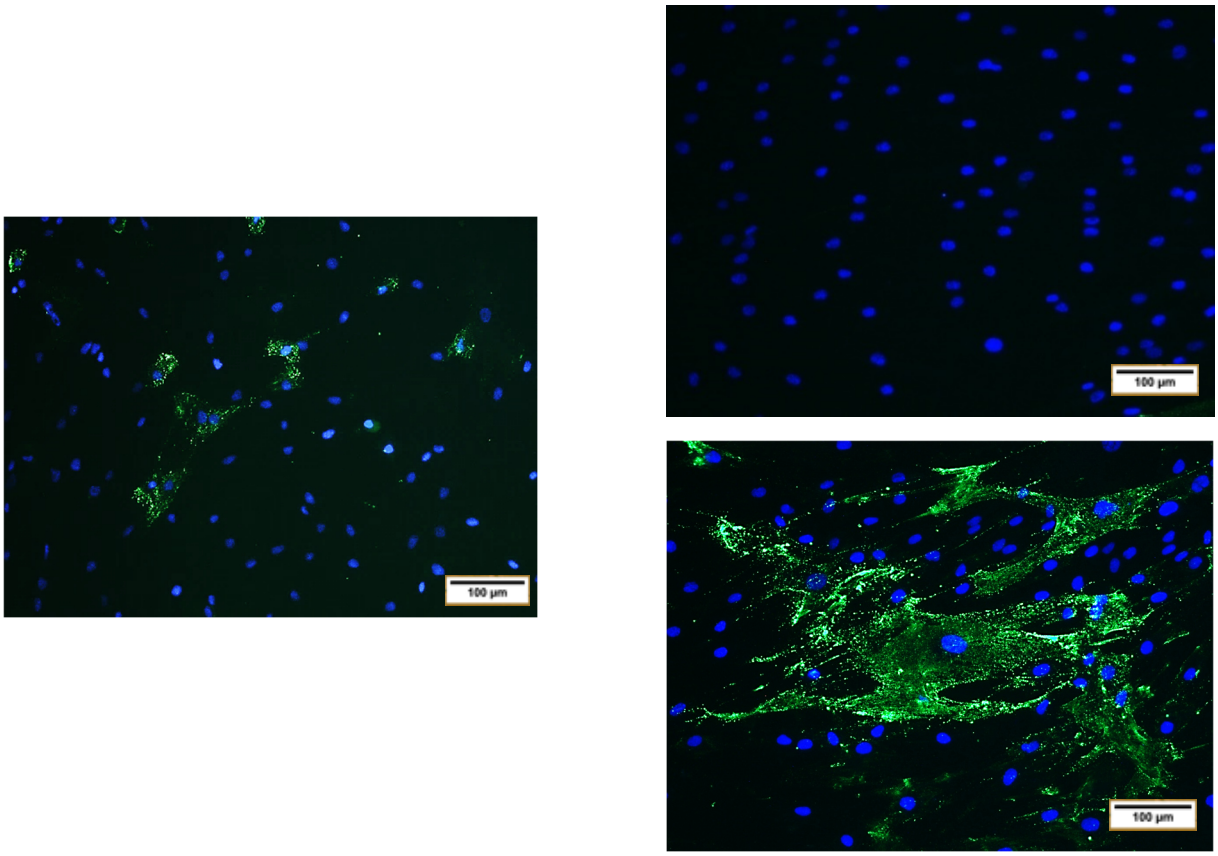


Figure 53: Fluorescence microscopy image of living BM-MSC stained with CD146-AlexaFluor488 (in green) and DAPI (in blue) to stain nucleus.
On the left: BM-MSC before sorting. On the right, up: negative fraction of CD146 sorted cells.
On the right, down: positive fraction after sort.
Microscope: Leica DMI3000B, scale bar: 100 µm.

To our knowledge, only one other study made CD146 magnetic sort from BM MSC after *in vitro* culture (174). They used both magnetic and FACS separation and obtained a better enrichment in CD146 expressing cells with FACS (enrichment factor 1.8 ± 0.62 with CD146 percentages). They used the same protocol for magnetic sorting, except that they did not precise in how many separation columns the labeled cells have been put in. In our work, the positive fraction retrieved after a first separation was put in a second column to obtain a better cell enrichment. In contrast to their results, the FACS sorting that was tried for "52" sample did not allow to obtain an increased enrichment compared to magnetic sorting. To the contrary, it was the less satisfying, CD146 percentage was not increased after FACS separation, but nMFI after sort was 1.4 times higher than before. They may have placed more restrictive thresholds. Another study used FACS from BM MSC in culture obtained excellent purity, but the number of cells in the sorted fractions was so low that

they could not use them for functional studies (271). A compromise must be found between cell number and the level of enrichment after sort. There are other studies where different kinds of MSC in culture were sorted according to CD146 expression, but always with FACS.

B. CD146 phenotype maintenance

CD146 phenotype evolution was followed during 3 successive passages on a part of the samples studied on unsorted and sorted cells **(figure 54)**.

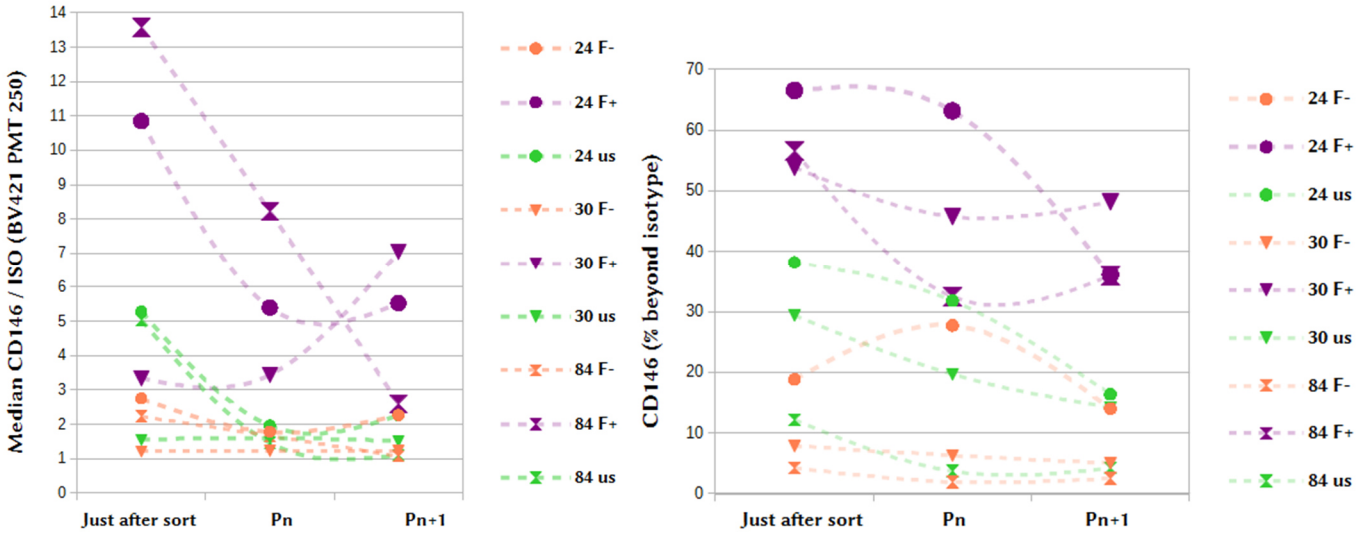


Figure 54: CD146 expression analyzed by flow cytometry along 3 passages for unsorted cells, in positive and negative fractions of sorted BM MSC of 3 samples.

Even when CD146 expression was low in the sample before sorting, a positive fraction of enriched cells could be obtained. This fraction of CD146^{high} cells remained enriched along 3 passages compared to the negative fraction and unsorted cells, even if some variation in CD146 expression level occurred. One of the samples showed an important decrease on CD146 nMFI in the positive fraction along passages, but it was not reflected in the percentage of CD146 expressing cells. This parameter can be more sensitive in some cases for very low expressed markers.

One of the research groups that tried to sort BM MSC in culture with FACS reported that CD146^{low} sorted cells started to re-express CD146 after a short period (271). Two other studies report an increase in CD146 expression after several passages in culture in the CD146^{low} sorted BM MSC after FACS sort from bone marrow mononuclear cells (60,69).

Another group studying intervertebral disc BM MSC reported a decrease of the CD146 mRNA in CD146^{high} sorted cells with time in culture. They used FACS on MSC in culture (164). In our study, CD146^{low} cells remained at a lower level of expression during at least two passages after the sort. The difference in the sorting method or culture conditions could explain these differences.

It may also be noted that the presence of the CD146 label with the magnetic beads on MSC could slightly reduce the fluorescence detected after CD146-BV421 label, maybe with an effect of saturation. The nMFI of cells without beads was in average 1.23 (± 0.16) times higher than samples labeled with CD146-beads (test made on n = 4 samples).

C. Other surface markers on CD146 sorted cells

Several surface markers were analyzed on sorted cells and compared to see if they were enriched or depleted together with CD146 **(figure 55)**.

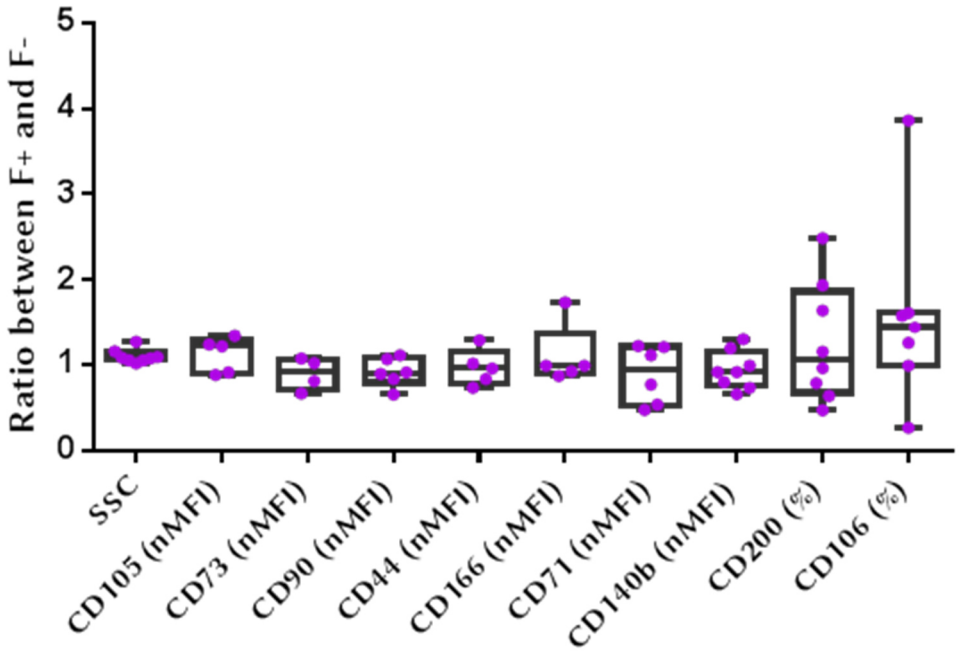


Figure 55: Ratio between surface marker expression on CD146^{high} and CD146^{low} sorted cells.

Values obtained in CD146^{high} cells (F+ fraction) were divided to the values obtained in CD146^{low} (F- fraction) cells to obtain the displayed ratio.
Results are displayed with "min to max" boxplots and with all the points showed.
"52" sample sorted with FACS was included.

The markers assessed did not show clear differences between CD146^{high} and CD146^{low} cells. An important variability was found for CD106 and CD200 compared to the other markers. The results for these markers were reported in percentages that can be more sensible. They are also known to be naturally variable and the difference observed is unlikely to be associated with sorting.

This is consistent with another study that looked at other surface markers on CD146 sorted cells (174). However, they did not search for subtype markers and only looked at classical MSC markers.

D. Functional properties of sorted cells

1. Proliferation

Cell proliferation of the samples used for CD146 sorting experiment was followed and compared in the different fractions obtained **(figure 56)**.

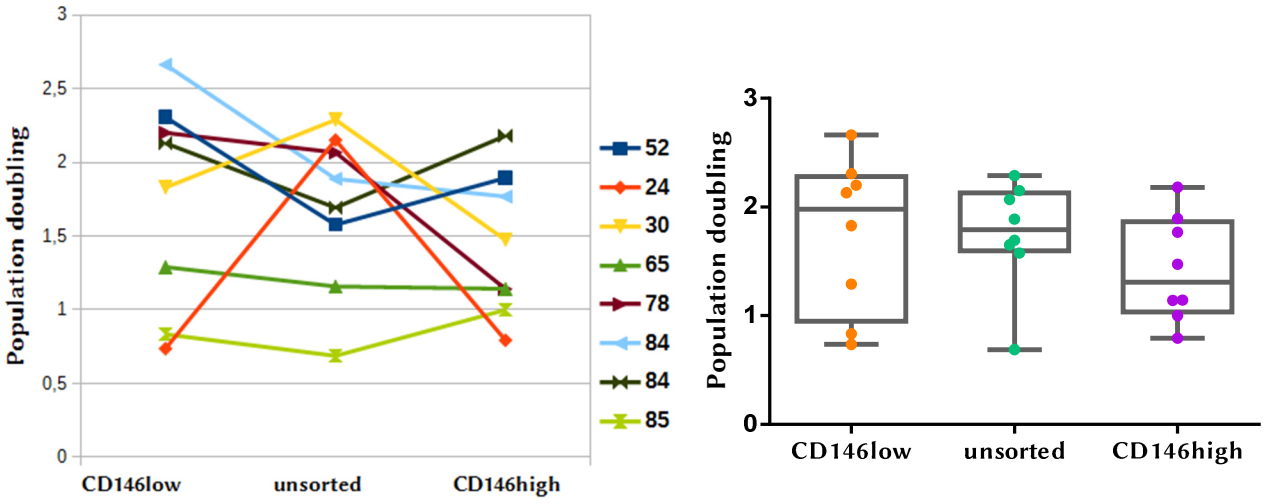


Figure 56: Population doubling calculated for unsorted cells, positive and negative fractions 1 passage after the sort (Pn+1).
On the right: results displayed in "min to max" boxplots.

The proliferation results were very variable and there were no clear differences of proliferation capacities between the sorted cells. The same observation was obtained with doubling time (data not shown). The results still tend towards a slower proliferation rate in the positive fraction.

These results appear in contrast with some studies on CD146 sorted BM MSC (172,174). However, there are even more reports that relate a decreased proliferation capacity in CD146^{high} sorted BM MSC compared to CD146^{low} cells (60,92,271). One study used a different strategy to study CD146 involvement on MSC behavior. They overexpressed or knocked down CD146 expression in human BM MSC. Contrastingly, they found that CD146 knocked down MSC had reduced proliferation capacities (166).

2. Colony formation

The capacity of cells to form CFU was also studied without consistent trend in results **(figure 57).**

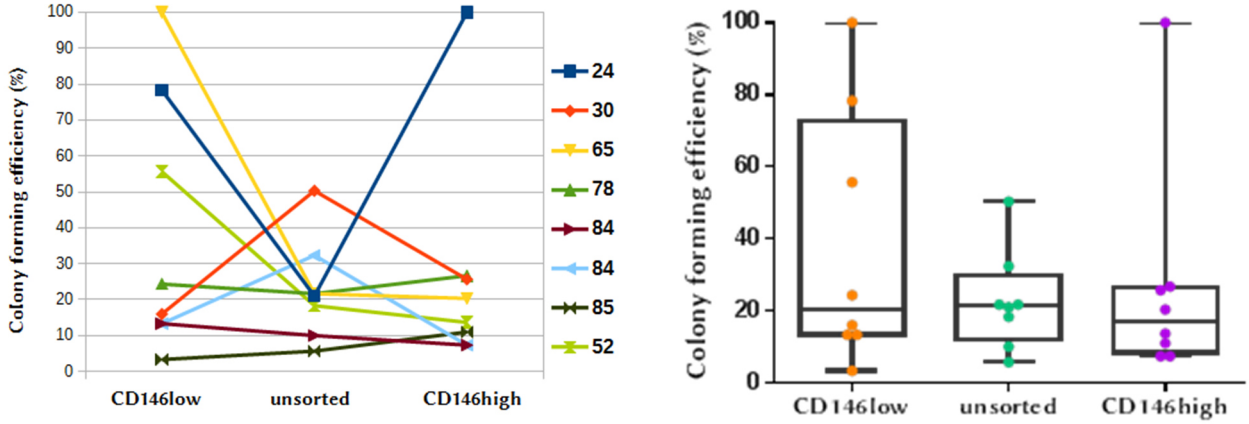


Figure 57: Colony forming efficiency of sorted cells.
To the right: results are displayed in "min to max" boxplots.

In a similar way to proliferation results, MSC capacity to form colony was not clearly different between the sorted cells. It still seems the negative fraction obtained better or at least similar results compared to CD146^{high} sorted cells.

An interesting study developed by Sacchetti in 2007 sorted cells with FACS from BM mononuclear cells and reported that only CD146^{high} sorted cells could generate colonies, while CD146^{low} cells could not, even plated at higher densities (20). The number of colonies found in CD146^{high} sorted cells was 2.6 ± 0.8 CFU for 100 cells (plated at 2 cells /cm²). The context of this study was very different, especially because the experiment was made just after sort from mononuclear cells, so it does not really appear to be in contradiction with the results presented here. A closer study with sorted MSC in culture was made with cells

derived from dental ligament. In two studies, an increased colony forming efficiency in CD146^{high} cells sorted with FACS was reported (179,180). Moreover, some of the studies performed with BM cells also failed to show any difference in colony formation between CD146^{high} and CD146^{low} sorted cells (60,173,271) (the first two after sorting mononuclear cells and the last after sorting BM MSC in culture).

For additional information, the magnetic particles used for CD146 sorting experiments were studied for their effect on MSC gene expression, proliferation, viability and even differentiation. It emerged in most cases that magnetic labeling had no effect on MSC (84).

3. Differentiation

In vitro differentiation capacity of CD146^{high} sorted cells was compared with the one of CD146^{low} cells obtained after the sort.

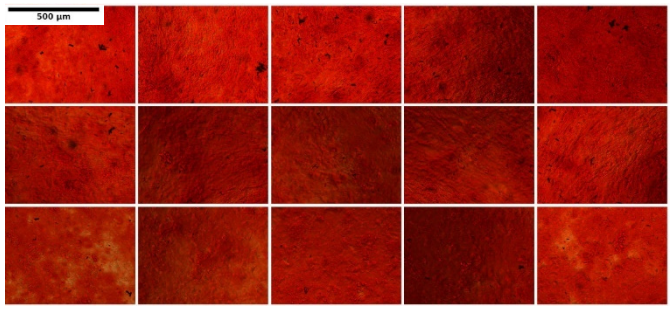
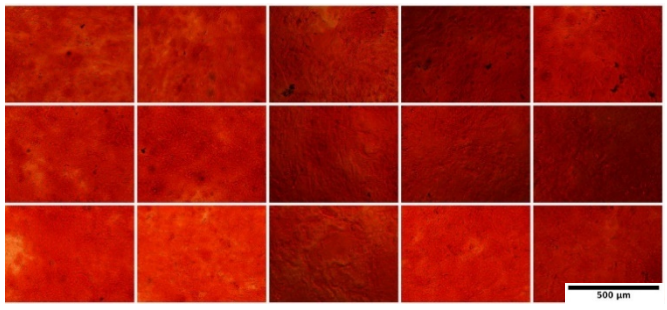
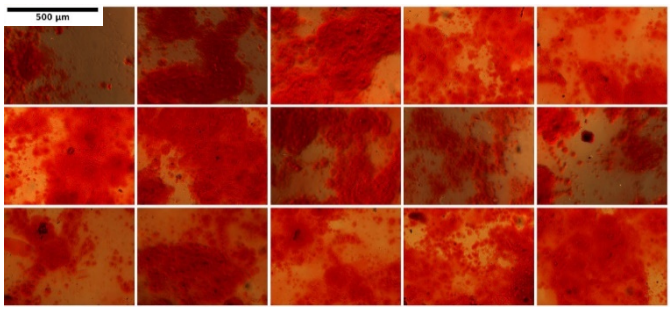
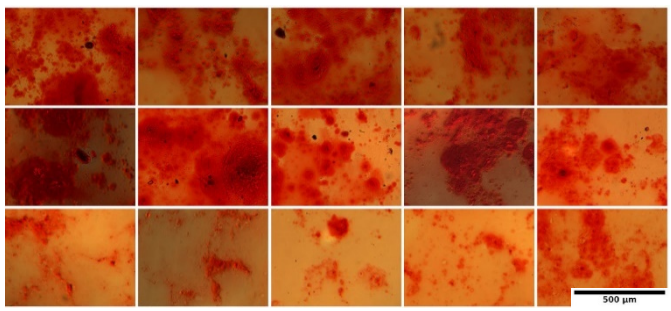
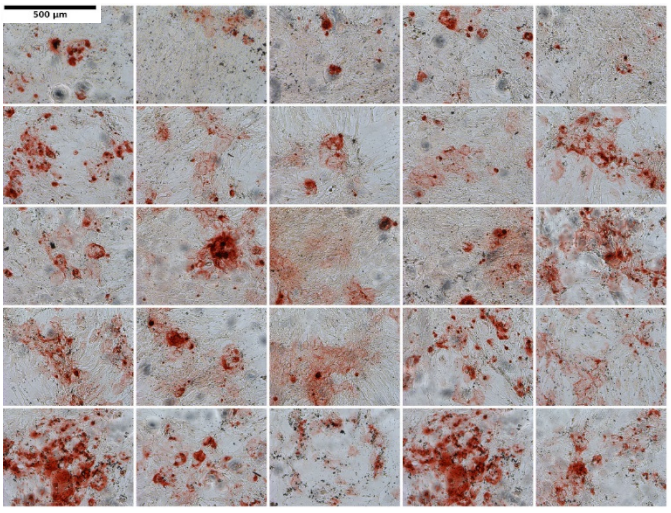
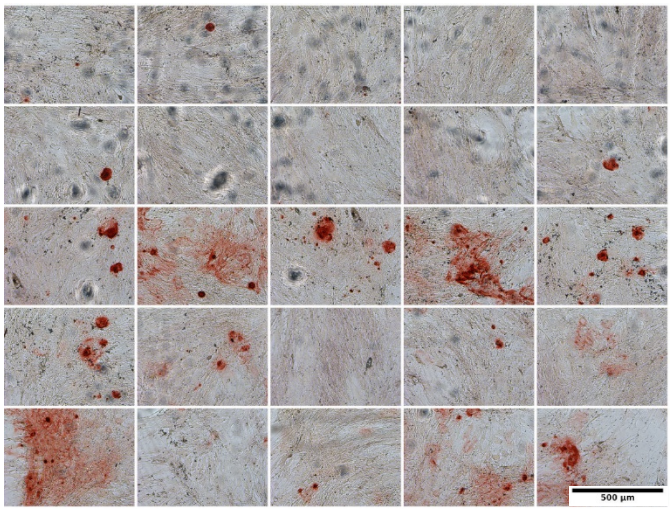
Most images taken under the microscope are shown **table 14**. Sample 52 sorted with FACS was not tested for differentiation. Furthermore, there were not enough cells remaining to test adipogenic differentiation for the sample from the 85-year-old donor.

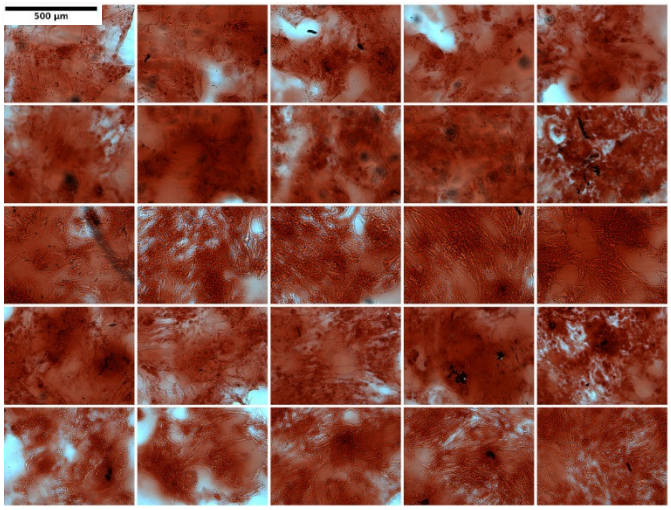
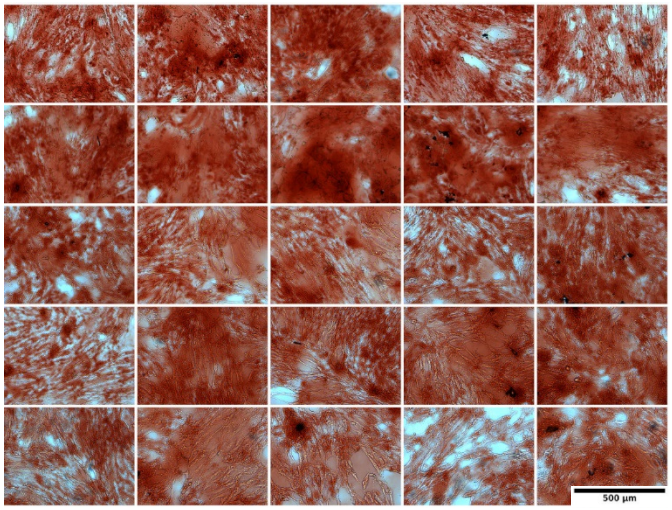
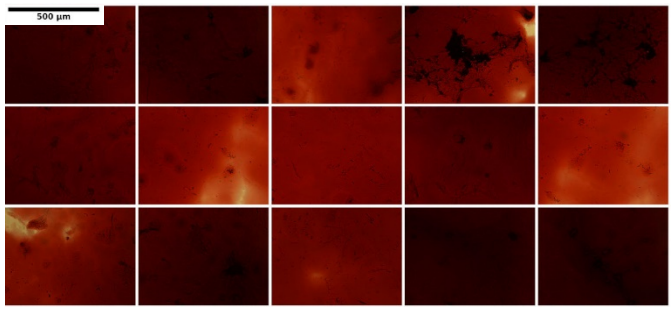
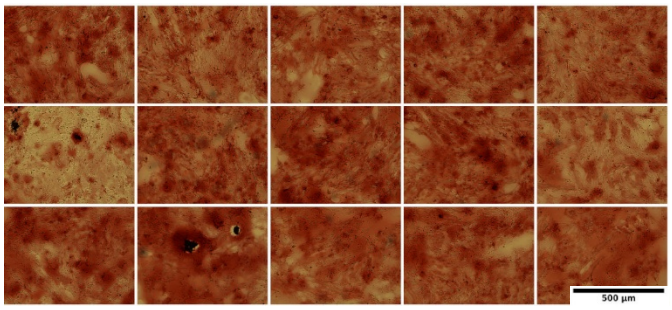
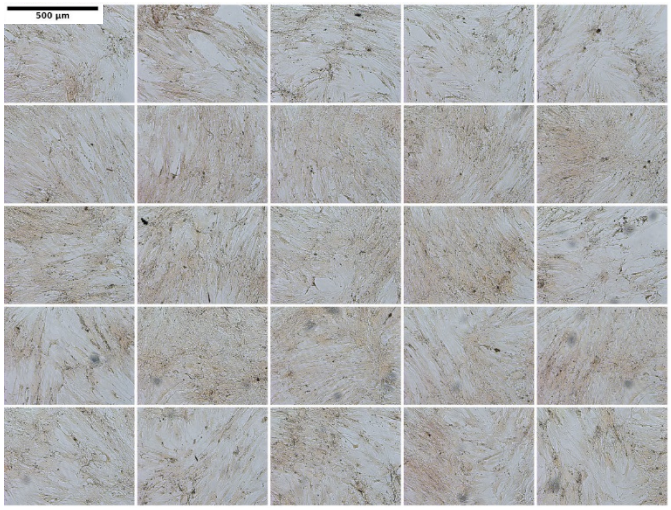
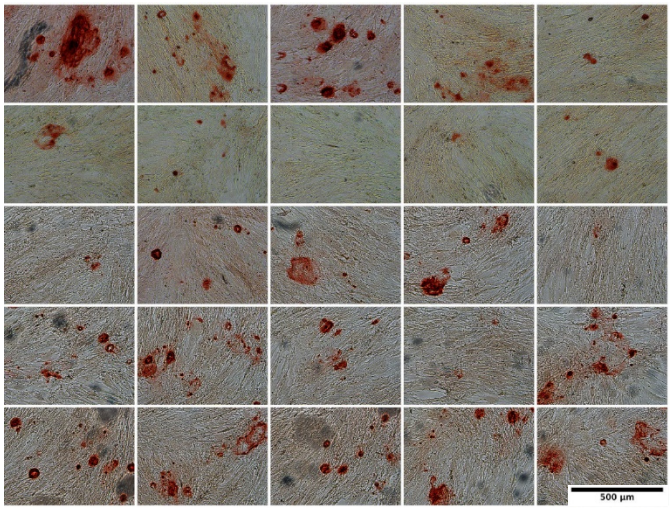
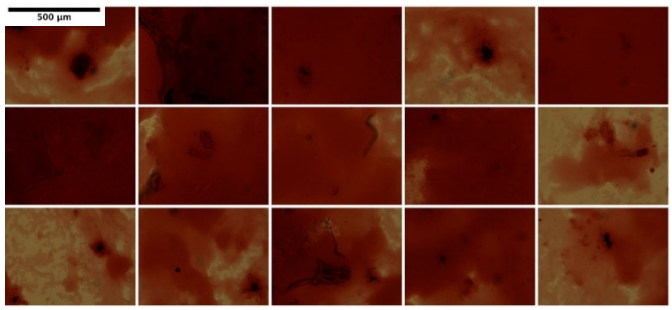
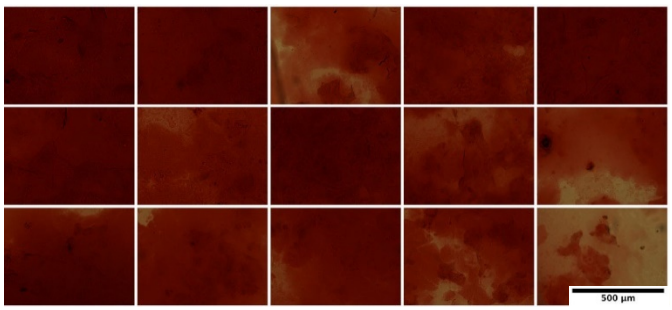
Qualitatively, a stronger staining was observed in CD146^{high} cells compared to CD146^{low} cells in most of the samples. However, the differences in staining between samples are stronger than the differences between sorted fractions.

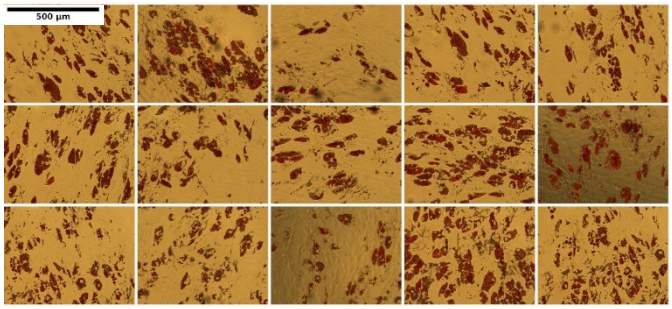
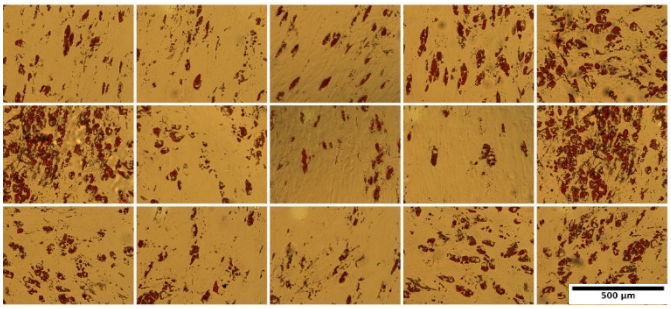
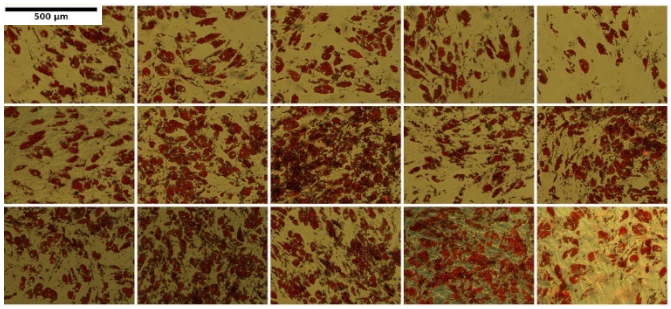
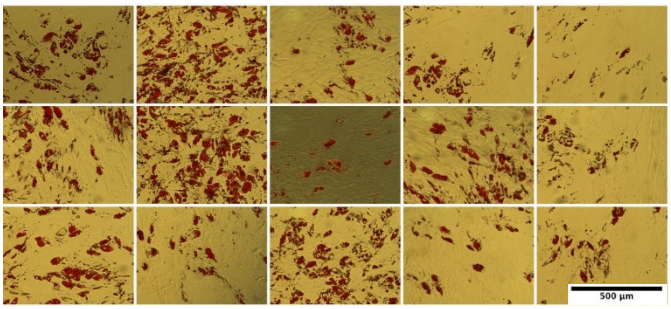
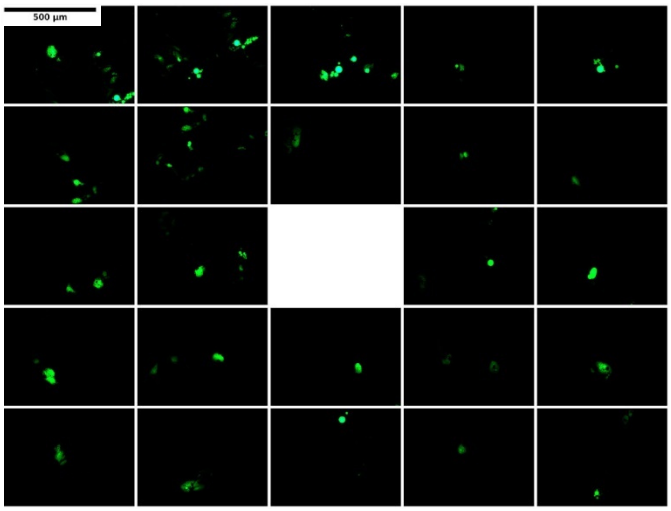
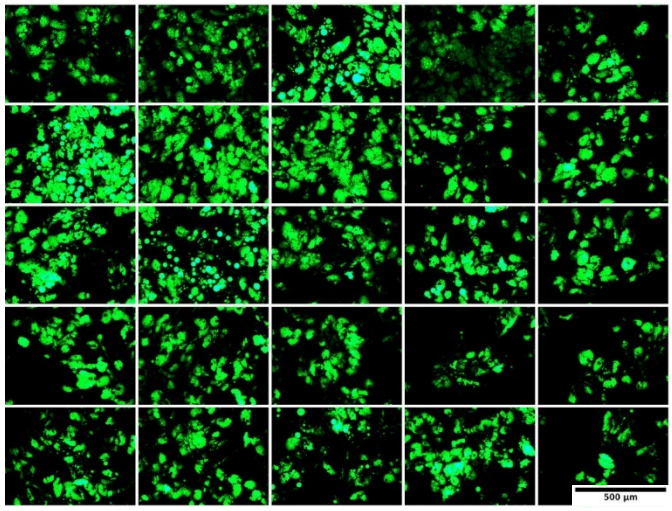
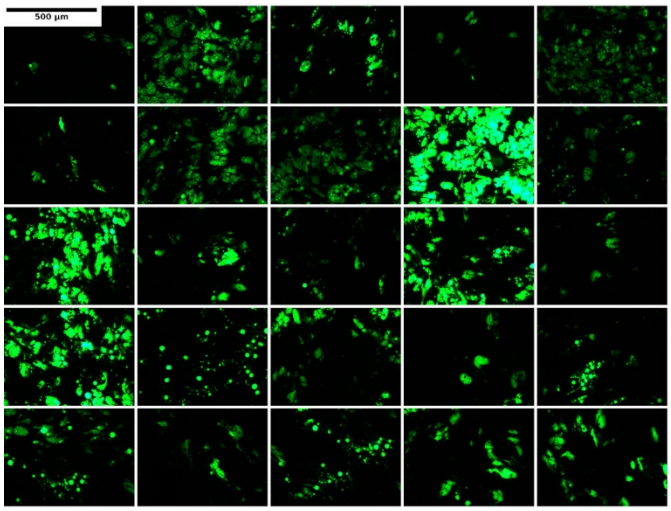
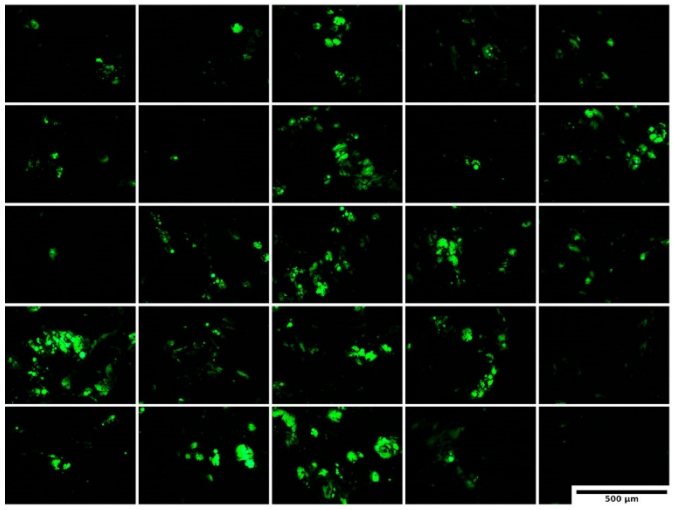
However, there were some exceptions. The staining of “24” and “85” samples after osteogenic differentiation was found to cover most of the culture surface in both fractions of sorted cells. Surprisingly low results were obtained with “65” sample at p4 after adipogenic differentiation and with “84” p6 sample after osteogenic differentiation, both in CD146^{high} sorted cells. For these samples, the difference between the staining obtained in CD146^{high} cells is very far from what was obtained with CD146^{low} cells, while for all the others samples, the differences were less pronounced. It is possible that the differentiation process did not go well for these samples, resulting in abnormally low results. These 2 samples were not included in the quantitative analysis performed by measuring the proportion of differentiated surface in each image with ImageJ software **(figure 58)**.

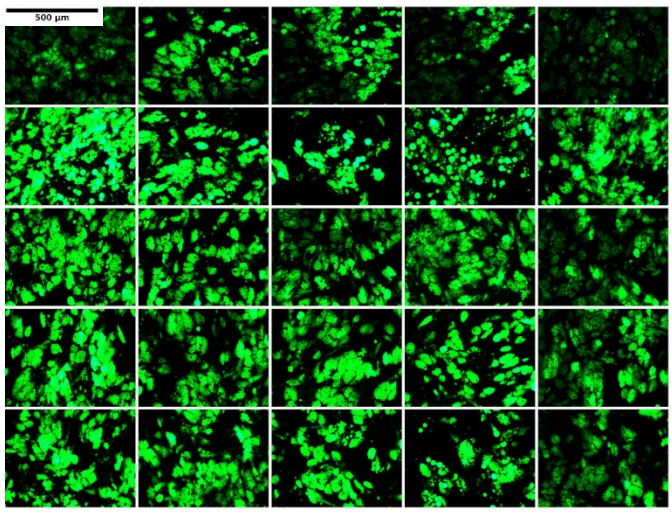
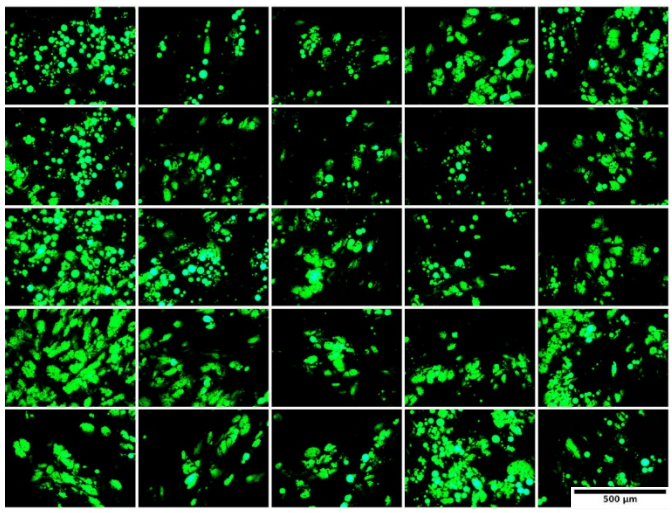
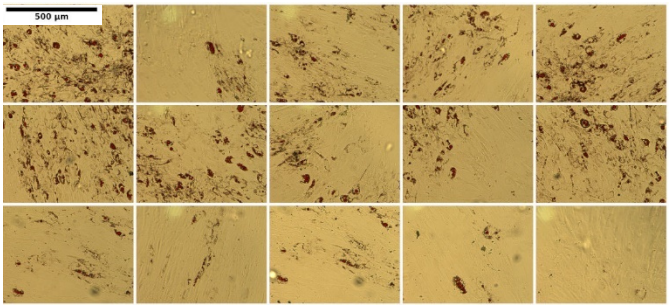
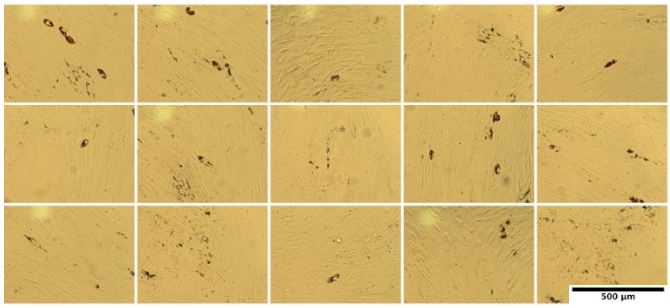
Table 14: Osteogenic and adipogenic differentiation made on CD146^{high} and CD146^{low} sorted cells.

Osteogenic differentiation was assessed after 3 weeks induction by alizarin red that stains calcium mineralization. Adipogenic differentiation was assessed 4 weeks after induction by Oil-Red-O staining or Adipored staining both allowing to highlight lipidic vesicles. Oil-red-O stains lipids in red and Adipored stains lipids in green fluorescence. Microscope used is Leica DMI 3000B, magnification x100, scale bar = 500 µm.

	CD146high	CD146low
24 p3		
30 p5		
65 p4		

78 p3		
84 p4		
84 p6		
85 p4		

	CD146high	CD146low
24 p3		
30 p5		
65 p4		
78 p3		

84 p4		
84 p6		

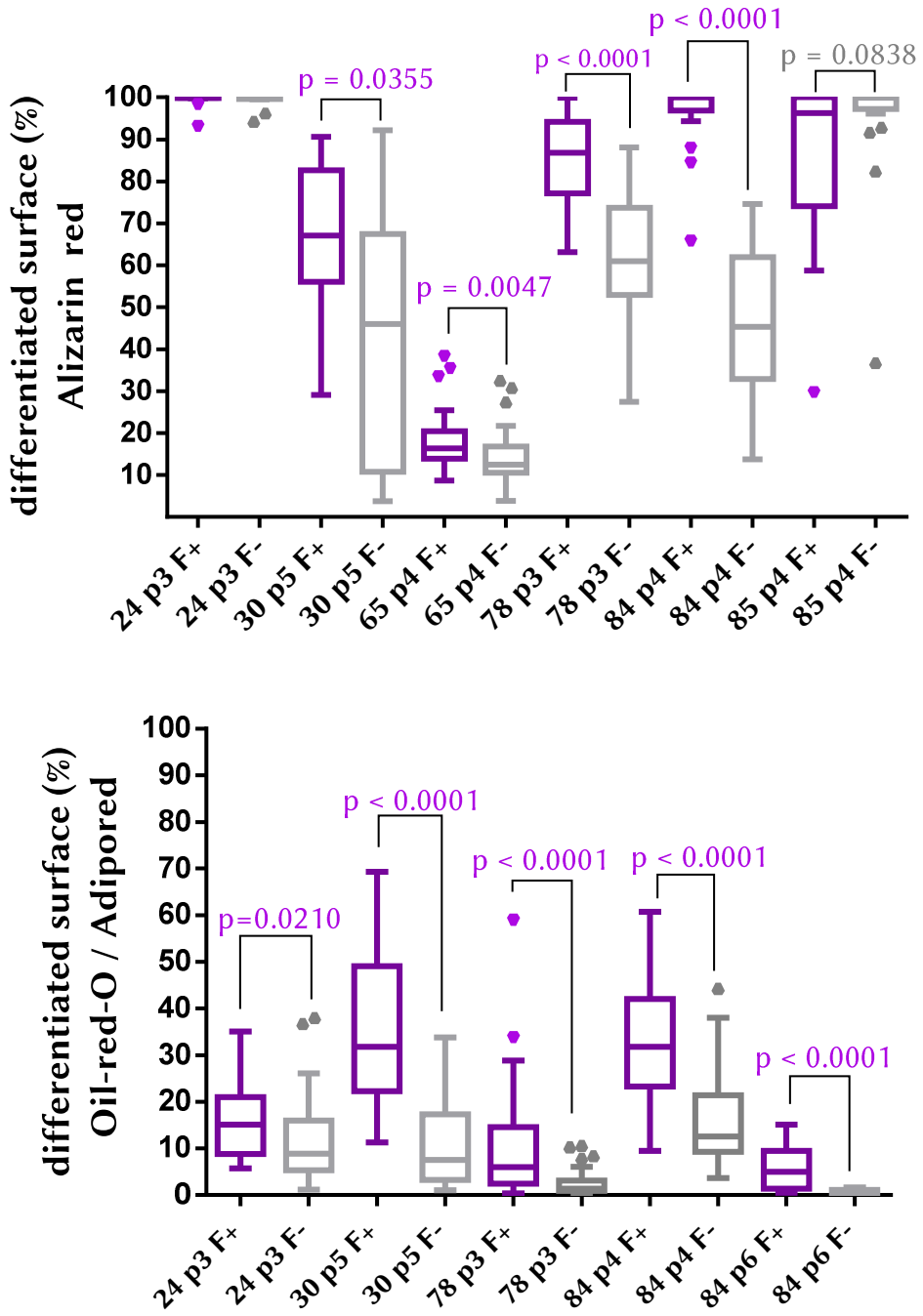


Figure 58: Differentiated surface quantification with ImageJ to estimate differentiation efficiency of BM-MSC after sorting.

On the left: results for adipogenic differentiation for 5 samples. On the right: results for osteogenic differentiation for 6 samples. Boxplots represent the gathering of the percentage of stained surface of at least 15 images.

P values displayed were calculated with Mann-Whitney test.

The quantitative analysis highlighted significant differences between $CD146^{high}$ and $CD146^{low}$ MSC for most of the samples included in the analysis, except the two with almost all the surface stained after osteogenic differentiation ("24" and "85" samples). A potential bias with this kind of assays is that for the experiment to work, the cells must proliferate enough to reach confluence.

Interestingly, the samples with the strongest differentiation were not those with the highest CD146 expression before sorting ("24" and "78" samples were the two that expressed CD146 the most). The samples that showed the better results when considering both osteogenic and adipogenic differentiation capacity were "30" p5 and "84" p4 samples. The level of expression may then not be so important, and the most relevant would be to distinguish different sub-populations in the sample.

These results are consistent with other studies that used different MSC sources. Two studies highlighted the higher multipotency of $CD146^{high}$ sorted cells *in vivo*. They showed their capacity to organize bone marrow formation and differentiate into bone marrow elements. For one, the cells were sorted from BM mononuclear cells (20) and for the other, from telomerized BM MSC in culture (167). With MSC from other sources, increased differentiation capacity and multipotency was also highlighted *in vitro* in $CD146^{high}$ sorted cells (periodental ligament MSC (179,180), placenta MSC (176), WJ MSC (175)).

Moreover, researchers using CD146 knocked down on BM and cord blood MSC found that these cells displayed significant decrease in osteogenic and adipogenic differentiation potential (166,270).

Several studies also highlighted the ability of $CD146^{high}$ sorted cells to undergo chondrogenic differentiation *in vivo* (173) (cells sorted from BM mononuclear cells) and *in vitro* (174) (cells sorted from BM MSC at p1). This capacity was often the most pronounced of the 3 differentiation assays usually performed (osteo-, adipo-, and chondrogenic differentiation).

4. Senescence

Senescence level of the sorted cells was assessed with β-galactosidase senescence associated staining **(figure 59)**.

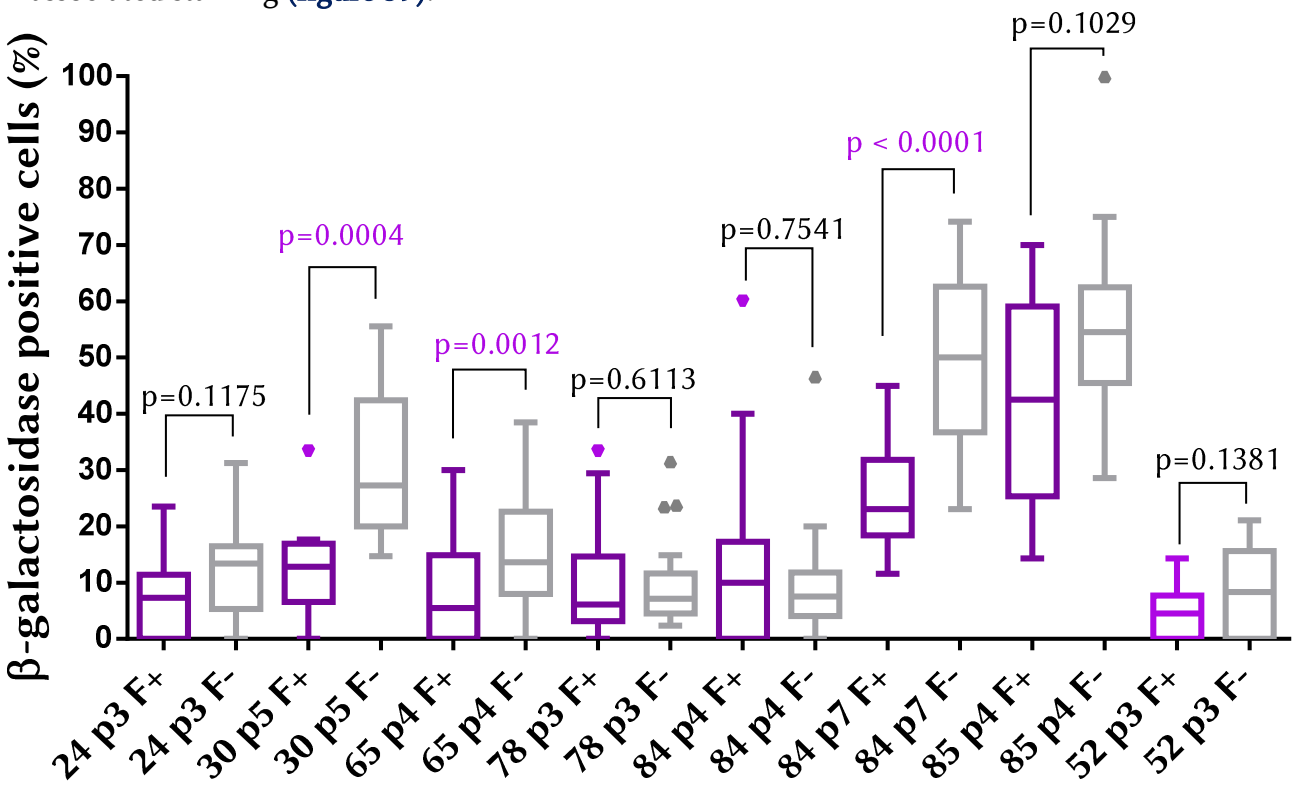


Figure 59: Percentage of senescent cells in CD146 sorted cells assessed by β-galactosidase senescence associated staining.

Percentages of positive cells displaying a blue staining were counted from at least 10 images.
Total number of cells present in each image was counted with DAPI staining.
Tukey boxplots were made with the gathering of result of all images for the sample.
p values were calculated with Mann-Whitney test.

In the samples with important baseline senescence levels, the fraction of cells with higher CD146 expression contained globally less senescent cells than MSC with low CD146 expression. The samples concerned are notably "30", "65" and "84" p7, with also a trend in "85" sample. The other samples displayed an overall weaker level of senescence in both fractions of cells, with slight tendencies towards less senescent content in $CD146^{high}$ sorted cells. One exception is "84" sample at p4 with the median value of $CD146^{high}$ sorted cells being slightly superior to the median value of $CD146^{low}$ cells. In addition, it can be noted that for "84" p7 and "85" p4 samples, the level of senescence remained relatively high even in $CD146^{high}$ sorted cells.

Senescence was not often assessed by other groups making CD146 cell sorting. However, some reports found a link between CD146 expression and senescence. Another

group found that CD146^{high} sorted cord blood MSC were less senescent than CD146^{low} cells (270). Furthermore, with CD146 knocked down BM MSC, more senescence was detected with senescence associated β-galactosidase staining than in unmodified MSC (166). This strategy was also used on cord blood MSC. Consistently, they found on MSC with CD146 knockdown a higher p16 expression (assessed by western blot) and β-galactosidase activity (270).

5. Migration

The capacities of MSC to migrate across small pores toward a chemoattracting signal was assessed for 2 samples with transwell assay **(figure 60)**.

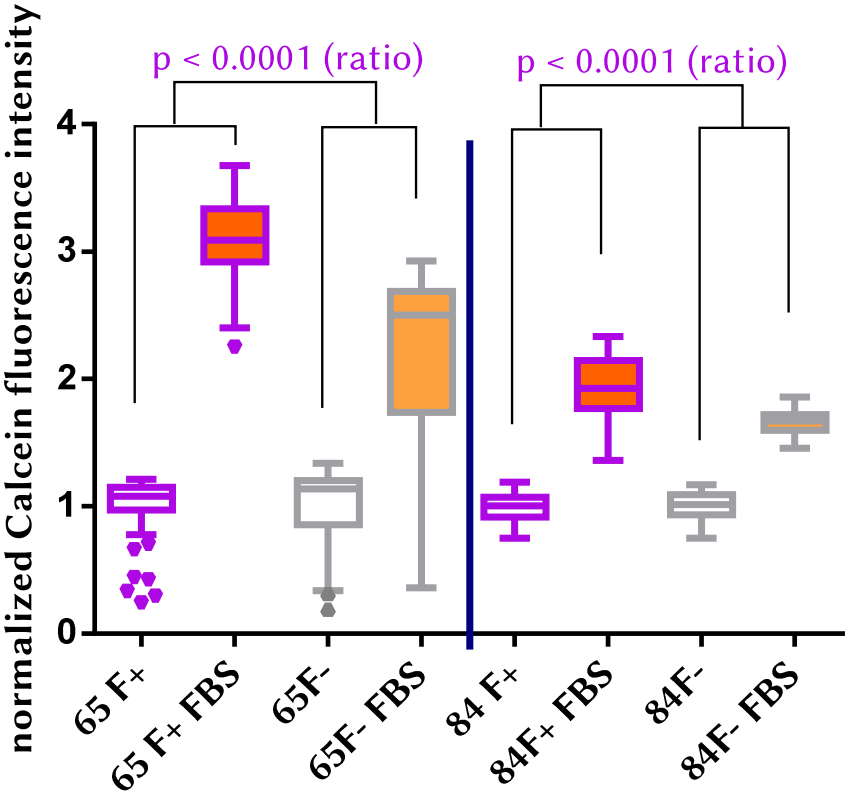


Figure 60: Comparison of the migration capacity of the sorted cells toward 30% FBS across 8 µm pores after 20 hours.

The samples were both at passage 4. Cells were stained with Calcein to detect living cells that were able to cross to the down part of the inserts. Boxplots gathers the results obtained at different points with the fluorescence reader (n=58 points, obtained from 2 separate inserts). Fluorescence intensity obtained is **normalized** with the one obtained from cells placed in medium without chemoattractant.

p values were computed with Mann-Whitney test of ratio.

For both fractions of sorted cells, MSC showed an increased capacity to answer to a chemoattractant signal by crossing the 8 µm pores compared to MSC placed with no chemoattractant. For the 2 samples studied, $CD146^{high}$ sorted MSC answered significantly more to the chemoattractant signal of FBS than $CD146^{low}$ MSC. However, for the sample "84", the basal level of migration was higher in $CD146^{low}$ cells, but the increase of migration in 30% FBS conditions was less pronounced. This test was also made with the sample "52" sorted with FACS, but the signals obtained were very similar between all the different conditions, even with and without FBS.

Another group also explored the migration capacities of MSC sorted according to CD146 expression. However, they used telomerized BM MSC. With transwell assay (n=2), they obtained twice more cells migrating with $CD146^{high}$ than with $CD146^{low}$ sorted cells in the 10% FBS condition. The baseline migration was assessed in 0,2% FBS and both fractions of sorted cells displayed similar results. They also looked at cell distribution after intravenous tail vein injection in 4 mice. They report that $CD146^{high}$ cells could better migrate away from the lungs and were more widely distributed in the mice body 6 days after injection. $CD146^{low}$ cells, for their part, remained in the lungs all over the experiment (167). Additionally, BM MSC with CD146 knockdown showed impaired migration capacities compared to control cells (166).

6. Secretions

The ability of MSC to secrete bioactive factor is crucial for their use in cell therapy. To have a global view of sorted MSC secretome, an array gathering more than 100 soluble proteins was used with 2 samples ("78" and "84") **(figure 61)**. A comparison of relative expression of the detected protein was made between the different fractions of sorted cells. The first observation was that a more important variety of proteins was detected in unsorted cells secretions. Among protein that were strongly expressed but did not differ between the sorted cells, there was pentraxin-3 and high levels of serpin. IL17 was secreted by all the fractions of one of the two samples studied, and was only found in the unsorted cells in the other sample. Proteins that were differentially expressed in $CD146^{high}$ and $CD146^{low}$ MSC in the 2 samples studied were then searched.

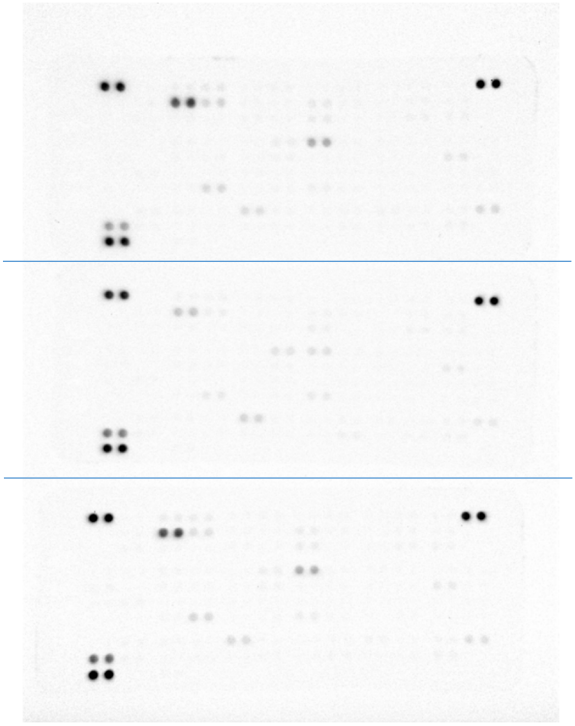


Figure 61: Stained membranes of the arrays obtained after exposure with gathered medium from unsorted (up), positive fraction (middle), and negative fraction (down) of sorted cells of one sample.
Spots density was analyzed with ImageJ software.

This principally revealed that chitinase-3-like-1 (**CHI3L1**) and insulin-like-growth-factor-binding-protein-3 (**IGFBP3**) were more expressed in $CD146^{low}$ MSC than in $CD146^{high}$ sorted cells. Another protein, macrophage-inhibitory-factor (**MIF**), was found in higher relative concentrations in $CD146^{high}$ MSC than in $CD146^{low}$ cells.

To confirm the results obtained with the array, ELISA technique was used to determine IGFBP3 and CHI3L1 concentrations secreted by the sorted cells **(figure 62)**. ELISA results confirmed that $CD146^{high}$ sorted MSC secreted less IGFBP3 and CHI3L1 than $CD146^{low}$ cells. A significant difference was only obtained for IGFBP3. The difference between the two fractions of sorted cells was reduced when MSC were stimulated by inflammatory cytokines.

Two other samples tested (“52” and “65” samples) in the same conditions provided contrasted results. Firstly, the concentration obtained were much weaker (no more than 800 pg / mL for IGFBP3 and 2500 pg / mL for CHI3L1). Then, by looking at the relative expression between the sorted cells, only the “52” sample stimulated with inflammatory cytokines reproduced the same difference in cytokine expression. In the other conditions, $CD146^{high}$ cells of the two samples showed a tendency to be equal or to secrete more IGFBP3 and CHI3L1 than $CD146^{low}$ cells, but the differences were very slight compared to what was seen with “78” and “84” samples.

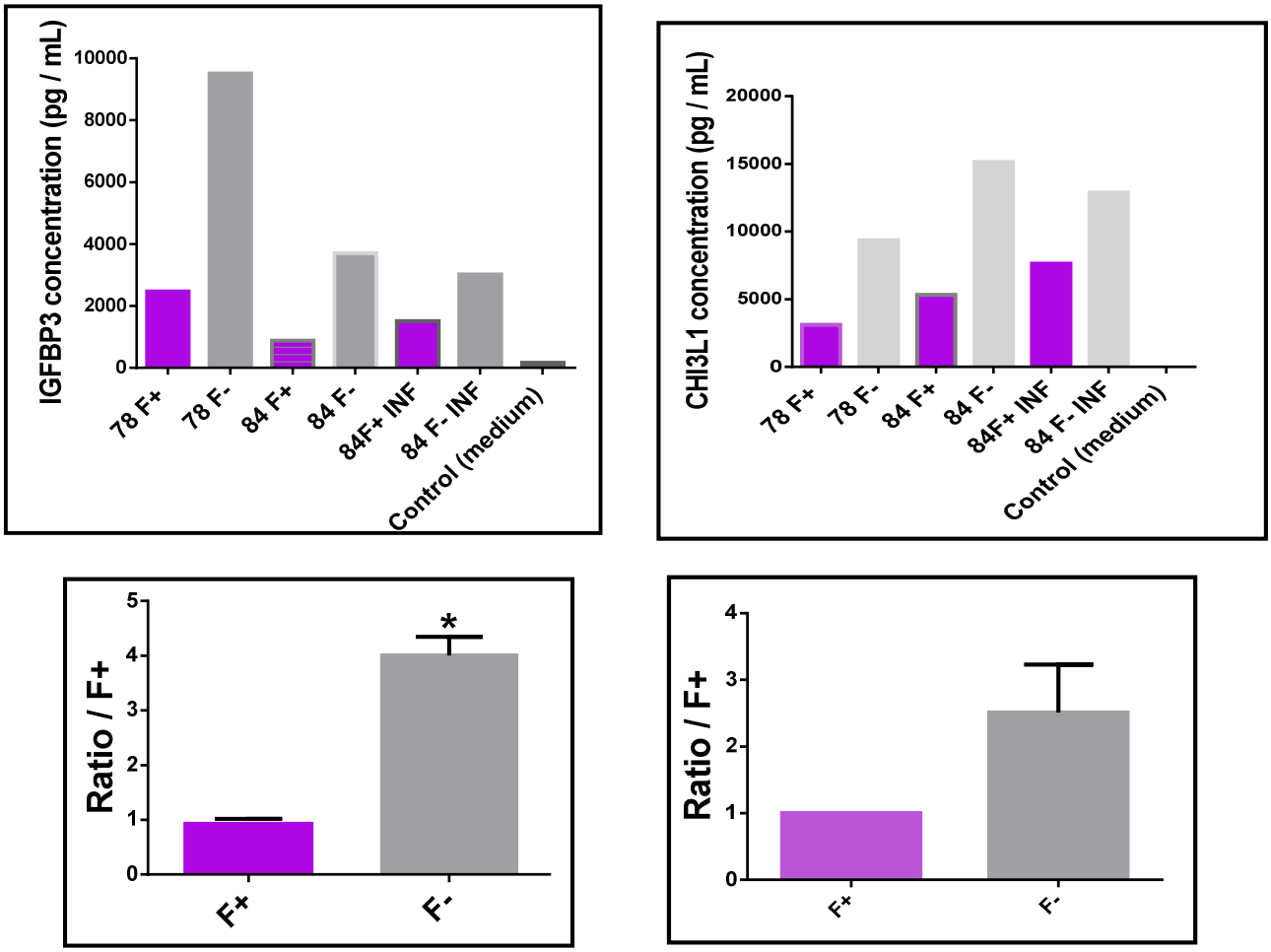


Figure 62: IGFBP3 (on the left) and CHI3L1 (on the right) concentration assessed by ELISA on MSC fractions obtained after magnetic sort.

Secretions were gathered after 48 h in medium without FBS.

INF : addition of 25 ng/mL TNFα and 20 ng/mL IFNγ in medium without FBS during 48 h.

For ratio (below): concentrations were normalized to the mean concentration obtained for F+ fraction.

*: p = 0.0286: significant difference between ratio / F+ of the sorted cells with Mann-Whitney test.

This should not be due to difference between the number of cells assayed because they were counted and were in equivalent quantities. The results obtained with the array cannot be generalized to the other samples.

The cytokines highlighted remain interesting targets for future studies on more samples.

Regarding the function of these molecules, it seems that CHI3L1 can bind to the receptors for IL13 and form a multimeric complex together with IL13 and IL13 receptor. It could regulate by this way the responses associated with oxidant injury, apoptosis, pyroptosis,

inflammasome activation, antibacterial response, melanoma metastasis, and TGFβ1 production (272). CHI3L1 is also upregulated in situation of chronic inflammation, tissue remodeling and human cancer (273).

IGFBP3 was first described to inhibit IGF1 action and is now studied for many other functions like inhibiting cell proliferation and promotion of apoptosis (274). With MSC, one study was found to mention IGFBP family molecules (183). They found that IGFBP4 and 7 were released by replicative senescent BM MSC. Furthermore, IGFBP3 was found in patients with various fibrotic conditions (275) and in marrow plasma of patients with myelodysplastic syndromes (276).

MIF role on BM MSC was explored by a study (277) but they found that exposure to recombinant MIF had a tendency to decrease MSC migration capacity. They hypothesize that this may retain MSC to the site of injury where MIF is upregulated as an inflammatory cytokine, but this is contradictory with our migration results. However, the exact secreted concentration of MIF was not measured and there may have a dose-dependent response. Furthermore, another study found that MIF addition in rat BM MSC culture (100 ng/ml, and treatment was for 1 h at 37˚C) could protect the cells from senescence induction by doxorubicin. It also increased the cells paracrine function and resistance to oxidative stress with superoxide dismutase upregulation. Another study investigated MIF function in human placental pericytes expressing NG2. These cells were shown to secrete a cocktail of chemoattractant molecules, including MIF. MIF was more specifically shown to mediate the recruitment of leukocytes (144) .

In the literature, few studies looked at CD146 sorted MSC secretions. No other study comparing CD146 sorted cells global secretion profile from BM MSC with proteomics or arrays was found. To our knowledge, the principal cytokine highlighted with the array were not described in association with MSC in general. A study showed that $CD146^{high}$ sorted WJ MSC secreted less IL6 than $CD146^{low}$ cells (92). Contrastingly, IL6 was not detected in any cell fraction with the array used in our study.

E. Global view with principal component analysis

With the various parameters gathered for all the samples during the study of CD146 cell sorting, it was not easy to have a clear and global view of the results. A principal component analysis (PCA) was then undertaken to try to bring the most important tendencies out, and to see how the samples position themselves compared to the others. The objective of this analysis was also to highlight the flow cytometry parameters that can be the most informative to make links with cell properties and tendency of evolution.

Variables included in the PCA are listed **table 15**. Dispersion parameters (standard deviation, coefficient of variation, …) were not used because the results obtained were inconsistent. Improvement in the method to gather these parameters is needed to allow their reliable use. Differentiation and other functional results were not included either because the data could not be obtained from all the samples tested. The number of samples included was already low for the PCA and it could not be reduced any further.

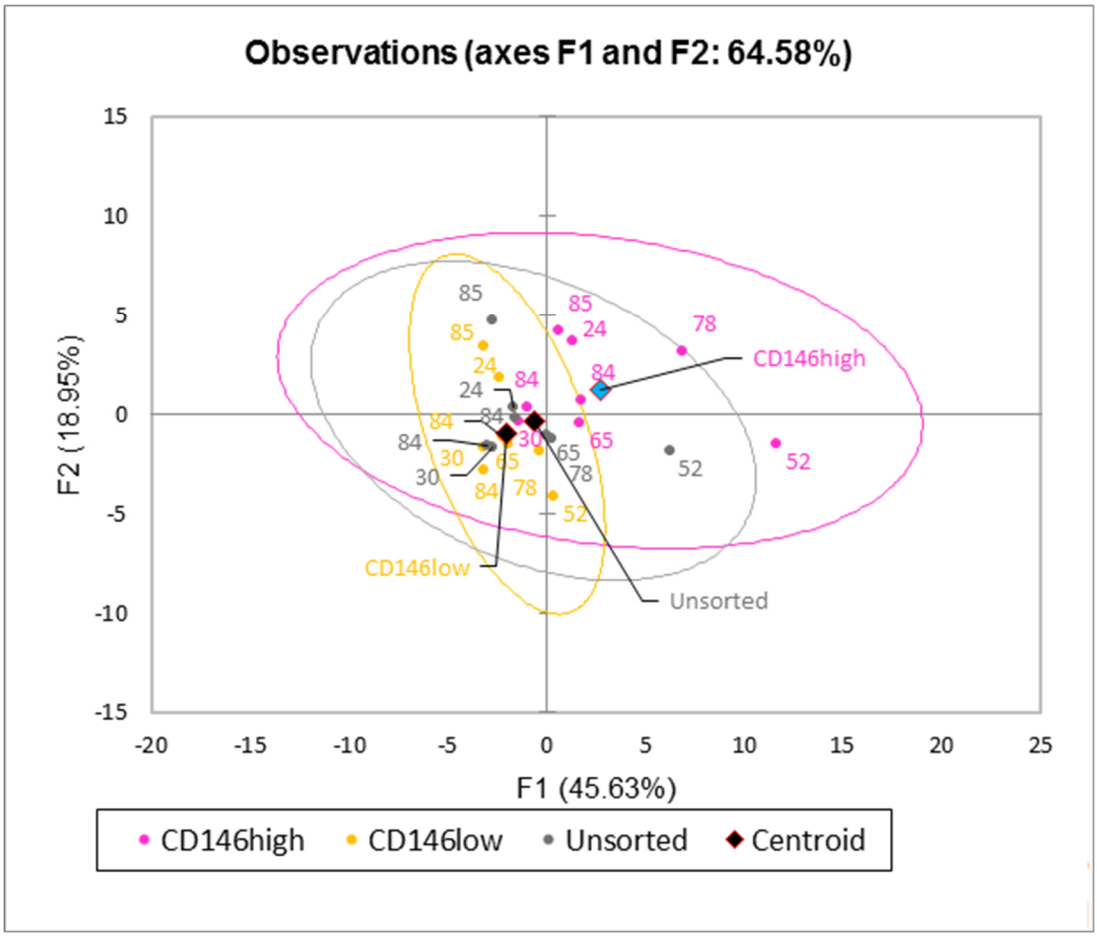
The chart illustrating PCA results are displayed **figure 63**. Only 65% of the total variability could be represented with the projection in two axes. The results must then be interpreted with caution.

As confirmed by the square cosines of the variables **(table 15)**, variables associated to CD146 expression were linked with F1 axis. Variable associated with cell size (FSC and SSC) and Population Doubling and Doubling Time were rather linked to F2 axis. Senescence, CPD and colony forming efficiency variables could not be well represented on F1 and F2 axes.

Table 15: Selected variables for principal component analysis.

The interest to gather all these parameters is explained in the chapter "Methods".

Variables	Square cosines in F1	Square cosines in F2
CD146 (% beyond ISO) just after sort	**0,404**	0,185
Median CD146 (RAW)	**0,901**	0,011
Median CD146 ratio ISO	**0,650**	0,150
Median CD146 delta ISO	**0,904**	0,015
Geometric Mean CD146 (RAW)	**0,908**	0,016
Geometric Mean CD146 ratio ISO	**0,540**	0,140
Geometric Mean CD146 delta ISO	**0,804**	0,006
Median SSC (RAW)	0,040	**0,497**
Median FSC (RAW)	0,086	**0,403**
Arithmetic Mean SSC (RAW)	0,078	**0,522**
Arithmetic Mean FSC (RAW)	0,100	**0,413**
Senescence level (%)	0,200	0,188
Colony Forming Efficiency (%)	0,008	0,001
Population Doubling	0,003	**0,635**
Cumulative Population Doubling	0,147	0,220
Doubling Time	0,002	**0,718**
CD146 (% beyond ISO) one passage after sort	**0,781**	0,005
Median CD146 (RAW) one passage after sort	**0,954**	0,001
Median CD146 ratio ISO one passage after sort	**0,886**	0,000
Median CD146 delta ISO one passage after sort	**0,958**	0,001
Geometric Mean CD146 (RAW) one passage after sort	**0,943**	0,003
Geometric Mean CD146 ratio ISO one passage after sort	**0,920**	0,000
Geometric Mean CD146 delta ISO one passage after sort	**0,960**	0,003
Median SSC (RAW) one passage after sort	0,094	0,383
Median FSC (RAW) one passage after sort	0,164	0,247
Arithmetic Mean SSC (RAW) one passage after sort	0,130	0,316
Arithmetic Mean FSC (RAW) one passage after sort	0,178	0,221
Yield	0,029	0,008



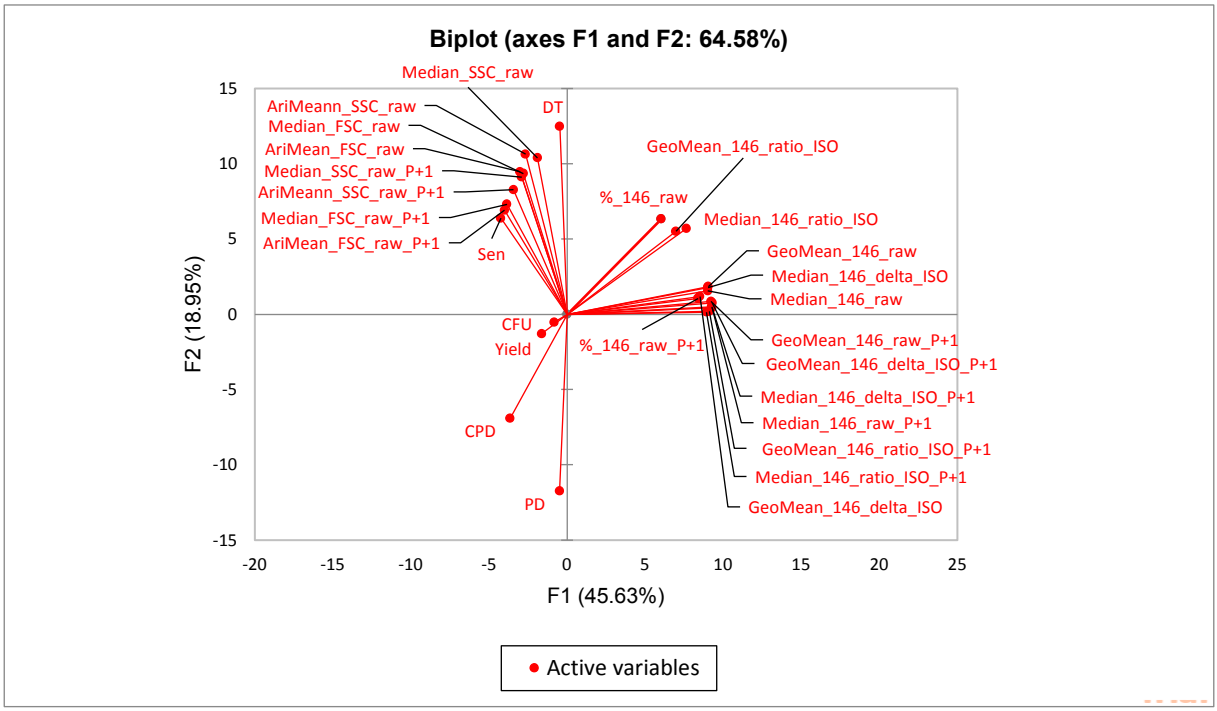


Figure 63: Chart obtained after principal component analysis.
Up: Observations position and clusters with F1 / F2 projection.
Down: projection of the initial variables.

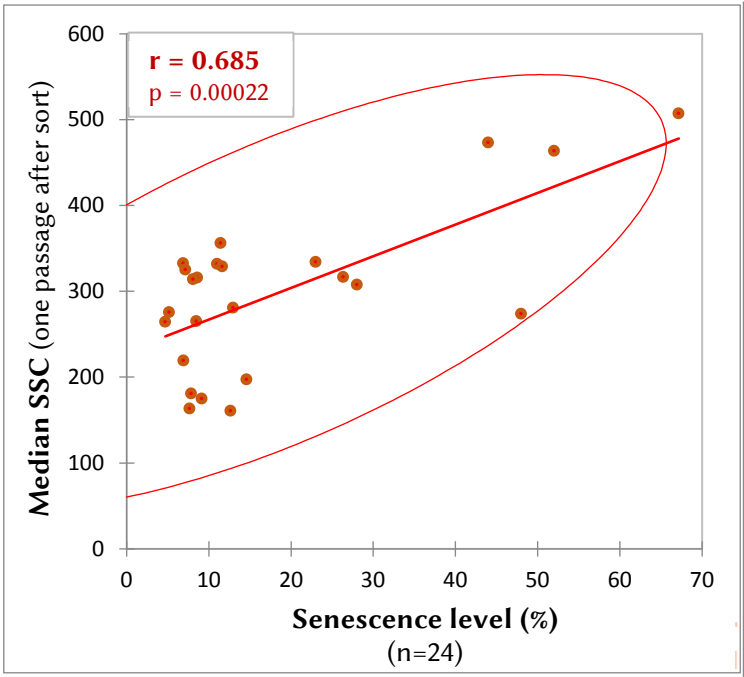
For the variables that could be linked with F1 or F2 axis, the position of the variables can be interpreted as follows:

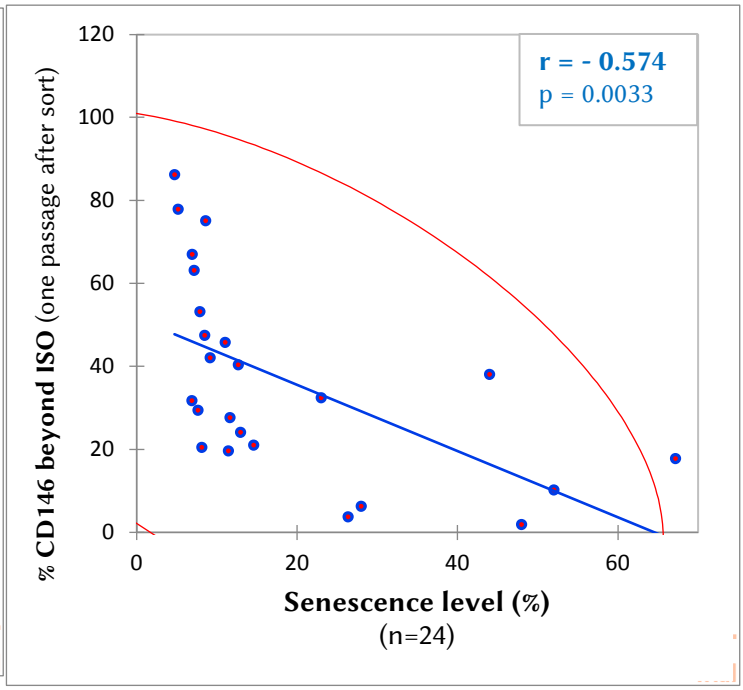
- the closer they are, the more they are positively correlated,
- they are not correlated if they are orthogonal,
- they are negatively correlated if they are on the opposite side.

CD146 percentage, normalized geometric mean and normalized median were closed to each other than to the other parameters that can be used in flow cytometry to measure CD146 expression. Doubling time variable was found close to parameters associated to cell size, notably SSC median.

On the chart with the samples **(figure 63)**, the cluster of each fraction of sorted cells were represented in different colors. It can be seen that $CD146^{low}$ group is the most homogeneous, while $CD146^{high}$ group was more dispersed. $CD146^{high}$ cells were consistently found to be more oriented toward CD146 expression parameters than others. They were also further away from proliferation variables.

The most relevant association observed in the Pearson correlation matrix computed with the PCA were selected and shown **figure 64**. A positive correlation was found between senescence level and SSC median. It means that the biggest cells are the most senescent. It could also validate the use of SSC parameter as indicative marker of cells condition. A negative correlation was found between senescence level and CD146 percentages. This global analysis confirms the previous results obtained with univariate analyses of sorted cells. CD146 percentages were also negatively correlated with cumulated population doubling. The association was also found with CPD before sort. However, there was no correlation with the population doubling obtained after sort. This means that CD146 expression may be rather linked to the differences between samples proliferation backgrounds than with the differences obtained between cell fractions after sort. Furthermore, the association were slightly stronger with CD146 percentages than with CD146 medians. For CD146, percentages then appeared as one of the most interesting parameters in the conditions of this study to make links with other variables.





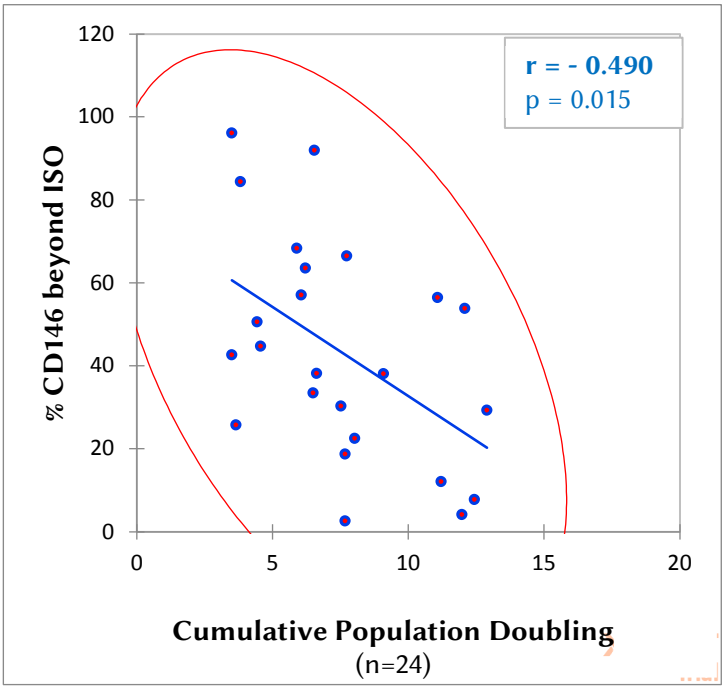


Figure 64: Strongest associations observed in the Pearson correlation matrix obtained after PCA.
Cumulative population doubling was calculated at the end of the passage of the sort.

A. Main points of part II

With the different samples used in this study:

- **CD146 enrichment was feasible and efficient** with immunomagnetic technique, even for CD146 low expressing samples. However, a large number of cells is needed to obtain a sufficient quantity in the positive fraction.

- **CD146 expression was maintained during at least 2 passages after sort**. It lets the time for applications where the cells could need to be amplified further, stimulated or placed in scaffolds.

- MSC sorting of $CD146^{high}$ cells did not allow to obtain cells with better proliferation capacities.

- $CD146^{high}$ sorted cells did not show improved capacity to form colonies.

- The results obtained with differentiation assays showed the **cells with stronger CD146 expression have a better capacity to produce differentiated cells**. However, other parameters influencing the samples capacities may have more impact than CD146 expression.

- Senescence associated β-galactosidase staining results suggest **$CD146^{low}$ cells populations contain more senescent cells**.

- $CD146^{high}$ cells answered significantly more to the chemoattractant signal of FBS in transwell assay.

- The difference in secretion profile between $CD146^{high}$ and $CD146^{low}$ cells deserve to be further studied to be able to conclude about its potential implication for cell therapy. IGFBP3, CHI3L1 and MIF appeared as interesting targets.

- The PCA allowed to include all parameters used to measure CD146 expression and gather proliferation, senescence, and colony formation efficiency data of all sorted samples. This approach highlighted the **link between senescence, CD146 expression and cell size**. It also underlined the **interest to use percentage to follow CD146 expression** and link it with other variables.

III. MSC senescence characterization

Senescence was described by many research groups to strongly influence MSC cultures and their resulting properties. A tendency for CD146^{high} sorted cells to contain less senescent cells was also reported in the previous part of this work. To go further in the understanding of MSC senescence, surface markers appeared as the most interesting targets. They enable to combine fundamental and applied approaches. For this study, a model of replicative senescence by serial passaging was chosen. Contrary to stress induced senescence, replicative is very close to the phenomena observed during MSC culture and with MSC obtained from older donors. The objective was to compare young cells containing a small portion of senescent cells with cells passaged until exhaustion containing a majority of senescent cells. For this, BM MSC from 3 young donors were used and studied by proteomics and flow cytometry. The cells were also characterized to follow functional changes occurring in replicative senescent MSC.

A. Replicative senescence model

Before addressing the surface markers studies, the changes of MSC state undergoing replicative senescence are first described in this part.

1. Proliferation and senescence associated β-Galactosidase

MSC cultures were maintained until the proliferation capacity of the cells was too low to support cell growth. **Figure 65** shows the progressive changes of MSC proliferation capacity.

The decline in MSC proliferation capacity occurred in a progressive manner. Interestingly, a kind of resurgence can occur after a decline, it was observed for the three samples, but it never reached the levels of the first 2 passages. The samples “24 C” and “33” could reach passage 10 and only the sample named “24 A” was able to reach passage 14 with a sufficient number of cells to be cultivated.

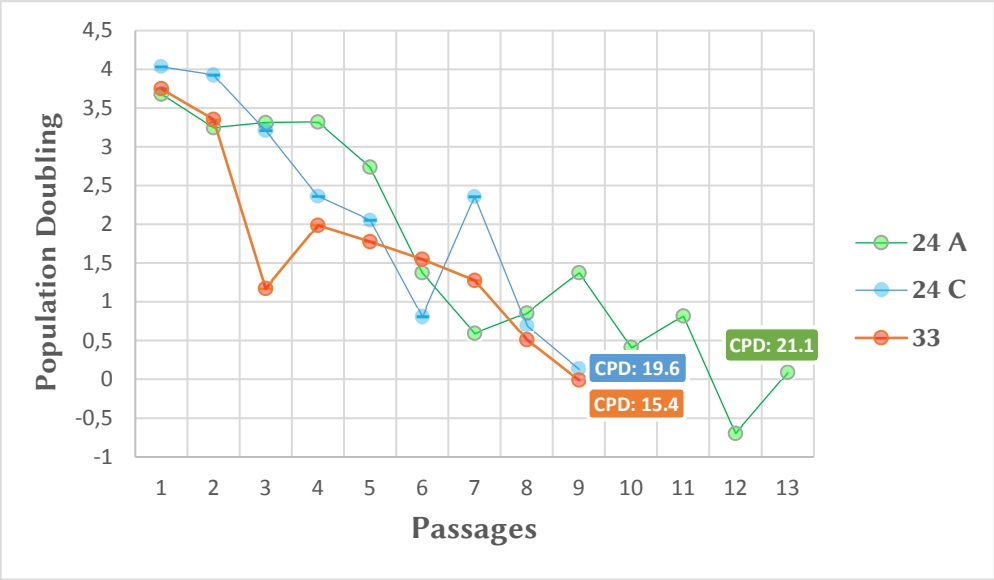


Figure 65: Proliferation capacity reflected by population doubling values determined at each passage for the three samples studied until replicative senescence.

CPD: Cumulative population doubling at p13 for sample "24 A" (in green) and at p9 for samples "24 C" (in blue) and "33" (in orange).

The samples displayed different global proliferation capacity, with sample "24 A" being able to reach the highest cumulative population doubling at the end of culture, the sample "24C" being intermediate and "33" displaying the lowest production of cells. These differences between the samples could already be observed with the cumulative population doubling at passage 5 (respectively 16.3; 15.6; 12.0).

The last passages are not shown **figure 65** because all cells were seeded at higher density to obtain a sufficient number of cells to be compared with their younger counterparts for proteomics and functional tests. Cell seeding density is known to change MSC proliferation dynamics and the results of population doubling could not be gathered with previous results. In this study, what could be observed is that cells regained some semblance of proliferation capacity when seeded at higher density, allowing to use these late passage cells for different experiments. However, this effect did not last and was not sufficient to push the culture forward.

Senescence associated β-galactosidase staining associated to EdU proliferation staining were also made along MSC cultures. Examples of the obtained images can be seen **figure 66**.

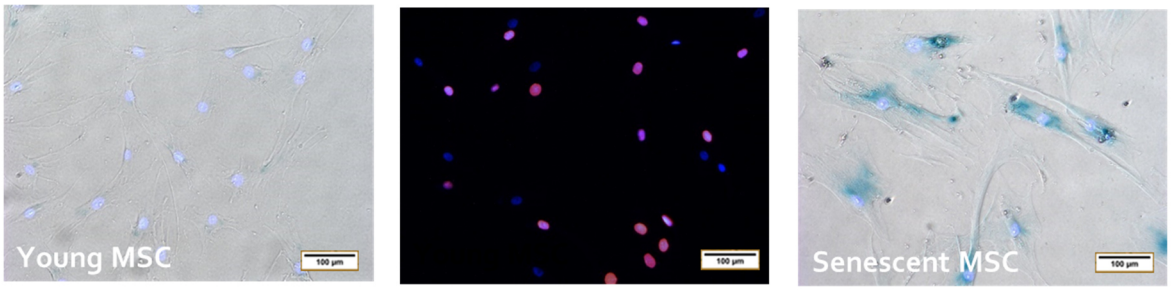


Figure 66: Examples of images obtained by microscopy after β-galactosidase staining associated to EdU and DAPI staining.
When β-galactosidase is overexpressed, a blue compound is formed after staining and can be seen in blue in bright field. EdU is incorporated when cells replicate their DNA and is revealed by AlexaFluor594 fluorescence, represented in red. All nucleus are stained with DAPI, fluorescent compound that binds A-T regions in DNA, represented in bright blue.
On the left: representative bright field image of BM MSC coming from a young donor at passage 3. An overlay with the image of DAPI staining allows to see the cells location.
In the middle: Representative overlay of EdU and DAPI staining.
On the right: representative bright field image of the same BM MSC sample at replicative senescence at passage 10.
Microscope used is Leica DMI3000B, scale bar is 100 µm.

The rates of senescent and proliferative cells were quantified, the results obtained for each sample are displayed **figure 67**.

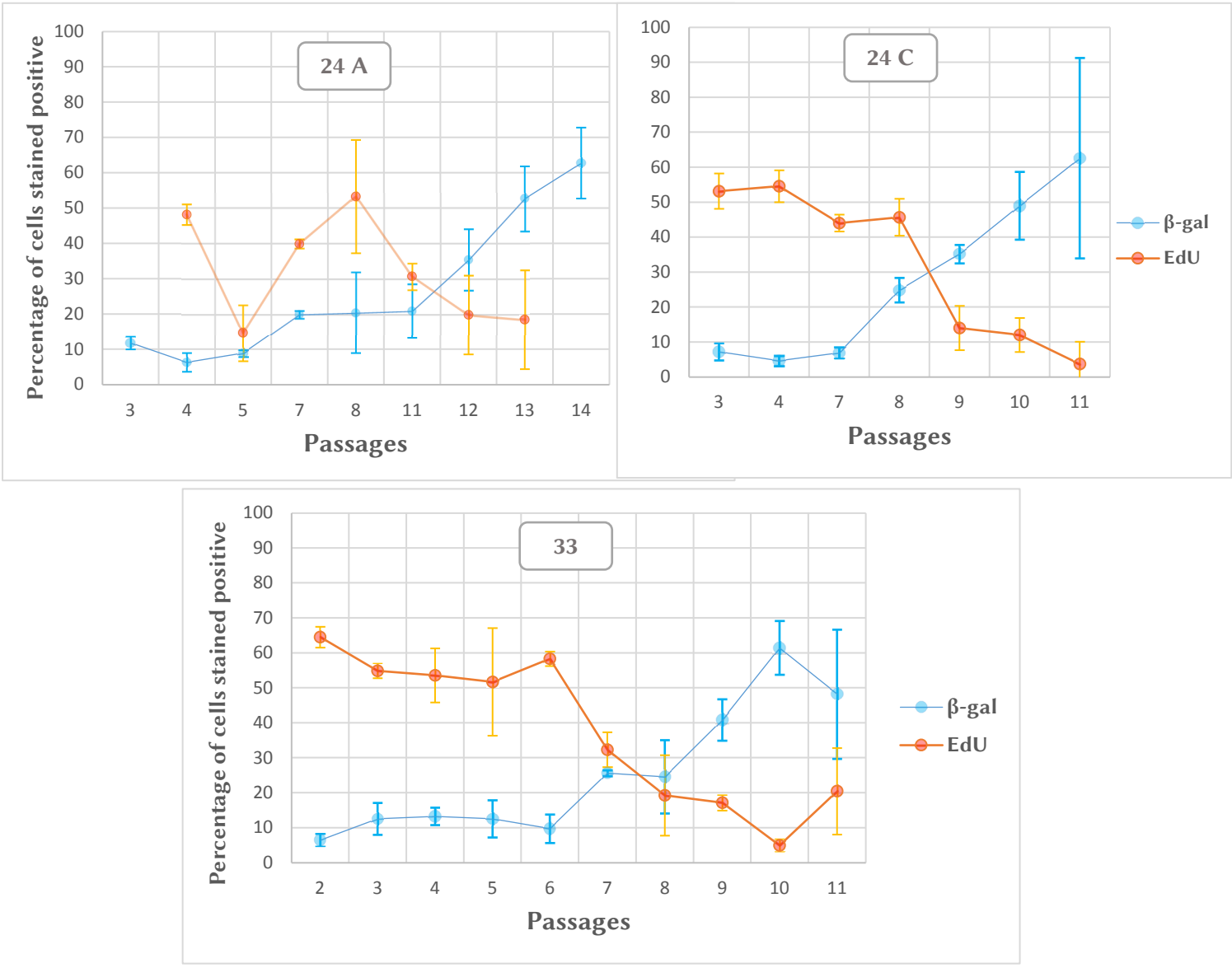


Figure 67: Proportions of senescent and proliferative MSC assessed with β-galactosidase and EdU stainings along culture.

The percentage of cells identified as positive for β-galactosidase staining is represented in blue.
The percentage of MSC that incorporated EdU is represented in orange.
The results shown in this figure are **means +/- standard deviation from n=3** wells for each sample.
The lines between points are represented to show the tendency of evolution with advancing passages.
For "24 C" and "33" samples, the results could be obtained until p11 because very few cells are needed for this experiment.

These curves show the dynamic of increase in senescent cell content in the samples. After a relatively stable step, the proportion of senescent cells increased and became more important than the proportion of proliferating cells. This step occurred between passage 7 and 8 for "33" sample, between passage 8 and 9 for "24 A" sample and between passage 11 and 12 for "24 A" sample. The baseline levels at early passage were

also different between the samples. The “33” sample for example displayed globally more percentage of β-Gal positive cells at early passage than the others.

At the end of the culture, more than half of the cells inside the samples were β-Gal positive and less than 20% were positive with EdU staining. From this point on, MSC culture becomes very difficult with more cell loss than cell renewal. These results can give an idea of the threshold of senescent cells that a sample can handle to support cell growth. At the last passages of these culture, some little group of cells were still in good shape and able to proliferate, but their capacity was outpaced by the influence of senescent cells, even if they were not dividing anymore.

The trends obtained with β-Galactosidase and EdU staining were globally consistent with the population doubling results, but they displayed less fluctuations.

The count of percentage of positive cells with senescence associated β-galactosidase staining can be drastically different in different teams and even between different investigators. With the association to EdU staining and the attention made to reduce false positive results, percentage of β-Galactosidase stained cells showed in this manuscript were lower than what is found in the literature. In another report, they could expand BM MSC during 12 passages in 84 days, with progressive decrease in proliferation rate (74). At the end of the culture, they report that all cells were positive with β-Galactosidase staining. This is similar to the report made in another study with 80% of β-Galactosidase positive cells when MSC reached their maximal lifespan in culture (71). Another study reports the culture of BM MSC during 50 to 90 days before they stopped to proliferate. The cumulative population doubling they found at the culture end varied between 7 and 22 (39). In a similar way to what has been observed in our study, a study also report that BM MSC proliferation slowed down after 5 to 7 passages and stopped after 8 to 12 passages. However, the cumulative population doublings their cells reached were higher, between 25 and 40 (136).

2. Colony forming efficiency

BM MSC ability to form colony and to grow while seeded at very low density was also followed along the culture of the 3 samples brought to replicative senescence **(figure 68)**. The samples content in cells able to form a colony globally decreased with advancing passages but it also showed fluctuations, with steps of resurgence. It is interesting to see that all three BM MSC samples retained some capacity to grow and form colonies, even at very late passage when their population doubling was negative for some samples. However, the colony size remained smaller at later passages than the ones produced by early passage cells.

Another study followed MSC colony forming efficiency (CFE) at different passages. At the start of the culture, CFE was between 15 and 20%. The highest CFE levels were then reached between p3 and p7 (25 to 35%) and decreased progressively to almost no colony formed at the end of cells lifespan (136).

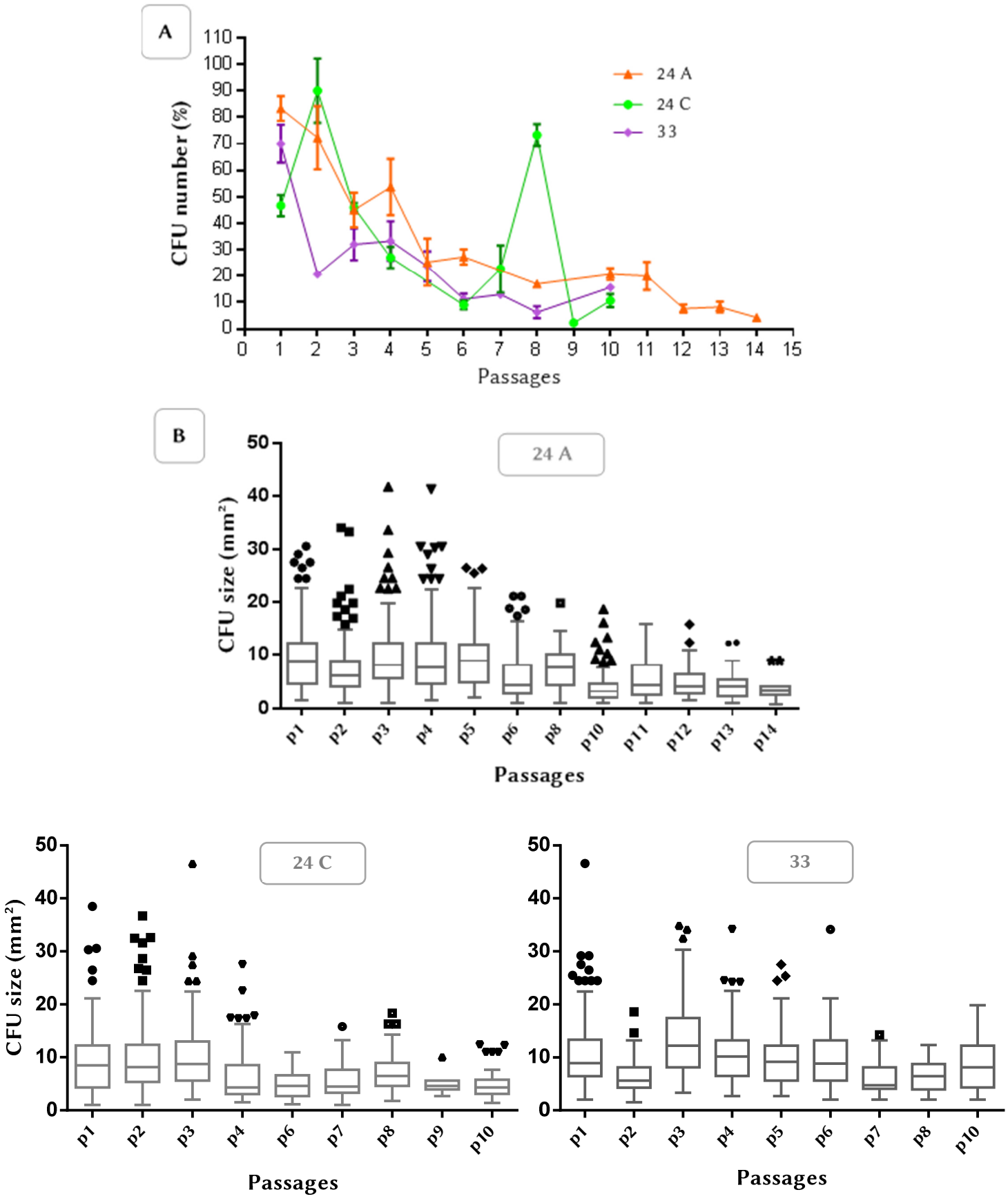


Figure 68: Colony forming efficiency and colony size evolution along passages for "24 A", "24 C" and "33" samples.

For each passage, the results showed were obtained from 3 petri dishes that were seeded with 100 cells in each (less than 2 cells / cm²).

A: Percentage of MSC that could form a colony. Results are presented as **means +/- standard deviation.**

B: Colony size was measured with ImageJ, with the parameter area indicated here in mm². Results are presented in Tukey boxplots (GraphPad Prism).

3. Fluorescence microscopy

To complete cell characterization and obtain first indications of the mechanisms implicated in MSC replicative senescence, several intracellular markers known to be potentially associated to senescence were searched **(table 16)**.

The label of actin fibers of the cytoskeleton with phalloidin confirmed cell enlargement at later passage. For its part, nuclear laminA, did not show evident change of structure in replicative senescent cells.

The proliferation marker Ki67 was more present in early passage BM MSC but it could still be found in later passage cells. It can be noted that less proliferating cells could be detected with Ki67 than with EdU staining.

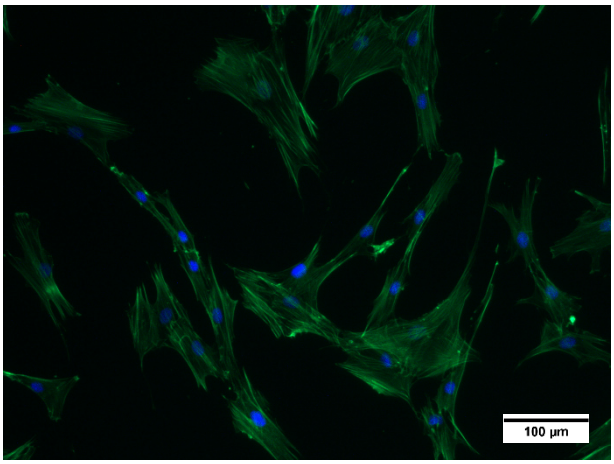
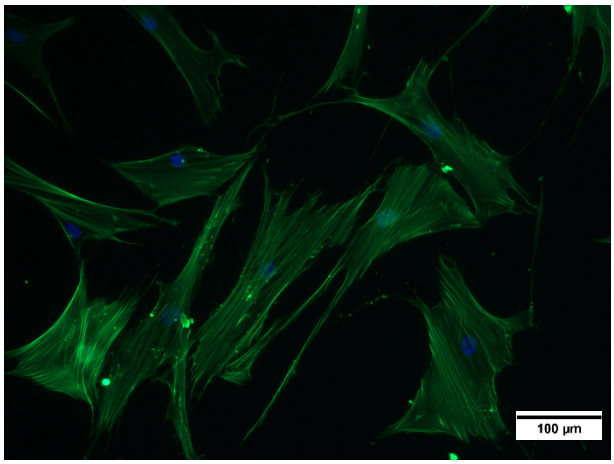
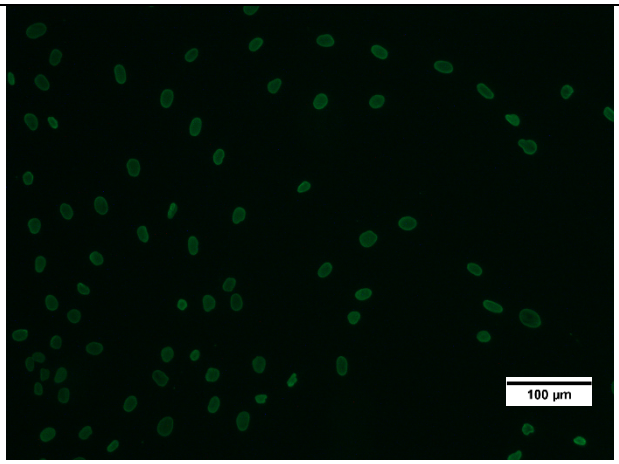
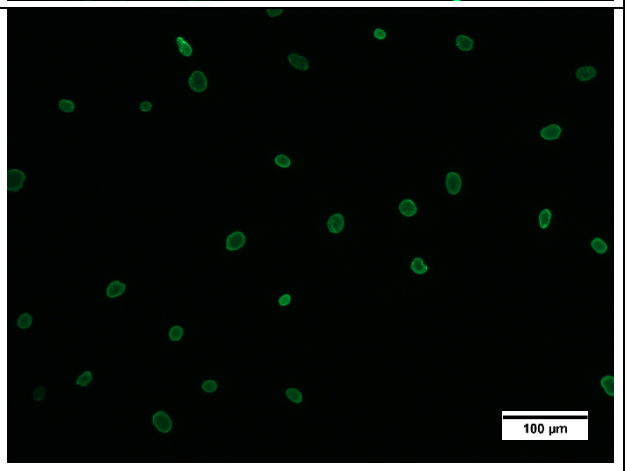
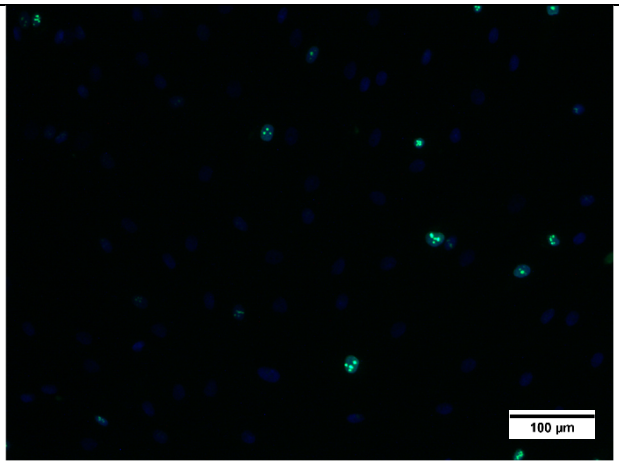
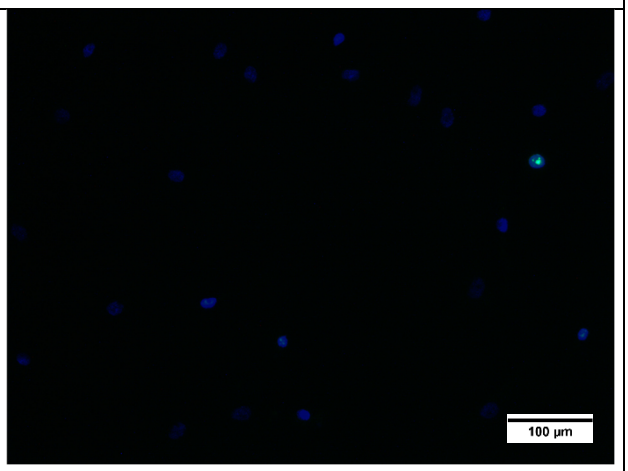
The antioxidant enzyme SOD2 was almost not visible in early passage cells whereas its presence was clearly detected in the cytoplasm of several cells at later passage. A diffuse fluorescence is more visible in all cells and some of them showed numerous bright spots.

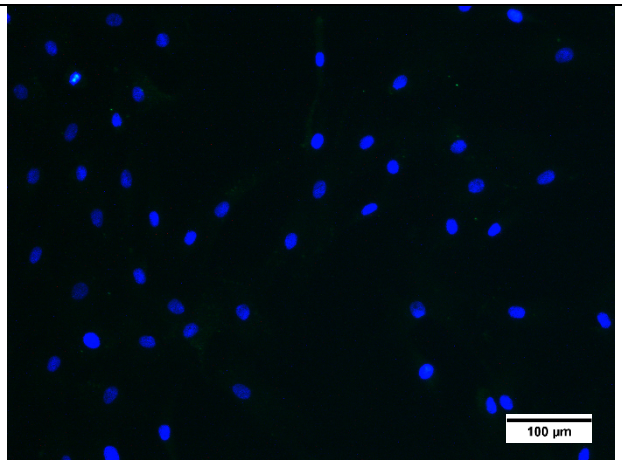
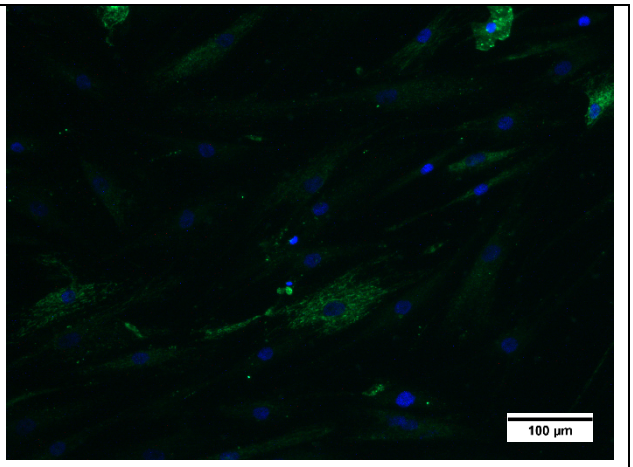
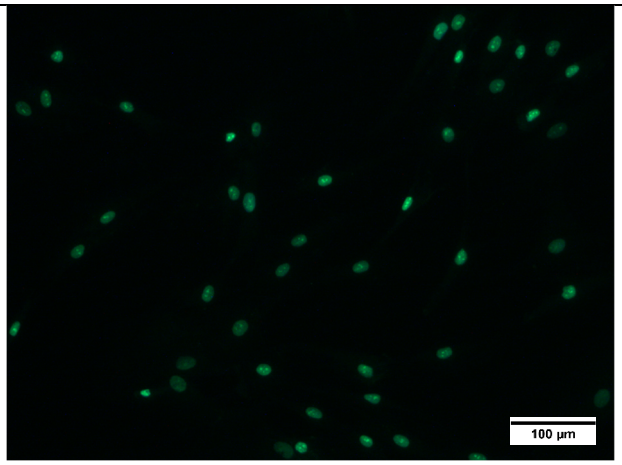
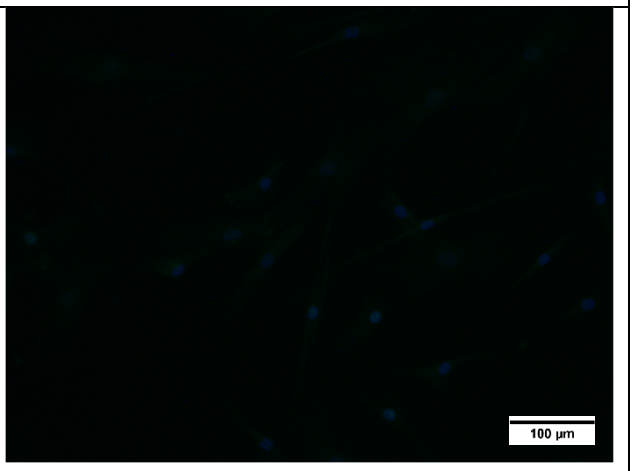
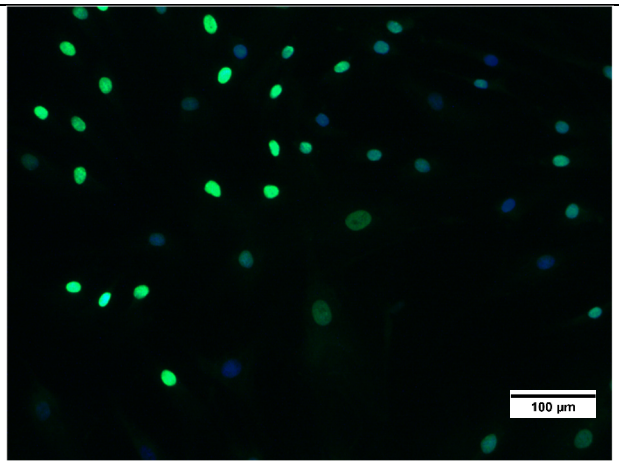
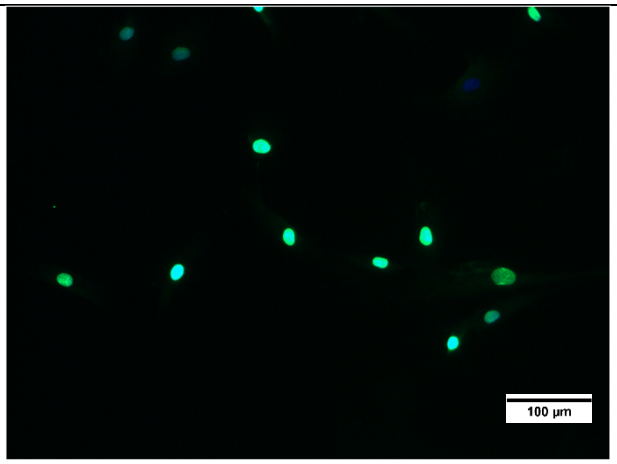
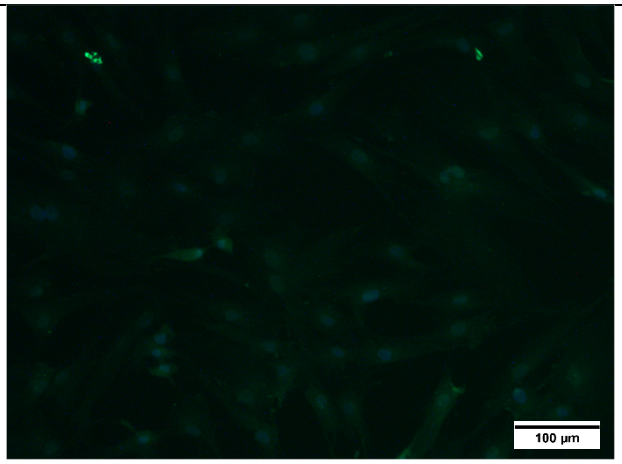
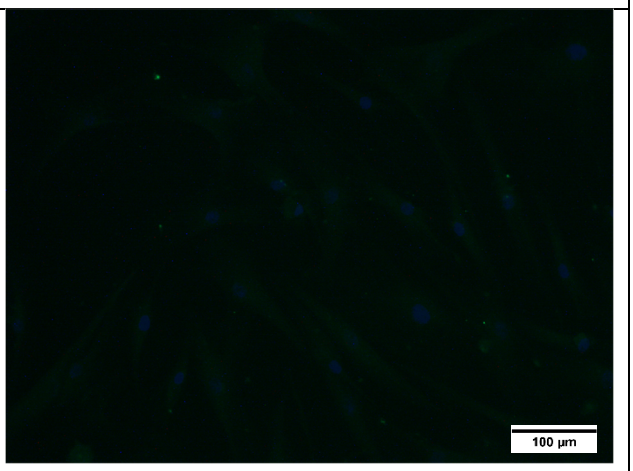
HMGB1 is normally present in the nucleus. It was reported to be secreted outside the cell in other senescence models and is associated with chronic inflammation and aging (200). This change of localization was also observed with the BM MSC studied. Early passage cells showed clear label of the nucleus and almost nothing in the cytoplasm. The label inside the nucleus was homogeneously lost in late passage cells and more diffuse fluorescence in the cytoplasm could be seen.

H3me3K9 is a repressive histone mark. This methylation was previously followed in several models of senescent cells and was found in senescence associated heterochromatin *foci*. In replicative senescent cells, it was reported to be associated with non-methylated regions in fibroblasts and BM MSC (197). Here, the observed fluorescence was brighter in some of late passage cell nucleus, possibly suggesting its accumulation in cells closer to replicative senescence. For both early and late passage cells, the label was faded in the nucleus of some cells, letting out the blue color from DAPI fluorescence. The meaning of the low level of this methylation in some cells remains unclear, but it could be consistent with the proportion of proliferating cells highlighted with Ki67 staining.

Table 16: Comparison of intracellular labels by fluorescence microscopy between late and early passage BM MSC.

The molecules presented can be linked to senescence (see part VI. C. "Senescence markers"). The sample used for most images is "24 C". Only H3me3K9 staining was made on the sample "24 A" at P3 and P14. The fluorescence of the targeted molecule is represented in green. Nucleus stained with DAPI are shown in blue. Microscope used is Leica DMI3000B, the same parameters were used for early and late passage cells and images taken in parallel. Overlays were made with Leica Application Suite or with Gimp. Scale bar is 100 µm.

	P3	**P9** (P14 for H3me3K9)
Actin (phalloidin)		
LaminA		
Ki67		

SOD2		
HMGB1		
H3me3K9		
p16		

The classical gold standard for senescence assessment is the increase of the expression of the cell cycle inhibitor p16. During the thesis work, the results obtained with multiple tests remained mitigated and could not be exploited. The staining was always diffuse and, surprisingly, an accumulation of fluorescence was visible in the nucleus of cells at early passage and seemed to disappear from the nucleus of late passage cells. In the literature, the most common way p16 is followed is by western blot in senescence studies, it is rarely reported with fluorescence microscopy images. Some studies could still be found with interesting p16 images. For example, a report could show clearly the increase in the observed brightness in the nucleus area of replicative senescent fetal lung fibroblasts compared to cells at earlier passage (278). Some reports also highlighted the lack of specificity of some commercial p16 antibodies (199). Two different p16 antibodies were tried in the studies made for this thesis work. It would be interesting to test yet another antibody before concluding with the results. To note, intracytoplasmic p16 labeling was also tested by flow cytometry. A strong signal could be obtained with Hela cells, a robust cell line known to express p16. Unfortunately, this kind of analysis could not be done on BM MSC samples during the time of the thesis. It required a very high number of cells compared to the study of surface marker. This is notably due to cell loss with the additional fixation and permeabilization steps, associated to several necessary washings. This is also the reason that precluded the study of the level of expression of SOD2 by flow cytometry. For further studies, this could be interesting to obtain single cell results, notably because the expression of SOD2 was seen in the microscopy images to be heterogeneous within the samples.

4. Migration

Cells undergoing replicative senescence were also tested in comparison with early passage cells with functional experiments such as migration assessment with transwells. Cell size being and important parameter associated with senescence, different pore sizes, 1µm, 3µm and the classical 8µm, were tested for 2 of the 3 samples (figure 69-71).

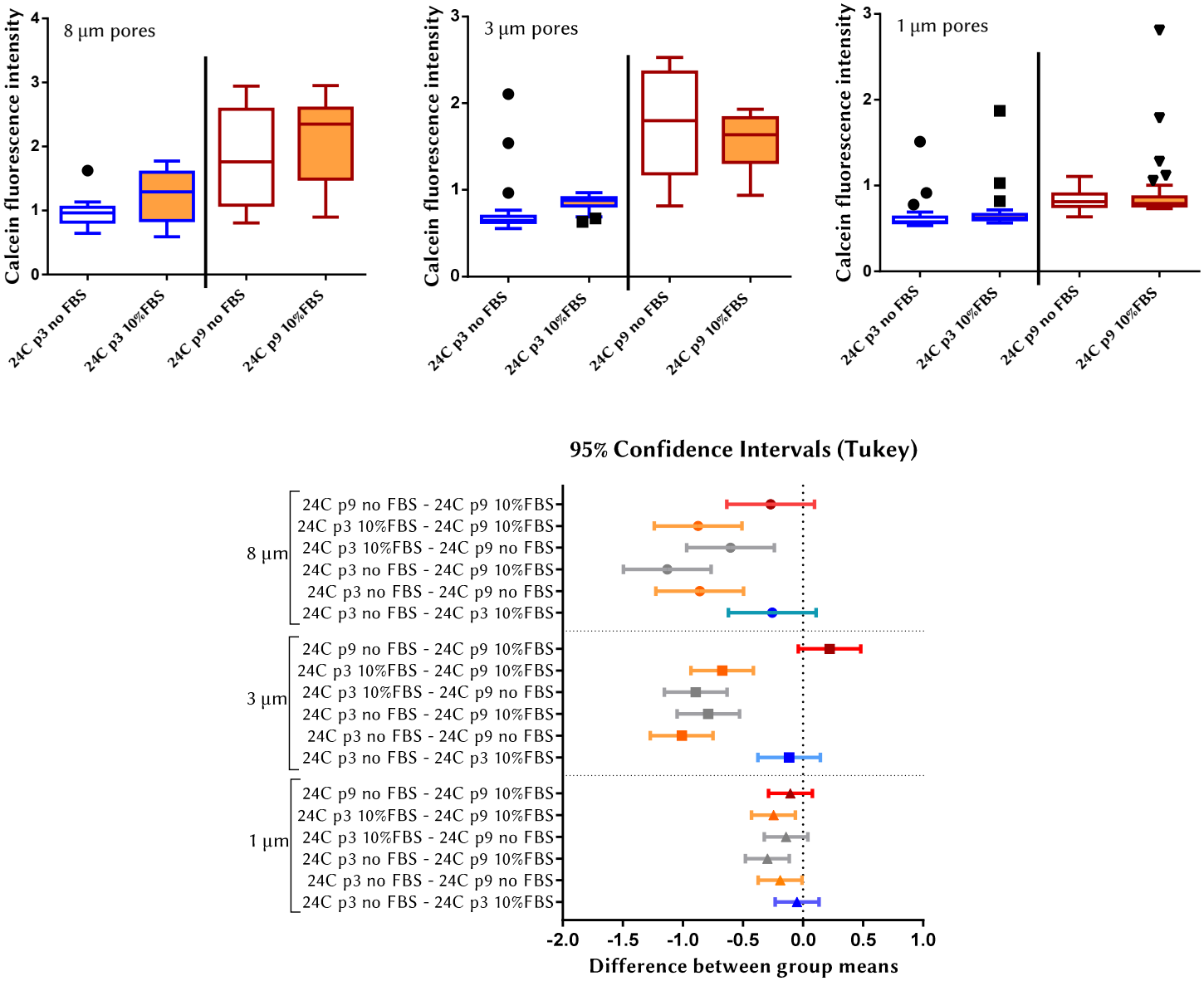


Figure 69: Comparison of the migration capacity of 24 C p3 and 24 C p9 cells toward 10% FBS across 8, 3 and 1 µm pores after 15 hours.

Cells were stained with Calcein to detect living cells that were able to cross to the down part of the inserts. Up: Boxplots gathers the raw results obtained with the fluorescence reader (n=29 points analyzed for each boxplot). Below: representation of the results of one-way ANOVA with Tukey's multiple comparisons test. When the confidence intervals cross the vertical line, the difference between the means is not considered significant.

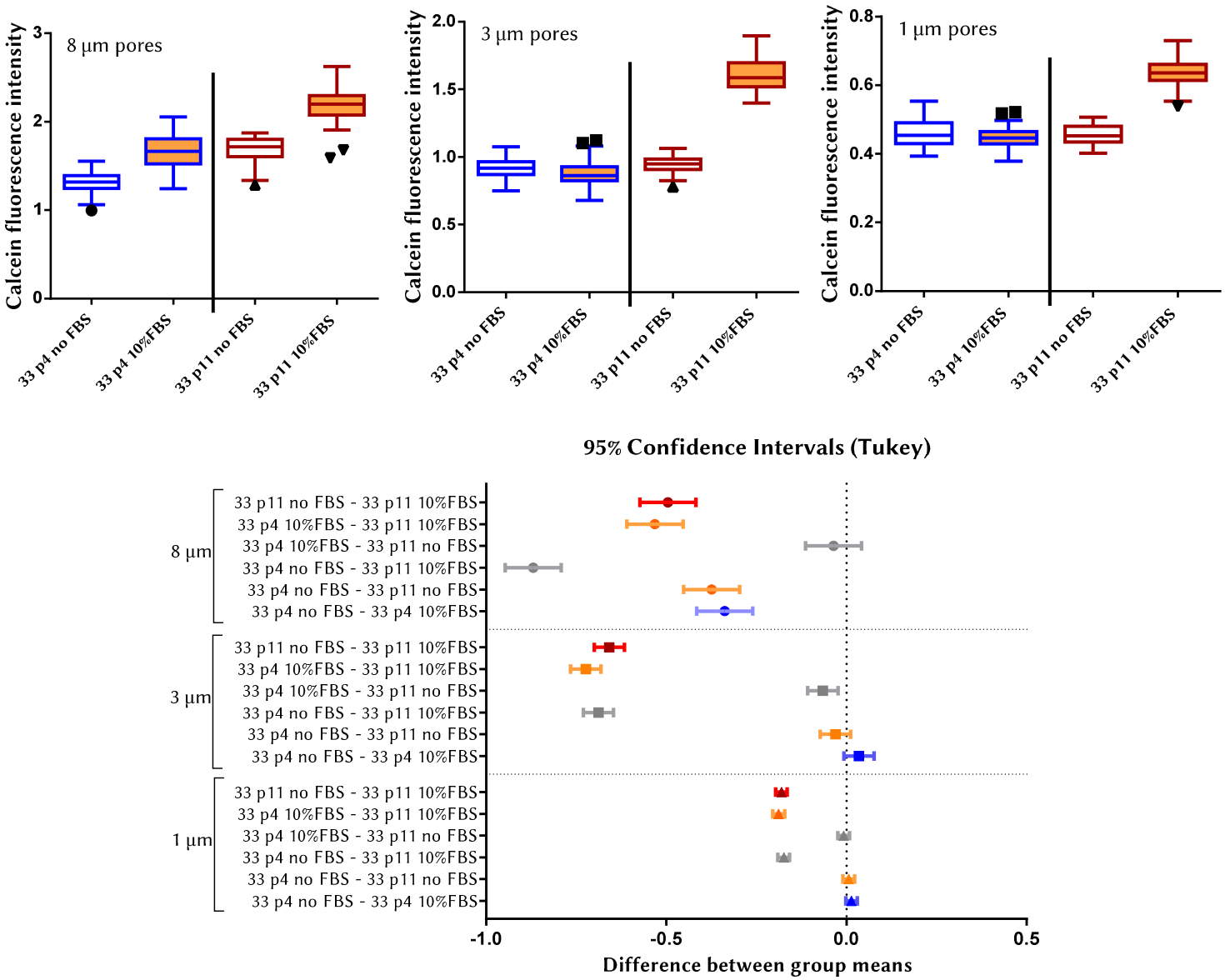


Figure 70: Comparison of the migration capacity between 33 p4 and 33 p11 cells toward 10% FBS across 8, 3 and 1 µm pores after 15 hours.

Cells were stained with calcein to detect living cells that were able to cross to the down part of the inserts. Up: Boxplots gathers the raw results obtained with the fluorescence reader (n=58 points obtained from 2 inserts). Below: representation of the results of one-way ANOVA with Tukey's multiple comparisons test. When the confidence intervals cross the vertical line, the difference between the means is not considered significant.

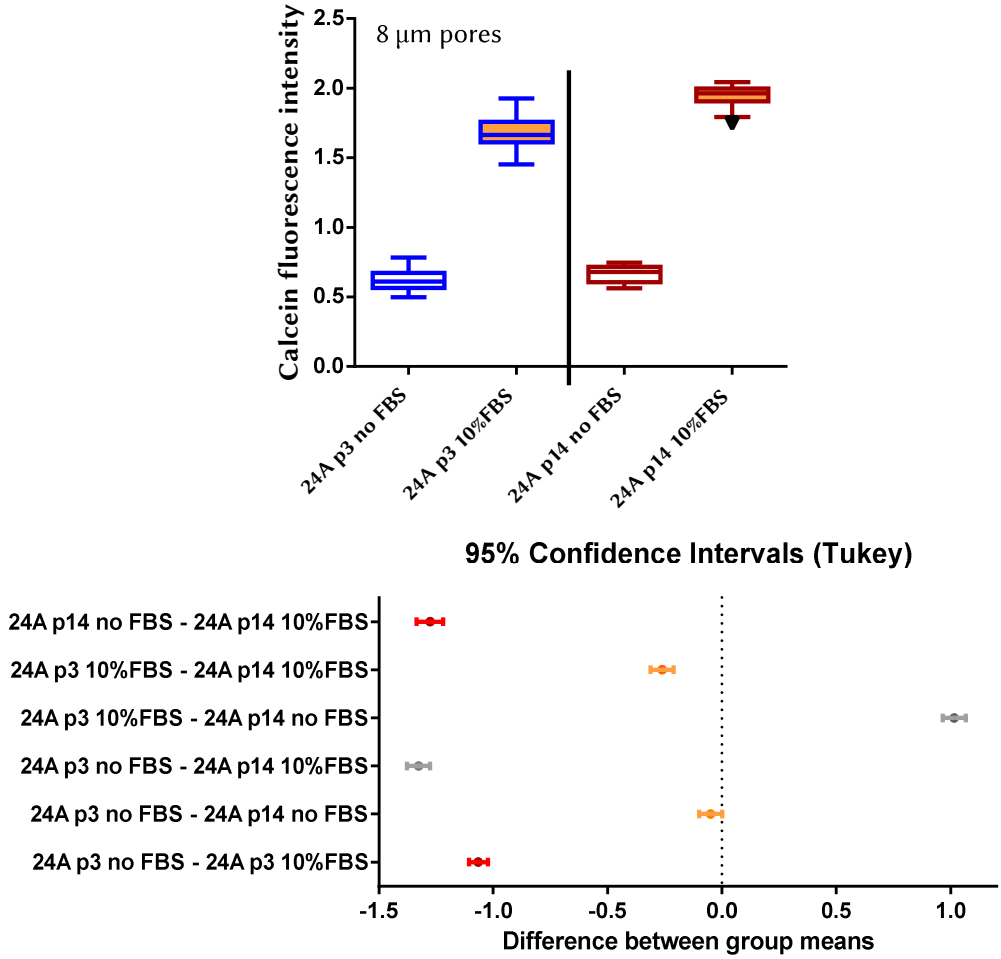


Figure 71: Comparison of the migration capacity of 24 A p3 and 24 A p14 cells toward 10% FBS across 8 µm pores after 15 hours.

Boxplots gathers the raw results obtained with the fluorescence reader (n=58 points analyzed from 2 inserts for p3 boxplots and n=29 points for p14 boxplots).

Below: representation of the results of one-way ANOVA with Tukey's multiple comparisons test. When the confidence intervals cross the vertical line, the difference between the means is not considered significant

A part of the cells of all samples tested were able to cross the pores of different sizes until 1 µm. Interestingly, in a general manner, MSC samples containing more senescent cells displayed greater capacity to migrate across the different sizes of pore. For "24 C" sample, the baseline level of migration was already higher in p9 cells compared to p3 cells, even with no chemoattractant. For this sample, there are no significant differences with and without FBS, but significant differences were seen between p3 and p9 cells for all pore sizes. For the sample "33", the baseline level of migrating cells was equivalent or higher in p11 cells compared to p4 cells. Late passage cells of this sample also answered more markedly chemoattractant FBS signals, especially in inserts with 3 and 1 µm pores membranes. Significant differences could be observed between p4 and p11 cells for all pore sizes in the condition with 10% FBS. For "24 A" sample, only 8 µm inserts were used because

there was not enough cell remaining at later passage. Here again, replicative senescent MSC, even at p14, showed a slightly better capacity to migrate in this study model. The baseline levels here were almost equivalent, but with FBS, late passage cells migrated significantly more than p3 cells (Tukey's multiple comparisons test). For this sample, the difference between the conditions with and without FBS were more marked.

These results can be surprising. Samples at later passage contained larger cells and it could be thought that they became too big to cross the pores, especially the sizes below 8 µm, but it seems, on the contrary, that they have an enhanced capacity to do it. This capacity may be related to the SASP (senescence associated secretory phenotype) factors senescent MSC release.

In these experiments, there was a high variability in the response to FBS. FBS was chosen because it was reported in protocols to be the solution that was attracting MSC to migrate the most across inserts. This condition was chosen to compare early and late passage cells with the best conditions, not to test specific answers to defined molecules. It is difficult to say why in several cases, MSC did not migrate more with FBS than without. In the results presented, it can be seen notably in the experiment with "33" sample. While the different conditions and inserts were tested simultaneously, with early passage cells, it was only with 8 µm inserts that they migrated more in presence of FBS. With this kind of assay, disturbance of the plate or of some inserts during the experiments can cause the solution in the upper part of the insert to mix with the lower part too fast, resulting in an ineffective gradient and could explain the lack of stimulation to move for the cells. Attempts to resolve this with membrane coating (to avoid too fast diffusion of FBS) or with other kind of assay allowing more stable gradient such as agarose spot assays (279,280) were unsuccessful. With membrane coated with collagen, too many cells accumulated on the underside of the membranes in every condition. Agarose spots containing chemoattractant medium, for their part, could not be used reliably and detached too often from the surface.

The cells were also observed by fluorescence microscopy **(figure 72)**. A possible bias may be that senescent cells that are larger appear brighter and were considered falsely as more numerous. Calcein label does not allow to count the number of cells precisely and the

classical nuclear labels such as DAPI are not compatible with the Fluoroblok inserts used. For 1 μm inserts, it is not certain whether what was observed to cross the pores were only some parts of cytoplasm or entire cells. Globally, microscopy images still tend to confirm that more cells went through the pores in the samples brought to replicative senescence compared to early passage cells.

Another study tested the migration capacity of replicative senescent MSC. Their experiment was also made with 8 μm inserts, but with transparent membranes and instead of FBS, they used mouse macrophage-conditioned media, obtained after macrophages were stimulated by lipopolysaccharide. In these conditions, replicative senescent MSC showed 75% reduction in their migration capacity compared to less senescent cells.

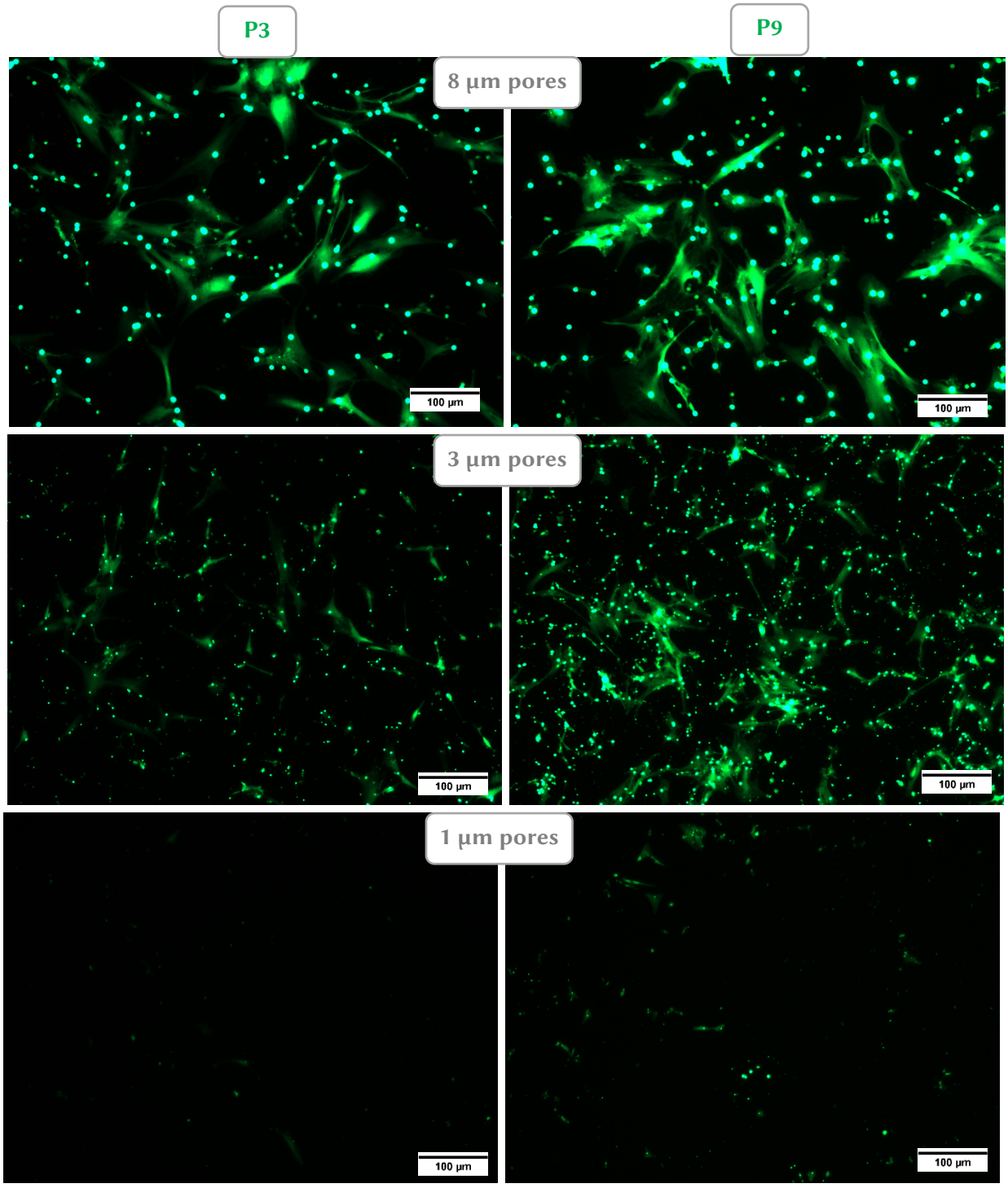


Figure 72: Fluorescence microscopy images of the underside of the inserts with 8, 3 and 1 µm pores after letting early and late passage MSC 15h, with a solution of 10% FBS as chemoattractant.

The images shown here were obtained with "24 C" sample at passage 3 (left column) and passage 9 (right column). Microscope used is Leica DMI3000B, the same parameters were used for early and late passage cells and images taken in parallel. Scale bar is 100 µm.

5. Immunosuppression and immunogenicity

Secretions of senescent cells were reported to contain high levels of pro inflammatory factors. Immunomodulation is one of the major functions for which MSC are used in therapeutics. Thus, a simple functional test of immunosuppression and immunogenicity was tried to compare MSC at passage 3 and MSC undergoing replicative senescence to obtain early indications of senescent MSC behavior (table 17).

All MSC tested showed a suppressive effect on activated CD4 proliferation. For the two samples tested, MSC containing more senescent cells could suppress more CD4 proliferation than their early passage counterpart. The difference in population shape in flow cytometry and in the percentage of proliferating CD4 is relatively pronounced.

The results can be considered equivalent for immunogenicity, both young and senescent cells causing no CD4 proliferation when there was no activation.

Inflammatory cytokines such as TNFα and IFNγ were not used to prime MSC before coculture, while it is commonly used to enhance MSC suppressive effect on lymphocytes. Senescent MSC may have been naturally primed with the senescence associated secretory phenotype they secreted, resulting in superior immunosuppression. Late passage MSC were also less busy with proliferation and maybe more active for secretions and interactions with other cells. These results were similar to migration results in the way that senescent cells displayed a stronger functional response than early passage MSC. These results appear in contradiction with the idea that MSC potential is better predicted by MSC fitness and proliferation capacity.

Another study assessing immunosuppression on senescent BM MSC reported a reduced, yet still significant, suppressive effect compared to “normal” cells (244), but it was made with MSC exposed to ionizing radiations (10 Gy) and not replicative senescent MSC. Another study, with replicative senescent MSC this time, also found a decrease in their capacity to inhibit T cells proliferation. This study was made with similar culture conditions to the ones used for the experiment of the thesis, except that the culture medium contained

between 15 to 20% FBS and their co-culture experiment was made with total PBMC instead of sorted CD4 T cells (210).

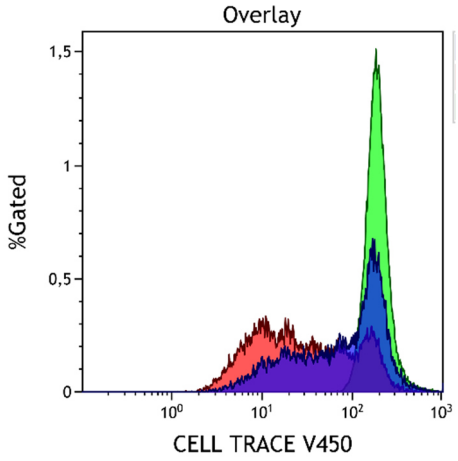
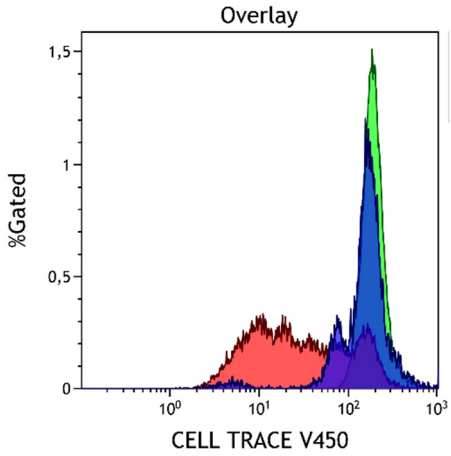
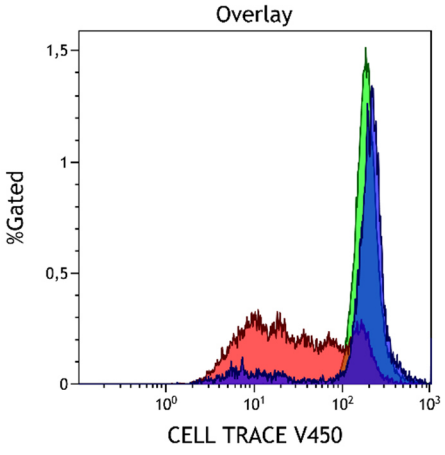
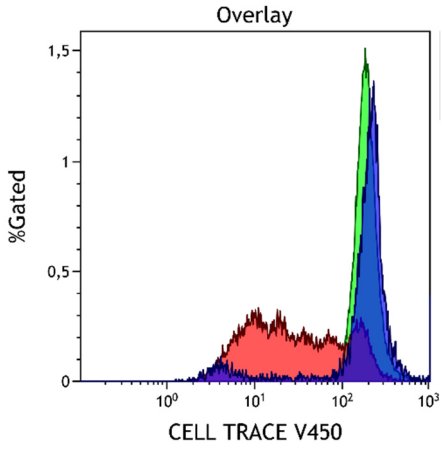
Interestingly, with WJ MSC, it was also reported that the immunomodulatory abilities of late passage cells (p15) were enhanced compared to early passage ones (p3) (215). This experiment was conducted with total PBMC stimulated by phytohemagglutinin. The superior capacity of the samples containing more senescent cells was potentially linked with increased IL6 production.

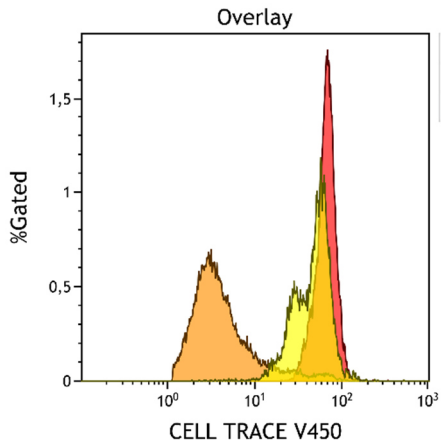
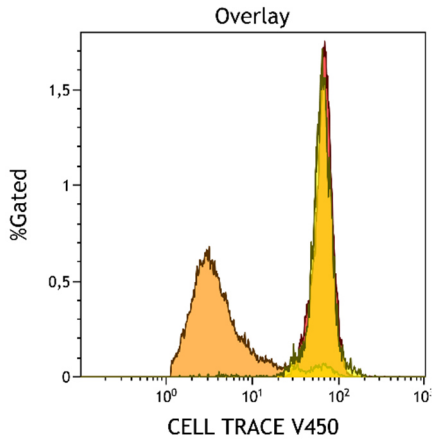
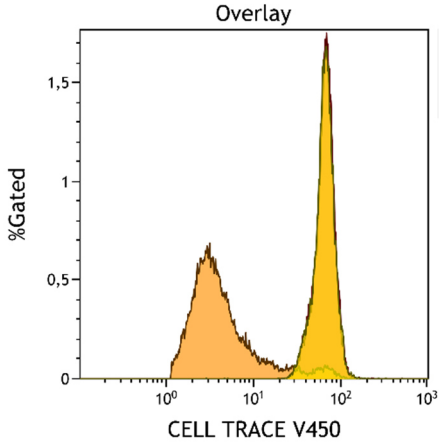
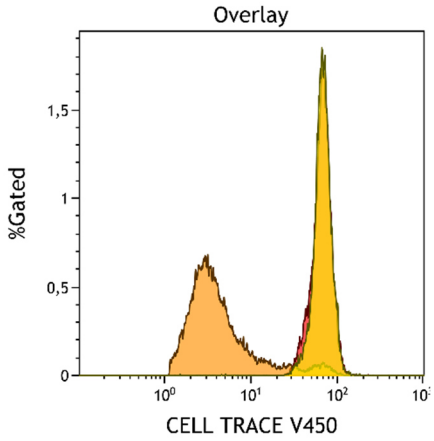
Table 17: Overlay of flow cytometry histograms showing CD4 proliferation alone, after activation and after contact with MSC at p3 and p10 for the samples "24 C" and "33".

CD4 proliferation was followed with CellTrace(V450), each cycle of proliferation is associated with a decrease of fluorescence.

For "24 C", non-activated CD4 alone are represented in green, activated CD4 are represented in red and CD4 in coculture with MSC are in blue.

For "33" sample, non-activated CD4 alone are represented in red, activated CD4 are represented in orange and CD4 in coculture with MSC are in yellow.

	24 C P3	24 C P10
Activated CD4 Immunosuppression	 Activated CD4 alone: 78.3% Activated CD4 after MSC coculture: **48.5%**	 Activated CD4 alone: 78.3% Activated CD4 after MSC coculture: **18.4%**
CD4 (not activated) Immunogenicity	 CD4 activated spontaneously: 0.9% CD4 activated after MSC coculture: **12.8%**	 CD4 activated spontaneously: 0.9% CD4 activated after MSC coculture: **11.4%**

	33 P3	33 P10
Activated CD4 Immunosuppression	 Activated CD4 alone: 96.4% Activated CD4 after MSC coculture: **24.6%**	 Activated CD4 alone: 96.4% Activated CD4 after MSC coculture: **3.7%**
CD4 (not activated) Immunogenicity	 CD4 activated spontaneously: 1.1% CD4 activated after MSC coculture: **1.4%**	 CD4 activated spontaneously: 1.1% CD4 activated after MSC coculture: **0.9%**

The sample “24 A” was also tested but there was a problem with CD4 activation and there were not enough cells remaining to reproduce the experiment.

B. Surface markers with flow cytometry

Classical and subtypes markers that were selected to study MSC phenotype were also followed at different passages during the culture of cells until replicative senescence. It provides interesting insight about the fluctuations of expression of these markers and the possible interplay between the sample composition in different subtypes and in senescent cells. With only 3 samples analyzed, this part does not intent to generate conclusions about the way these markers are supposed to evolve with advancing passages, but it shows the possibilities of MSC phenotype change in the conditions tested in this study.

1. MSC classical markers

a. Highly expressed markers

The intensity of expression of MSC markers that are constantly and strongly expressed was studied at different passages for the 3 samples brought to replicative senescence. SSC results, as approximation of cell size, were also followed and showed here **(figure 73)**.

In most studies, these highly expressed surface markers were reported to be very constant despite important variations in MSC behavior and capacities. They were not viewed as interesting marker to characterize MSC state, and served only as additional indicators that the cells could be defined as MSC instead of another cell type. Here, MSC revealed some strong fluctuations in the level of expression of these markers according to the passage. Their expression however, remained very high with near 100% of the cell population in the positive gate. One exception with “24 A” sample with CD90 marker, notably at passage 0 where the percentage found was 74%, and then fluctuated between 81 and 96%. For the other markers, the percentage were always at least at 90% on the 3 studied samples.

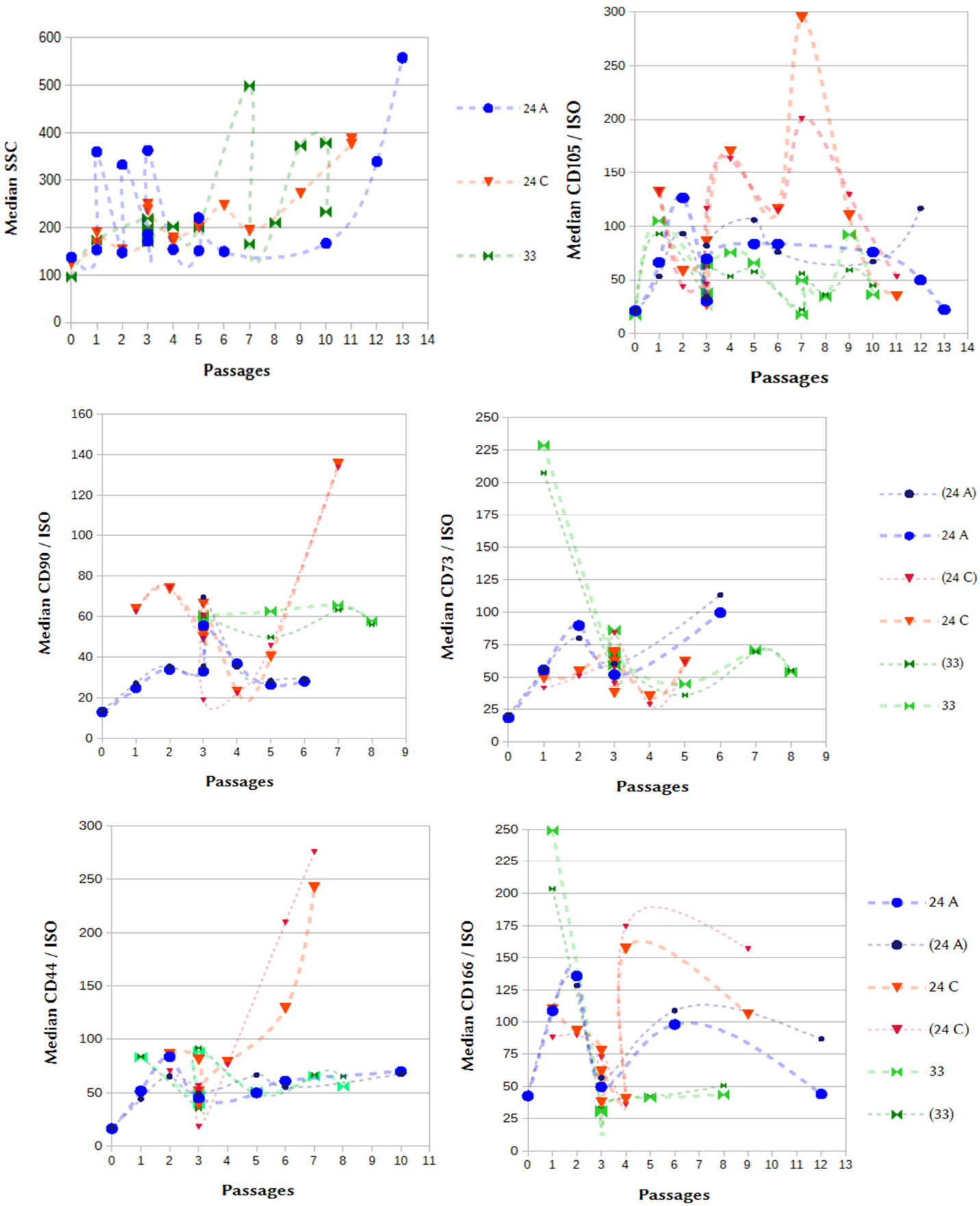


Figure 73: Median fluorescence intensity measured by flow cytometry of MSC surface marker at different passages until replicative senescence: SSC and highly expressed surface markers.

Between brackets in the legend, and represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size between all passages and avoid the bias that bigger cells might appear brighter.
These data were obtained from different runs of culture for each sample. In some cases, it can happen that one passage has been studied several times for the same sample. All the data obtained were displayed.
Dotted curves are shown only to help to view the tendency of variation of each sample along passages, but do not represent true data or models.

When looking at SSC, reflecting cell size, it also fluctuated a lot for the 3 samples studied. For "24 A" and "24 C", the highest point was reached at the end of the culture. For "33", the highest SSC points were also reached at passage 7, 9 and 10 but in a different run, the SSC values remained similar to the ones obtained at early passage. "24 A" also showed such fluctuations, the SSC value at passage 10 and 12 were not very different from what could be obtained at early passage. It was only at passage 13 that the median SSC value became higher.

It could be possible that the biggest cells in late passage were excluded from the analysis with the viability dye (because they were brighter or more sensible to handling and more dying), causing underestimation of SSC at these passages. The median parameter may also not be sensible enough to reveal the presence of bigger cells with SSC. However, it seemed, with several experiments in microscopy and in flow cytometry, that senescent MSC were effectively bigger (figure 66 page 267 and figure 74). This effect may not be as strong as it seemed to be, and it is also possible that a small number of very big cells made a strong impression.

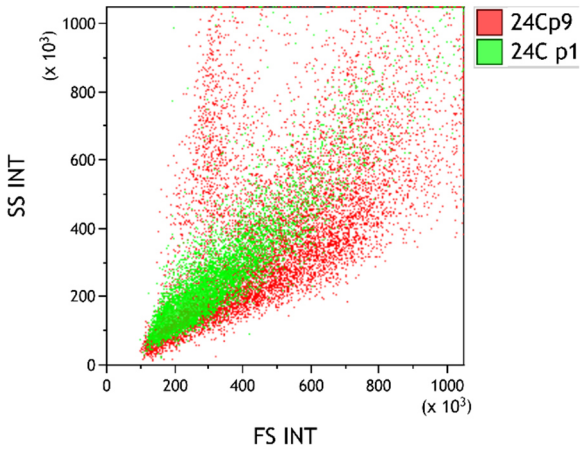


Figure 74: Overlay of Forward Scatter (FS) versus Side Scatter (SS) flow cytometry charts of passage 1 MSC (in green) and passage 9 MSC (in red).

CD105 marker expression was relatively stable despite many little fluctuations for "24 A" and "33" samples. "24 C" sample however, showed very high difference of expression between passages, the highest level being reached at passage 7 and then declined at passage 9 to reach the lowest point at passage 11.

Among the highly expressed markers studied, CD90 expression appeared as the weakest. Less variations were seen for CD90 expression along passages, except for "24 C" sample. Its lowest level was at passage 4 and it increased a lot afterwards, reaching the highest point again at passage 7.

CD73 was also found to be relatively stable for the three samples along different passages, except for one very high point with the “33” sample at passage 2.

CD44 displayed one of the most constant and similar expression between the 3 samples, except for “24 C” sample, with strong increase in expression at passage 6 and 7 compared to all other points.

Even with less passages analyzed than for the other markers, CD166 showed very high fluctuations in expression levels for the 3 samples.

b. Negative or lowly expressed surface markers

The surface proteins that are usually checked on MSC to confirm the absence of contaminating cells were also followed at different passages **(figure 75)**. With the use of normalized median fluorescence intensity, the level of expression of these markers would remain negligible or very low, while the presence of small population of cells that were expressing these markers could be seen in flow cytometry histograms. The percentage parameter was used instead to better reflect these results.

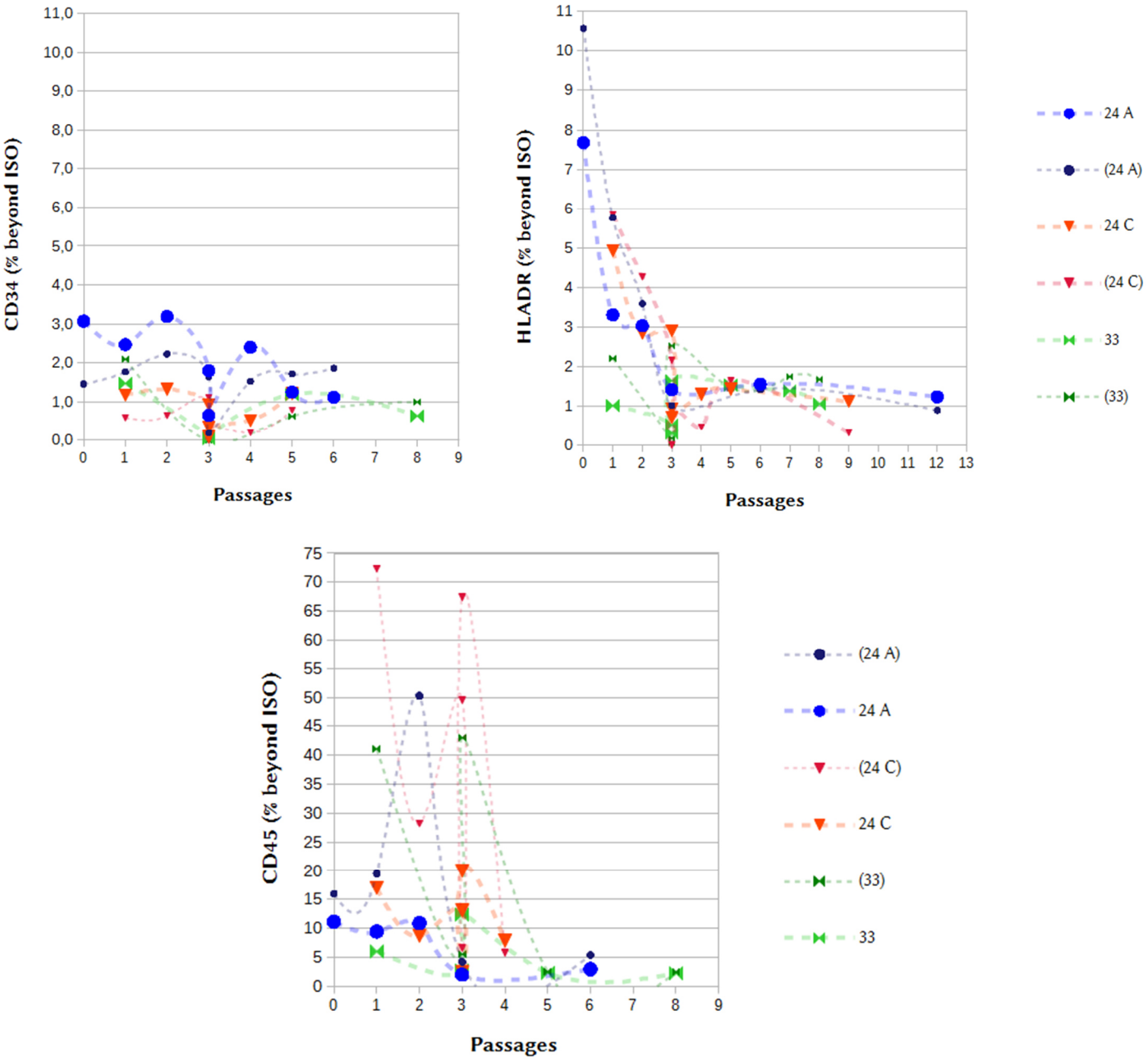


Figure 75: Percentages associated to MSC negative or lowly expressed surface marker expression measured by flow cytometry at different passages until replicative senescence.

The results are displayed in percentage for these markers. This parameter is more easily biased but also more sensible. It allows to detect the presence of small population of cells with low expression of some markers, where median fluorescence intensity of the tested cells remains too close to the results obtained with isotype control.

Between brackets in the legend, and represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size between all passages and avoid the bias that bigger cells might appear brighter.

These data were obtained from different runs of culture for each sample. In some cases, it can happen that one passage has been studied several times for the same sample. All the data obtained were displayed.

Dotted curves are shown only to help to view the tendency of variation of each sample along passages, but do not represent true data or models.

CD34 expression remained negligible and close to the results obtained with isotype control in the 3 samples studied along the culture.

HLADR expression was negligible after passage 3 for the 3 samples. However, for the first passages, its expression could be detected on a small portion of cells in “24 A” and “24 C” samples.

For its part CD45 expression revealed interesting results. Before passage 4, its expression was found on some population of cells of the 3 samples studied. More particularly, CD45 expression could be clearly seen on the small FSC / SSC population of cells selected to compare cells approximatively of the same size between the different samples and passages. This population selected in the FSC / SSC restricted area often displayed a cleaner result in isotype control. This could explain the high percentages reported, but the level of expression remained weak (the highest level reached of normalized MFI was around 3). As the only marker to show these results, it would be intriguing that non-MSC cells persist until p3 and express all the other MSC markers. The possibility of the presence of macrophages should still be considered. The addition of CD14 assessment, as recommended by ISCT, might help for the analysis of such results. Another explanation could be that MSC may display some scarce CD45 expression, similarly to what was reported by some studies on BM MSC with CD34 and CD45 (63).

2. CD146

Among all the surface markers studied during this thesis, CD146 was the most monitored. However, different fluorochromes and parameters were successively used to improve its detection, and all the results cannot be gathered together. This had still the advantage to highlight on the same cells the differences between the results obtained in these different conditions. **Figure 76** shows for each sample the evolution in CD146 expression observed with advancing passages and with these different protocols.

The order of magnitude of CD146 level of expression were quite different between the 3 samples, even when studied with the same protocol. “24 C” displayed the strongest expression, followed by “33” sample and then “24 A”. This is also reflected in the percentages results.

The difference between the results obtained with FITC and BV421 fluorochromes could show that FITC may fail to detect the presence of CD146 expressing cells, both with MFI and percentages. It can be seen with “24 A”, between passage 3 and 6 notably. The few results made with BV421 fluorochrome with low voltage (250) were slightly superior to FITC, but the best conditions were with BV421 fluorochrome with high voltage (510) of the detector. This protocol was then kept for the largest part of this work and will be used for future studies.

Concerning the fluctuations observed with passages, with less sensible conditions with FITC-conjugated antibody, the tendency of CD146 expression to decrease with passages was clear. However, with BV421, the tendencies of variation were less linear. The highest level of expression reached were again at passage 7 for “24 C” sample, and at passage 4 for “33”. For these 2 samples, the lowest level of expression was still at the end of the culture at later passages. For “24 A” sample, CD146 expression effectively decreased between p3 and p10, but it increased at p12 and p13. Interestingly, the variation in CD146 expression (with nMFI) between p3 and p9 for “24 C” sample were of similar shape than CD105 variation of expression.

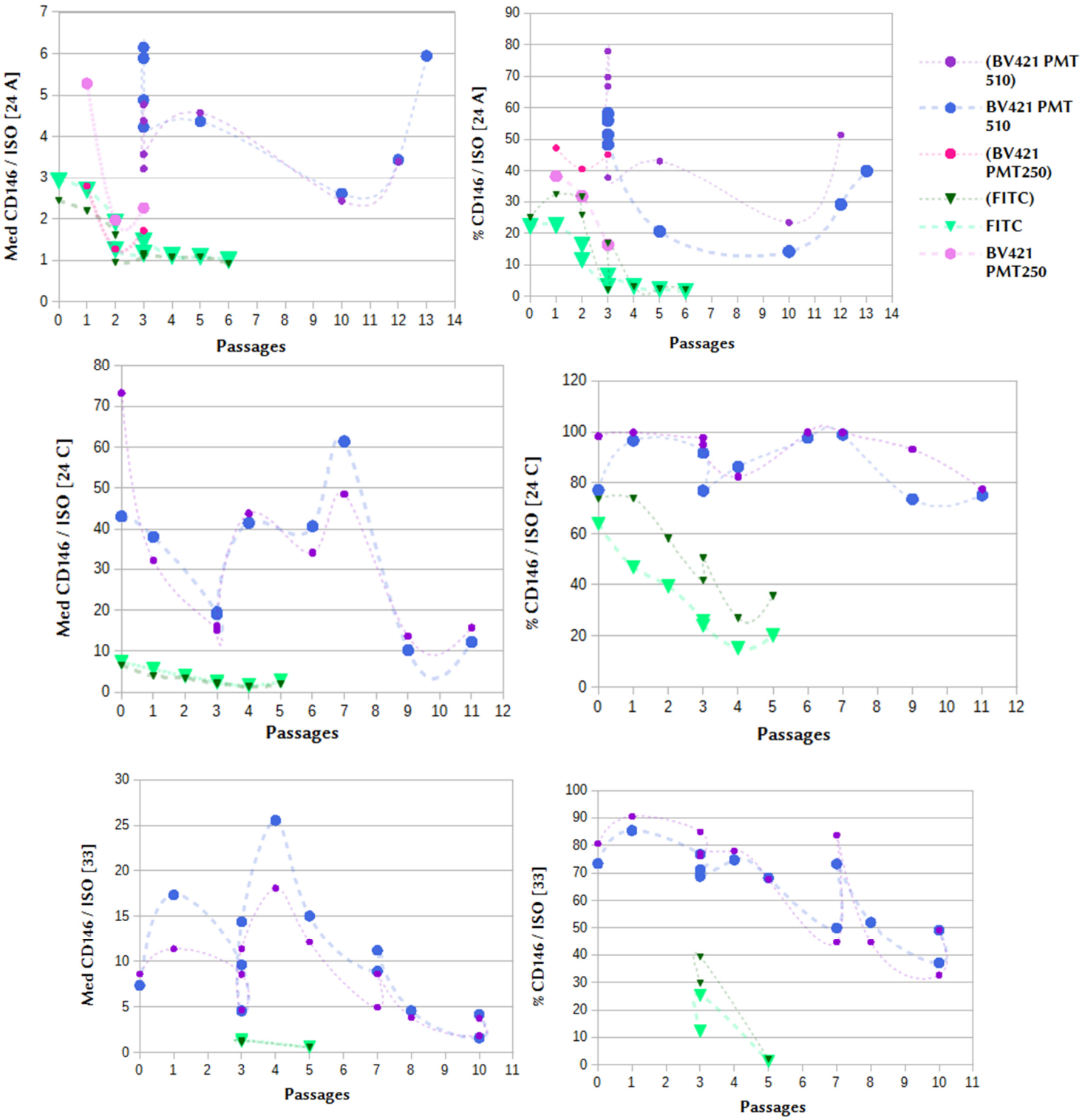


Figure 76: CD146 median fluorescence intensity measured by flow cytometry at different passages until replicative senescence.

The results are displayed in normalized MFI (on the left) and percentages (on the right).
For a clearer rendering, the results of each sample were shown in separated charts
(24 A at the top, 24 C in the middle, 33 at the bottom)
Several fluorochromes and detector voltage were used and are represented in different colors.
These data were obtained from different runs of culture for each sample. In some cases, some passages have been studied several times, even with the same fluorochrome.
All the data obtained were displayed to show results variability.
Between brackets in the legend, and represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size between all passages and avoid the bias that bigger cells might appear brighter.
Dotted curves are shown only to help to view the tendency of variation of each sample along passages, but do not represent true data or models.

Cell size could in some case also account for the results obtained. It can be seen when the results coming from the FSC / SSC restricted area are far from the results of the whole living population, for examples with the percentages of "24 A" sample. The restricted population detached better from the signal obtained with isotype control, resulting in higher percentages. For the "33" sample, the fluorescence intensity detected was weaker in the restricted population than in the whole living population including bigger cells.

To better visualize the diversity of CD146 expression within samples, examples of population distribution are shown **figure 77**. This figure shows how CD146 can be expressed differently, already from early passage, in different samples.

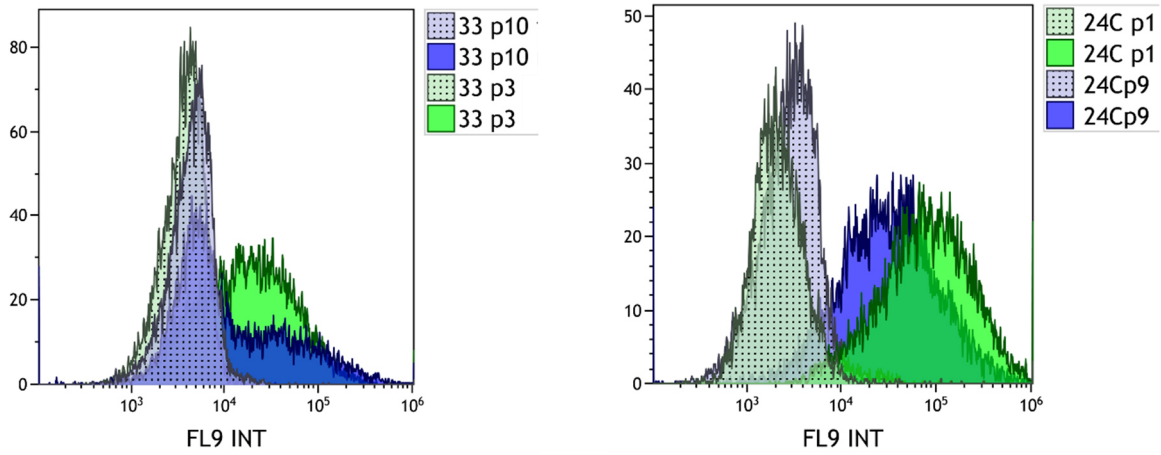


Figure 77: Overlay of monoparametric histograms of fluorescence intensity associated to CD146-BV421 detected by flow cytometry, in early and late passage BM MSC.
Isotype controls are shown with lighter and dotted color.

"33 sample" displayed a clear subpopulation of cells negative for CD146, detaching itself from a smaller other subpopulation of CD146 expressing cells. With advancing passages, the global shape remained similar, but the proportion of CD146 expressing cells was reduced. In such cases, median parameter is not ideal and the analysis of each subpopulation should be made separately. However, the way CD146 was expressed among the cells of "33" sample was more unusual than the shape of the distribution observed in "24 C" sample. In its case, no clear subpopulation was observed, almost all the cells were expressing CD146 but at different levels. In this case, the median of the whole population remains the most robust parameter to describe CD146 expression. Here also, it is interesting to see that distribution shape remained similar between early and late passage, even if

expression level changed a little. No clear subpopulation of MSC negative for CD146 appeared in “24 C” sample, even after 9 passages.

The other studies following CD146 variation of expression in MSC at different passages reported disparate results. A study reported a decrease in percentage of CD146 of BM MSC at passage 10 (74). It is not clear if the decrease observed was progressive or if it was only monitored at one early and one late passage. Several studies making CD146 cell sorting also followed the evolution of this marker over time. One of them with BM MSC reported a decrease of CD146 in $CD146^{high}$ sorted cells with time in culture, but it was only at the mRNA level (164). Two other studies found an increase in CD146 expression after several passages in culture, but in the $CD146^{low}$ sorted cells this time (60,69). On another side, CD146 phenotype of adipose MSC was found to stay stable after 9 passages (178). These discrepant results might also have been caused by the sensibility of CD146 expression to culture conditions. With the question of CD146 expression according to the number of passage or time in culture, the level of confluence could notably impact the results. It is often considered and managed differently between teams and confluence alone was reported to downregulate CD146 expression (150).

3. Other subtypes markers

In this part, the results concerning CD71, CD140b, CD200 and CD106 are exposed figure 78.

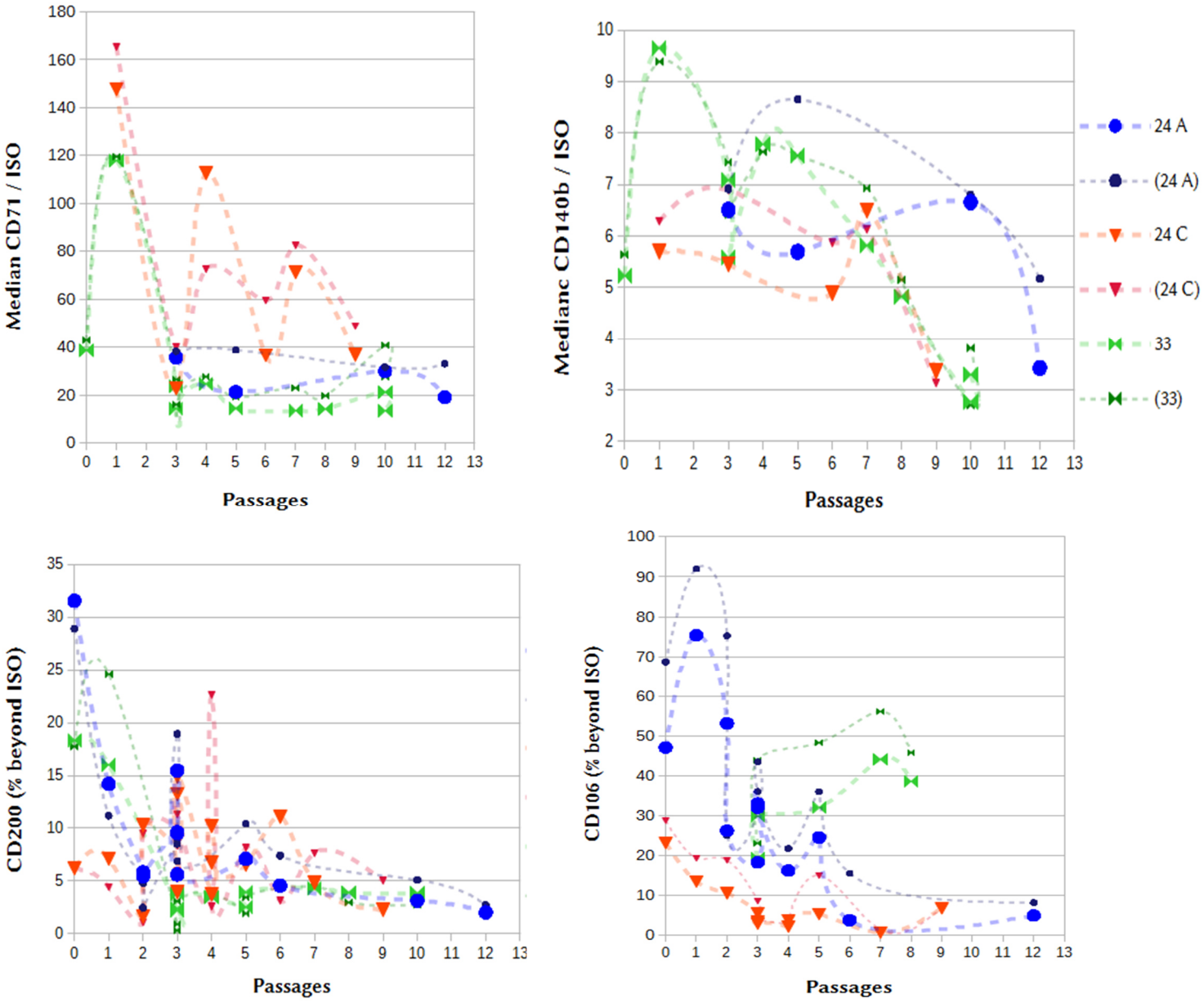


Figure 78: MSC subtypes markers assessed by flow cytometry at different passages until replicative senescence.

The results are displayed in normalized MFI for CD71 and CD140b and in percentages for CD200 and CD106.
These data were obtained from different runs of culture for each sample. In some cases, some passages have been studied several times. All the data obtained were displayed to show results variability.
Between brackets in the legend, and represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size between all passages and avoid the bias that bigger cells might appear brighter.
Dotted curves are shown only to help to view the tendency of variation of each sample along passages, but do not represent true data or models.

The highest level of expression observed for CD71 and CD140b were at early passage, notably at passage 1, and after various fluctuations, this expression reached the lowest level at the end of the culture. Even with these variations, CD71 remained a marker strongly expressed all along the culture, close to what can be obtained with classical highly expressed MSC markers. Flow cytometry histograms showing the distribution of CD71 expression in early and late passage cells are proposed **figure 79**.

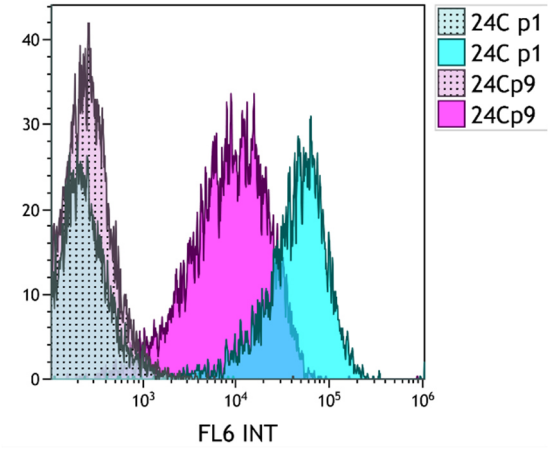


Figure 79: Overlay of distribution histograms showing fluorescence intensity of CD71-APC detected by flow cytometry on early and late passage MSC.
Isotype controls are shown with lighter color with black dots.

CD140b expression was not very strong from the start and reached very low levels at later passages.

CD200 and CD106 were found to be very low expressed by the studied MSC. Variations of their expression were better seen with percentages. The percentage reported for CD200 were very variable according to the run and to the passage, but the highest values were obtained at early passage, while it was only observed below 5% at later passages. Some low values were also obtained at early passage thought. The variations of the percentages associated to CD106 were more sample-dependent, with marked differences. For “24 C” it started at more than 20% at p0, then progressively decreased and stayed at less than 10% from passage 3. For “24 A” it reached more than 70% at passage 1 and then decreased to lower value, below 10% from passage 6. Fewer values were available for “33” sample, and its evolution was different. It increased from near 30% to around 40% between p3 and p8.

Few other studies followed these subtype markers at different levels of cell cultures.

An interesting study monitored 20 surface markers over time in culture and report a relatively stable expression pattern until passage 12. In particular, they found a strong decrease of CD146 expression at p10 and a slight increase of CD141 from p1 to p9. The other markers decreased over time (74).

CD71 was found by another research group to be less expressed among MSC undergoing replicative senescence (74).

CD106 was followed on BM MSC between p3 and p7 in another study that reported oscillations of CD106 expression (194).

C. Relative quantification of surface membrane proteins by proteomics

To go further in the study of MSC senescence, and potentially to find new markers specific to senescent MSC, the plasma membrane proteins of the cells were analyzed by proteomics.

Surface membrane proteins of the 3 BM MSC samples obtained from young donors at passage 3 and at replicative senescence were purified and analyzed with LC-MALDI system (261). A selection of results is displayed **figure 80.**

Additional name / information		Donor 1 (24 yo)				Donor 2 (24 yo)				Donor 3 (33 yo)			
		Fold Change	pept nb	CV	p	Fold Change	pept nb	CV	p	Fold Change	pept nb	CV	p
Thy-1	**CD90**	0,2	6	18	0,004	0,41	6	9	0,04	1,41	6	17	0,2
HCAM	**CD44**	0,54	7	21	0,1	1,29	10	29	0,5	1,04	10	25	0,5
Endoglin	**CD105**	0,12	1		0,01	0,36	5	50	0,2	0,37	4	35	0,03
ALCAM	**CD166**	0,1	6	48	0,02	0,43	8	37	0,1	0,62	7	27	0,27
PDGF-Rβ	**CD140-b**	0,18	3	21	0,08	0,37	7	28	0,04	1,05	7	11	0,4
MCAM	**CD146**					0,11	6	43	0,1	0,75	5	12	0,3
BST-1	**CD157**	3,68	2	18	0,06	3,13	2	25	0,02	2,6	2	23	0,05
Endosialin	**CD248**	0,16	6	27	0,02	0,4	9	21	0,05	0,62	10	18	0,2
	ANP-R	0,09	1		0,01	0,18	3	14	0,002	0,74	2	13	0,6
4F2	**CD98**	0,43	6	34	0,1	0,55	7	48	0,2	1,47	6	28	0,3
Galectin 1	**GAL-1**	0,12	6	31	0,01	0,21	7	44	0,08	0,61	7	52	0,4
	MYADM	0,13	2	45	0,1	0,52	2	8	0,2	0,57	2	15	0,3
	Vasorin	0,18	3	18	3E-04	0,45	4	76	0,008	0,66	5	34	0,1
integrin alpha 5	**IT-α5**	0,09	5	13	0,3	0,19	5	56	0,01	0,62	7	21	0,3
integrin alpha V	**IT-αV**	0,22	8	45	0,06	0,44	9	50	0,09	0,83	7	24	0,4
integrin beta 1	**IT-β1**	0,12	13	40	0,05	0,47	16	57	0,1	0,84	15	19	0,5
class1	**HLA-A2**	0,37	6	44	0,2	0,25	12	53	0,03	0,52	7	28	0,09
class1	**HLA-B38**	0,37	2		0,1	0,32	9	13	0,07	0,27	10	49	0,06

Figure 80: Selection of results of the relative quantification of surface proteins between replicative senescent and young MSC.

In the upper part, from CD90 to CD146, surface markers already known and also studied by flow cytometry are presented. Then, the proteins shown were those displaying the greatest differences between young and senescent MSC of the 3 samples.

"pept nb" column contains the number of peptides used to identify and quantify the corresponding protein. CV is the variation coefficient between results obtained from different peptides. A CV inferior to 20 is considered acceptable.

"p" is the statistical p value.

Globally, two samples displayed similar results and the third sample (33) had a different behavior, with less difference between young and senescent cells. In a general manner, most of the detected proteins showed a tendency to decrease in later passage cells. Only CD157 consistently increased in the 3 samples of replicative senescent MSC. The other proteins listed after showed a tendency to decrease in the 3 samples but the results obtained were less reliable. It is important to note that this experiment needed many steps for sample preparation. This could generate important variations and affect the resulting statistics obtained with this kind of set up.

Concerning the surface markers that were already known and followed by flow cytometry, it can be interesting to see that CD73 was not detected with this technique, while it is one of the classical highly expressed MSC marker. CD146 results were also interesting, it was detected in “24 C” and “33” samples but not in “24 A”. It seems consistent with the fact that “24 A” sample was the one with the weakest expression. The markers CD105 and CD166 were found for the 3 samples to decrease in the replicative senescence cells, but with low statistical strength.

It can also be interesting to see that for all the samples, early and late passages, the list of proteins was the same and there was no appearance or complete disappearance with prolonged culture.

The surface phenotype of senescent cells was already studied by a similar technique, but it was with other cell types and the proteins found were different (281).

The CD157 marker stood out and priority was placed to develop its study further. CD157 is an ecto-enzyme associated with NAD+ metabolism, but also a receptor linked with adhesion to extracellular matrix proteins and signaling (282,283).

D. CD157 flow cytometry

After CD157 was highlighted by the proteomics study, an extensive study of the expression of this marker was started.

1. CD157 changes along culture

CD157 assessment could be made by flow cytometry on some different samples than the one used for the senescence study, between passage 0 and passage 2. These results are shown in a first part before the details of CD157 expression evolution with replicative senescence for the same samples used in the proteomics study.

a. CD157 expression changes during the first passages

The results of CD157 expression in the first passages of cultures could be obtained from 4 samples **(figure 81)**.

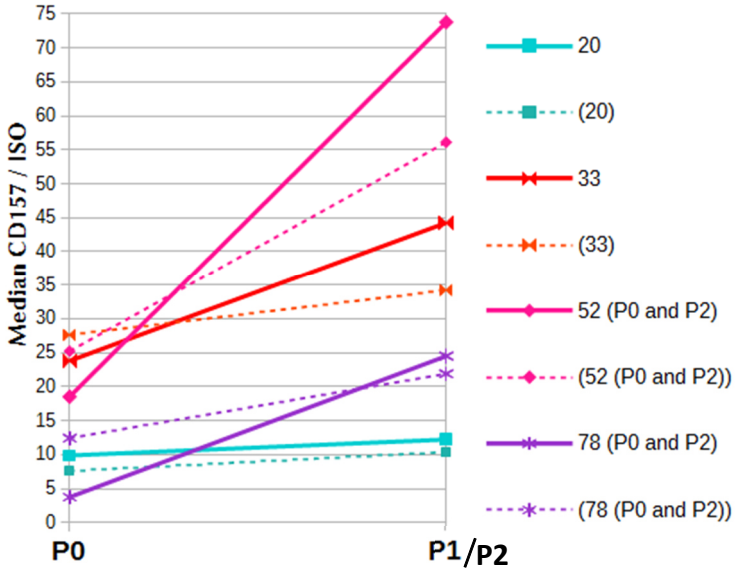


Figure 81: Evolution of the normalized median fluorescence intensity of CD157 detected in 4 samples between passage 0 and passage 1 or 2.
Between brackets in the legend, and represented by dotted lines: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size and limit the bias that bigger cells might appear brighter.

Already at the start of the culture, CD157 expression was found to increase at varying degrees between p0 and p1 or p2 for the 4 samples studied.

b. CD157 expression changes between early and late passages

The 3 BM-MSC samples at passage 3 and replicative senescence were also studied by flow cytometry to validate proteomics results **(figure 82)**. For this first validation, only

one early and one late passage were compared for each sample, in a similar set up than for proteomics.

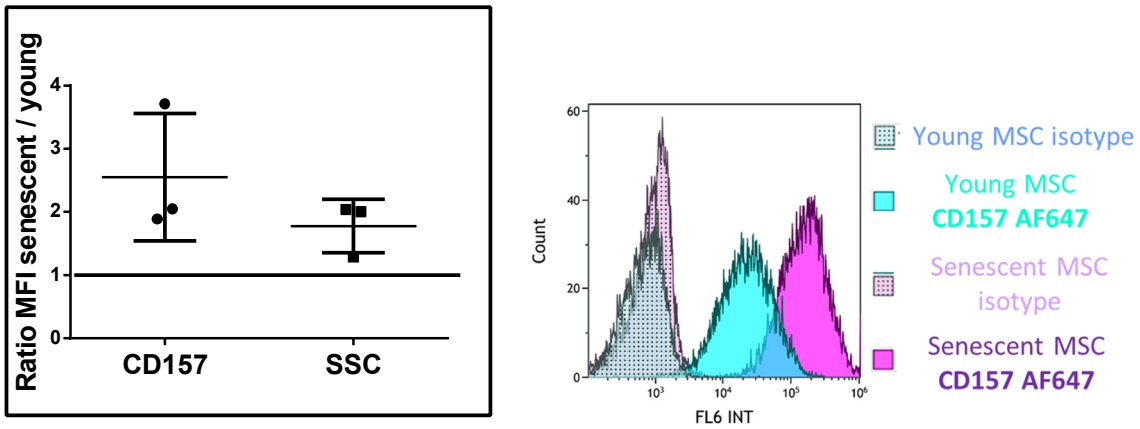


Figure 82: Ratio of CD157 expression and SSC parameter reflecting cell size between replicative senescence and passage 3 MSC (on the left).
On the right: overlay of one-parameter histograms of CD157 in"24 C" young and senescent cells.

Flow cytometry results confirmed proteomics results, with an increased expression of CD157 in replicative senescent MSC of around 2 times for 2 samples ("24 A" and "33") and of more than 3 times for "24 C" sample. One-parameter histograms show that senescent MSC population stand out from young MSC, but an overlap is present. Cell size reflected by SSC was also increased in senescent MSC.

After this, a more complete study was undertaken to observe the expression of CD157 at several intermediate passages **(figure 83)**. The results were displayed both with raw and normalized MFI because there were important differences between the two. This could notably be due to the variability of the MFI of APC isotype controls (between 0.24 and 3.05) observed for this set of experiments. This variability was not linked to the passage. Cell size also had an important influence on the results. Those obtained from the FSC / SSC restricted area were for some points very far from those of the whole living population, but not in an equivalent manner for all.

In any case, with more passages taken into account, the evolution of CD157 expression was revealed to be less linear and strong fluctuations were highlighted.

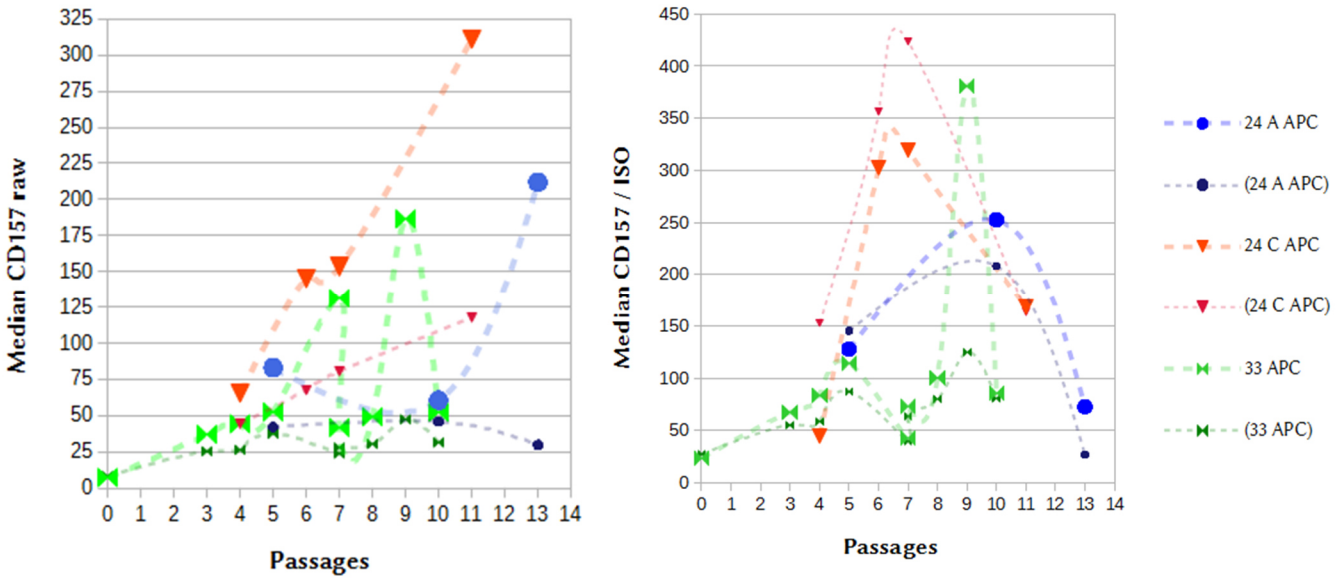


Figure 83: CD157 median fluorescence intensity measured by flow cytometry at different passages until replicative senescence.

The results are displayed in raw MFI (on the left) and normalized MFI (on the right). These data were obtained from different runs of culture for each sample. In some cases, some passages have been studied several times. All the data obtained were displayed to show results variability. Between brackets in the legend, and represented with smaller and darker points in the charts: the results obtained with a population of cells gated in a restricted FSC / SSC area, the gate being placed on the exact same place for all the samples. This allows to compare cells of similar size between all passages. Dotted curves are shown only to help to view the tendency of variation of each sample along passages, but do not represent true data or models.

Both with raw and normalized results, the increase of CD157 with passage was not clearly seen.

The highest point at passage 9 for "33" sample also seems to be associated with the presence of bigger and brighter cells and may not show a real increase of CD157 expression for the cells. This effect can also be seen for "24 A" sample at p13 (raw data).

The raw data for "24 C" sample showed a clear and linear increase, even in the cells from the FSC / SSC restricted area. However, with the normalized MFI, the point at passage 11 appears diminished. This effect is linked to the MFI of isotype controls, (0.15 for 24 C p7 and 1.85 for p11). Several other seemingly contradictory variations are associated to this.

Even for results with more easy interpretation, the tendency was not clear. For example, when comparing passage 5 and 10 of "33" sample, the results of CD157 expression were very close.

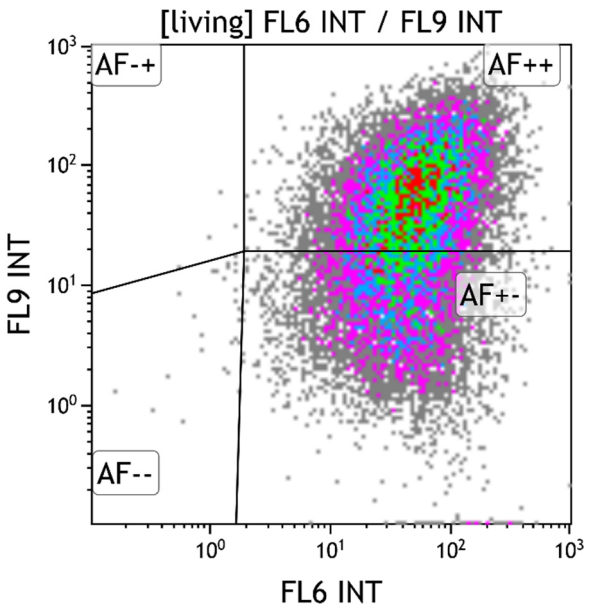
More points are needed to reveal reliable links between CD157 level of expression at **these different passages** and other MSC characteristics. It could also be necessary to refine the method to obtain a more reliable parameter representing CD157 expression in flow cytometry. For now, for the 3 samples studied, the results obtained with CD157 did not follow the same trends as with population doubling and senescence-associated β-Galactosidase staining results. The latter showed more stability at early-medium passage and more regular increase at later passages.

These results allow to weigh the conclusion that could have been taken rashly with the study of only two time points. CD157 remains an interesting marker to follow to better understand MSC, but once again the impact of senescence is not as obvious. CD157 expression might increase more clearly during the first passages, but there were not enough cells remaining at passage 3 and before for the 3 samples followed until replicative senescence.

The results obtained by other groups were also disparate. A group found a decrease of CD157 expression in BM MSC with extended culture, but culture conditions were very different (different kind of medium and splitting ½ at each passage) (284). Another study reported an increase of CD157 genetic expression with BM MSC at passage 7 compared to cells at passage 3 (285).

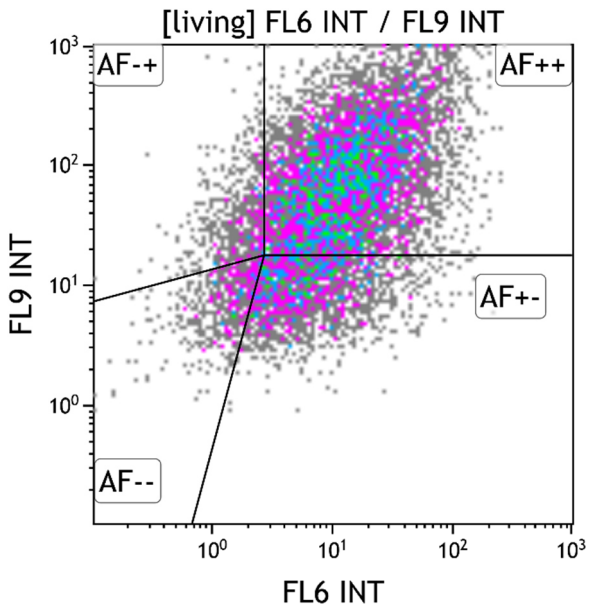
c. CD157 heterogeneity within one sample

The data obtained on CD157 expression were reviewed more precisely to observe the distribution of expression of this marker inside the cell population of a sample. Density plots combining CD157 and CD146 expression results from 2 representative samples are shown **figure 84**. A large range of levels of expression could be observed for CD157, but not as widespread as the results with CD146 label. This dispersion could still be sufficient to separate $CD157^{high}$ from $CD157^{low}$ cells.



Gate	Number	%Gated	X-Med	X-GMean
All	18 996	100,00	46,48	44,00
AF--	27	0,14	1,45	1,10
AF-+	4	0,02	1,23	1,02
AF+-	7 180	37,80	35,73	34,58
AF++	11 785	62,04	53,61	51,45

Gate	Y-Med	Y-GMean
All	31,33	26,41
AF--	5,78	5,21
AF-+	30,41	28,36
AF+-	6,98	5,93
AF++	62,29	65,87



Gate	Number	%Gated	X-Med	X-GMean
All	9 731	100,00	9,92	9,68
AF--	585	6,01	1,39	1,23
AF-+	541	5,56	1,79	1,60
AF+-	2 064	21,21	6,09	6,98
AF++	6 541	67,22	14,57	14,98

Gate	Y-Med	Y-GMean
All	45,53	44,68
AF--	7,57	6,95
AF-+	34,33	39,86
AF+-	9,44	8,75
AF++	82,72	89,12

Figure 84: Density plots with CD157-APC (FL6) and CD146-BV421 (FL9) fluorescence intensity data.

Left: 52 p2 sample. Right: 20 B p1 sample.

These results were obtained from a homogeneous population in FSC and SSC dot plot.

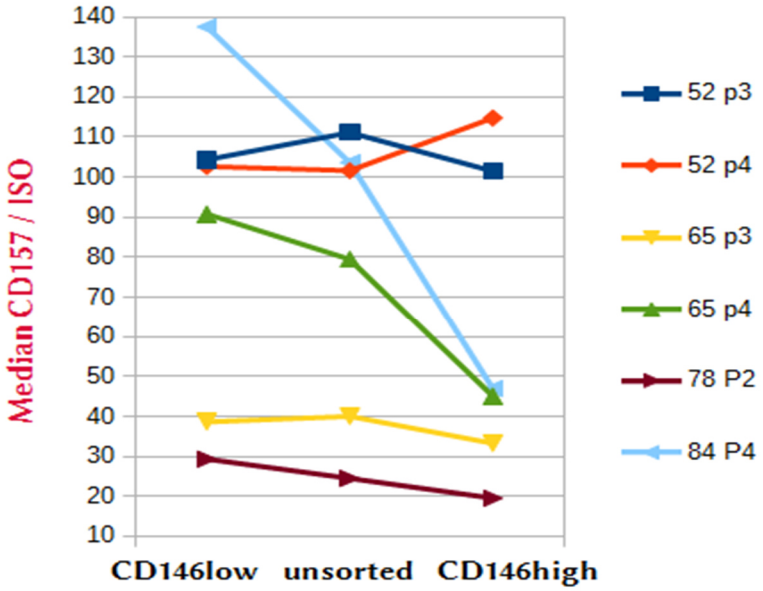


Figure 85: CD157 associated normalized median fluorescence intensity of CD146^{high}, CD146^{low} sorted cells and unsorted cells.

To go further, CD157 expression was also assessed on cells from the CD146 sorting experiment **(figure 85)**. Except for the sample "52" at p4, CD146^{high} cells tended to display lower level of CD157 expression than CD146^{low} sorted cells, at varying degrees. The "84" sample showing the biggest difference between cell fractions also showed a marked difference in senescent cell content, with less senescent cells found in CD146^{high} cells.

2. CD157 expression change with donor age for MSC at passage 1

CD157 expression was also assessed between BM MSC coming from donors of different ages **(figure 86 and 87)** to compare replicative and chronological aging effects.

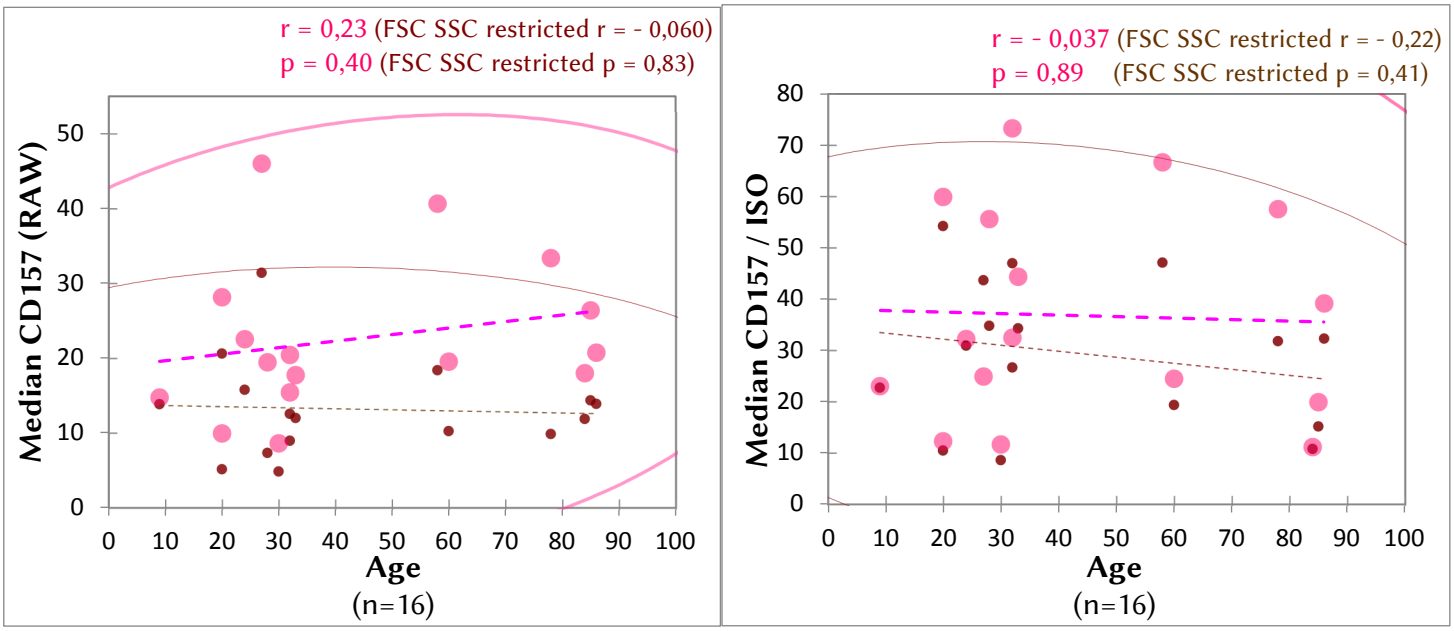


Figure 86: Scatter plots of raw and normalized median fluorescence intensities obtained for CD157-APC on 16 BM MSC samples at p1 coming from donors of different ages.
Raw MFI are on the left and normalized MFI on the right side.

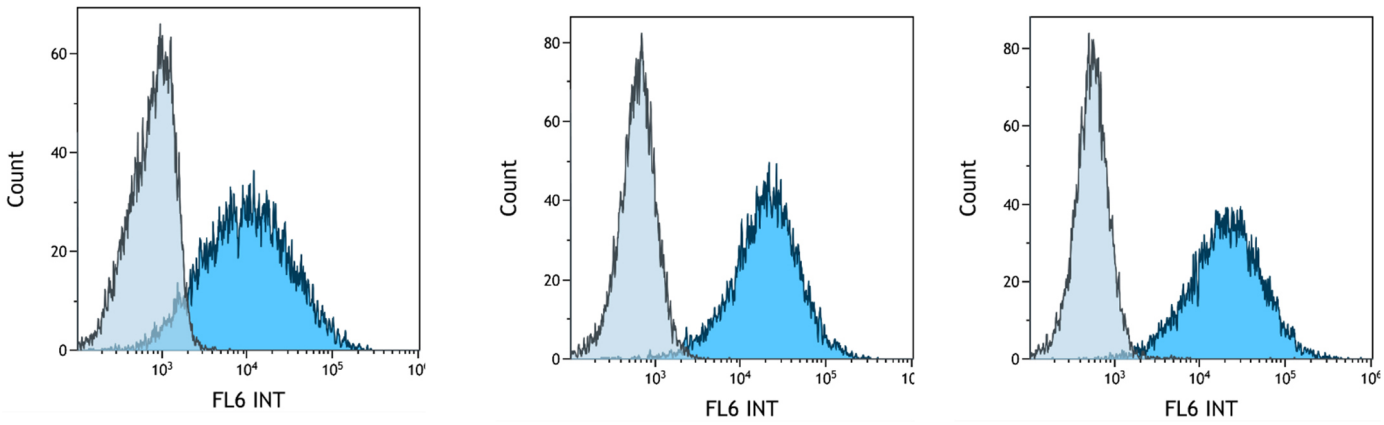


Figure 87: One parameter flow cytometry histograms of CD157-APC expression among BM MSC from different donors.
Superposition of control isotype in transparent light color and CD157-APC fluorescence in deep blue color.
The data were obtained from BM MSC at passage 1 from 20, 32 and 86 years old donor (from left to right).

There was no correlation between the level of CD157 expression and donor age, either with raw or normalized MFI. To note, variations in MFI results due to cell size were also observed here.

No other group highlighted CD157 association with donor age, but another study addressed the link with donor pathology. CD157 protein was found to be overexpressed in BM MSC from patients with rheumatoid arthritis and it was shown to promote pre-B-cell maturation (286). This is an interesting direction to better understand CD157 functioning.

3. CD157 expression change with MSC proliferation capacity at p1

The association between CD157 expression and MSC proliferation at passage 1 was also explored **(figure 88)**.

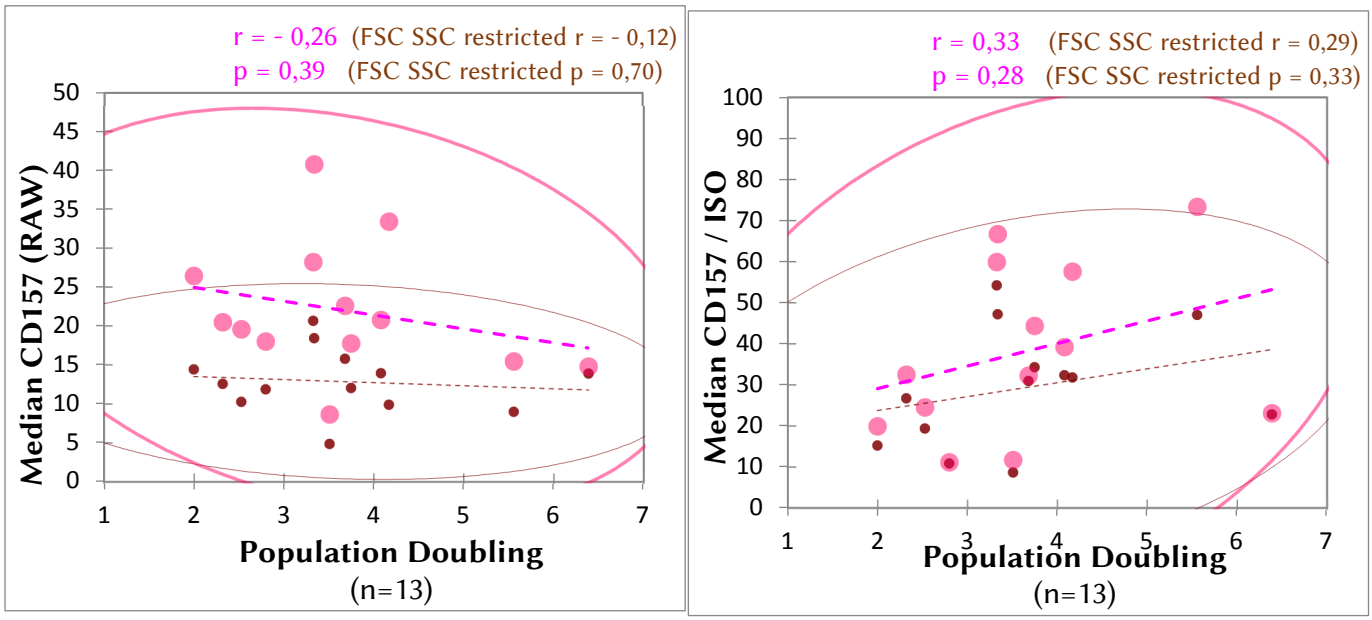


Figure 88: Scatter plots of raw and normalized median fluorescence intensities obtained for CD157-APC on 13 BM MSC samples at p1 at different population doubling levels.
Raw MFI are on the left and normalized MFI on the right side.

Less data was available than with donor age. In the end, no clear tendency of association could be identified between CD157 and population doubling either.

In contrast, 2 other studies highlighted CD157 in association with proliferation and other MSC functions. The first group reported that the signaling pathway associated with CD157 receptor and its ligand SCRG1, that can be secreted by MSC, was implicated in MSC *self-renewal*, migration and osteogenic differentiation, especially to maintain these

functions at high passages (287). The second group reported the results of a large-scale study of BM MSC which allowed them to draw a network of genes controlling MSC proliferation. They highlighted CD157 along with CD200 transcripts as important factors at the plasma membrane level (65). Their expression was found to be downregulated after differentiation process.

E. Main points of part III

- Samples obtained after 10 to 14 passages were characterized for senescence markers. Late passage cells showed strongly reduced proliferation and increased levels of β-Galactosidase activity. By fluorescence microscopy, they were also seen to release HMGB1 from the nucleus and to display increased size and SOD expression. This confirmed that replicative senescence MSC were enriched in late passage samples.

- **Senescent samples displayed interesting capacities for migration and immunosuppression**.

- Along MSC culture until replicative senescence, MSC undergo strong fluctuations in surface marker expression levels, even for surface markers thought to be constantly and highly expressed. The fluctuations were also very sample-dependent.

- **Proteomics study highlighted CD157 increase in replicative senescent MSC** while other detected surface proteins tended to decrease.

- Flow cytometry study of CD157 revealed **strong fluctuations** of its expression. The evolution of CD157 expression did not follow the progressive increase of other senescence markers.

General discussion

By their biological properties, mesenchymal stromal/stem cells provide interesting tools for the development of cell-based therapies for the treatment of several kind of diseases (81). However, despite many clinical trials developed to date using MSC, clinical outcomes showed limited success (50,82,83), despite the evidence obtained by *in vitro* but also *in vivo* experiments. To overcome that, different questions have emerged, some of them linked to cell quality and changes induced during *in vitro* expansion, in particular related to MSC heterogeneity and senescence.

To provide knowledge on these issues, this work focused on MSC surface markers with the aim to:

- describe initial MSC variability between and within different samples and study eventual associations between phenotype, donor age and MSC basic properties,

- develop and test a sorting strategy to separate MSC subsets according to CD146 expression,

- search for specific surface markers on senescent MSC.

The first part of this work presents results linked to surface markers expression on BM MSC. First of all, some parameters that must be considered in the analysis of surface markers by flow cytometry were studied. Indeed, impact of freezing cells, culture time and conditions were highlighted as elements that could affect the observed expression of markers. This allowed to set the conditions needed to compare the cells and to observe the possible range of variability using flow cytometry. To minimize bias, all experiments presented in this part were performed with frozen cells after one passage in culture.

The study of surface markers expression developed using flow cytometry made it possible to differentiate between proteins expression at constant or variable levels on the cell surface. Each surface marker seems to have its own dynamic (different variations, different level of expression) and no evident similarities were observed among them. In general terms, expression of those expressed at constant level (CD90 and CD73) do not correlate with donors age or sample proliferation capacity. On the contrary, proteins expressed at

variable level between samples (CD146, CD71 and CD105) were correlated with donors age and/or sample proliferation capacity.

The studies exploring the potential of surface marker to reveal MSC functionality often offer discrepant results in the literature (60,92,172,174,271). Plasma membrane proteins are markers that must be considered with caution. To be able to interpret their variation, it is essential to place it in the context of the other cell characteristics and culture conditions. Threshold of expression levels are also very difficult to place and can be very variable between samples and even between close time points for the same sample. More studies are necessary to make reliable links between the level of expression of a surface protein and MSC properties and functionality.

Even if some associations could be highlighted between donor parameter or cell specific capacities and surface markers, a high level of heterogeneity remained. For this, cell sorting strategies have emerged to decompose heterogeneous populations into mixtures of simpler subpopulations. CD146 expression results obtained by flow cytometry and microscopy, combined with the highlighted links with donor age and cell proliferation, but also data from the literature (20,74,167), validated this marker as one of the best candidates for cell sorting. The second part of this work was, therefore, the optimization of a sorting method based on CD146 expression on MSC.

For clinical use, the choice of the sorting strategy is crucial. It must be practical and should not induce cell damage while allowing efficient sorting. To do that, two main strategies exist: FACS and magnetic cell sorting. FACS sorting methods spend more time than magnetic ones, leading potentially to cells alterations (288). Nevertheless, this strategy enables the use of several markers in parallel. On the contrary, magnetic sorting is less time consuming and easier to set up. However, with magnetic sorting, only one positive selection is possible, eventually combined with several depletions. Furthermore, the more restrictive the sorting is, the fewer cells will be recovered. Sometimes it could be necessary to pool cells from different donors (227). In this work, magnetic sorting was chosen to obtain two subtypes: $CD146^{low}$ and $CD146^{high}$ cells.

Results presented in this report showed that the magnetic sorting method devoted to obtain samples expressing CD146 enabled efficient $CD146^{high}$ cells enrichment even from samples with low expression. The average recovery of $CD146^{high}$ expression cells was about 14% of the initial cell population. Then, depending on the desired application, a large number of cells will be needed to obtain a sufficient quantity of cells in the positive fraction. This could represent a limitation for the use of sorted cells in clinical applications. Instead, local applications requiring less cells could however be considered (289).

The comparison of functional properties between high and low expressing CD146 cells showed some differences. While no significant differences were found in terms of proliferation or clonogenicity, CD146 highly expressing cells showed better differentiation capacity and less senescence than CD146 lowly expressing ones. These results are in concordance with several studies found in the literature obtained with MSC coming from different tissues (20,60,92,166,167,173–176,179,180,270,271).

Finally, high heterogeneity remained between the samples, and CD146 sorting was not enough to gather a homogeneous cell subtype that would be similar between different MSC samples. Then, the state of the sample at the moment of harvest seems to be more important.

As CD146 marker is found on other cell types such as endothelial cells (290,291), it is not a sufficient surface marker by itself for MSC identification and prospective isolation from BM samples. However, unlike other markers proposed for that purpose, such as CD271 (116), it has the advantage of being better maintained in culture. Its use to select a subpopulation of MSC may therefore be interesting from *in vitro* amplified cells. Moreover, for clinical applications, instead of using CD146 as a marker to sort cells, it will also be interesting to determine whether CD146 can be used as a predictive marker of MSC quality.

Senescence is a physiological cell process in response to stress or increased replication cycles. As senescence could interfere with MSC properties and, in particular, with the secretory ones, its presence within samples intended for clinical applications need to be tracked. Thus, the third part of the work focuses on MSC senescence. With the aim to find surface markers related to MSC senescence, a replicative senescence model was used because it was shown to be representative of *in vivo* aging (34,39,196).

Senescence development on MSC was gradual and accompanied by a decrease in cells proliferation. Several markers known to be involved on senescence were detected in senescent MSC. Comparison of senescent and non-senescent MSC in our study model showed that senescence affects several functions of MSC like proliferation and clonogenicity. However, it did not impact other functions like migration or immunosuppression. Interesting properties that could be exploited were even revealed.

Then, the study was focused on surface markers related to MSC senescence. This work represents the first study where replicative senescent and early passage BM MSC surface proteins were compared by a proteomics approach. Results revealed changes in cell surface phenotype between senescent and non-senescent cells. In particular, CD157 expression was shown to increase in senescent cells. Results obtained by flow cytometry were consistent with proteomics results. However, with further study including intermediate points between early and replicative senescent cells, it was seen that CD157 expression oscillated a lot and there was no linear increase with passage. CD157 could be an interesting marker to be explored but it is maybe not so linearly associated with time in culture or senescence than preliminary data could have made us think initially. Even if the link with senescence is not straightforward, it remains interesting to investigate the potential role of CD157 in MSC function. A study notably highlighted its potential role in proliferation and migration (287).

Conclusion and perspectives

I. Conclusion

The research conducted during this PhD has yielded extensive data on the variability of surface markers expression in BM MSC. These data include flow cytometry analyses of MSC surface marker expression by flow cytometry of a set of classical MSC markers (CD105, CD90, CD73, CD44, CD166) combined with subtype surface markers (CD146, CD71, CD140b, CD200, CD106). SSC was also monitored as a reflection of cell size. The level of heterogeneity of these markers was observed between different runs, along successive passages and between MSC samples of different proliferation capacity from donors of different ages. Despite surface markers sensitivity to variations with culture, some associations stood out with donor age for CD146, CD71, CD105, CD44 and SSC and with proliferation rate for CD146, CD140b and CD71. When looking at cell population distributions, CD146 was also the marker the most heterogeneously expressed. It was then selected for cell sorting experiment to compare different MSC subpopulations defined by their CD146 expression.

An efficient magnetic cell sorting method was optimized. MSC functionality was assessed *in vitro* by following MSC proliferation and senescence, their capacity to form colonies, to differentiate, to migrate, and to secrete bioactive factors. The results of CD146 sorting experiment were consistent with the presence of different subtypes with different *in vitro* functionalities within MSC samples. In particular, $CD146^{high}$ cells displayed less senescence, different secretion profile, better capacity to produce differentiated cells and to migrate toward chemoattractant signal than $CD146^{low}$ sorted cells. However, the variability between samples exceeded the differences between sorted fractions.

MSC samples can be strongly influenced by the presence of senescent cells. MSC brought to replicative exhaustion were characterized by monitoring senescence markers, cell proliferation and capacity to form colonies, to migrate, and to suppress CD4 cell proliferation. The model of replicative senescence established allowed to enrich the sample in a majority of senescent cells between p10 and p14. However, it emerged that the samples

containing the most senescent cells displayed interesting capacities, in particular for migration and immunosuppression. From these characterized cells, a relative quantification of surface proteome of young and senescent BM MSC revealed an interesting increase of only one protein in senescent MSC, CD157. CD157 was then assessed by flow cytometry along with the set of classical and subtypes markers already studied. This showed that along MSC culture until replicative senescence, MSC undergo strong fluctuations in surface marker expression levels, even for surface markers thought to be constantly and highly expressed. The evolution of CD157 expression did not follow the progressive increase of other senescence markers. The optimized flow cytometry protocol including CD157 was integrated in MSC monitoring in the research team for further analyses.

II. Perspectives

The results presented in this report raise many different perspectives. Some of them are proposed here.

A. Complementary studies

Firstly, the study of surface markers expression needs to be continued by the inclusion of others samples in order to verify the trends identified. Moreover, the study of the expression of these markers need to be extended to other cell sources like WJ MSC but also under other culture conditions like hypoxia, platelet lysate instead of FBS or large-scale expansion in bioreactors.

In a preliminary study, few WJ MSC samples were studied and more samples are currently being under study. Important differences in phenotype between BM MSC and WJ MSC were observed. The analyzed WJ MSC were smaller and globally more homogeneous, with a stronger expression of CD71 and CD200, and almost no expression of CD140b and CD106 compared to BM MSC. The increased expression of CD200 on WJ MSC could be associated with interesting capacities for immunomodulation. For CD146, only a global tendency seems to indicate WJ MSC display a stronger expression. However, even if BM MSC showed weaker expression most of the time, a great heterogeneity was described,

and some samples could surpass WJ MSC. In relation with the use of platelet lysate for culture, for both WJ and BM MSC, CD146 is lower and CD200 more heterogeneous than when cells are cultured in media complemented with FBS. Following these results, changes in culture conditions (media composition, O_2 tension, ...) could be interesting to guide MSC phenotype depending on the desired function.

In addition to that, some of the markers studied, or a combination of them, if found to be associated to particular functions, could be proposed as quality or prognostic biomarkers linked to cell behavior.

To go further into the characterization of BM MSC senescence, the study of surface markers needs to be continued. CD157 appeared to be linked to replicative senescence even if fluctuations were observed. The role of CD157 on MSC and its relationship with senescence must to be clarified. It could be achieved by knocking down CD157 expression on MSC and test cell behavior.

Other paths to find senescence markers may be considered. One of them includes senescence associated secreted proteins. It is well known that senescent cells secrete specific proteins, mainly pro-inflammatory, but it will be relevant to study if particular proteins are secreted by senescent MSC. That could enable to follow MSC cultures and avoid the occurrence of senescent cells. Another one includes oxidative stress linked markers that are suspected of being increased with senescence. In this work, preliminary results showed an increase of SOD expression in senescent MSC. Another proteomics study is also envisaged to compare young and senescent MSC secretions and to refine the results of surface proteome with a more sensitive instrument.

Furthermore, our results showed increased immunosuppressive properties on 2 samples containing senescent MSC. However, more samples need to be tested to confirm this trend. Then, the mechanisms involved on senescent MSC mediated immunosuppression could be investigated. To verify the effect of senescent cells, it could be also interesting to test the effect of senescent MSC on GvHD or other animal models. Moreover, the effect of MSC samples depleted on senescent cells would be worth testing.

Finally, cell sorting strategies with combination of several markers are possible and could be developed. For example, it could be a good option to select SSC^{low} $CD146^{high}$ and $CD157^{low}$ cells. This strategy could select small cells with low senescence.

B. Methodological proposals

To make these complementary studies, suggestions for method improvements have been identified.

1. Flow cytometry

To obtain precise and reliable results of surface markers expression levels, the use of quantitative flow cytometry should be considered instead of the current relative fluorescence measurement (292). This would require more cells and limit the possibility to assess several markers on the same cells, but it could increase the robustness of the study to compare experiments conducted in different conditions and on cells of different sizes.

There would also have a need of quantitative parameters that could provide a precise assessment of the shape of population distributions, related to intra-sample heterogeneity. For this, kurtosis and skewness appeared as relevant parameters to follow. Skewness indicates the amount of deviation from horizontal symmetry. Kurtosis is associated to the height and sharpness of the central peak compared to standard bell curve. This is itself related to the distribution "tails", higher kurtosis meaning "more of the variance is the result of infrequent extreme deviations, as opposed to frequent modestly sized deviations". These two parameters are already used for normality testing (293).

More research on classical dispersion parameters like variation coefficient should also be considered. The use Median Absolute Deviation (MAD) and its alternatives should also be investigated and may be better suited to describe fluorescence intensity results obtained by flow cytometry (294,295).

To improve the analysis of heterogenous samples that may contain different cell subtypes, the use Gaussian Mixture Models (GMM) to cluster multidimensional data

according to their distribution also appeared as a relevant technique to investigate. It was already used by research teams studying stem cells heterogeneity (1).

As an easy start, skewness, kurtosis, median absolute deviation and gaussian mixture models can be easily computed with XLSTAT software. Examples of the use of gaussian mixture models on flow cytometry results obtained during the doctoral work can be found figure 89. However, XLSTAT is a proprietary software and, without further research, it does not make it possible to appropriate the methods used. A collaboration with statisticians and the use of Python or R would be an ideal perspective.

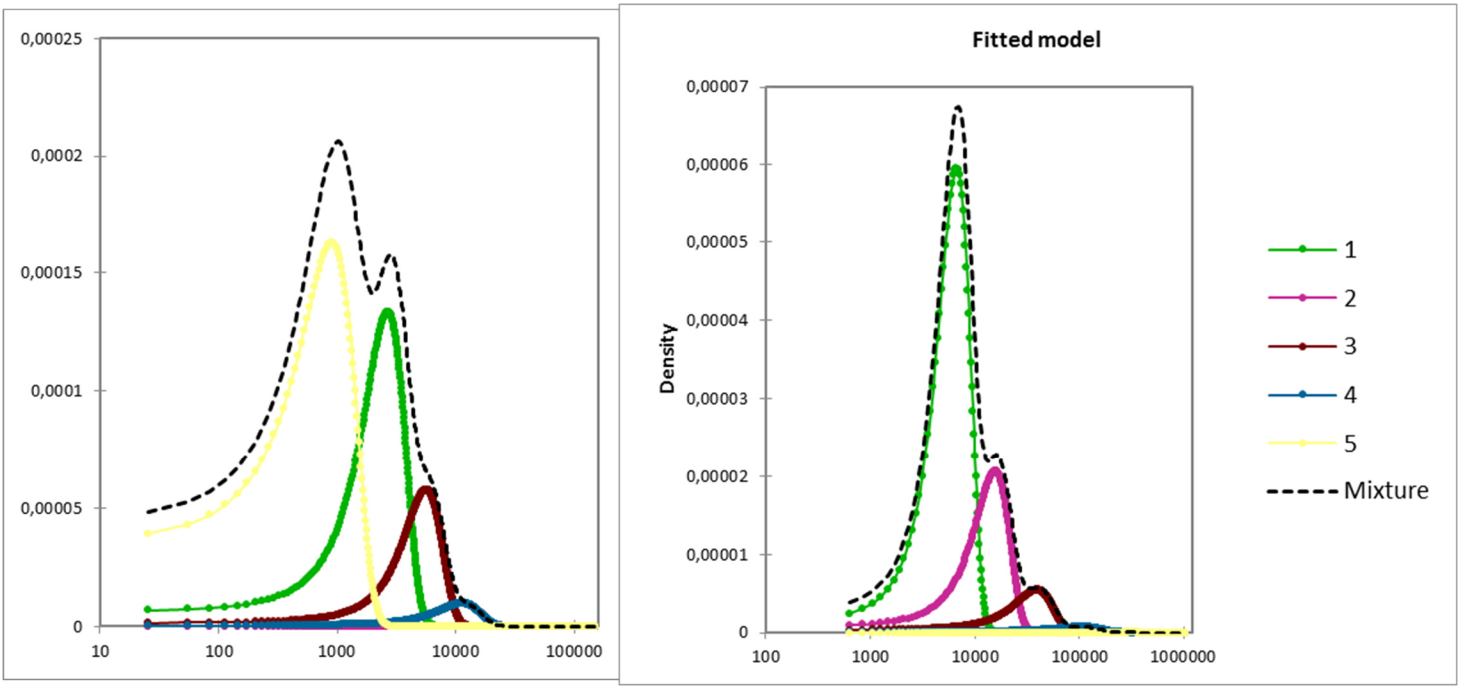


Figure 89: Example of the proposition obtained with gaussian mixture model function of XLSTAT for CD146-BV421 (78 B p1 sample) on the left and CD200-PE distribution (52 p2 sample) on the right.

2. Reproducible research tools

To limit unavoidable biases and help better practices for more reliable studies, there are already various solutions available, but they are still little known.

One of these solutions is to preregister studies through platforms such as "Cos.io/prereg". This can be done at different moments. The most common is to do it before data collection, but it can also be done before analyzing an existing data set or after more data are asked in peer review. The justification of the study, its experimental design and statistical analysis that will be conducted are described and officially registered before the

study is conducted. This process is guided and allows to efficiently plan new research and increases results credibility. It also opens the possibility, with one of the 211 scientific journals accepting this system, to have a study published according to the seriousness of the approach and not by the orientation of the results. In this case, peer review is made before from the registered report before the results are observed. If the report is validated, the journal gives an agreement in principle that the study will be accepted for publication without considering the orientation of the results. This process also allows to notify earlier the development of an idea by a team. It also encourages the distinction between exploratory and confirmatory research in order to use the most appropriate methods according to the mode of research. In particular, an exploratory approach will stay open for unexpected discoveries and minimize false negatives to generate new hypotheses. On the contrary, a confirmatory approach will test an existing hypothesis and minimize false positives.

Another interesting solution is the use of advanced electronic notebooks instead of the paper laboratory notebook. In particular, Jupyter notebooks are themselves software that can document procedures by simple writing but also integrate data analysis with Python or R. They are displayed in an internet browser and can be saved as HTML, PDF or other file format. This allows to precisely trace the analyses carried out and it also limits the common problem of data loss. These notebooks have already been used with success to conduct research with large and complex data set. It was used, among other things, to improve image analyses (296,297) and for metabolomics (298). These tools also allow to limit the problem of data transfer between incompatible proprietary software and facilitates the exchange of protocols and data. Jupyter notebook, together with the numerous packages freely accessible in Python and R to analyze and generate graphics, can replace current limited routines (299).

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Mesenchymal Stromal / Stem Cells (MSC) hold great potential and are currently the most used in clinical trials with cell-based treatments. MSC quality and therapeutic effectiveness are influenced by *in vitro* expansion but also by other factors such as donor parameters. To ameliorate the success rate of MSC therapies, this study focused on MSC heterogeneity. To put together cell characterization and ways to act when facing cell heterogeneity, this work was oriented toward the study of surface markers that can be monitored on living cells, and can serve to sort them.

The first objective was to describe initial MSC surface markers variability between and within different bone marrow MSC samples from donors of different ages. The second objective was to develop a sorting method to separate MSC according to CD146 expression and compare the sorted cells. The third objective was to widen MSC surface markers knowledge by focusing on senescent MSC. Surface markers of early passage and replicative senescent cells were compared with proteomics and flow cytometry.

Flow cytometry results on MSC were shown to be submitted to strong fluctuations. However, some regularities were strong enough to stand out. A group of surface markers were found to be associated with donor age: CD146, CD71, CD105, CD44. CD146, CD140b and CD71 were also correlated with proliferation rate. CD146 expression had the particularity to be relatively stable in culture and turned out to be the most heterogeneously expressed when looking at cell population within the samples.

Cultivated MSC from bone marrow coming from donor of different ages and at different culture steps were sorted successfully according to CD146 expression with immunomagnetic method. MSC behavior remained heterogeneous after sort but it could still be observed that most $CD146^{high}$ cells had more often better differentiation and migration capacities and were less senescent than their $CD146^{low}$ counterpart.

Proteomics study showed that almost all surface proteins expression tended to decrease on replicative senescent MSC, except one marker that increased: CD157. MSC at different stages of culture until replicative senescence were then studied by flow cytometry. This study revealed strong fluctuation in marker expression between different passages, highlighting again the variability of MSC behavior and the difficulty to predict it.

CD146, CD71, CD140b, CD157 and SSC deserve to be followed for MSC quality control.

**Keywords: Mesenchymal stromal / stem cells,
heterogeneity, surface markers, flow cytometry, cell subtypes, senescence.**

Les Cellules Stromales / Souches Mésenchymateuses (CSM) ont un grand potentiel pour de nombreuses applications. Elles sont les cellules les plus utilisées pour les essais cliniques développant des thérapies cellulaires. L'efficacité thérapeutique des CSM est influencée par leur amplification *in vitro* et d'autres facteurs tels que les paramètres liés au donneur de cellules. Pour améliorer les thérapies à base de CSM, cette étude a ciblé l'étude de leur hétérogénéité grâce à leurs marqueurs de surface, qui permettent de mutualiser la caractérisation des cellules et la possibilité de trier des cellules vivantes.

Le premier objectif de ce travail a été de décrire la variabilité des CSM entre et au sein d'échantillons venant de moelle osseuse de donneurs d'âges différents. Le deuxième objectif a été de développer une méthode de tri pour séparer les CSM selon leur expression de CD146 et de comparer les cellules triées. La troisième partie du travail a eu pour objectif de mieux connaître les marqueurs de surface des CSM sénescentes.

Les résultats de cytométrie en flux sur les CSM sont soumis à de fortes fluctuations, mais certaines régularités ont pu être mises en évidence. Un ensemble de marqueurs de surface a pu être associé avec l'âge des donneurs : CD146, CD71, CD105, CD44. De plus, des liens entre l'expression de CD146, CD140b et CD71 ont été observés avec la capacité de prolifération des CSM.

Des CSM de moelle osseuse provenant de donneurs d'âges différents et à différentes étapes de culture ont pu être triées avec succès selon leur expression de CD146 par une méthode immunomagnétique. Le comportement des CSM triées était encore hétérogène mais il a pu être observé que les CSM exprimant plus fortement CD146 avaient plus souvent de meilleures capacités de différentiation et de migration et étaient moins sénescentes que les cellules exprimant plus faiblement CD146.

Une étude protéomique a montré que la plupart des protéines de surface détectées avaient tendance à être moins représentées sur les cellules sénescentes à l'exception de CD157. Les CSM à différentes étapes de culture jusqu'à la sénescence réplicative ont ensuite été suivies par cytométrie en flux. Cette dernière étude a révélé d'importantes fluctuations entre les différents passages, soulignant la difficulté associée à l'utilisation de ces marqueurs.

Les marqueurs CD146, CD71, CD140b et CD157 méritent d'être suivis pour le contrôle qualité des CSM provenant de moelle osseuse.

**Mots-clés : Cellules stromales / souches mésenchymateuses, hétérogénéité,
marqueurs de surface, cytométrie en flux, sous-types de cellules, sénescence.**