It's a Jungle Out There: Myoblasts, Matrix, and MMPs

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The undersigned, appointed by the dean of the Graduate School,

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Presented by the Biological Sciences Department

A Candidate for the Degree of

Doctoral of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

Mom and Dad this dissertation is dedicated you. Thank you for raising me to be a respectable young man, I appreciate how you have shaped my view of the world and how to interpret it. I will always strive to make both of you proud.

I love you.

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My loving animals I grew up with. When times are hard, I can close my eyes and think of all the great moments I have had with my animal companions. The most important, Cody, has a special place in all of the hearts that knew her.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

CHAPTER 2

CHAPTER 2 ADDENDUM

CHAPTER 3

It's a Jungle Out There: Myoblasts, Matrix, and MMPs

Dane Lund

Dr. D.D.W. Cornelison, Dissertation Supervisor

ABSTRACT

Extracellular Matrix: Mammalian skeletal muscle is notable for both its highly ordered biophysical structure and its regenerative capacity following trauma. Critical to both of these features is the specialized muscle extracellular matrix (ECM), comprising both the multiple concentric sheaths of connective tissue surrounding structural units from single myofibers to whole muscles and the dense interstitial matrix that occupies the space between them. ECM-dependent interactions affect all activities of the resident muscle stem cell population, the satellite cell, from the maintenance of quiescence and stem cell potential to the regulation of proliferation and differentiation. This review will focus on the role of the extracellular matrix in muscle regeneration, with a particular emphasis on regulation of satellite cell activity.

Cell Invasion: The twenty-five known matrix metalloproteases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteases (TIMPs), mediate cell invasion through the extracellular matrix (ECM). In a comparative 3D assay, we analyzed human and mouse satellite cells' competence to invade an artificial ECM (collagen I). We identified a single MMP that: 1) is expressed by human muscle satellite cells; 2) is induced at the mRNA/protein level by adhesion to collagen I; and 3) is necessary for invasion into a collagen I matrix. Interestingly, murine satellite cells neither express this MMP, nor invade the collagen matrix. However, exogenous human MMP-14 is not sufficient to induce invasion of a collagen matrix by murine cells, emphasizing species differences.

CHAPTER 1:

Enter the Matrix: Shape, Signal, and Superhighway

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Modified from (Lund and Cornelison, 2013)

ABSTRACT

Mammalian skeletal muscle is notable for both its highly ordered biophysical structure and its regenerative capacity following trauma. Critical to both of these features is the specialized muscle extracellular matrix (ECM), comprising both the multiple concentric sheaths of connective tissue surrounding structural units from single myofibers to whole muscles and the dense interstitial matrix that occupies the space between them. ECM-dependent interactions affect all activities of the resident muscle stem cell population, the satellite cell, from the maintenance of quiescence and stem cell potential to the regulation of proliferation and differentiation. This review will focus on the role of the extracellular matrix in muscle regeneration, with a particular emphasis on regulation of satellite cell activity.

INTRODUCTION

Our approximately six hundred and forty individual skeletal muscles, comprising roughly 40% of an adult human's total body mass, are collectively responsible for maintaining posture and balance, for respiration, and for nearly all movements from the most delicate microsurgeries and brushstrokes to marathon running and power-lifting. Possibly due to the potential for damage implicit in such diverse and critical functions, skeletal muscle is one of the most highly regenerative tissues in the body; such regeneration requires the activity of a population of tissue-specific adult stem cells referred to as satellite cells.

Briefly, muscle satellite cells are the obligate tissue-specific stem cells of skeletal muscle: if the satellite cell population (defined by expression of the satellite cell marker Pax7 (Seale et al., 2000) is genetically ablated after maturity in the mouse, muscle regeneration fails (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). Satellite cells are derived from a somitic lineage during development, and are thought to disperse throughout the developing musculature concomitantly with the myoblasts that will contribute to embryonic and fetal myogenesis (Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). They are maintained in a quiescent state in the absence of physiological signals of damage, overuse, or disease in a minimal niche consisting of the cell membrane of the multinucleate, differentiated myofiber they are associated with and its overlying basal lamina (described further below.) When activated from quiescence by stress or damage, satellite cells will enter the cell cycle and proliferate extensively to form a population of replacement myocytes, which will fuse with each other or existing myofibers to reconstitute the muscle (reviewed in (Hawke and Garry, 2001; Shi and Garry, 2006).) Due in large part to their potential as either vectors or targets for cell therapy of

human myopathies, particular Duchenne muscular dystrophy, satellite cells have been the focus of intensive research to try to unravel the molecular mechanisms governing their maintenance and activity *in vivo*, in both healthy and pathological tissue. We will describe briefly the role the extracellular matrix plays in the structure and function of healthy skeletal muscle, then address key interactions with the ECM during satellite cell-mediated muscle regeneration, with particular emphasis on potential new and emerging areas of research.

Structure of the skeletal muscle extracellular matrix

The architecture of each individual skeletal muscle is maintained in part by concentric arrangements of connective tissue layers, which give the muscles both structure and strength (Berendse et al., 2003; Grounds et al., 2005); a recent review (Gillies and Lieber, 2011) describes the muscle ECM in detail with respect to its composition, ultrastructure, and interconnectivity. The myofiber plasma membrane is referred to as the sarcolemma; it encases each myofiber and forms the most proximal layer of the myofiber basal lamina. The sarcolemma and its associated basal lamina are a key feature of muscle fibers: first described in Bowman's classic monograph "On the Minute Structure and Movements of Voluntary Muscle" in 1840 (Bowman et al., 1840) as a thin, tough, transparent membrane that remained after hypercontracture of the contents of a myofiber, Bowman's tubes "would seem not improbably to consist of a very close and intricate interweaving of threads, far too minute for separate recognition." The basal lamina and its associated interstitial matrix are of particular interest for the purposes of this review due to their physical proximity and intimate interactions with quiescent and activated satellite cells and the sarcolemma of myofibers. In fact, the "gold standard" definition of a quiescent satellite cell is as originally described by Mauro in 1961 (Mauro, 1961), and elaborated on in further electron microscopy-based studies (Muir et al., 1965): small, bipolar, cells with a heterochromatic nucleus, scanty cytoplasm, and cytoplasmic processes of up to 25 μm, located beneath the basal lamina but outside the sarcolemma.

The basal lamina is also referred to as the endomysium, and is the smallest unit of the ECM in muscle. It overlays the sarcolemma and includes a tight sheath of laminin α2β1γ1 (also known as laminin-2 or merosin) connected to a mesh-like layer of collagen IV (reviewed in (Gillies and Lieber, 2011; Sanes, 2003).) Deposition of

these components of the basal lamina requires the interactions of myoblasts with muscle fibroblasts (Kuhl et al., 1984; Sanderson et al., 1986), consistent with the recent observation that muscle connective tissue fibroblasts are absolutely essential for muscle development (Mathew et al., 2011) and regeneration (Murphy et al., 2011): laminin is secreted by myofibers (Vilquin et al., 1999) while collagen IV is secreted by muscle fibroblasts (Kuhl et al., 1984). This dense, tough lamina surrounds and protects the myofiber, transduces contractile force to the muscle unit, and forms the apical side of the quiescent satellite cell niche (Grounds et al., 2005). Electron microscopy studies (Purslow and Trotter, 1994; Trotter and Purslow, 1992) of myofibers and their associated endomysium highlight the intimate associations of each muscle fiber with the three-dimensional network of the endomysium; the endomysium also harbors capillaries and axons serving each individual muscle fiber (Buchthal and Schmalbruch, 1980).

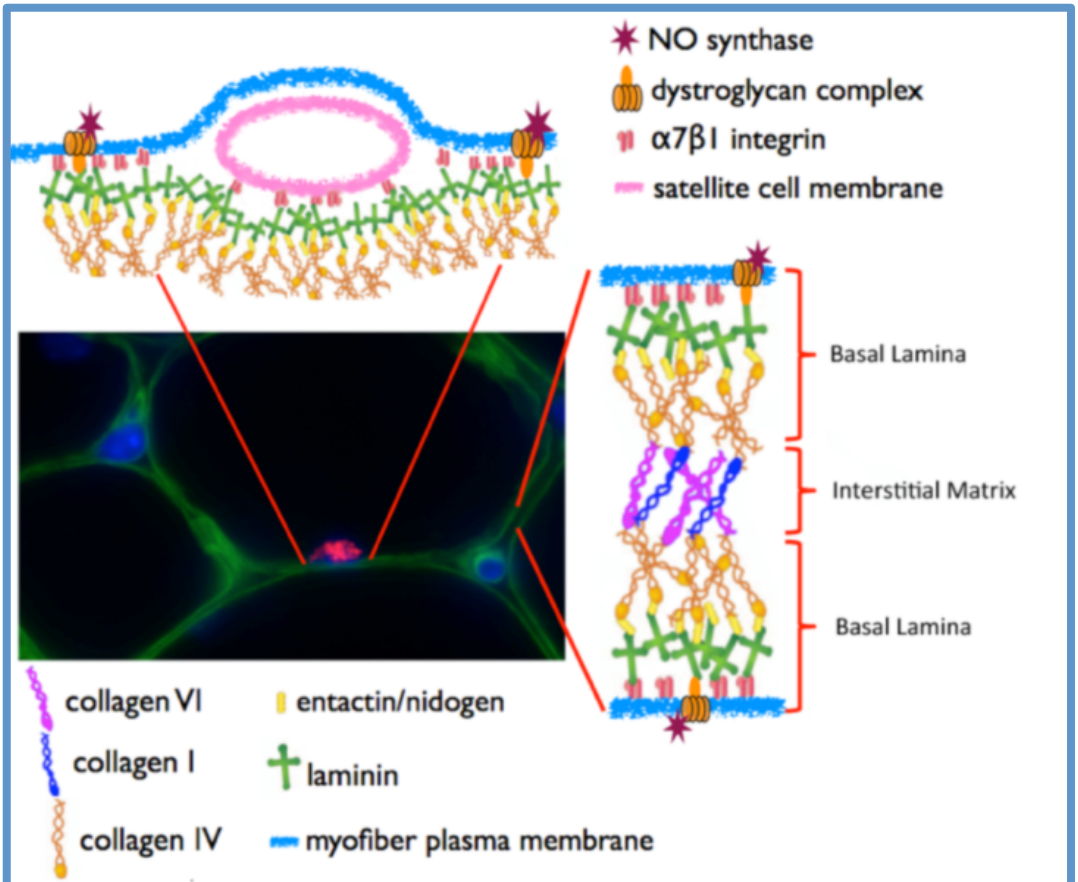

Figure 1-1: The Structural Architecture of a Quiescent Satellite Cell and the Extracellular Matrix. An activated satellite cell (identified by CD34 staining, red) on an isolated mouse myofiber 18 hours post-isolation; myofiber basal lamina is stained for laminin (green.) Left, schematic representation of the local matrix interactions during multiple phases of satellite cell activity *in vivo*.

The cytoskeleton of each myofiber is physically attached to the basal lamina, mainly through integrins and dystrophin-glycoprotein complexes (Mayer, 2003; Michele and Campbell, 2003); these connections are continued via additional crosslinking molecules such as entactin, nidogen, and agrin to the interstitial matrix between myofibers (Reed, 2009) (Figure 1-1.) It is disruptions in this linkage that are most often responsible for human myopathies, including Duchenne's muscular dystrophy (Cohn and Campbell, 2000). The interstitial matrix that surrounds myofibers accounts for 1-10% of muscle tissue and fills all of the space between muscle fibers while maintaining mechanical continuity with tendons. The major roles of the interstitial ECM are to transduce mechanical force from the muscles and to serve as a structural support for the muscle and its associated blood vessels and nerves. It is more porous than the basal lamina, and is composed of fibrous components providing tensile strength (primarily collagens) and proteoglycans (including chondroitin, heparan, and dermatan sulfates), which comprise 10% of the weight but 90% of the volume of the matrix and provide both a labile environment for other cells and components to move within and a sink for other matrix-associated molecules (proteases, chemokines, cytokines, mitogens, and growth factors) (reviewed in (Sanes, 2003).) The most significant fibrous components of the interstitial matrix are the collagens: collagen comprises roughly 90% of the protein mass of the ECM. Local fibroblasts secrete collagen VI into the interstitium (Zou et al., 2008) where the triple-helical subunits form double-beaded collagen VI microfibrils by end-to-end association. Collagen VI in the interstitial matrix acts to bind the muscle ECM into functional units and provide structure and stability.

Bundles of muscle fibers bounded by epimysia are then formed into fascicles, which are surrounded by the perimysium; this layer contains and organizes larger

blood vessels and nerves as well as connecting to the interstitial matrix. The whole muscle is then surrounded by the epimysium, which will broaden and flatten at the extremities of each muscle to form the myotendinous junction. The perimysium and epimysium differ from the endomysium in their composition (primarily collagen I and III instead of IV and VI, and different complements of proteoglycans) and function; their specific role in preservation of muscle architecture and function is also somewhat less well defined (reviewed in (Gillies and Lieber, 2011).) The epimysium, perimysium, and endomysium are linked by interconnecting collagen fibers to evenly and effectively distribute contractile forces without damaging the muscle structure during movement (Tidball, 1991).

As would be expected, loss or mutation of basal lamina or interstitial matrix components frequently leads to muscle pathologies (Carmignac and Durbeej, 2012): approximately 50% of congenital muscular dystrophies are the result of deficiencies in laminin-2 (congenital muscular dystrophy type 1A, or MDC1A), mutations in collagen IV have pleiotropic effects which include some forms of myopathy, and defects in collagen VI lead to Ullrich (the second most frequent form of congenital muscular dystrophy) and Bethlem myopathies (Bonnemann, 2011). While myopathies caused by defects in the structure of the endomysium itself are the most common in patients, disruptions in the connectivity between the myofiber cytoskeleton and the endomysium, including Duchenne's muscular dystrophy, collectively represent a larger class of mutations. In all of these cases, a direct molecular connection to satellite cell biology is unclear: structural proteins of mature muscle such as these are not expressed by undifferentiated satellite cells, but as the cells responsible for muscle repair and homeostasis they are necessarily affected by the chronic over-activation, inflammation, and alteration of the muscle environment itself that attend muscle

disease. As described below, there remain significant gaps in our understanding of what signals satellite cells receive from the muscle ECM and how they respond, even in healthy tissue; it is important to bear in mind that parallel studies on the signals prevalent in pathological muscle are also being conducted, although not discussed further here.

Signaling to satellite cells by the extracellular matrix

It has been argued that ECM signals are at least as important as soluble signals in regulating cellular determination, differentiation, proliferation, survival, polarity, and migration (Hynes, 2009). In the case of satellite cell-ECM signaling interactions, there are published examples of regulation and presentation of 'soluble' factors by the ECM, specific adhesion signaling, and biophysical stress or stiffness-induced signals that are each critical for satellite cell activity and function. It is likely that not only are all three signaling modalities directly affecting satellite cell activity, but that they are cooperative and interactive.

The niche of the quiescent satellite cell is composed of the sarcolemma of the host muscle fiber and the interior side of the basal lamina, thus any niche factors involved in maintenance of quiescence must derive from these two matrix sources: during quiescence, satellite cells are isolated electrically and chemically from both the myofiber cytoplasm and the extracellular environment (Bader et al., 1988). Because of the difficulties in studying quiescent satellite cells *in situ*, comparatively little is known regarding the input of the niche to satellite cell activity, however it has been established that disruption of the matrix components of the quiescent niche will negatively affect satellite cell activity (Ross et al., 2012) while preservation of the niche in cell transplant will dramatically enhance engraftment and function (Hall et al., 2010).

In contrast to the limited understanding of ECM signaling during quiescence, the critical role of the matrix in the earliest events of the satellite cell response has been well-established. Initial activation of the satellite cell occurs downstream of physical stretch (Tatsumi et al., 2006; Tatsumi et al., 2001) or rupture of the myofiber membrane and basal lamina (reviewed in (Wozniak and Anderson, 2007).) Nitric

oxide synthase-1 (NOS1 or N-NOS) is anchored to the myofiber sarcolemma by association with the dystrophin-glycoprotein complex and has mechanosensory as well as enzymatic functions: following myofiber stretch, a bolus of nitric oxide (NO) is released locally (Wozniak and Anderson, 2009). This leads to release of the active form of hepatocyte growth factor/scatter factor (HGF) (which is itself sequestered within the extracellular matrix (Tatsumi and Allen, 2004)), potentially through the activity of the matrix metalloprotease MMP-2 (Yamada et al., 2008; Yamada et al., 2006). HGF remains the only protein factor with the ability to directly activate satellite cells from quiescence (Allen et al., 1995; Tatsumi et al., 1998), thus these matrix-mediated events are critical to satellite cell activity.

Once activated, satellite cell proliferation and differentiation are modulated by multiple extracellular signaling pathways, the majority of which include a matrix component; however, this aspect of 'soluble' factor signaling is rarely taken into account in *in vitro* studies. For example, the binding of FGFs to their receptors requires specific sequences of heparan sulfates as components of the ternary complex (Rapraeger et al., 1994); transforming growth factor-β (TGF-β) ligands must bind to proteoglycans in the matrix to be 'presented' to their cellular receptors (Shi and Massague, 2003); and HGF complexes with its receptor c-Met can include fibronectin or vitronectin as well as integrins (Rahman et al., 2005). In particular, a requirement for ligand binding and presentation by proteoglycans, in *cis* or in *trans*, introduces an additional level of specificity to the organism, and analytical difficulty to the experimenter: carbohydrate chains are added and modified post-translationally via multiple different steps at different locations within the cell. The length, sequence, epimerization, and sulfation pattern of the sugars comprising the carbohydrate chains of matrix proteoglycans are extremely dynamic (reviewed in (Lindahl and Li, 2009))

and are also highly specific in their capacity for interaction with growth factors such as FGF (Walker et al., 1994). Thus, the colocalization of growth factor, receptor, and proteoglycan does not necessarily, or even usually, imply either ligand-receptor binding or downstream signaling activity (Allen and Rapraeger, 2003). Proteoglycan side chains are by far the most complex biopolymer, however current technology for analyzing even the sequence of carbohydrates lags dramatically behind that for DNA, RNA or protein (reviewed in (Li et al., 2012)). Unfortunately, realistic integration of this aspect of satellite cell-ECM signaling *in vivo* is therefore beyond the current capacity of the field. However, progress made primarily *in vitro* has identified roles for ECM molecules in directing the satellite cell response to soluble growth factors. Work in the Brandan lab has identified roles for the ECM proteoglycans decorin, biglycan, and dermatan sulfate in modulating the bioavailability and signaling potential of key growth factors including FGF-2 and HGF (Villena and Brandan, 2004), and TGF-β (Droguett et al., 2006); other groups have identified similar effects on myostatin (Zhu et al., 2007). It is important to note that these interactions are with extracellular proteoglycans that would presumably act in *trans*, unlike the interactions in *cis* that characterize cell-surface proteoglycans expressed by satellite cells themselves such as syndecans and glypican (Casar et al., 2004; Cornelison et al., 2001; Cornelison et al., 2004; Velleman et al., 2004).

In addition to ECM molecules produced by either muscle fibroblasts or differentiated myofibers, quiescent and activated satellite cells have themselves been shown to be a source of ECM components that impinge on multiple aspects of satellite cell activity. Most recently, an increase in fibronectin expression by satellite cells immediately following activation has been implicated in active remodeling of the local ECM to promote self-renewal by facilitating Wnt7a binding to a complex of

its receptor and the transmembrane heparan sulfate proteoglycan syndecan-4 (Bentzinger et al., 2013).

Another modality of signaling from the ECM is via integrins, with or without coordinated signaling of a growth factor receptor. Adhesion signaling to myoblasts/myocytes has been shown to be necessary for myogenesis, particularly in the case of differentiation. Hauschka and Konigsberg were the first to demonstrate a requirement for a specific ECM factor (collagen I) for differentiation of chick myoblasts (Hauschka and Konigsberg, 1966), and many studies since have highlighted the importance of both complete ECM and its components for differentiation of myogenic cell lines and primary myoblasts from developing chick and mouse (Clark et al., 1997; Grefte et al., 2012; Melo et al., 1996; Osses and Brandan, 2002; Stern et al., 2009). Integrins are heterodimeric transmembrane adhesion receptors with specificity for different ECM components based on the identity of the α and β chains they are comprised of (reviewed in (Humphries et al., 2006).) While satellite cells appear transcriptionally competent to express nearly all known integrin chains (Siegel et al., 2009), only a limited number of functional dimers have shown a biological activity or phenotype. Of these, the laminin receptor integrin α7β1 is the most prominent (reviewed in (Grounds et al., 2005).) Integrin α7β1 is localized throughout the sarcolemma, myotendinous junctions, and neuromuscular junctions of myofibers (Burkin and Kaufman, 1999) where it mediates adhesion to laminin as well as interacting with the dystroglycan-glycoprotein complex, syndecans, and sulfatides (reviewed in (Carmignac and Durbeej, 2012; Grounds et al., 2005; Mayer et al., 1997).) It is also considered a molecular marker for the satellite cell population even in quiescence (Conboy et al., 2010), where its primary role appears to be in migration (Chazaud et al., 1998; Echtermeyer et al., 1996; Siegel et

al., 2009), discussed further below. Of the other integrins expressed by satellite cells and their progeny, most have not yet been correlated with a molecular function, although activity in proliferation, differentiation, and fusion have been suggested. Making analysis more complicated is the key role many of these same integrins play in the maintenance and function of differentiated muscle: not only do integrin a7b1 deficiencies result in myopathy (Mayer et al., 1997), but changes in integrin a7b1 expression are associated with muscle pathologies caused by many other different mutations as well (Echtermeyer et al., 1996). Unraveling the roles of specific integrins *in vivo* within a tissue in which myofibers and satellite cells both coexist and co-express the same integrins will most likely require cell type-specific genetic deletions and careful downstream analysis, as affected satellite cells will give rise to affected and/or chimeric myofibers.

An aspect of satellite cell/ECM interactions that has only recently been closely examined is the biophysical influence of substrate stiffness and organization on satellite cell behavior. While physical stress has been widely understood to influence muscle differentiation and organization and the role of transient stretch in activation of satellite cells is well-established (see discussion of NOS above), the effects of two-dimensional versus three-dimensional culture (Grabowska et al., 2011) and varying substrate rigidity on satellite cell physiology are an emerging area of inquiry. Of particular interest, the Blau lab has recently described the effects of gradually altering substrate rigidity on multiple satellite cell activities including maintenance of stem cell potential and capacity for differentiation using single satellite cells cultured on a wide array of PEG hydrogels (Gilbert and Blau, 2011; Gilbert et al., 2010). Since the great majority of *in vitro* studies are still done in two dimensions on rigid surfaces which are either uncoated or coated with purified recombinant matrix factors, these

new systems may serve to highlight areas where our current models of potential *in vivo* signaling events may be incomplete or incorrect. This will be particularly important in understanding the differences in satellite cell activity associated with either pathological or aged muscle tissue (Leiter and Anderson, 2010; Yablonka-Reuveni and Anderson, 2006), since fibrosis and changes in matrix stiffness are characteristic of both of these conditions.

Experimental paradigms that should be of increasing interest in establishing physiologically-relevant biophysical influences on satellite cell activity include simple three-dimensional culture systems using hydrogel or collagen, three-dimensional culture in reconstituted native ECM, and decellularized tissue that can be seeded with live cells. While all three of these methods are currently in use, there are significant drawbacks to each: inert or single-component hydrogels are easily created and manipulated, but are necessarily lacking the majority of ECM-derived signals discussed above; purified and reaggregated muscle matrix lacks the ordered arrangement of matrix proteins as well as their carbohydrate side chains; and even decellularized muscle tissue implanted *in vivo* fails to provide an enhanced environment for myogenesis (Wolf et al., 2012). In this respect, the muscle field lags behind studies of other tissues in the ability to produce bioactive, biomimetic scaffolds for either research or clinical use, such as have been described for cartilage and bone (Kuo et al., 2006; Nooeaid et al., 2012; Ofenbauer et al., 2012). For many of the reasons outlined here, further development of three-dimensional systems replicating both the biochemical and biomechanical properties of native muscle matrix will be a key step in developing both physiologically relevant *in vitro* systems and therapeutically useful clinical tools.

Role of the extracellular matrix in satellite cell motility

As both the surface that motile cells adhere to and travel on and the substance that they must travel through, the different components of the ECM are critical influences on cell migration. Myogenic precursor cell motility is required for muscle development, during which emigration form the somites to the presumptive muscle fields is dependent on signaling of HGF via c-Met (Brand-Saberi et al., 1996). Members of the FGF and PDGF families have also been implicated in myoblast motility during development, and as discussed above all of these growth factors have requirements for matrix presentation and/or interaction to signal, however the role of the matrix in embryonic muscle development has not been widely addressed. Thus, this area represents the increasingly rare case in which cellular and molecular mechanisms are better understood in the context of regeneration than development. Once activated, satellite cells leave their quiescent position beneath the basal lamina then proliferate and migrate in the interstitial space before differentiating and fusing with a new or damaged myofiber (Figure 1-2.) This exit from the sublaminar space appears to rely on physical force rather than protease activity to create a tear in the basal lamina, as it can be blocked by preventing adhesion to laminin in *ex vivo* fiber culture using blocking antibodies but not by exposure to the broad-spectrum matrix metalloprotease inhibitor GM6001 (Siegel and Cornelison, unpublished results.) This results in both a change in the aspect of the basal lamina the satellite cells are in contact with, and exposure to the interstitial matrix with the accompanying potential for signaling. Laminin is the preferred substrate for satellite cell motility *in vitro* (Ocalan et al., 1988; Siegel et al., 2009), and engagement of laminin by α7β1 integrin is necessary for satellite cell motility on the surface of the myofiber (Siegel et al., 2009).

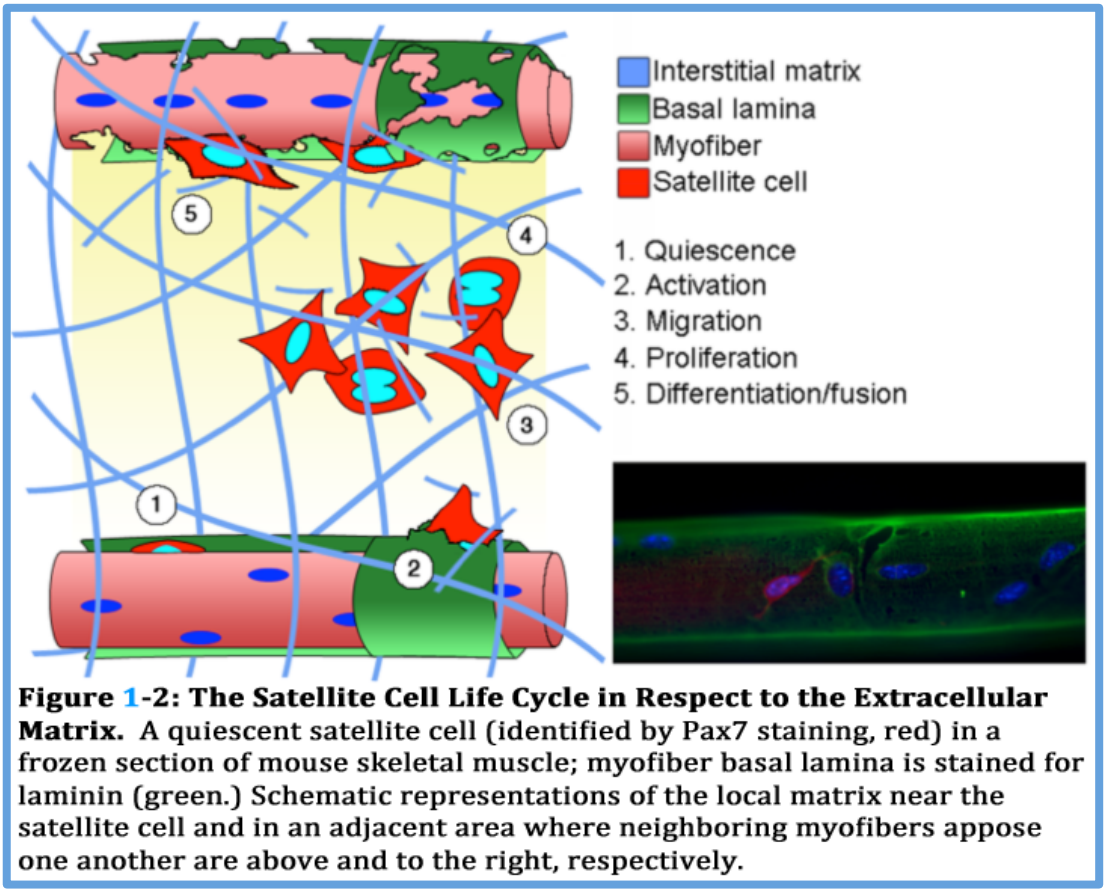

Figure 1-2: The Satellite Cell Life Cycle in Respect to the Extracellular Matrix. A quiescent satellite cell (identified by Pax7 staining, red) in a frozen section of mouse skeletal muscle; myofiber basal lamina is stained for laminin (green.) Schematic representations of the local matrix near the satellite cell and in an adjacent area where neighboring myofibers appose one another are above and to the right, respectively.

Once satellite cells have exited the niche and adhered to the exterior of the myofiber of the basal lamina, they are capable of extensive motility under *ex vivo* conditions: time-lapse observations have recorded cell velocities of up to 250 mm/hour (Siegel et al., 2009). However, it is important to note that this does not completely or accurately mimic an *in vivo* situation, because the single myofiber system does not include the interstitial matrix through which cells are required to navigate *in vivo*, additional cells present in a regenerating muscle, or any matrix or soluble factors not derived from the host myofiber.

The fact that satellite cells can and do move both along and between myofibers during regeneration *in vivo* is well-established (Hughes and Blau, 1990), however the question of whether muscle precursor cell migration is *necessary* for regeneration *in vivo* (as it is in development) remains open. While there are theoretically adequate local populations of satellite cells available to restore lost myonuclei without recruiting cells from more distal areas (Zammit et al., 2002), it is possible that the regeneration response could be both accelerated and enhanced by mobilizing additional cells. A role for long-range motility of satellite cells, including recruitment of cells from distal uninjured muscle (Klein-Ogus and Harris, 1983), was suggested based on early *in vivo* analyses of muscle regeneration. Evidence for long-distance activation and motility of satellite cells includes: detection of BrdU positive satellite cells in uninjured areas of the TA distal to a needle injury (Garrett and Anderson, 1995) and movement of satellite cells from distal uninjured areas towards a focal crush site (Schultz et al., 1985). In addition, after free grafting of large muscles, satellite cells will migrate from the central necrotic areas toward the periphery (Schultz et al., 1985) then back again after revascularization (Phillips et al., 1987),

and have been observed to migrate from the viable half of a longitudinally split autograft into the dead half (Phillips et al., 1990).

Several groups have described soluble factors that promote satellite cell motility and migration *in vitro* (reviewed in (Siegel et al., 2009).) Of these, the small chemokine SDF-1 is particularly intriguing: a key mediator of stem cell homing in several other tissues (reviewed in (Kucia et al., 2004; Lapidot et al., 2005; Miller et al., 2008)) it is expressed following injury by muscle-derived fibroblasts and is chemotactic to satellite cells (Ratajczak et al., 2003). The transmembrane heparan sulfate proteoglycan syndecan-4, which is expressed by satellite cells during both quiescence and activation (Cornelison et al., 2001), is the obligate coreceptor for SDF-1 along with CXCR4 (Charnaux et al., 2005), which is also a marker for satellite cells (Conboy et al., 2010): all three components of the ternary complex are implicated in satellite cell motility (Griffin et al., 2010), donor cell engraftment (Parker et al., 2012) and enhancement of muscle regeneration *in vivo* (Brzoska et al., 2012). HGF may also have a role in addition to initial activation: it has been characterized as a potent motogen in many systems including both developing and adult muscle (Bischoff, 1997; Brand-Saberi et al., 1996), once having been named 'scatter factor' for this activity (Gherardi and Stoker, 1991). It stimulates cell motility in developmental and physiologically normal contexts as well as being one of the primary signaling pathways hijacked during tumorigenesis and metastasis (reviewed in (Gao and Vande Woude, 2005; Trusolino et al., 2010).) This makes the HGF/c-Met axis a frequent target for therapeutic down-regulation or inhibition (Gherardi et al., 2012), and many tools have been developed in the context of tumor therapy that may also be useful for evaluating the role and requirement for HGF in satellite cell biology and motility.

The requirement for digestion and remodeling of the interstitial matrix to facilitate satellite cell migration *in vivo* is suggested by experiments in which matrix-modifying enzymes such as urokinase plasminogen activator (uPA) (reviewed in (Suelves et al., 2005),) MMPs (matrix metalloproteases) (Bedair et al., 2007; El Fahime et al., 2000; Pichavant et al., 2011; Torrente et al., 2000; Wang et al., 2009; Zimowska et al., 2012) or their inhibitors (TIMPS) (Lluri and Jaworski, 2005; Lluri et al., 2008) are experimentally manipulated to yield a positive effect on satellite cell spread and/or muscle regeneration. This would be consistent with the idea that mobilization and relocalization of satellite cells is either beneficial or necessary for successful muscle regeneration. However, while these results have been promising, the overall mechanism(s) by which matrix-modifying enzymes act to enhance satellite cell motility or activity remain elusive due to the promiscuous nature of their extracellular functions: indeed, the question of whether MMP activity should be enhanced or diminished is still highly dependent on the context of the *in vivo* experiment and the particular enzyme being studied. Significantly more work focusing on both endogenously-released soluble motogens and chemoattractants and matrix remodeling enzymes therefore remains to be done before a definitive role for satellite cell migration can be assigned. However, even in the absence of such a role during normal muscle regeneration, the difficulty in achieving adequate spreading of therapeutically-engrafted satellite cells or their progeny (Peault et al., 2007) makes a better understanding of local factors affecting satellite cell movement through the muscle tissue an important goal.

Signposts?

While it was not the authors' intent at the outset, a primary theme that emerged in writing this review is the emphasis of areas in which understanding the matrix's place in muscle regeneration is incomplete, potentially inaccurate, or currently impossible. Although the ECM clearly must play multiple critical roles in satellite cell-mediated myogenesis, its influence is rarely taken into account in *in vitro* studies, and is somewhat cryptic in many *in vivo* situations. While mimicking the properties of the muscle ECM is difficult, it should not be impossible: advances in decellularized organs (Murphy and Atala, 2013; Ofenbauer et al., 2012), purification of native muscle matrix for seeding myoblasts (DeQuach et al., 2012; Stern et al., 2009), and three-dimensional biomaterials-based culture systems (Gilbert and Blau, 2011; Grabowska et al., 2011) are promising developments that will ideally soon resolve many of our unanswered questions (and suggest new ones!)

FIGURE LEGENDS

Figure 1-1: The Structural Architecture of a Quiescent Satellite Cell and the Extracellular Matrix. A quiescent satellite cell (identified by Pax7 staining, red) in a frozen section of mouse skeletal muscle; myofiber basal lamina is stained for laminin (green.) Schematic representations of the local matrix near the satellite cell and in an adjacent area where neighboring myofibers appose one another are above and to the right, respectively.

Figure 1-2: The Satellite Cell Life Cycle in Respect to the Extracellular Matrix. An activated satellite cell (identified by CD34 staining, red) on an isolated mouse myofiber 18 hours post-isolation; myofiber basal lamina is stained for laminin (green.) Left, schematic representation of the local matrix interactions during multiple phases of satellite cell activity *in vivo*.

CHAPTER 2:

MMP-14 is necessary but not sufficient for invasion of three-dimensional collagen by human muscle satellite cells

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Modified from (Lund et al., 2014)

ABSTRACT

The twenty-five known matrix metalloproteases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteases (TIMPs), mediate cell invasion through the extracellular matrix (ECM). In a comparative 3D assay, we analyzed human and mouse satellite cells' competence to invade an artificial ECM (collagen I). We identified a single MMP (MMP-14) that: 1) is expressed by human muscle satellite cells; 2) is induced at the mRNA/protein level by adhesion to collagen I; and 3) is necessary for invasion into a collagen I matrix. Interestingly, murine satellite cells neither express this MMP, nor invade the collagen matrix. However, exogenous human MMP-14 is not sufficient to induce invasion of a collagen matrix by murine cells, emphasizing species differences.

INTRODUCTION

Mammalian adult skeletal muscle is composed of parallel, syncytial myofibers derived from the fusion of differentiated myocytes during fetal and postnatal development. Because the resulting myonuclei are terminally postmitotic, muscle growth, repair or regeneration requires muscle satellite cells, a population of muscle-specific precursor cells located between the sarcolemma and the basal lamina in uninjured muscle (Hawke and Garry, 2001; Mauro, 1961; Shi and Garry, 2006). Satellite cells are the obligate stem cell of skeletal muscle (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011): after activation by damage-induced local or systemic signals they will expand to generate a pool of differentiation-competent myocytes, which will then fuse to each other or to existing myofibers to replace or repair damaged muscle tissue. Although the soluble factors influencing satellite cell activation, proliferation, and to a lesser extent motility are increasingly well described (Cornelison, 2008), our understanding of the roles of signals derived from cell-matrix interactions and matrix remodeling associated with or required for satellite cell activity *in vivo*. The ECM in skeletal muscle is made up of both interstitial matrix (composed of different multiple collagen isoforms, fibronectin, hyaluronic acid, and proteoglycans such as perlecan) and myofiber-associated basement membrane (composed of organized layers of collagen IV, collagen VI, and laminin) (Gillies and Lieber, 2011; Grounds et al., 2005; Sanes, 2003). Collagen type I in particular is dynamically expressed in muscle by multiple different resident cell types, including fibroblasts, satellite cells, and differentiated myofibers (Goldberg and Green, 1967; Grounds, 1991). During skeletal muscle regeneration, satellite cells first exit their niche between the lamina and the sarcolemma, then transit on the myofiber lamina as they reposition to the site of injury. This would suggest that satellite cells

have the potential to traverse the interstitial matrix, and indeed satellite cells have been observed protruding from the lamina into the interstitial matrix as well as relocating between myofibers (Hughes and Blau, 1990).

Cell-matrix interactions are frequently bidirectional: binding of ECM proteins by cellular adhesion receptors such as integrins activates intracellular signaling pathways leading to changes in cell proliferation, cell shape, and cell motility (Hynes, 1999), but the ECM is also actively remodeled by secreted and cell-surface proteases during processes such as wound healing, fibrotic diseases, and scar formation. This remodeling not only changes the physical properties of the ECM, it liberates cryptic cleavage products that are then capable of signaling to local cells, including satellite cells (Agrawal et al., 2011). In addition to degradation and remodeling, deposition of new matrix proteins by local cells will modify the local signaling environment, and thus change the kinetics of tissue regeneration and/or remodeling. For example, recent work suggests that active production of fibronectin by satellite cells modulates their activity and stem cell status by altering their local niche signaling (Bentzinger et al., 2013).

Matrix metalloproteases (MMPs) are zinc-dependent endopepsidases that play a major role in the constructive proteolysis of the ECM, including collagen type I (Brinckerhoff and Matrisian, 2002; Visse and Nagase, 2003), and are required for cellular invasion through the ECM (Stetler-Stevenson et al., 1993; Werb et al., 1977). Twenty-five members of the MMP family have been identified; most are secreted, but six are membrane-type MMPs (MT-MMPs). MMPs are historically grouped as collagenases, gelatinases, stromelysins and matrilysins based on their respective specificity for ECM substrates (Egeblad and Werb, 2002). MMPs are responsible for the activation and processing of secreted molecules during ECM remodeling,

including chemokines, cytokines, and growth factors (Roy et al., 2009; Van Lint and Libert, 2007), which can in turn reciprocally activate MMPs (Allen et al., 2003; Hernandez-Barrantes et al., 2002; Stamenkovic, 2003; Woessner, 1991). In general, pathological activation of MMPs is implicated in inflammation, angiogenesis, and cell death, as well as in kidney disease, tumor proliferation and metastasis (Carmeli et al., 2004; Woessner, 1991). In skeletal muscle, MMP activity is implicated in both homeostasis and regeneration, and impinges on myofiber integrity, satellite cell activation and the adult as well as myoblast proliferation, migration and fusion during development (Chen and Li, 2009; El Fahime et al., 2000; Fukushima et al., 2007; Kherif et al., 1999; Ohtake et al., 2006; Vinarsky et al., 2005; Yagami-Hiromasa et al., 1995). Therefore, as would be expected, overall MMP activity increases upon injury, correlating with a decrease in intact ECM proteins (Chen and Li, 2009), and inhibition of MMPs also inhibits skeletal muscle regeneration (Vinarsky et al., 2005). In cell transplant studies, MMP activity has been implicated in migration of exogenous myoblasts, and is therefore a potential therapeutic target (El Fahime et al., 2000; Torrente et al., 2000).

In this study we show that human, but not mouse, satellite cells are competent to invade a 3-dimensional collagen type I matrix *in vitro*. We also show that expression of the membrane-type metalloprotease MMP-14 (also known as MT1-MMP) is necessary for this invasion. Interestingly, primary murine satellite cells neither express MMP-14 nor invade a collagen I matrix, highlighting the importance of MMP-14 activity for ECM invasion and myoblast migration *in* vivo. These data highlight one of several differences between species, and may identify an area of potential concern for the translation of murine results into human therapeutic perspective. In particular, these data might be useful in interpretation of satellite cell

transplant experiments in cases in which human and murine grafts have given differing results.

RESULTS

Human, but not mouse, satellite cells invade a 3D collagen type I matrix

We compared the potential of primary adult murine satellite cells and adult human satellite cells immortalized by expression of telomerase and cdk4 (Zhu et al., 2007) to invade a 3D collagen matrix, as an *in vitro* model of cellular movement through muscle tissue *in vivo*, murine and human satellite cells were seeded on the top of a 2% collagen type I matrix and cultured for ninety hours, after which the matrices were fixed, sectioned, and stained with Alexa 594-phalloidin to visualize satellite cell cytoarchitecture. Under these conditions, we observed that human satellite cells invade the collagen matrix an average of 87 μm (Figure 2-1A) based on at least five sets of independent runs (see Materials and Methods.) In contrast, primary murine satellite cells failed to invade any detectable distance (Figure 2-1B). The same result was seen in both cell types when the cells were first adhered to the culture plate then overlaid with 3D collagen I and challenged to invade 'up' (data not shown), opposite in terms of direction from the 3D collagen I invasion assay shown in Figure 2-1A.

To test whether poor adhesion to the collagen I prevented murine cells from initiating invasion, we compared adhesion of human and murine cells to both collagen and laminin. We have previously shown that murine satellite cells detectably express mRNAs for all integrin monomers except αE and αL, and that while laminin is the preferred substrate for primary mouse satellite cells as well as immortalized muscle cell lines they adhere to and migrate over collagen I as well (Siegel et al., 2009). Consistent with these data, murine satellite cells plated on collagen have a rounder morphology and fewer focal adhesions on collagen or gelatin than on laminin (Figure 2-1C), but are nonetheless adhered. Murine cells also failed to invade matrices

composed either of collagen I copolymerized with 50% or 75% laminin, or Matrigel, which is ~60% laminin (data not shown).

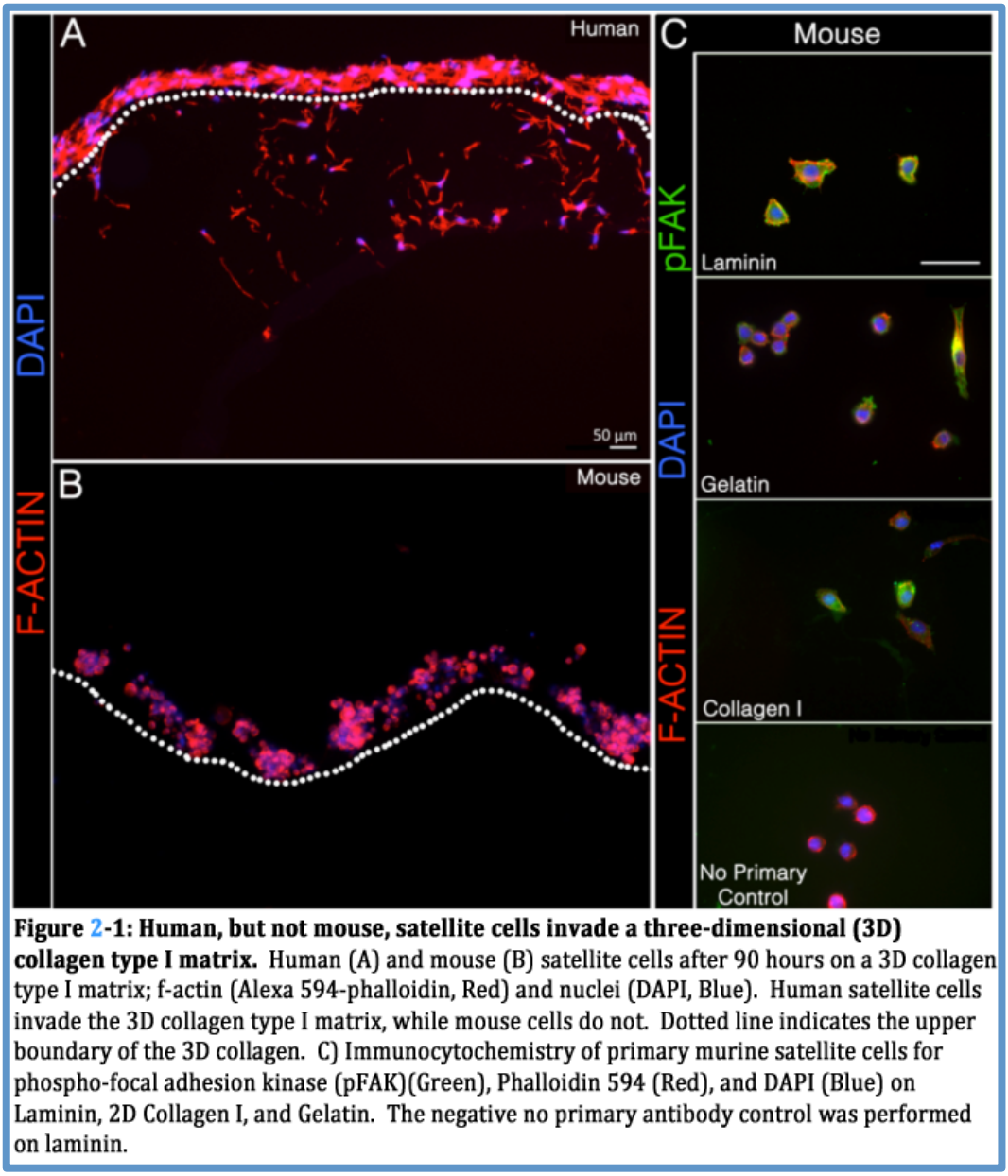

Figure 2-1: Human, but not mouse, satellite cells invade a three-dimensional (3D) collagen type I matrix. Human (A) and mouse (B) satellite cells after 90 hours on a 3D collagen type I matrix; f-actin (Alexa 594-phalloidin, Red) and nuclei (DAPI, Blue). Human satellite cells invade the 3D collagen type I matrix, while mouse cells do not. Dotted line indicates the upper boundary of the 3D collagen. C) Immunocytochemistry of primary murine satellite cells for phospho-focal adhesion kinase (pFAK)(Green), Phalloidin 594 (Red), and DAPI (Blue) on Laminin, 2D Collagen I, and Gelatin. The negative no primary antibody control was performed on laminin.

Invasion of collagen I by human satellite cells is MMP-dependent

While the primary mechanism of mesenchymal cell invasion is via matrix metalloproteases (MMPs), other proteases are expressed in muscle and could be active in remodeling the ECM and in the process of invasion of the matrix. To determine the specific participation of MMPs in satellite cell invasion, we treated human satellite cell cultures as above with a global MMP inhibitor, GM6001. This treatment completely prevented their invasion into the collagen matrix (Figure 2-2), indicating that one or more MMPs are essential for invasion. To validate that invasion is MMP-dependent, we treated human satellite cells in 3D cultures with recombinant Tissue Inhibitors of MMPs (TIMP-1, TIMP-2, and TIMP-3), the endogenous inhibitors of MMPs (Takino et al., 1995). Each TIMP binds to specific MMPs at a different rate and affinity, and blocks their catalytic activity: in general, TIMP-1 inhibits soluble MMPs, while TIMP-2 and TIMP-3 inhibit both soluble MMPs and membrane-type MMPs. TIMP-1 did not significantly inhibit invasion, but TIMP-2 and TIMP-3 both did, implicating membrane-type rather than soluble MMPs in this process (Figure 2-2).

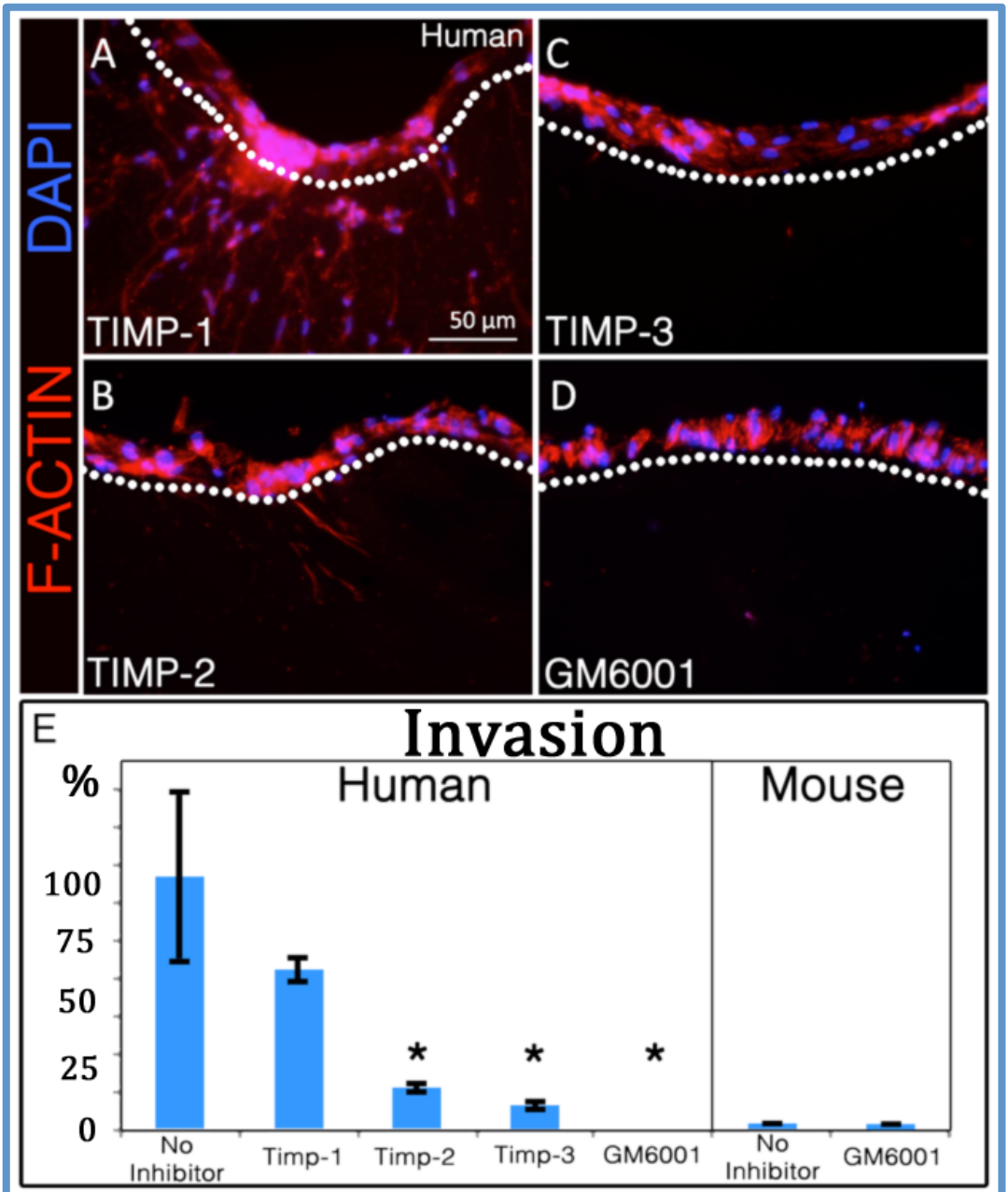

Figure 2-2: Matrix metalloprotease (MMP) inhibitors differentially inhibit human satellite cell invasion. Human satellite cell invasion in the presence of TIMP-1 (A), TIMP-2 (B), TIMP-3 (C), or GM6001 (D); all but TIMP-1 significantly inhibit invasion. Quantitative comparison of human and mouse satellite cell invasion (E) based on the fraction of cells invading beneath the surface of the collagen matrix, compared to all cells in the section, normalized to the no inhibitor control (100%) (n≥3). *P<0.05, **P<0.005. Statistically significant P values were based upon comparison of protease inhibitors to the no inhibitor positive control.

Human, but not mouse, satellite cells express MMP-14 when adhered to collagen I

The primary MMPs expressed in skeletal muscle are MMP-2, MMP-9, and MMP-14 (Balcerzak et al., 2001; Chen and Li, 2009). In other systems, MMP-2 and MMP-9 are required for invasion of angiogenic, endothelial, and metastatic cells through the ECM (Coopman et al., 1998; El Bedoui et al., 2005; El Fahime et al., 2000; Zhou et al., 2012). MMP-14 (also known as MT1-MMP) is more broadly associated with invasion by fibroblasts, endothelial cells, cancer cells, inflammatory cells, and leukocytes in addition to myogenic cells (El Bedoui et al., 2005; Galvez et al., 2004; Lee et al., 2007; Stetler-Stevenson, 1999; Stratman et al., 2009) and acts not only to degrade the ECM but also to activate other MMPs including MMP-2 (Werb, 1997) and MMP-9 (Toth et al., 2003). When we screened human and murine satellite cells for their expression of MMP-2, MMP-9, MMP-13, and MMP-14 mRNA using primers designed to amplify sequences from both species individually, and a cross-species spanning primer control (GAPDH), we found that while both express MMP-2, only mouse cells express MMP-9 and MMP-13, and only human satellite cells express MMP-14 mRNA (Figure 2-3A).

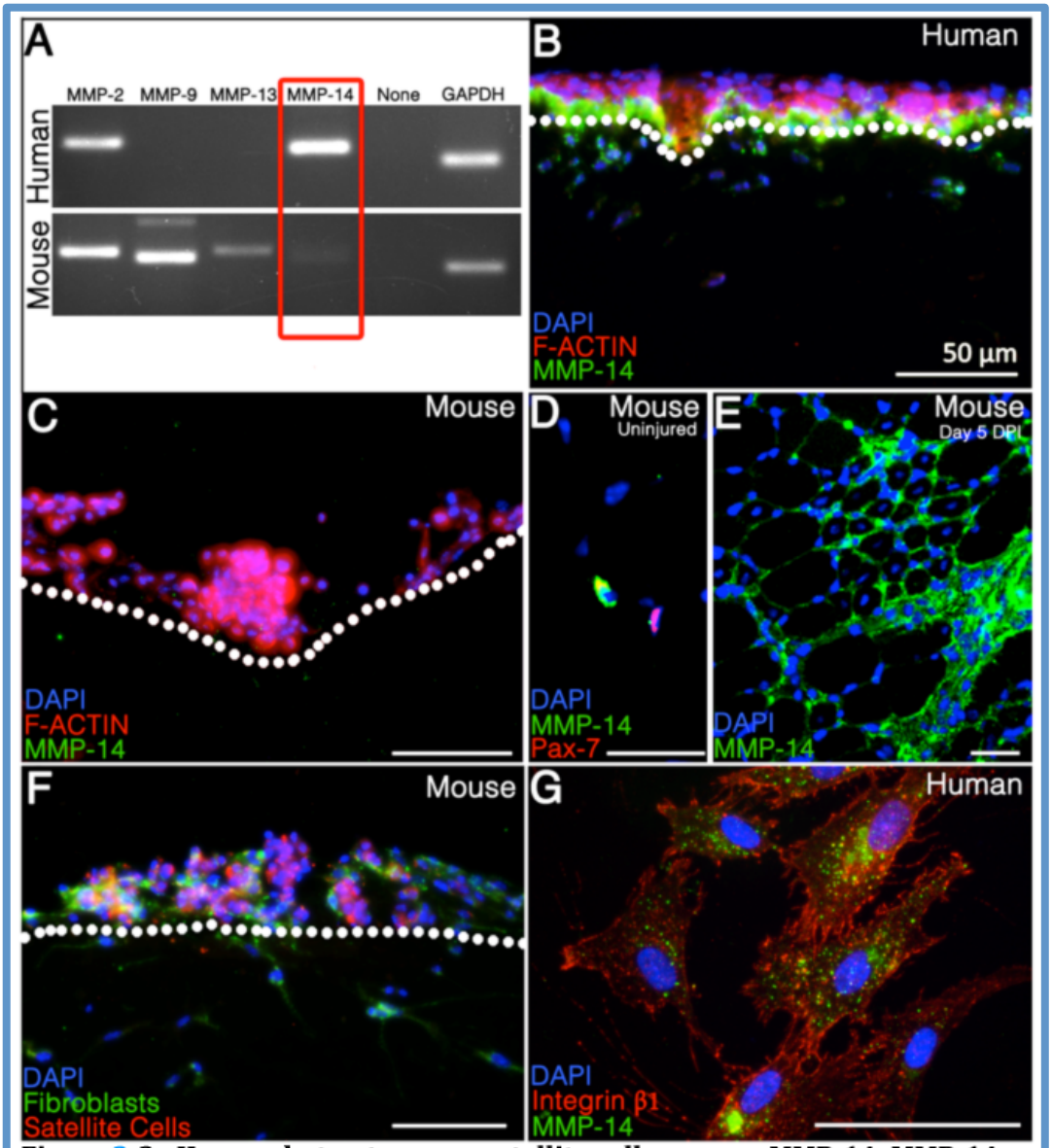

Figure 2-3: Human, but not mouse, satellite cells express MMP-14; MMP-14 expression in human satellite cells is dependent on contact with collagen I. A) RT-PCR for candidate MMP mRNAs and GAPDH as a positive control. B, C) Cross-sections of collagen I matrices on which human (B) or mouse (C) satellite cells were seeded and allowed to invade for 90 hours. Sections were stained for MMP-14 (green) and then labeled with Phalloidin 594 (red) and DAPI (blue); note the differential MMP-14 expression in human cells based on location adjacent to the collagen matrix. D) Cross-section of uninjured mouse tibialis anterior (TA) stained for Pax7 (red), MMP-14 (green), and DAPI (blue). E) Cross-section of mouse TA five days after BaCl injury showing high local expression between nascent myofibers in the area of injury. MMP-14 (green), DAPI (blue). F) Co-culture of ROSAmTmG mouse satellite cells (red) and unlabeled mouse fibroblasts on a collagen I matrix; all cells are stained for MMP-14 expression (green.) G) Human satellite cells were seeded onto gelatin-coated coverslips and analyzed for MMP-14 (green) and integrin β1 (red) colocalization.

Because of this exclusive expression of MMP-14 by human cells, we considered it a potential mediator of their invasive phenotype. To confirm protein expression and test for its localization, we stained sectioned matrices as above for MMP-14 by immunohistochemistry. Consistent with the mRNA results, human cells on 3D collagen I show robust expression of MMP-14 protein (Figure 2-3B) but murine satellite cells do not (Figure 2-3C). We confirmed that the antibody detects murine MMP-14 on uninjured muscle sections (Figure 2-3D) co-stained with antibodies to MMP-14 (green) and the satellite cell marker Pax7 (red), as well as on sections of hindlimb muscle five days after barium chloride-induced injury (Figure 2-3E).

We noted Pax7-negative, MMP-14-positive interstitial cells, possibly muscle fibroblasts, in the uninjured sections, raising the possibility that non-myogenic cells might act as a local source of MMP-14 in regenerating mouse muscle to compensate for the lack of expression by satellite cells. *In vitro*, primary mouse fibroblasts express MMP-14 protein (Rowe et al., 2011), so we asked whether co-culture with fibroblasts would enhance murine satellite cells' ability to invade collagen I. We isolated primary adult mouse muscle fibroblasts (Murphy et al., 2011) from a wild type mouse and co-cultured them with genetically labeled satellite cells isolated from a ROSA^{mTmG} mouse (Muzumdar et al., 2007) constitutively expressing membrane-localized tdTomato, to differentiate between the two cell types. The mixed cell population was plated as above on a collagen I matrix, and while the muscle fibroblasts both express MMP-14 and invade, the satellite cells do neither (Figure 2-3F). This suggests that primary mouse satellite cells do not invade collagen type I even in the presence of secreted MMPs and cleaved collagen fibers generated by other cell types.

Interestingly, the MMP14 protein expression in human cells is not homogeneous in that only cells in direct contact with the collagen matrix show significant protein expression. 3D collagen-dependent MMP-14 expression has previously been described *in vivo* and *in vitro* in lung fibroblasts, endothelial cells, and metastatic cells (Sabeh et al., 2004; Sakai et al., 2011; Toth et al., 2003); in at least one cell type where it was tested this expression is independent of both integrin activity and substrate stiffness (Sakai et al., 2011). Consistent with those data, we found that MMP-14 and β1 integrin do not co-localize on human satellite cells (Figure 2-3G). In addition, neither antibody neutralization of β1 integrin nor treatment with cyclic RGD peptides significantly inhibited invasion (data not shown). Thus, our data support an integrin-independent mechanism for invasion of 3D collagen by human satellite cells.

To ensure that the difference we observed between human and mouse satellite cells is not an artifact of the immortalized human satellite cell line, we repeated invasion assays using two different samples of non-clonal, non-transformed human primary myoblasts, as well as two immortalized murine myoblast cell lines. Primary human myoblasts generated at the Institute of Myology (http://www.institut-myologie.org/), one from a 5-year-5-day-old male (Figure 2-4A) the other from a 73-year-old female (Figure 2-4B), both express MMP-14 and invade into the collagen matrix. C2C12 myoblasts, the most commonly-used murine myoblast cell line (Yaffe and Saxel, 1977), also express MMP-14 and display a remarkable capacity for invasion into collagen (Figure 2-4C), MM14 myoblasts (Linkhart et al., 1980), which on the basis of morphology, gene expression, and myogenic capacity are the most similar to primary mouse satellite cells, did not invade although they do express low levels of MMP-14 (Figure 2-4D). C2C12 cells rapidly proteolyse 3D collagen I, as

indicated by cleavage-induced activation of fluorescent collagen I (Figure 2-4E, Supplemental Movie 2-1; summarized before the discussion). These characteristics may correlate with the tendency of C2C12s to form tumors when engrafted into host muscle (Rando and Blau, 1994).

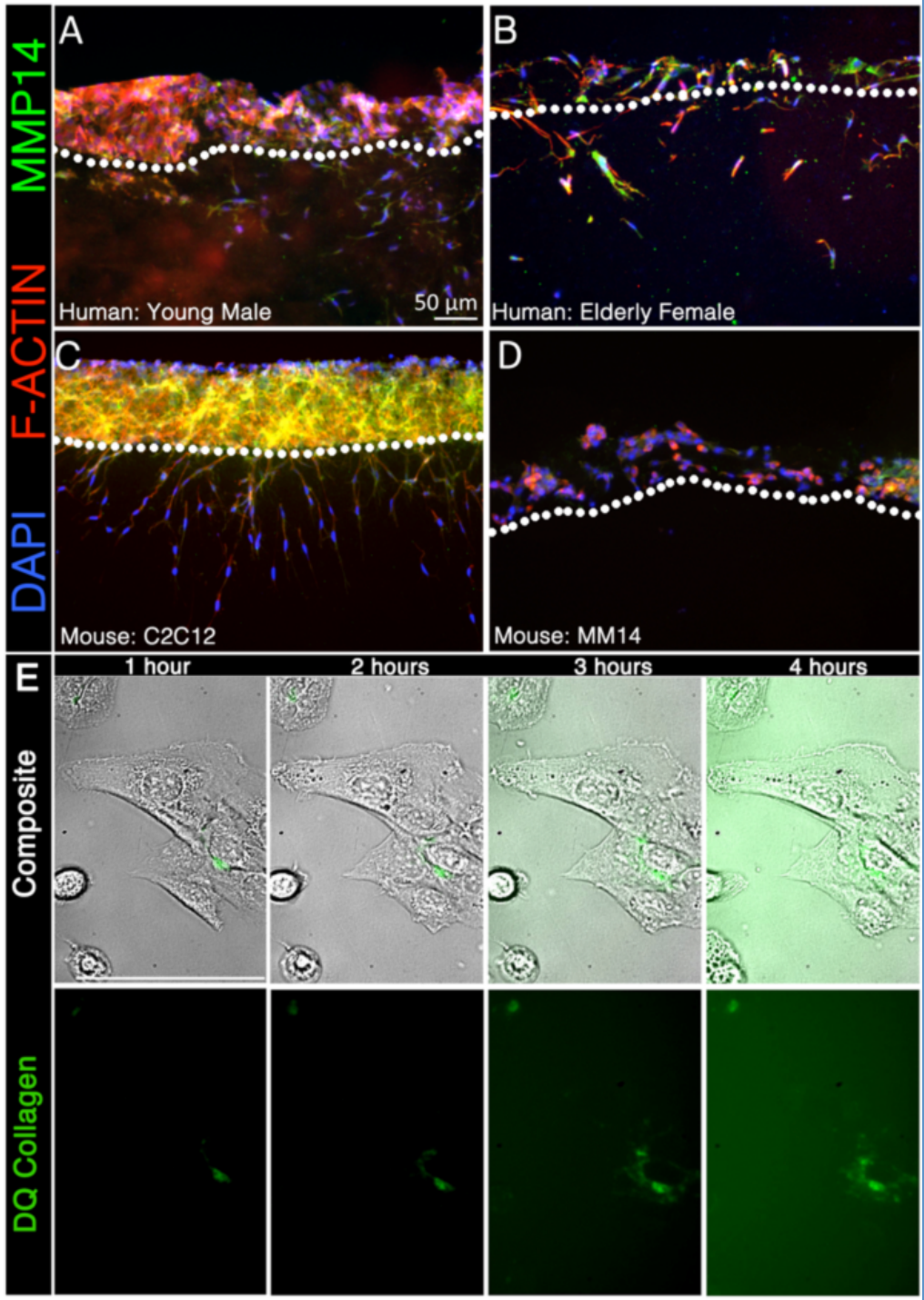

Figure 2-4: All human satellite cell populations tested express MMP-14 and invade collagen I, while murine cell lines differ from primary cells and each other. Two primary (nonclonal, nontransformed) human myoblast samples from a 5-year-5-day old male (A) and a 73-year-old female (B) and two immortalized murine myoblast cell lines (C2C12, C and MM14, D) were seeded on a 3D collagen type I matrix for 90 hours as above and stained for MMP-14 (green) with phalloidin 635 (red) and DAPI (blue). E) Images are reference stills from Supplemental Movie I. The panel illustrates collagen proteolysis (green) by C2C12 myoblasts over four hours. A composite (top) of the FITC (bottom) and bright field channels shows localized proteolysis respective to the cell. C2C12 satellite cells adhered to the bottom of a 48-well plate were challenged with 3D collagen I co-polymerized with DQ Collagen I {1mg/mL}(Green.)

MMP-14 is necessary but not sufficient for invasion of collagen I

To determine whether MMP-14 activity is necessary for human satellite cell invasion, we knocked down MMP-14 expression in human cells using targeted siRNA (Invitrogen). Knockdown was confirmed by immunocytochemistry and Western blot, and transfection efficiency (98%) was verified through BLOCK-iT™ Alexa Fluor® Red Fluorescent Control Oligo. Transfection with MMP-14-specific (Figure 2-5A,B) but not scrambled (Figure 2-5C,D) siRNA significantly reduced expression of MMP-14 protein as well as human satellite cell invasion through collagen I, and this reduction is dose-dependent (Figure 2-5E-G). These data indicate that MMP-14 is necessary for invasion of collagen I by human satellite cells, which could be either direct cleavage of matrix substrates or proteolytic activation of other collagenases, or both.

Reduction of MMP-14 activity also reduces the level of active MMP-2 when assayed by gelatin zymography (Figure 2-6A), raising the possibility that the requirement for MMP-14 is through MMP-2. Human satellite cells also displayed an elevated amount of TIMP-2 mRNA when compared to primary murine satellite cells (Figure 2-6B), and it is important to note that TIMP-2 is required for activation of MMP-2 in conjunction with MMP-14 dimerization (Bernardo and Fridman, 2003).

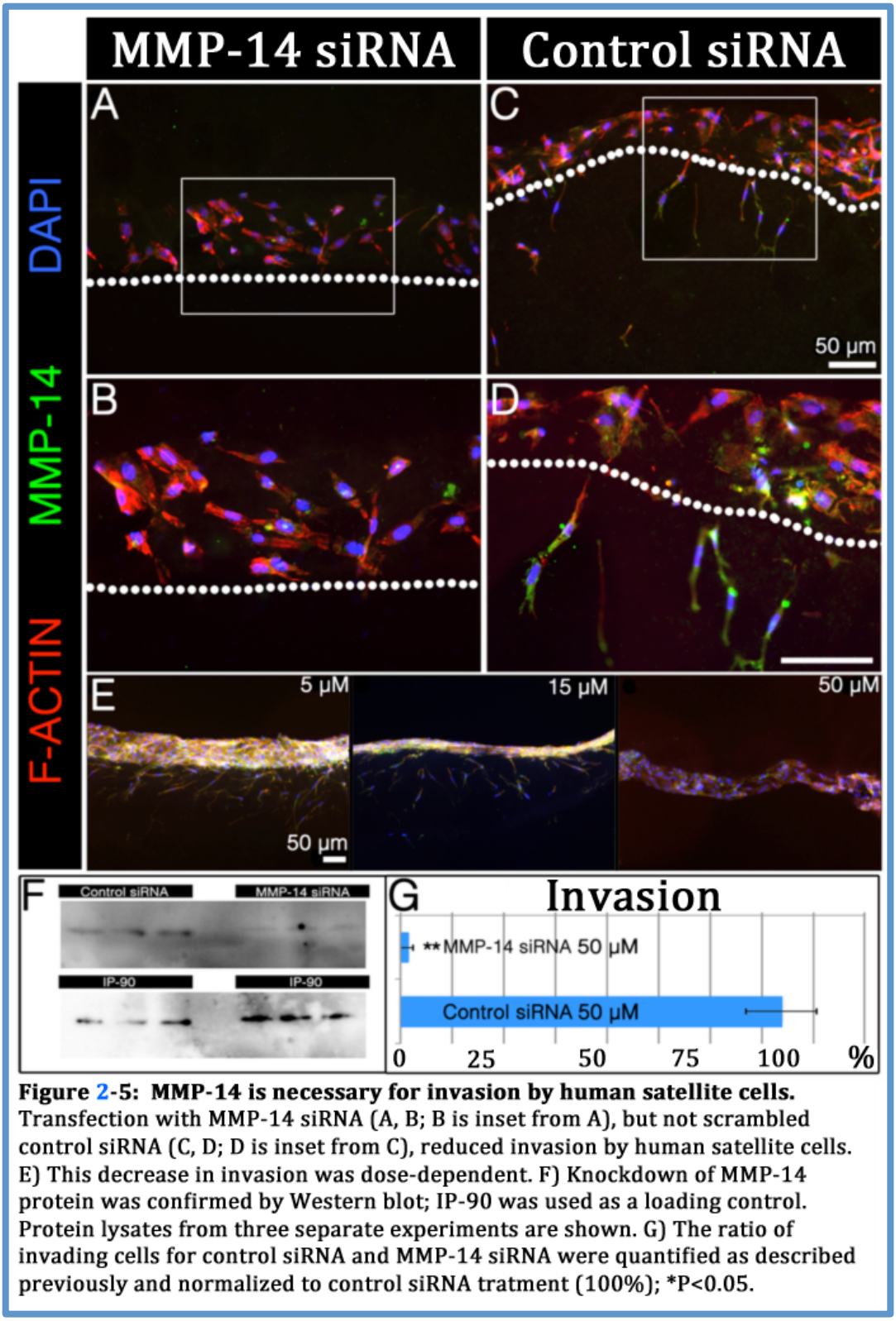

Figure 2-5: MMP-14 is necessary for invasion by human satellite cells. Transfection with MMP-14 siRNA (A, B; B is inset from A), but not scrambled control siRNA (C, D; D is inset from C), reduced invasion by human satellite cells. E) This decrease in invasion was dose-dependent. F) Knockdown of MMP-14 protein was confirmed by Western blot; IP-90 was used as a loading control. Protein lysates from three separate experiments are shown. G) The ratio of invading cells for control siRNA and MMP-14 siRNA were quantified as described previously and normalized to control siRNA tratment (100%); *P<0.05.

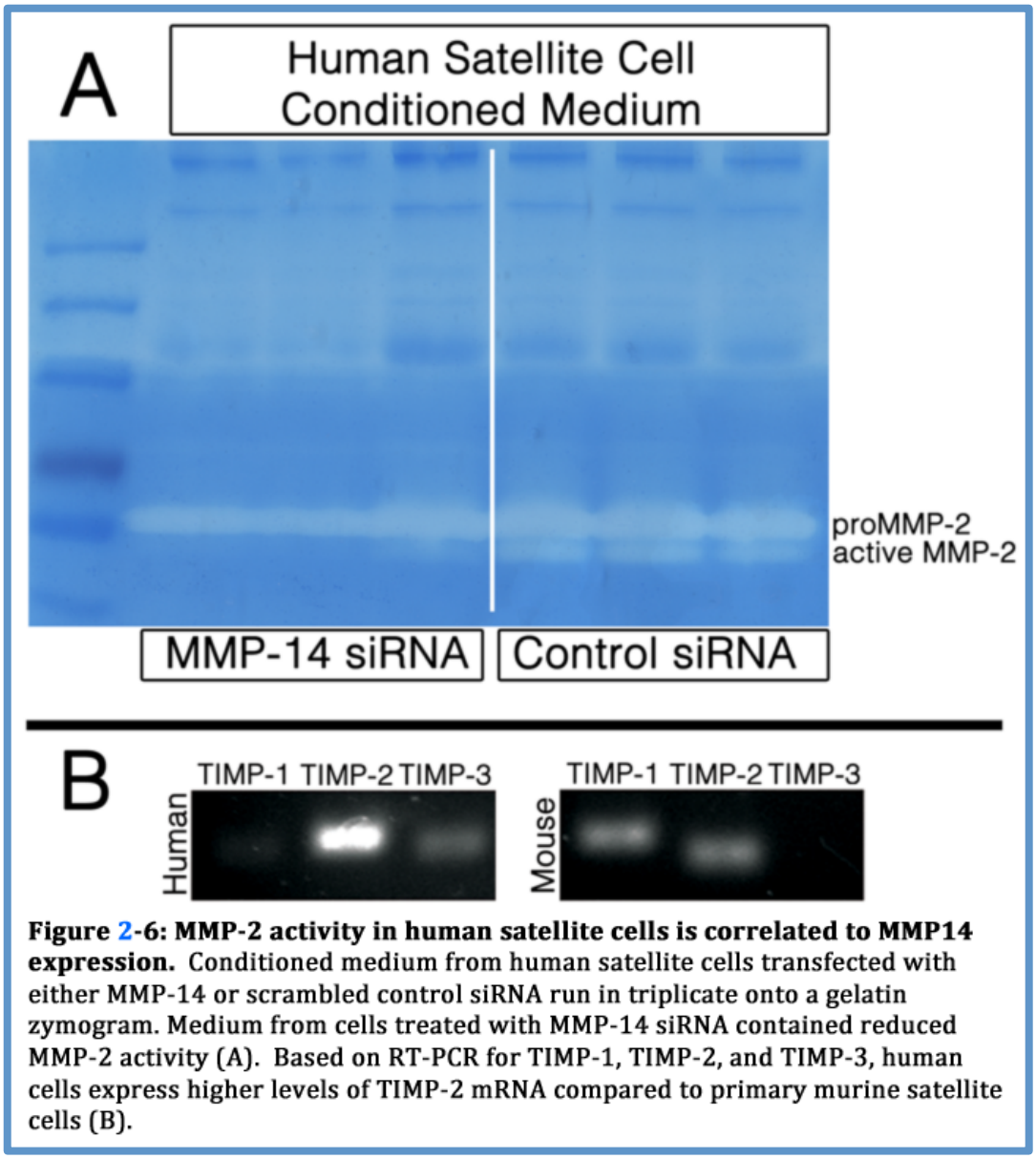

Figure 2-6: MMP-2 activity in human satellite cells is correlated to MMP14 expression. Conditioned medium from human satellite cells transfected with either MMP-14 or scrambled control siRNA run in triplicate onto a gelatin zymogram. Medium from cells treated with MMP-14 siRNA contained reduced MMP-2 activity (A). Based on RT-PCR for TIMP-1, TIMP-2, and TIMP-3, human cells express higher levels of TIMP-2 mRNA compared to primary murine satellite cells (B).

To test whether MMP14 is sufficient to permit invasion of 3D collagen, we asked whether ectopic expression of MMP-14 in primary murine satellite cells would confer an invasive phenotype. Interestingly, even primary murine satellite cells expressing human MMP14 did not invade (Figure 2-7). Coupled with the lack of invasion by MMP-14-expressing MM14 myoblasts noted above, we conclude that MMP-14 is necessary but not sufficient in this system. It is possible that the decreased levels of TIMP-2 we noted in murine cells may contribute to the lack of invasion.

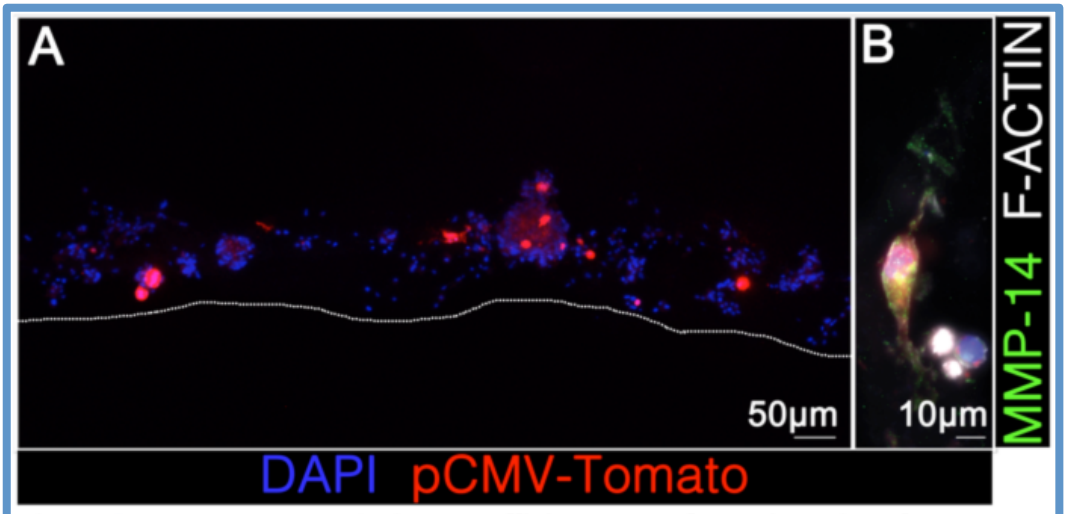

Figure 2-7: Human MMP-14 is not sufficient to confer an invasive phenotype on primary murine satellite cells. Primary murine satellite cells were transfected with human MMP-14 or empty vector (control) with pCMV-TOMATO (red) as a transfection marker. Phalloidin 633 (white, B) and DAPI (blue) (A, B) were used to visualize whole cells seeded onto the matrix.

DISCUSSION

Surprisingly, given the key role played by satellite cell-ECM interactions during muscle homeostasis and repair (Lund and Cornelison, 2013a), analysis of the suite of proteins secreted by satellite cells for matrix modification *in vivo* is not yet well characterized. To date, the only published secretome for murine myoblasts was made using the C2C12 cell line (Henningsen et al., 2010), which is not an ideal model for primary myoblasts (Grabowska et al., 2011; Lindsell et al., 1995). The secretome from differentiating human myocytes was recently published (Le Bihan et al., 2012): of the 253 soluble secreted proteins identified by gel-free nano-flow LC-MS/MS analysis, 72 are matrix modifying enzymes or structural matrix proteins- for comparison, in the same set, there are only 25 secreted growth factors represented. The presence of both constructive and destructive modulators of the ECM suggests a dynamic interplay between the local matrix and satellite cells, which we interpret to be remodeling and not simply proteolysis.

Matrix remodeling by MMPs contributes to adult tissue homeostasis and stem cell-mediated regeneration (Alwayn et al., 2008; Chen and Li, 2009; Ding et al., 2011; Filous et al., 2010; Hsu et al., 2008; Visse and Nagase, 2003); MMP-14 in particular is required for regeneration in multiple tissues (Atkinson et al., 2007; Chan et al., 2012; Ding et al., 2011; Ohtake et al., 2006; Watanabe et al., 2001). In the context of skeletal muscle, other membrane type MMPs (MMP-2 and MMP-9 as well as MMP-14) have been implicated in muscle homeostasis (Ohtake et al., 2006) as well as in satellite activation after damage (Fukushima et al., 2007; Kherif et al., 1999) and motility *in vivo* (El Fahime et al., 2000). MMP-14 is directly or indirectly required for activation of membrane-type proteases (i.e. MMP-2 and MMP-9), secreted proteases (i.e. MMP-13) (Sabeh et al., 2004; Seomun et al., 2008; Yaffe and Saxel, 1977) and also activates multiple chemokines, cytokines, and growth factors

that would be expected to impinge on muscle regeneration (Hernandez-Barrantes et al., 2002; Stamenkovic, 2003). Thus, MMP-14 has the potential to affect muscle regeneration by multiple distinct mechanisms, and would not be limited to directly promoting matrix invasion.

It is important to note that these *in vitro* experiments do not fully represent multiple aspects of muscle regeneration *in vivo*, and accordingly care should be taken in their interpretation. We did not assess satellite cell activity on or in response to a bona fide muscle ECM *in vivo*, which is not simply a more complex mix of proteins but is also organized, cross-linked, and polarized in patterns that are difficult to replicate *in vitro* (Lund and Cornelison, 2013b). Attempts to modify the experimental system by using Matrigel (a more complex ECM mixture) instead of collagen I produced the same results (data not shown.) However, our own data suggest that murine satellite cells that have been continually in contact with a native matrix or their host myofiber may transit through 3D collagen (Siegel et al., 2009). This may be a contributing factor to the dramatically enhanced engraftment and spread of satellite cells when single fibers, rather than monoculture satellite cells, are injected into host muscles (Hall et al., 2010).

Muscle connective tissue fibroblasts, which are a local source of both extracellular matrix components and matrix-modifying enzymes *in vivo* and are necessary for robust satellite cell-mediated muscle regeneration (Murphy et al., 2011), were included in coculture experients to ask if they provided a key component of the *in vivo* environment. Although they did not enhance myoblast invasion in our experimental system, a role for fibroblast-specific effects on the matrix *in vivo* is still strongly suggested by the work of other groups. Inflammatory cells including leukocytes and macrophages express MMPs including MMP-14 (Bar-Or et al., 2003;

Newby, 2008) and pericytes, which may participate in muscle regeneration independently of satellite cells (Dellavalle et al., 2007), require MMP-14 for key processes during tissue growth and remodeling (Stratman and Davis, 2012); these cell types were not tested in our *in vitro* model. Our inability to induce invasion by murine cells with ectopic expression of MMP-14 also does not rule out the possibility that other membrane proteins are necessary in an MMP-14 containing proteolytic complex, and thus MMP-14 alone would not be sufficient.

Our results also serve to reinforce the need for caution in generalizing from animal models to human physiology. It is well-established that muscle regeneration differs between mice and humans, particularly in the context of disease (reviewed in (Boldrin et al., 2010)) or therapy (Bachrach et al., 2004; Boldrin et al., 2012; Galvez et al., 2006; Partridge, 2013). Potential cell-based therapies are frequently tested either by engrafting murine satellite cells into immunodeficient mice (El Fahime et al., 2000; Morgan et al., 2010; Riederer et al., 2008) or by doing the same with human cells (Riederer et al., 2012; Silva-Barbosa et al., 2008; Silva-Barbosa et al., 2005); our data suggest caution both in interpreting the results of these experiments and in generalizing mouse-mouse or human-mouse grafts to human cells engrafted in human patients. One of the confounding difficulties in developing such therapies to date has been failure of engrafted cells to spread away from the point of injection (Peault et al., 2007), thus a key area of interest is in enhancing migration and invasion. In published studies of simple 2D motility, murine satellite cells are significantly more motile than human (Jansen and Pavlath, 2006; Siegel et al., 2009), however in the current study murine cells are also significantly less competent to invade a three-dimensional matrix; neither set of results adequately explains the differences observed between species in satellite cell transplantation studies.

We have not tested for species-specific differences in MMP-14 expression and activity in nonmuscle cell types. Because both its matrix remodeling and intracellular signaling activites play key roles in multiple physiological and pathological processes including angiogenesis and tumor metastasis (Carmeli et al., 2004; Woessner, 1991), it would be important to know whether any such differences exist in other contexts. If the phenotype is widespread, it may necessitate a re-examination of existing research outside the muscle field as well.

CHAPTER 2 ADDENDUM:

Dystrophic and Healthy Human Satellite Cells Behave Identically in their Interaction with a 3D Collagen Type I Matrix and MMP Expression

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INTRODUCTION

As noted previously, satellite cells are required for skeletal muscle regeneration and homeostasis (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011).. In diseased muscle, Duchenne muscular dystrophy (DMD) specifically, myogenesis is not compensatory to atrophy; eventually gradual atrophy of skeletal muscle results in premature death, usually due to fiber loss in the diaphragm or cardiac problems (Goldstein and McNally, 2010). DMD is the most common form of muscular dystrophy: it is an early-onset autosomal recessive disease effecting roughly 1 in 3,600 males (Emery, 1991). Muscle fibers are lost due to loss, truncation, or mutation in the dystrophin protein, which provides a link between the muscle cell membrane and the extracellular matrix (Cox et al., 1993; Petrof et al., 1993; Rybakova et al., 2000). Loss of this connection leads to a lack of structural support required for proper muscle function as well as changes in cell signaling. Importantly, when muscle fibers are lost, the area is repopulated with dense fibrotic tissue (fibrosis), or adipose tissue (Klingler et al., 2012). The accumulation of fibrotic tissue is the characteristic of many different dystrophies and myopathies. Much of the fibrotic tissue is composed of dense regions of collagen proteins, predominantly collagen type I (Mann et al., 2011). Alleviating fibrosis has shown great promise in delaying myopathy progression (Bo Li et al., 2012), but while this approach has been used to reverse cardiac and diaphragm abnormalities in the mouse model for DMD (the *mdx* mouse), there has been comparatively little work done with human cells. Satellite cell function in this pathogenic environment is expected to be abnormal as well (Boldrin et al., 2015; Emery, 2002; Morgan and Zammit, 2010). In Chapter 1, we highlighted the ability of the ECM to alter/direct cell behavior. We hypothesize that chronic exposure to a progressively more fibrotic environment may also perturb

satellite cell function. Specifically, the mechanisms and pathways by which satellite cells sense and respond to the surrounding ECM may have changed due to either adaptation or exhaustion. If such changes occur, they would potentially affect the efficacy of therapies aimed at simply replacing dystrophin in satellite cells and/or differentiated muscle fibers, without also modifying other aspects of satellite cell function. To look for changes in satellite cell/ECM interactions, we compared the gene expression profiles, invasive capacity into 3D collagen type I, and proteolytic capacity of satellite cells derived from DMD patients with wild type satellite cells. Surprisingly, we found no significant differences in these areas, suggesting that there may be no intrinsic changes in the ECM-modifying activity of human satellite cells from patients with even highly advanced dystrophy.

RESULTS

Satellite cells from dystrophic and healthy individuals have identical expression of MMP/Collagen mRNAs

We compared the expression of 23 of the 25 known MMPs, all three TIMPs, and nine collagen chains associated with various myopathies (Jobsis et al., 1996) via RT-PCR in human satellite cells from a nondystrophic control (KM155C25Dist) and three patients with dystrophy [DMD5-13515C15:37 (DMD), KM571DMD10FLCL1:48 (DMD with specific loss of exon 52), and KM390BMD196CL2:41 (Becker's Muscular Dystrophy, BMD)]. Primers used to detect MMP mRNA were published, validated primers that had been generated to screen for expression in primary human cancer cells (Kohrmann et al., 2009). Out of the 35 genes screened, we noted no significant differences (Figure 2-8), suggesting that satellite cells from dystrophic individuals maintain their proteolytic machinery for ECM negotiation during invasion, despite their long-term exposure to a fibrotic environment.

Satellite cells from dystrophic and healthy individuals have identical expression of MMP-14 in 2D culture

In Chapter 2, we demonstrated the requirement for MMP-14 in human satellite cell invasion into 3D collagen type I matrices (Lund et al., 2014). We first compared expression and localization of MMP-14 protein in the control and dystrophic myoblasts. We found that each cell line has very similar localization/trafficking of MMP-14 (red) around concentrated regions of the nucleus to the extending edges of their respective filopodia (Figure 2-9). This expression is considered a defining characteristic of invasive filopodia, now known as invadopodia (Machesky, 2008). While cells from each line cells vary slightly in morphology (Figure 2-9) and proliferate and move at varying rates (data not shown), there appears to be no significant difference in their expression or localization of MMP-14 (Figure 2-9).

Satellite cells from healthy and dystrophic individuals invade a 3D collagen type I matrix

Based on the differences in invasive capacities we observed in Chapter 2 (Lund et al., 2014), we also chose to quantify invasion into 3D collagen by dystrophic myoblasts. Potentially, they could either have increased invasiveness (adaptation) or decreased invasiveness (exhaustion) as a consequence of chronic exposure to a fibrotic ECM environment in the patients they were derived from, although we had not detected molecular signs of either at the mRNA or protein expression level. However, we saw that each dystrophic myoblast cell line examined displayed an equal capacity to traffic MMP-14 to their respective invadopodia while completely embedded in the 3D matrix (Figure 2-10) and invaded into the collagen matrix to the same extent as the control cells.

In contrast, when we compared MMP activity in conditioned medium collected from each human satellite cell line grown on 3D collagen, we noted differences that correlate with the presence and severity of the condition. As reported in Chapter 2, healthy human satellite cells express active and latent MMP-2, and MMP-2 activation is inhibited when MMP-14 expression is reduced via siRNA. Interestingly, DMD5-13515C15:37 (DMD) and KM571DMD10FLCL1:48 (DMD with specific loss of exon 52) show qualitatively lower levels of active MMP-2 when compared to KM390BMD196CL2:41 (Becker's Muscular Dystrophy, BMD) and KM155C25Dist (healthy satellite cell) (Figure 2-11).

Taken together, these data suggest that dystrophic human satellite cells maintain a similar ability to interact with and invade through the ECM, despite long-term exposure to a fibrotic environment, further highlighting that perturbed

myogenesis in myopathies is not solely based on the intrinsic abilities of the satellite cells, but the microenvironment itself.

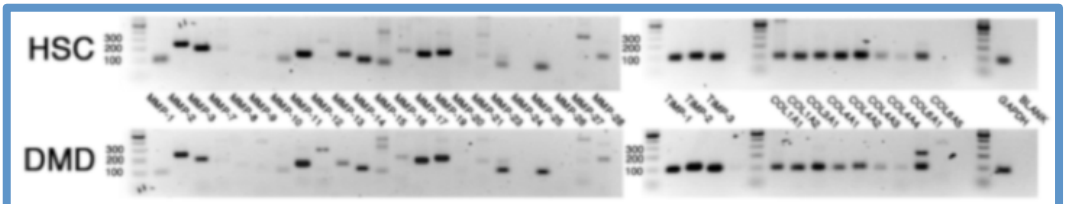

Figure 2-8: Satellite cells from dystrophic and healthy individuals have identical expression of MMP/Collagen mRNA. Various healthy and dystrophic human satellite cells' candidate MMP/Collagen mRNA visualized via RT-PCR.

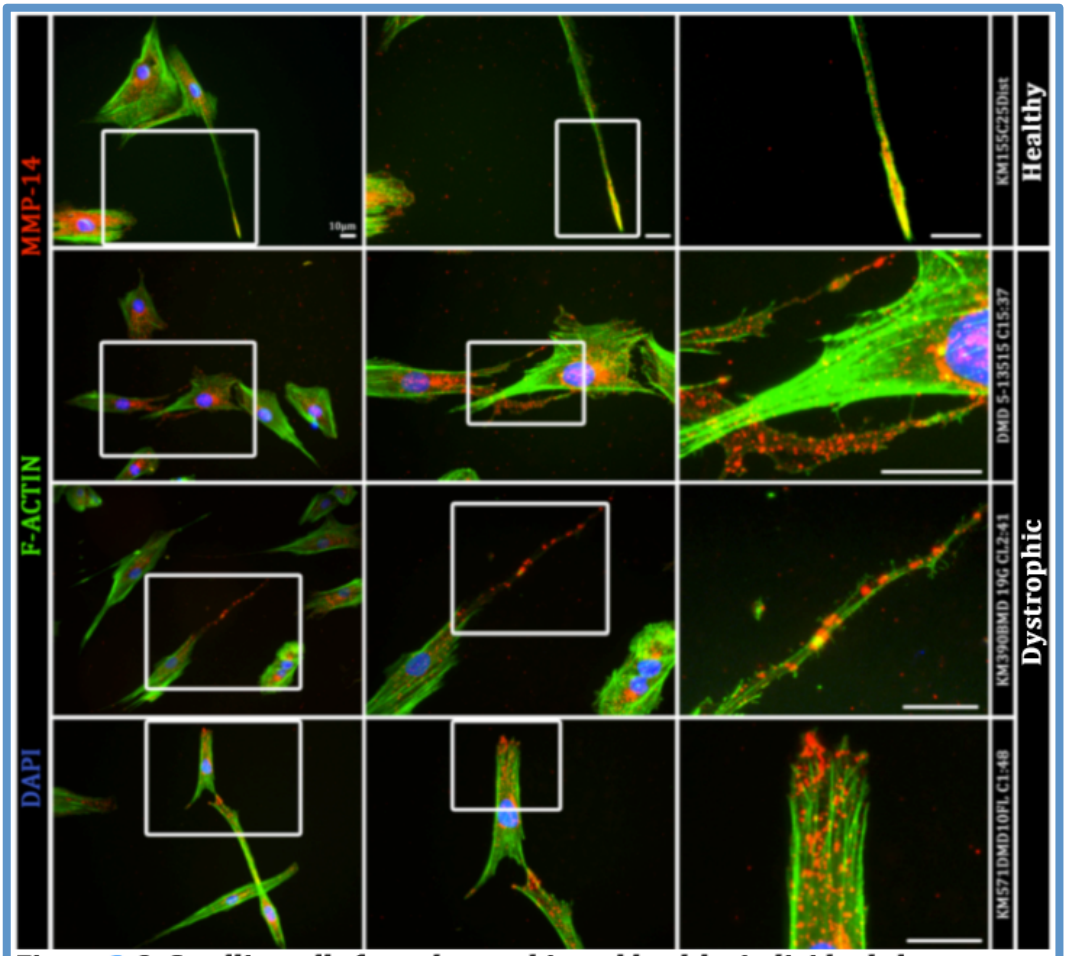

Figure 2-9: Satellite cells from dystrophic and healthy individuals have identical expression of MMP-14 in 2D cultrue. Various healthy and dystrophic human satellite cells were placed on gelatin-coated coverslips; MMp-14 (red), f-actin (green), and nuclei (DAPI, blue) are illustrated through immunocytochemistry. All immunocytochemistry was performed in coordination with no primary antibody negative controls.

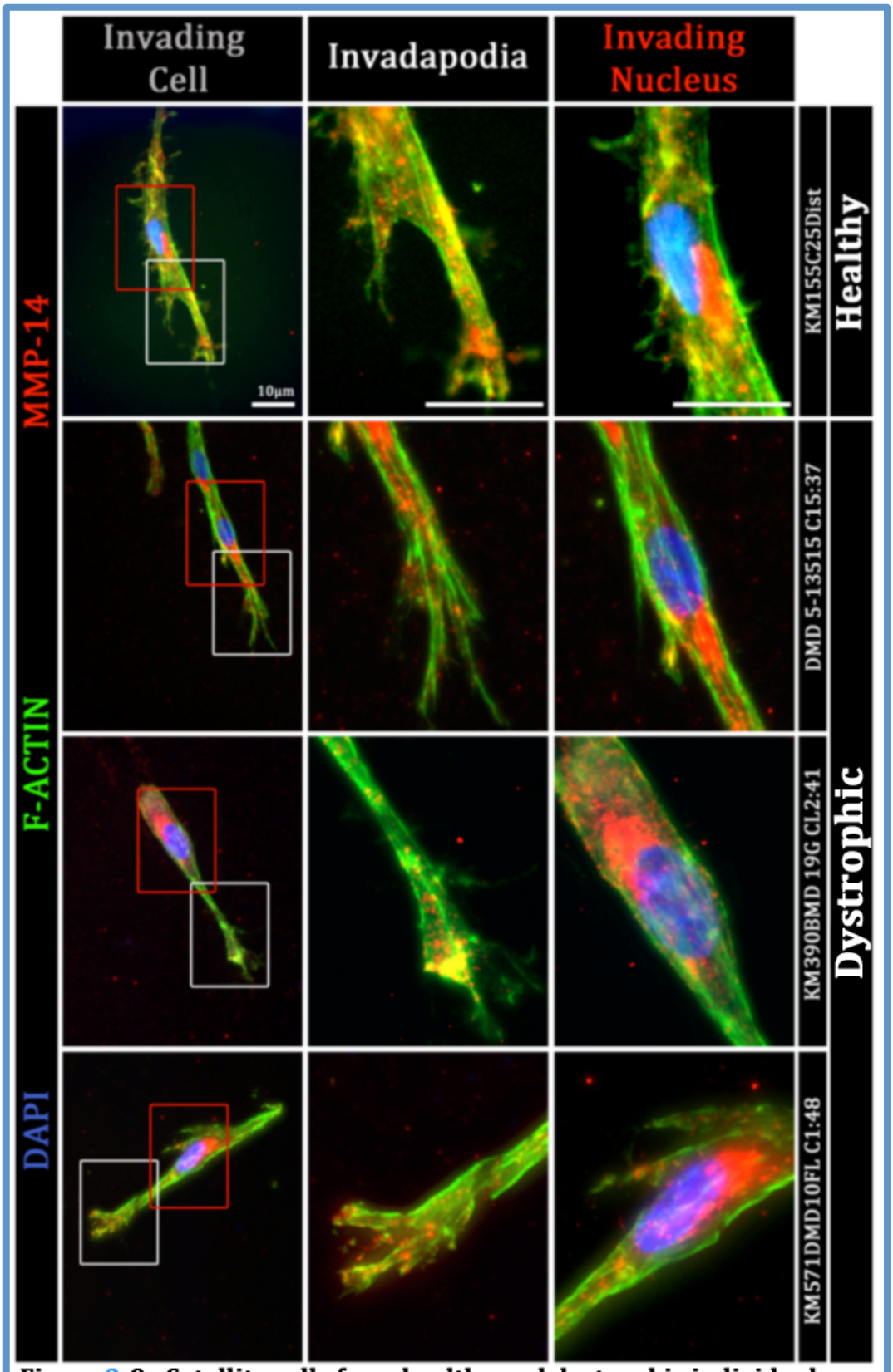

Figure 2-9: Satellite cells from healthy and dystrophic individuals invade a 3D collagen type I matrix. Various healthy and dystrophic human satellite cells were seeded on top of a 3D collagen type I matrix for 90 hours. Images depict invading cells of these respective samples. Highlighted are the invadapodia and nuclei (blue) of these respective cells.

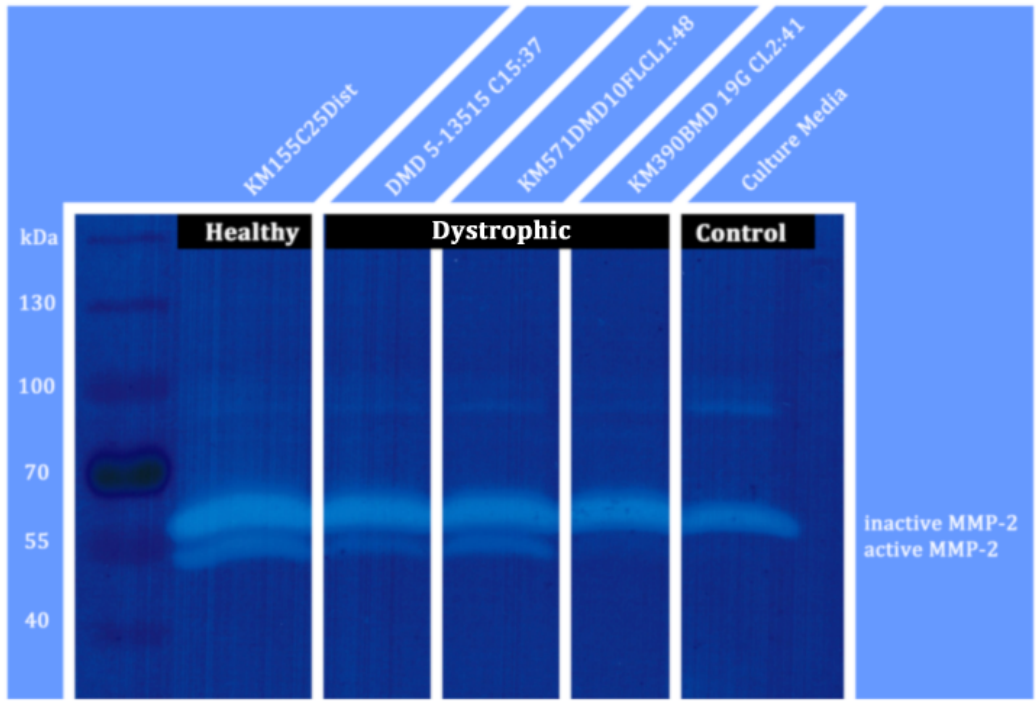

Figure 2-11: Satellite cells from dystrophic and healthy individuals have variable soluble MMP-2 activity. Conditioned medium from human satellite cells , dystrophic and healhty, run on a gelatin ymogram. MMP-2 enzyme can be seen in white, while the gelatin is blue.

DISCUSSION

Fibrosis has long been attributed with myopathy, but it is unclear how the perturbed ECM effects satellite cell function (Mann et al., 2011). We chose to compare cells from DMD and BMD patients with control cells based on the prevalence of fibrotic tissue in dystrophic muscle environments. Due to the correlation of abundant matrix accumulation and lack of myogenesis, the overlap between myopathy and ECM diseases, and the ability of the ECM to modulate cell behavior we hypothesized that myoblasts taken from dystrophy patients may have pathologically altered interactions with the ECM. that would contribute to the decreased muscle regenerative capacity in dystrophic individuals (Hughes and Blau, 1990; Stetler-Stevenson et al., 1993). Surprisingly, we found very limited differences between control and dystrophic cells. This is consistent with other reports suggesting that satellite cells from a dystrophic individual engrafted into a non-pathological environment have a regenerative capacity equivalent to wild type cells (Boldrin et al., 2015). A potential extension of these experiments would be to generate an artificial dystrophic/pathologic ECM to test the role of altered matrix effects on myogenesis *in vitro*.

FIGURE LEGENDS

Figure 2-1: Human, but not mouse, satellite cells invade a 3D collagen type I matrix.

Human (A) and mouse (B) satellite cells after 90 hours on a 3D collagen type I matrix; f-actin (Alexa 594-phalloidin, Red) and nuclei (DAPI, Blue). Human satellite cells invade the 3D collagen type I matrix, while mouse cells do not. Dotted line indicates the upper boundary of the 3D collagen. C) Immunocytochemistry of primary murine satellite cells for phospho-focal adhesion kinase (pFAK)(Green), Phalloidin 594 (Red), and DAPI (Blue) on Laminin, 2D Collagen I, and Gelatin. The negative no primary antibody control was performed on laminin.

Figure 2-2: MMP inhibitors differentially inhibit human satellite cell invasion. Human satellite cell invasion in the presence of TIMP-1 (A), TIMP-2 (B), TIMP-3 (C), or GM6001 (D); all but TIMP-1 significantly inhibit invasion. Quantitative comparison of human and mouse satellite cell invasion (E) based on the fraction of cells invading beneath the surface of the collagen matrix, compared to all cells in the section, normalized to the no inhibitor control (100%) ($n \geq 3$). $^{*}P<0.05$, $^{**}P<0.005$. Statistically significant *P* values were based upon comparison of protease inhibitors to the no inhibitor positive control.

Figure 2-3: Human, but not mouse, satellite cells express MMP-14; MMP-14 expression in human satellite cells is dependent on contact with collagen I.

A) RT-PCR for candidate MMP mRNAs and GAPDH as a positive control. B, C) Cross-sections of collagen I matrices on which human (B) or mouse (C) satellite cells were seeded and allowed to invade for 90 hours. Sections were stained for MMP-14 (green) and then labeled with Phalloidin 594 (red) and DAPI (blue); note the differential MMP-14 expression in human cells based on location adjacent to the collagen matrix. D) Cross-section of uninjured mouse tibialis anterior (TA) stained for Pax7 (red), MMP-14 (green), and DAPI (blue). E) Cross-section of mouse TA five days after BaCl injury showing high local expression between nascent myofibers in the area of injury. MMP-14 (green), DAPI (blue). F) Co-culture of ROSA^{mTmG} mouse satellite cells (red) and unlabeled mouse fibroblasts on a collagen I matrix; all cells are stained for MMP-14 expression (green.) G) Human satellite cells were seeded onto gelatin-coated coverslips and analyzed for MMP-14 (green) and integrin β1 (red) colocalization.

Figure 2-4: All human satellite cell populations tested express MMP-14 and invade collagen I, while murine cell lines differ from primary cells and each other.

Two primary (nonclonal, nontransformed) human myoblast samples from a 5-year-5-day old male (A) and a 73-year-old female (B) and two immortalized murine myoblast cell lines (C2C12, C and MM14, D) were seeded on a 3D collagen type I matrix for 90 hours as above and stained for MMP-14 (green) with phalloidin 635 (red) and DAPI (blue). E) Images are reference stills from Supplemental Movie I. The panel illustrates collagen proteolysis (green) by C2C12 myoblasts over four hours. A composite (top) of the FITC (bottom) and bright field channels shows localized proteolysis respective to the cell. C2C12 satellite cells adhered to the bottom of a 48-well plate were challenged with 3D collagen I co-polymerized with DQ Collagen I {1mg/mL}(Green.)

Figure 2-5: MMP-14 is necessary for invasion by human satellite cells.

Transfection with MMP-14 siRNA (A, B; B is inset from A), but not scrambled control siRNA (C, D; D is inset from C), reduced invasion by human satellite cells. E) This decrease in invasion was dose-dependent. F) Knockdown of MMP-14 protein was confirmed by Western blot; IP-90 was used as a loading control. Protein lysates from three separate experiments are shown. G) The ratio of invading cells for control siRNA and MMP-14 siRNA were quantified as described previously and normalized to control siRNA treatment (100%); $^{*}P<0.05$.

Figure 2-6: MMP-2 activity in human satellite cells is correlated to MMP14 expression.

Conditioned medium from human satellite cells transfected with either MMP-14 or scrambled control siRNA run in triplicate onto a gelatin zymogram. Medium from cells treated with MMP-14 siRNA contained reduced MMP-2 activity (A). Based on RT-PCR for TIMP-1, TIMP-2, and TIMP-3, human cells express higher levels of TIMP-2 mRNA compared to primary murine satellite cells (B).

Figure 2-7: Human MMP-14 is not sufficient to confer an invasive phenotype on primary murine satellite cells. Primary murine satellite cells were transfected with human MMP-14 or empty vector (control) with pCMV-TOMATO (red) as a transfection marker. Phalloidin 633 (white, B) and DAPI (blue) (A, B) were used to visualize whole cells seeded onto the matrix.

Figure 2-8: Satellite cells from dystrophic and healthy individuals have identical expression of MMP/Collagen mRNA. Various healthy and diseased human satellite cells' candidate MMP/Collagen mRNA visualized via RT-PCR.

Figure 2-9: Satellite cells from dystrophic and healthy individuals have identical expression of MMP-14 in 2D culture. Various healthy and diseased human satellite cells were placed on gelatin-coated coverslips; MMP-14 (red), f-actin (green), and nuclei (DAPI ,blue) are illustrated through immunocytochemistry. All immunocytochemistry was performed in coordination with no primary antibody negative controls.

Figure 2-10: Satellite cells from healthy and dystrophic individuals invade a 3D collagen type I matrix. Various healthy and diseased human satellite cells were seeded on top of a 3D collagen type I matrix for 90 hours. Images depict invading cells of these respective samples. Highlighted are the invadopodia and nuclei (blue) of these respective cells.

Figure 2-11: Satellite cells from dystrophic and healthy individuals have variable soluble MMP-2 activity.

Conditioned medium from human satellite cells diseased and healthy, run on a gelatin zymogram. MMP-2 enzyme can be seen in white, while the gelatin is blue.

satellite cells were seeded on top of a 3D collagen type I matrix for 90 hours. Images depict invading cells of these respective samples.

Supplemental Movie 2-1: Live-cell proteolysis of DQ Collagen I by C2C12 satellite cells.

A) C2C12 satellite cells adhered to the bottom of a 48-well plate were challenged with 3D collagen I co-polymerized with DQ Collagen I {1mg/mL}(Green). Images were taken every 15 min for six hours. Composite (Left) and FITC (right) provides a side-by-side visualization of the live-cell proteolysis.

METHODS

All methods involving live animals were performed under approval from the University of Missouri Animal Care and Use Committee.

Murine Cell Culture

C2C12 cells were cultured in DMEM (GIBCO) supplemented with 15% fetal bovine serum. Mouse satellite cells were harvested from hindlimb muscles of adult (90–120 days) females using our established protocol (Cornelison et al., 2004). Mouse muscle fibroblasts were harvested simultaneously and enriched by differential plating 24 h after harvest. Primary cells and MM14s were cultured on gelatin-coated plates (Nunc) in Ham's F-12 media (GIBCO) supplemented with 10% horse serum (HS) and 5 nM fibroblast growth factor 2 (FGF2) (Olwin and Rapraeger, 1992).

Human Cell Culture

Immortalized human satellite cells were originally isolated from a muscle biopsy taken from a 25-yr-old male donor, obtained from Myobank, affiliated with EuroBioBank, in accordance with the French legislation and EU regulation (Bigot et al., 2009). They were grown in a one-fourth volume of 199-DMEM media respectively supplemented with 2.5 ng/ml hepatocyte growth factor (HGF; Miltenyi), 10^{-7} M dexamethasone (Invitrogen), and 20% fetal calf serum (GIBCO) as previously described (Mauro, 1961). Primary human satellite cells were grown under the same conditions, except for the 5-yr-5-day-old clone, which was not supplemented with HGF or dexamethasone. All human cultures were also challenged in an invasion assay in which they were preconditioned for 24 h then plated on the 3D collagen matrix in murine growth medium (Ham's F-12 + 10% HS + FGF2); this did not affect their invasion.

3D Collagen Culture

One hundred microliters of acid-extracted rat-tail type I collagen (Sigma) (2 mg/ml in growth medium) was added per well in 96-well plates (Corning) and allowed to rapidly polymerize at 37°C. When used, recombinant tissue inhibitors of metalloproteases (TIMPs) 1, 2, or 3 (R&D Systems) at a final concentration of 3.5 nM (IC_{50} provided by the supplier is 2.2, 2.5, and 3.0 nM, respectively) or GM6001 (Calbiochem) at a final concentration of 10 μM was added to the collagen prior to polymerization. Satellite cells (50,000) suspended in 100 μl growth medium were added and cultured for 90 h. All conditions in each experiment were repeated in triplicate, in at least five independent runs. The matrices were fixed in 4% paraformaldehyde, equilibrated into 50% sucrose, and snap frozen. Cryosections (40 μm) were labeled with 1 μg/ml phalloidin-594 or -635 (Invitrogen) and Vectashield containing DAPI (Vector) for analysis. Invading cells were quantified using the cell counting function of μManager (www.micro-manager.org); criteria to score a cell in later statistical analysis required the entire nucleus and cytoplasm to be present in the section.

Live-cell Imaging

C2C12 myoblasts adhered to a 48-well plate (Corning) and overlaid with 3D collagen type I (previously described) copolymerized with DQ collagen type I (1 mg/ml; Life Technologies) were assayed via time-lapse microscopy. Images were automatically collected from each field every 15 min for 6 h using IPLab (Scanalytics) and analyzed via μManager. Images are representative of triplicate wells.

Immunostaining

Cells were immunostained as described previously (Stratman et al., 2009). Rabbit monoclonal (EP1264Y) anti-MMP-14 (Abcam) was used at 1:250; this antibody recognizes both the inactive and active forms of both human and mouse MMP-14. Mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank) was used neat. Samples were stained with fluorescently labeled secondary antibodies (Invitrogen) at 1:500 and/or fluorescently labeled phalloidin (Invitrogen) at 1 μg/ml concentration. Nuclei were visualized with DAPI (Vector.) Slides were imaged with an Olympus BX61 microscope using SlideBook (Intelligent Imaging Innovations) and μManager software. Z-stacks were generated for each image, and Z-projections were used for analysis and quantification of cell invasion.

Injury

Adult mice were anesthetized with 2,2,2-tribromoethanol (Avertin; Sigma) and injected intramuscularly with 50 μl of 1.2% barium chloride in sterile solution. Images shown are of muscles harvested 5 days after injury.

RT-PCR

Total RNA from **human** (25-yr-old male clone) or primary **mouse** satellite cells cultured on collagen-coated 10-cm plates was reverse-transcribed into cDNA (SuperScriptIII, Invitrogen). Four hundred nanograms of each cDNA sample was used as template; primer sequences used were as follows:

Gene	Forward primer	Reverse primer
MMP-2	GGAGAAGGCTGTGTTCTTCG	GCATCTACTTGCTGGACATCAG
MMP-9	CAGAGGTAACCCACGTCAGC	GGGATCCACCTTCTGAGACTT
MMP-13	CCTGGACAAGCAGTTCCAA	GCCAGTGTAGGTATAGATGGGAAA
MMP-14	GGACTGAGATCAAGGCCAAT	GCCCACCTTAGGGGTGTAAT
TIMP-1	TACGCCTACACCCCAGTCAT	ATGTGCAAATTTCCGTTCCT
TIMP-2	AGGTACCAGATGGGCTGTGA	GTCCATCCAGAGGCACTCAT
TIMP-3	CCACGTGCAGTACATTCACAC	TGTACATCTTGCCTTCATACACG
MMP-2	TATGGCTTCTGCCCTGAGAC	CACACCACATCTTTCCGTCA
MMP-9	TCGTCATCCAGTTTGGTGTC	ATGGGCGTCTCCCTGAAT
MMP-13	ATTTGATGGGCCCTCTGG	GCAACAAGAAACAAGTTGTAGCC
MMP-14	GGCAAATTCGTCTTCTTCAAA	GAGCAGCATCAATCTTGTCG
TIMP-1	CTGTTGTTGCTGTGGCTGAT	AACTTGGCCCTGATGACG
TIMP-2	AGAAGAGCCTGAACCACAGG	TGACCCAGTCCATCCAGAG
TIMP-3	CCCATGTGCAGTACATCCATAC	CCATCATAGACGCGACCTG
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
	MOUSE	
	HUMAN	
	BOTH SPECIES	

Primer sequences used for diseased **human** satellite cells are listed as follows:

Gene	Forward primer	Reverse primer
MMP-1	TCGATGCTGCTCTTTCTGAG	GATAACCTGGATCCATAGATCGTT
MMP-2	TTTCCATTCCGCTTCCAGGGCAC	TCGCACACCACATCTTTCCGTCACT
MMP-3	GGCTTTCCCAAGCAAATAGC	GTGCCCATATTGTGCCTTCT
MMP-7	TCCAACCTATGGAAATGGAGA	GGAGTGGAGGAACAGTGCTT
MMP-8	TCTGCAAGGTTATCCCAAGG	ACCTGGCTCCATGAATTGTC
MMP-9	TCGTCATCCAGTTTGGTGTC	ATGGGCGTCTCCCTGAAT
MMP-10	TTGATGATGATGAAAAATGGACA	AAGCTTCAGTGTTGGCTGAGT
MMP-11	GGGGATGTCCACTTCGACTA	CAGTGGGTAGCGAAAGGTGT
MMP-12	GGAATCCTAGCCCATGCTTT	CGTGAACAGCAGTGAGGAAC
MMP-13	AACATCCAAAAACGCCAGAC	GGAAGTTCTGGCCAAAATGA
MMP-14	GGCAAATTCGTCTTCTTCAAA	GAGCAGCATCAATCTTGTCG
MMP-15	TACCAGTGGAAGGACGTTGA	AGAGGCTGGGTAGGCTGTG
MMP-16	GACATGCTCTGGGATTGGAG	TCATTTTTCCTTGGGTCAGC
MMP-17	GGAGCTGTCTAAGGCCATCA	CGACAGGTTCCTCTTGTTCC
MMP-19	CAGCCTCGTTGTGGCCTAGA	ACCAGCCTGCACCTCTTGGA
MMP-20	CGACAATGCTGAGAAGTGGA	ATCTTTGGGGAGGTGGAATC
MMP-21	GACGACGACGAGCACTTCAC	TTTCCTGTCTGACCAGTCCA
MMP-23	GGACCACTTCAACCTCACCTA	GGACACGTCGCTCCACAT
MMP-24	GAACCTGTGGGCAAGACCTA	TGACAACCAGAAACTGAGCG
MMP-25	CTCCGGGGACACTCACTTT	TGGCCAAACTCATGGACAG
MMP-26	CACTGTGGGGTGCCTGAT	TGGCTTCATATCATGTGGGTAA
MMP-27	TTGTTTCTTGTGGCTGCTCA	GCTAAGCCAAAGGAACCCAC
MMP-28	CACCTCCACTCGATTCAGCG	AAAGCGTTTCTTACGCCTCA
TIMP-1	CTGTTGTTGCTGTGGCTGAT	AACTTGGCCCTGATGACG
TIMP-2	AGAAGAGCCTGAACCACAGG	TGACCCAGTCCATCCAGAG
TIMP-3	CCCATGTGCAGTACATCCATAC	CCATCATAGACGCGACCTG
COL1A1	CTGGACCTAAAGGTGCTGCT	GCTCCAGCCTCTCCATCTT
COL1A2	AAAGGTCATGCTGGTCTTGC	TTCCACCTTGAACACCCTGT
COL3A1	CCTGGTTCCCCTGGATCT	GGACCAGATGGACCTATAGCA
COL4A1	GAGATGGTGTTGCAGGAGTG	AGCCGCAAGTCGAAATAAAA
COL4A2	GATACCTGGGCGCAAAAG	CGATGTCTCCCTTGACTCCT
COL4A3	TGGAAAGGATTTTCATTCATCA	GGCTGGCTCGGAATTCTT
COL4A4	GTTCCTGAAAAGGGGTCTCG	TCTCTCCTGAAAGCCCAATG
COL6A1	TCGTCGTCAAGGTCATCG	GCTCCTGCATCTGGCTGT
COL6A5	TGTCTCAGGAGAACCAGGAAA	TTCTCCTTTTCTGCCAGGATT

siRNA Transfection

MMP-14 siRNA (15, 25, or 50 μM/well; Invitrogen) or control siRNA was transfected into human satellite cells cultured on gelatin-coated six-well plates (Nunc) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols prior to seeding on 3D collagen I matrices. All samples were cotransfected with Alexa Fluor 555-labeled Red Fluorescent Oligo (Invitrogen) to identify transfected cells. Ninety hours after transfection, cells were fixed with 4% paraformaldehyde for analysis.

Immunoblotting

Ninety hours after siRNA transfection, total cell lysates were collected in Allen buffer (50 mM Tris, 10 mM EDTA, 5 mM EGTA pH 7.4 with 1 × Roche Protease Inhibitor, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 μg/μl pepstatin A, and 1% Triton X-100.) Ten micrograms of each lysate were loaded onto 4–8% gradient polyacrylamide gel (Invitrogen), transferred to polyvinylidene difluoride membranes, and blocked in StartingBlock (TBS) blocking buffer (Fisher). Membranes were incubated overnight at 4°C with primary antibodies to MMP-14 (EP1264Y, Abcam) at 1:2,000 and IP90 (AB10286, Abcam) at 1:2,000 in StartingBlock, washed, and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 1 h at room temperature. Chemiluminescent substrate (Pierce SuperSignal West) was detected with a LAS3000 imager (Fujifilm).

MMP-14 Overexpression

Ninety-six hours after isolation, primary mouse satellite cells on gelatin-coated 10-cm plates (Nunc) were transfected with 5 μg of human MMP-14 expression vector (GenBank accession no. BC064803 cloned into pCMV-SPORT6, Open BioSystems) and 5 μg pCMV-Tomato reporter using 15 μl Fugene HD (Promega); control cells were transfected with 5 μg pCDNA4 and 5 μg pCMV-Tomato reporter. After 8 h the cells were seeded onto the 3D collagen type I matrices and cultured for an additional 90 h. Collagen matrices were then fixed, sectioned, immunostained, and imaged for analysis as above.

Zymography

Forty micrograms of protein lysate were separated on 8% acrylamide gels polymerized with 2 mg/ml gelatin. After electrophoresis, gels were renatured in 2.5% Triton X-100 for 1 h at room temperature then incubated in developing buffer (50 mM Tris·HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35) at 37°C for 12 h. Gels were rinsed in distilled H_2O and stained with Coomassie R-250 for 30 min, then destained in methanol:acetic acid:water (50:10:40) and imaged on a flatbed scanner

Diseased Human Satellite Cell Lines

DMD5-13515C15:37 (DMD), KM571DMD10FLCL1:48 (DMD with specific loss of exon 52), KM390BMD196CL2:41 (Becker's Muscular Dystrophy, BMD), and KM155C25Dist (healthy satellite cell) were isolated from primary muscle biopsies ,obtained from Myobank, affiliated with EuroBioBank, in accordance with the French legislation and EU regulation (Bigot et al., 2009).

CHAPTER 3:

The Matrix Reloaded: Loose Ends

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TRANSITION - RHABDOMYOSARCOMAS

Cancer cells can be highly invasive/metastatic, and research identifying molecular mechanisms involved in three-dimensional movement may provide novel targets for therapy. Importantly, cancer cells have been shown to use MMPs, particularly MMP-14, during invasion, and targeting MMPs specifically has been shown to perturb their 3D motility *in vitro* and *in vivo*. We applied the techniques and expertise developed in investigating satellite cell invasion (Chapter 2) to assess Rhabdomyosarcoma (RMS) invasion into a 3D collagen type I matrix and MMP-14 expression/localization.

INTRODUCTION

Soft-tissue sarcomas (STSs) are rare mesenchymal tumors [less than 1% of adult human cancer and 15% of pediatric malignancies (Borden et al., 2003)] commonly originating from skeletal muscle, adipose tissue, or connective tissue. Rhabdomysarcomas (RMS), first described in a five-year old child in 1839 (Stafford, 1839), are a heterogeneous STS seldom found in adults but the most common soft tissue malignancy in children and adolescents (Anderson et al., 1999; Miller et al., 1995). Commonly arising in or near skeletal muscle tissue, RMS are often generalized as skeletal muscle tumors that manifest from aberrant muscle development (Langenau et al., 2007; Wagers and Conboy, 2005). RMS express common skeletal muscle lineage markers: skeletal muscle actin and myosin, desmin, myoglobin, Z-band protein, MYOD, and myogenin [reviewed in (Marshall and Grosveld, 2012)]. Their expression of the classic markers for myogenic precursor cells [PAX3, PAX7, and MyoD family members (Anderson et al., 1999)] is consistent with their characterization as a satellite cell-derived skeletal muscle cancer. However, it has also been suggested that they arise from mesenchymal cells, not satellite cells (Charytonowicz et al., 2009; Lisboa et al., 2008; Shinkoda et al., 2009). RMS can be divided into four subtypes: embryonal, alveolar, botryoid, and pleiomorphic. The two most prevalent and well-characterized forms are embryonal (ERMS) and alveolar (ARMS). Both of these subtypes arise in the viscera and axial soft tissues more frequently than in the extremities (Donaldson, 1989), but they exhibit considerable histological, molecular, and developmental origin variability (Hettmer and Wagers, 2010).

The most prevalent RMS, ERMS (Punyko et al., 2005), is histologically similar to embryonic skeletal muscle (Parham, 2001) and often exhibits the

morphological phases of myogenesis: undifferentiated mesenchymal cells to elongated myoblasts, multinucleated myotubes, as well as fully differentiated myofibers. ERMS frequently develop in the head, neck or genitourinary tract of young children (Anderson et al., 1999; Meza et al., 2006; Puri et al., 2006). More aggressive, alveolar rhabdomyosarcomas (ARMSs) morphologically resemble alveoli in the lung, primarily form in the extremities and trunk (Meza et al., 2006; Puri et al., 2006; Takahashi et al., 2004; Xia et al., 2002), and have experienced chromosomal translocations [either a *t*(2; 13) or a *t*(1; 13)] generating *PAX3:FKHR* or *PAX7:FKHR* fusion genes, the former being clinically illustrated as more aggressive (Anderson et al., 1999; Sorensen et al., 2002). Both embryonal and pleomorphic RMS have no pathogenetic translocation, but frequently experience inactivation of the p53 tumor suppressor pathway (Borden et al., 2003) as well as other pro-cancer mutations.

New treatments for RMS over the last 30 years have resulted in a significant decrease in mortality in some tumor types (Crist et al., 1995; Koscielniak et al., 1999; Oberlin et al., 2008; Stevens et al., 2005), however ARMS in particular can be highly metastatic, often spreading to the lungs, brain, skin, breast, and bone marrow. Metastatic RMS patients, while rare (15%), have a dramatic decrease in cure rate (Breitfeld et al., 2001; Breneman et al., 2003; Carli et al., 1999; Koscielniak et al., 1999; Koscielniak et al., 1992; Maurer et al., 1988; Maurer et al., 1993; Raney et al., 1988; Sandler et al., 2001): despite a high response rate to chemotherapy, five-year survival is approximately 25% (Breneman et al., 2003; Carli et al., 2004; Crist et al., 1995; Koscielniak et al., 1992; Maurer et al., 1988; Maurer et al., 1993; Raney et al., 1988; Sandler et al., 2001). Thus, increased understanding of how RMS acquire and maintain invasive capacity could potentially impact treatment and cure rate.

Recently, down-regulation of c-MET and CXCR has been shown to decrease the metastatic potential of RMS cells (Miekus et al., 2013). This was of particular interest to us due to the involvement of c-MET and CXCR signaling in satellite cell motility. In addition, as mentioned in Chapter 2, c-MET signaling has been directly linked to MMP-2, MMP-9, and MMP-14 activation during invasion (Ozen et al., 2012). With the emergent role of MMP-14 in metastasis and the increased interest in targeting MMP-14 in cancer therapeutics (Ulasov et al., 2013; Ulasov et al., 2014), we thought it would be useful to evaluate MMP-14's role in RMS. In Chapter 3, we describe a variance in motility between genetically distinct RMS cell lines, in terms of 2D motility, 3D invasive potential, and MMP-14 expression.

RESULTS

Primary derived rhabdomyosarcoma cells exhibit different invasive capacities

We acquired cell lines derived from transgenic Myf6Cre/*Pax3-Foxo1*/$p53^{-/-}$ mice (U23674 and U48484, ARMS) and Myf6Cre/$p53^{-/-}$ mice (U33915 and U57810, ERMS) from the Keller lab (Keller et al., 2004; Rubin et al., 2011). All were derived from primary tumors except for U48484, which was derived from a pulmonary metastasis of a different tumor-bearing animal (Aslam et al., 2014). We analyzed RMS metastasis using the same techniques we previously used to study satellite cell invasion. When we seeded four RMS lines on top of a 3D collagen type I matrix, only the U48484 metastasis-derived cells invaded into the matrix (Figure 3-1). The other RMS lines' invasive behavior was limited to small protrusions from cells that still appeared to be in contact with the other cells on top of the collagen (data not shown). Thus, it appears that the metastasis-derived cells have acquired one or more secondary mutations that confer a more invasive phenotype. We therefore compared mRNA and protein expression of MMP-14 between the lines to ask if it correlates with the invasive phenotype. However, we found that all four lines express MMP-14 at the RNA level (Figure 3-2), including the U57810 ERMS line that did not have detectable protein expression.

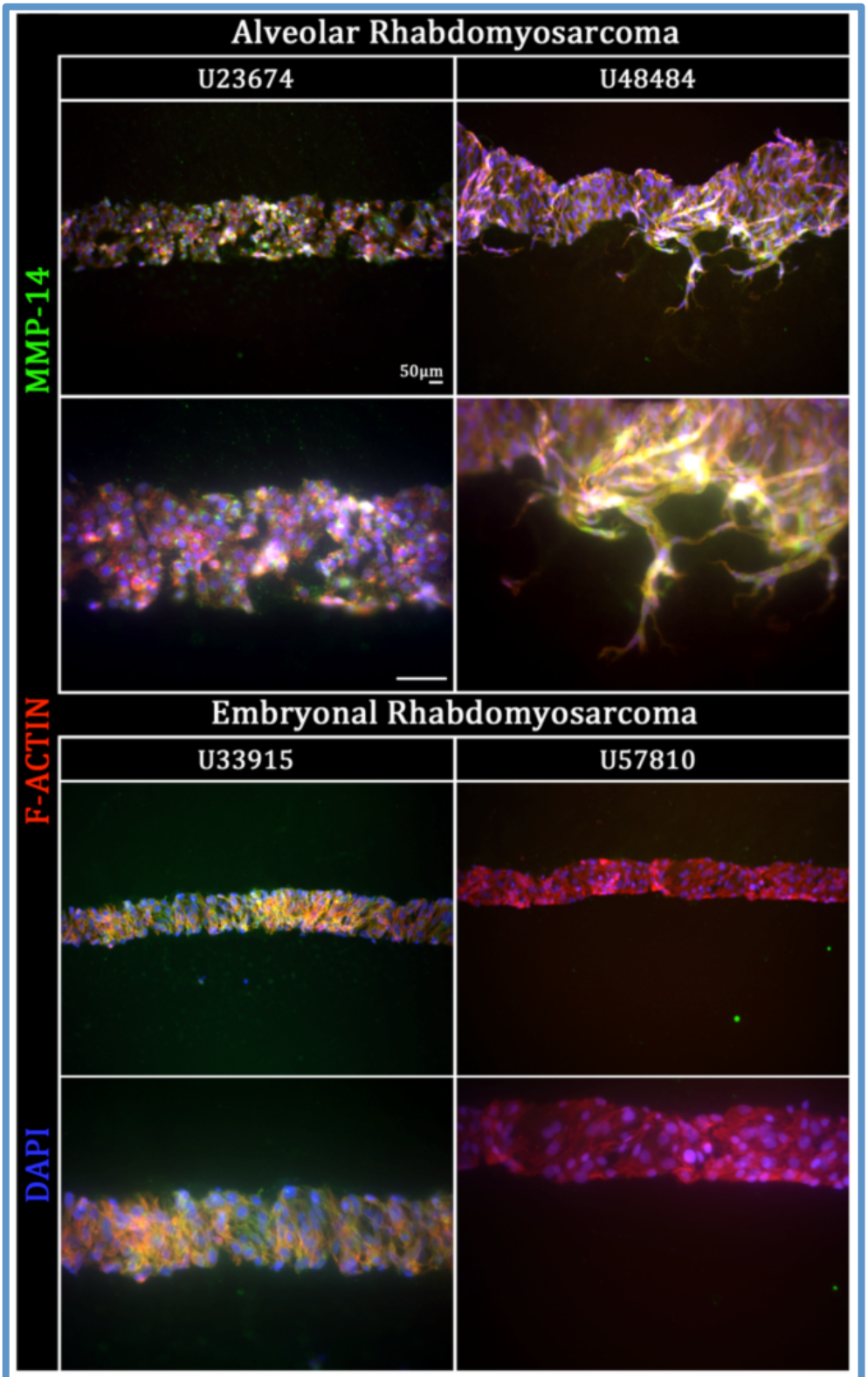


Figure 3-1: ARMS and ERMS invasion into 3D collagen I. U23674, U48484 (ARMS), and U33915, U57810 (ERMS), cells after 90 hours on a 3D collagen type I matrix; f-actin (Alexa 594-phalloidin, Red), nuclei (DAPI, Blue), and MMP-14 (green) (n=3). Three images of each sample were used to give a qualitative representation of invasive capacity. Positive MMP-14 expression was verified through no primary antibody controls on each sample.

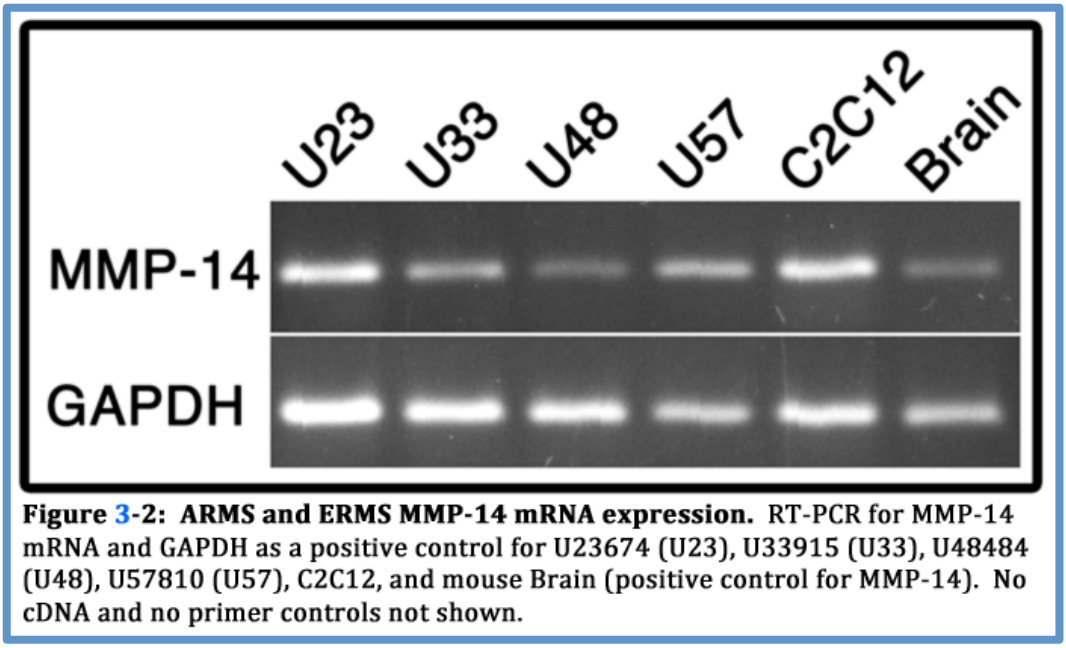
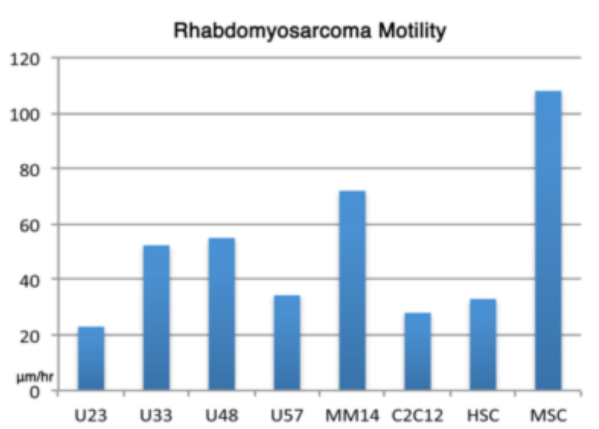


Figure 3-2: ARMS and ERMS MMP-14 mRNA expression. RT-PCR for MMP-14 mRNA and GAPDH as a positive control for U23674 (U23), U33915 (U33), U48484 (U48), U57810 (U57), C2C12, and mouse Brain (positive control for MMP-14). No cDNA and no primer controls not shown.



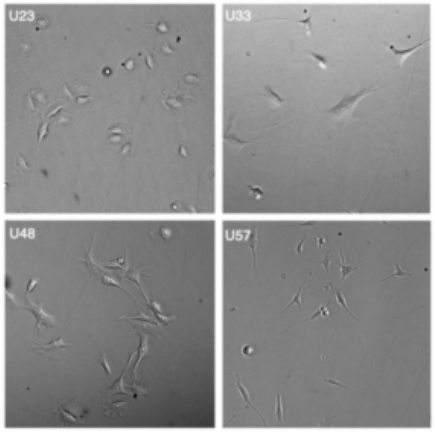


Figure 3-3: ARMS and ERMS 2D motility and morphology. U23674 (U23), U33915 (U33), U48484 (U48), and U57810 (U57) RMSs show no correlation to motility and genetic origin, as seen on the bar graph to the right (n=2). MM14 myoblasts, C2C12 myoblasts, primary mouse satellite cell (MSC), and human satellite cell (HSC) motility analyzed independently but under the same experimental parameters was used a reference to the RMS motility. Image stills from each representative video, on the right, highlight unique morphological phenotypes of each RMS cell line.

Primary derived rhabdomyosarcoma cells exhibit different motility profiles

In addition to 3D motility, we assayed simple 2D motility in all four lines, reasoning that increased motility might be a characteristic of one or more tumor lines compared to normal myoblasts. However, all four lines were less motile than primary mouse myoblasts, by significant percentages (Figure 3-3); data for murine cell lines and primary myoblasts from (Siegel et al., 2009). Additionally, there appears to be no correlation of velocity with RMS subtype:. the U23674 and U48484 (ARMS) moved at 22 and 55 μm/hr respectively, while the U33915 and U57810 (ERMS) moved at 51 and 32 μm/hr (Figure 3-3).

These data seem puzzling, as one would initially think tumor cells should exhibit a robust potential to invade the extracellular matrix as well as heightened motility. Future work identifying the additional mutations that permit previously non-invasive RMS cells to invade could be instructive.

FIGURE LEGENDS

Figure 3-1: ARMS and ERMS invasion into 3D collagen I.

U23674, U48484 (ARMS), and U33915, U57810 (ERMS), cells after 90 hours on a 3D collagen type I matrix; f-actin (Alexa 594-phalloidin, Red), nuclei (DAPI, Blue), and MMP-14 (green) (n=3). Three images of each sample were used to give a qualitative representation of invasive capacity. Positive MMP-14 expression was verified through no primary antibody controls on each sample.

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RT-PCR for MMP-14 mRNA and GAPDH as a positive control for U23674 (U23), U33915 (U33), U48484 (U48), U57810 (U57), C2C12, and mouse Brain (positive control for MMP-14). No cDNA and no primer controls not shown.

Figure 3-3: ARMS and ERMS 2D motility and morphology.

U23674 (U23), U33915 (U33), U48484 (U48), and U57810 (U57) RMSs show no correlation to motility and genetic origin, as seen on the bar graph to the right (n=2). MM14 myoblasts, C2C12 myoblasts, primary mouse satellite cell (MSC), and human satellite cell (HSC) motility analyzed independently but under the same experimental parameters was used a reference to the RMS motility. Image stills from each representative video, on the right, highlight unique morphological phenotypes of each RMS cell line.

METHODS

Rhabdomyosarcoma Cell Culture

RMSs cells were all cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum. Cells were derived from Dr. Charles Keller, and allowed to grow in culture until reaching a healthy state before experiments were performed.

3D Collagen Culture

One hundred microliters of acid-extracted rat-tail type I collagen (Sigma) (2 mg/ml in growth medium) was added per well in 96-well plates (Corning) and allowed to rapidly polymerize at 37°C. RMS cells (50,000) suspended in 100 µl growth medium were added and cultured for 90 hr. All conditions in each experiment were repeated in triplicate, in three independent runs. The matrices were fixed in 4% paraformaldehyde, equilibrated into 50% sucrose, and snap frozen. Cryosections (40 µm) were labeled with 1 µg/ml phalloidin-594 or -635 (Invitrogen) and Vectashield containing DAPI (Vector) for immunocytochemistry analysis.

Immunostaining

Cells were immunostained as described previously (Stratman et al., 2009). Rabbit monoclonal (EP1264Y) anti-MMP-14 (Abcam) was used at 1:250; this antibody recognizes both the inactive and active forms of both human and mouse MMP-14. Samples were stained with fluorescently labeled secondary antibodies (Invitrogen) at 1:500 and/or fluorescently labeled phalloidin (Invitrogen) at 1 µg/ml concentration. Nuclei were visualized with DAPI (Vector.) Slides were imaged with an Olympus BX61 microscope using SlideBook (Intelligent Imaging Innovations) and µManager software.

RT-PCR

Total RNA from each RMSs cell cultured on collagen-coated 10-cm plates was reverse-transcribed into cDNA (SuperScriptIII, Invitrogen). Four hundred nanograms of each cDNA sample was used as template; primer sequences used were as follows: GAPDH forward (F), 5′-CAAGGTCATCCATGACAACTTTG-3′, reverse (R), 5′-GGGATCCACCTTCTGAGACTT-3′; mouse MMP-14 F 5′-GGACTGAGATCAAGGCCAAT-3′, R.

Time-lapse Capture

All conditions are represented by triplicate wells in every experiment. Multiple 10x fields are identified per well and marked for return; images are automatically collected from each field every 10 minutes using IPLab (Scanalytics, Rockville, MD, www.scanalytics.com).

Post-imaging Analysis

Stacked images generated by IPLab (Scanalytics) are imported into MetaMorph (Axon Instruments/Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com) and arranged in sequential order. Distance of migration is measured using digital pixel trace measurements. If a cell selected for tracking proliferates during the 24-hour collection period, one daughter cell is selected at random to continue the trace.

CHAPTER 4:

Full Circle

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CONCLUDING REMARKS

The work presented in this dissertation moves the boundaries of what was known about interactions between satellite cells and their local extracellular matrix. It also suggests future directions, including outstanding questions regarding if and how mouse satellite cells use MMP-14 for movement through the tissue in vivo, whether MMP-14 is a potential modifier for cell therapies, and what changes accrue in metastatic rhabdomyosarcoma cells to confer an invasive phenotype. In Chapter 2, we show that primary murine satellite neither invade a 3D collagen type I matrix nor express MMP-14. However, muscle regeneration in the mouse clearly involves satellite cell relocation, suggesting that mouse satellite cells must be able to negotiate and invade the ECM *in vivo*. How can we explain this? As noted in Chapter 1, the actual muscle matrix and soluble microenvironment are quite complex, and our system is necessarily much simpler; perhaps there are components necessary for invasive motility that are lacking in our culture system. Alternately, the process of isolating and expanding satellite cells in culture may have changed or prevented expression of genes necessary for invasion.

Another potential extension of this work is to test if overexpression of MMP-14 in human satellite cells would enhance their engraftment capacity/efficiency. As mentioned earlier, researchers have had great difficulty getting engrafted cells to spread from the point of injection. This obstacle has slightly been alleviated with the over-expression of MMP-2 and MMP-9. Importantly, MMP-14 activates both MMP-2 and MMP-9, making it a much stronger candidate for over-expression in this context. If MMP-14 overexpression could reduce this obstacle to therapeutic use of satellite cells, it would be a significant clinical advance.

Finally, the striking differences in invasiveness between cells derived from primary RMS tumors and a metastatic RMS tumor deserve to be followed up, to identify what secondary mutations led to the invasive/metastatic phenotype and which may suggest new druggable targets.

The worst part about concluding this narrative is knowing that all the other cool projects and findings that did not even make it into this document, and maybe the ones that are even here, may disappear into the universe. I collaborated with many scientists over the years on a variety of interesting questions. I will try briefly to list some of these projects: Wnt5B cell expression identity, automated cell tracking software, time-lapse analysis of myogenic deficient satellite cells, endorphin expression in rat brains, human satellite cell's response to eph and ephrins, 3D time-lapse microscopy optimization, epigenetic effects of stress on pregnancy, plant immune response, etc… Helping others reach their answers has been one of the most rewarding aspects to my graduate career. It lets me know the tools I have acquired are going to good use.

While bringing my research narrative to an end, I figure I would take a leap of faith and mention a few ideas that I have cultivated over my course of study. From my five years of researching satellite cells, the extracellular matrix, and MMPs; I am a strong believer in several avenues for our fundamental scientific questions. 1) Targeting MMP-14 will be used as a successful therapeutic treatment to inhibit metastasis. 2) The extracellular matrix holds the cures to aging, oncogene expression, and myopathy. Finally, 3) satellite cells will be directly targeted in the future to

enhance muscle growth/myogenesis for sports medicine. I feel as if I have made a significant contribution to the community, and I incredibly excited and optimistic about continuing the advancement of science through out my life.

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VITA

I was born on September first, 1987 at Children's Memorial Hospital in Chicago. Art and creativity were a focal point emphasized by my mother, Amy Lerner, from a very early age. Her father, Nathan Lerner, was a very innovative photographer, and is noted for finding the works of the most famous outsider artist in the world, Henry Darger. Around the age of seven my family moved to Grayslake, Illinois, it was nice open and child friendly. My brothers, Aaron and Ryan, and I, played about every sport imaginable. When I was not outside I was always building something (with either Legos or kinects), or drawing/painting. Shortly after our move to Grayslake I found a true calling, wrestling.

From the beginning I was a natural, winning nearly every wrestling match with little to no training. I soon became my clubs, the wrestling factory, first state champion. Actually one of my proudest moments was walking out in the spotlight with my father, Steven Lund, to win my second state championship against an opponent who had already beaten me twice. I know he and I will never forget that moment. My little brother Ryan was exceptional at wrestling too, and since we were roughly the same size you could imagine how many competitive sparing matches we had as kids. High school was rough; I became very rebellious around 16-17 and as a result strained family relationships as well as my wrestling career.

I decided to attend the University of Missouri based on their no essay application, my previous familiarity with their wrestling program, and pretty much

out of laziness. My high school troubles followed me to college, and I started struggling in school and with the law. I am not sure when, but I hit an epiphany and I began working harder than ever. I got two jobs, volunteered and dramatically began improving in my classes. I think I started with less than a 1.0 GPA and brought it to nearly a 3.0 by the time I graduated. I often became frustrated seeing myself work so hard to financially support myself and seeing other students in my class not have to worry about such a task, and receive more free time to study than I did. I knew I could do better than them. I knew I wanted to make something of myself after college I just was not sure what.

My junior year, I took Developmental Biology as my capstone with Dr. Cornelison and Dr. Garcia. I fell in love with the subject. It perfectly fit my inquisitive nature and often kept me up at night thinking of experiments. I established a great connection with both D and Mike and eventually asked D if she would be interested in taking me on as a graduate student. I figured if I learned so much in one semester I could not imagine the wealth of knowledge I would gather under her mentorship over five years. Luckily, she happily obliged.

This where I began my journey today; I loved the time I have spent here in graduate school, and I am proud to know I made strides to better human health and made a significant contribution to science.

Importantly, the way I have developed has opened my eyes to some of the best friends, strengthened my ties with my family, and allowed me to be a better partner.

It is difficult to summarize my life story in two pages. However, I know that my story, my upbringing, allowed me to push my self through many of life's obstacles that maybe could not have been overcome without the proper nurturing of others. It is

this nurturing that I am forever grateful for, since one does not simply make themselves.

As a scientist, I can not be trained to think, that comes innately.