Steroids and growth factors in oral squamous cell carcinoma: useful source of dental-derived stem cells to develop a steroidogenic model in new clinical strategies

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Abstract. – OBJECTIVE: Head and neck region is involved in a high percentage of malignant lesions, and oral squamous cell carcinoma (OSCC) is undoubtedly the most frequently found, accounting for over 90% of malignant tumors. Hormone receptor overexpression, like Estrogen Receptor (ER), Progesterone Receptor (PR) and Endothelial Growth Factor Receptor (EGFR), and signaling have been related to the pathogenesis of OSCC. For metastasis of OSCC, Cancer Stem Cells (CSCs) undergo epithelial to mesenchymal transition (EMT) under the influence of growth factors, cytokines, and regulation of cadherins from the tumor's microenvironment. In this context, the stem cells may become a potential therapeutic target for OSCC through modulation of cytokines and RAS pathway, which is involved in intracell signal transduction. The objective of this study was to suggest an experimental steroidogenic model for OSCC in translational research.
PATIENTS AND METHODS: Dental-derived Stem Cells (D-dSCs) have been obtained from apical papilla tissue that surrounds the developing tooth of healthy donors and cultured in vitro. The cells have been exposed to different concentrations of Estradiol (E2 - 10 nM and 40 nM) in order to verify their response. The number of cells and cell viability has been evaluated up to 96 hours of treatment.
RESULTS: The results showed that cell growth was increased under estradiol treatments compared with cells maintained without estradiol. Moreover, no significant difference in cell death levels was detected among treatments.
CONCLUSIONS: This work underlines as D-dSCs could represent a useful steroidogenic model for the development of the target and gene therapies in OSCC.

Key Words:
Oral squamous cell carcinoma, Stem cells, Dental-derived Stem Cells (D-dSCs), Estrogen receptors, Endothelial growth factor receptor.

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Introduction

Head and neck region is involved in a high percentage of malignant lesions; in particular, about half of these occur in the oral cavity. In this district, oral squamous cell carcinoma (OSCC) is the most frequently found, accounting for over 90% of malignant tumors[1]. Advancing age and with the abuse of risk factors, such as alcohol and tobacco, the prevalence increases considerably[1,2]. The progression of oral cancer is marked by well-known multifactorial and multifactorial dynamics; the step-wise transition from pre-malignant conditions to the tumor phenotype constitutes the multi-step model of oral carcinogenesis[3]. The addition of genomic alterations causes this transition to gradually progress, resulting in the transformation from normal mucosa, to dysplasia, to carcinoma *in situ*, and advanced cancer[4-6]. The long-term survival rate in patients with OSCC, despite the great efforts of the scientific community, is not yet the desirable percentage, especially when it is not possible to surgically intervene permanently, also because the anticancer drugs still show a low efficacy[7,8]. Thus, a better understanding of the molecular profile of tumors[8] and carcinogenetic processes should facilitate the development of more efficient targeted therapies[2,9,10]. Despite improvements in therapeutic and diagnostic techniques in oral diseases and related researches[11-14], OSCC remains a lethal disease with a five-year survival rate of approximately 50%, urging the need for novel treatment modalities[15]. An increase in the incidence of OSCC in young female patients without risk factors (as alcohol or tobacco abuse) has been reported, so the hypothesis that tumors could be hormonally induced seems to be plausible[2,16,17]. In this context, hormone receptors expression, like Estrogen Receptor (ER) and Progesterone Receptor (PR), have been related to the pathogenesis of OSCC[18]. Estrogens influence various physiological processes by regulating the growth and differentiation of cells. The effects are mediated through two different ER: Estrogen Receptor-alpha (ERα) and Estrogen Receptor-beta (ERβ). ER-mediated signals are involved in the development and progression of several hormone-related cancers. Particularly for breast cancer, rectal cancer, prostate cancer, and exposition to sources of free radicals, this causal relation is well-characterized[19-22]. Some authors demonstrated that, in OSCC specimens, expressions of ERα, ERβ, and Androgens Receptors (AR) transcripts are respectively higher and lower than in normal tissues[10,16]. The aim of this exploratory study was to create an experimental model for the OSCC through the exposure of cells taken from Dental-derived Stem Cells (D-dSCs) to various levels of Estradiol (E2) (10 nM and 40 nM), comparing the growth curve and the mortality of these cells.

Patients and Methods

Cell Lines and Culture Conditions

This study was conducted on Dental-derived Stem Cells (D-dSCs) obtained from healthy pediatric donors, as previously described[23]. All patient's parents and guardians gave permission after they signed a written informed consent following the Helsinki Declaration for the re-use of human bio-specimens in scientific research. The investigation was conducted following the current medical protocol, as described by the Italian Government's NIH legislation.

The cells were cultured under standard conditions in 95% air and 5% CO_2 at 37°C to retain the proliferative condition and grown respectively in Roswell Park Memorial Institute (RPMI) (in the absence of phenol red and serum medium containing 10% fetal bovine serum (FBS)), 2 mM, 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were collected, and quantification was performed using a hemocytometer chamber in order to seed an equal number of viable cells for each experiment. The cells were seeded at a final density of 10,000 cells/well, in 6-well culture plates. The *in vitro* experiments were performed in triplicate to ensure the reproducibility of results.

Drug Treatments and Protocols

Estradiol (E2, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol (vehicle), reaching the desired concentration with the final content of ethanol in cultures less than 0.1%.

D-dSCs were plated in 35-mm diameter multi-wall dishes with RPMI containing 5% FBS. The serum was treated twice with the charcoal-coated dextran prepared using a previously reported procedure[24]. Twenty-four hours after seeding, the cells were treated with 10 nM and 40 nM of E2 and monitored for 96 h. In particular, the cells were subjected to the following treatments: 1) basal medium supplemented with ethanol as a vehicle, which represents the control; 2) basal medium supplemented with 10 nM of E2; 3) basal medium supplemented with 40 nM of E2.

Procedures were conducted following our previous experience in the field and according to manufacturer specifications[23-26].

Quantification of Cell Number

The number of cells was performed using a hemocytometer chamber every 24 hours for four days.

Cell Viability Assay

To assess the effect of those compounds on our panel of cells, cell viability assay was performed. CellTiter-Glo® (Promega, Madison, WI, USA) is a Luminescent Cell Viability Assay based on the quantitation of the ATP present, an indicator of metabolically active cells for cell proliferation and cytotoxicity assays (Trypan blue Exclusion Protocol).

The assay procedure includes adding the single reagent (CellTiter-Glo® Reagent, Promega, Madison, WI, USA) directly to cells cultured in serum-supplemented medium. The system detects as few as 15 cells/well in a 384-well format in 10 minutes after adding reagent and mixing. The total of ATP is directly comparative to the number of cells present in the culture. The CellTiter-Glo® Assay generates a luminescent signal, which has a half-life superior to five hours, depending on cell type and the medium used. The assay was performed using a Victor™ X5 2030 (PerkinElmer; Waltham, MA, USA) Multilabel plate reader after treatment with Estradiol and compared with control.

Statistical Analysis

The differences between the mean number of cells at different concentrations of estradiol (cells treated with 10 nM E2, 40 nM E2, and controls) were determined by the One-way ANOVA and Tukey tests. Moreover, in order to assess the difference of cell death among the three different groups, the Kruskal Wallis test was performed. The software R was used for all computations, and p values ≤ 0.05 were considered statistically significant.

Results

Estradiol Stimulation of Dental-derived Stem Cells (D-dSCs) Cell Growth

As reported in Figure 1, the plated cells were grown in absence and in the presence of two concentrations of estradiol (E2) (10 nM and 40 nM).

The cell growth was increased under estradiol treatment, while the cell growth-related to plated cells maintained without estradiol was slow (control). One way ANOVA detected a significative difference between the groups (p=0.049). In particular, when the Tukey test for multiple comparisons was performed, the mean number of cells growth in presence of 40 nM of E2 was higher than the controls (p=0.038).

Cell Viability and Cytotoxic Assays

The Kruskal Wallis test was performed to assess the difference of cell death among the three different groups of treatment (control, E2 – 10 nM, E2 – 40 nM), and did not reveal any significant difference in cell death levels (p=0.427); despite that, the apoptotic effects seem to be greater in the control group which was not stimulated with E2; this would, then, emphasize the possibility of a protective effect of E2 on stem cells.

Discussion

Expression of ERα and ERÐ in Oral Squamous Cell Carcinoma (OSCC)

Colella et al[27] have highlighted, with a case-control study, the presence and the expression levels of sexual steroid receptors, in particular, ERα and AR transcripts, in ten OSCC using RT-PCR analysis. Their data indicate that ERα mRNA is more expressed in the OSCC than in the control tissues while opposite results have been obtained for AR; in fact, it is less expressed in the OSCC than in the control tissues.

With the aim of targeting these pathways as a new treatment strategy for head and neck cancer, their data also suggest that OSCC (presence of ER) may respond to hormonal stimulation. On the other hand, the results do not support their hypothesis of an inhibitory effect of estrogen in the growth of cancer cells, as suggested for the esophageal cancer[16] in order to explain the mystery of male predominance.

Grimm et al[16] investigated ERα and Progesterone (PR) expression, using breast cancer tissues as a representative positive control. ERα expression was found in four oral precursor lesions (squamous intraepithelial neoplasia, SIN I-III, n=4/35, 11%) and in five OSCC specimens (n=5/46, 11%) while no ERa expression was found in normal oral mucosa (n=0/5) and simple hyperplasia (n=0/11), but PR expression was not found in normal oral mucosa (n=0/5), oral precursor lesions (simple hyperplasia,
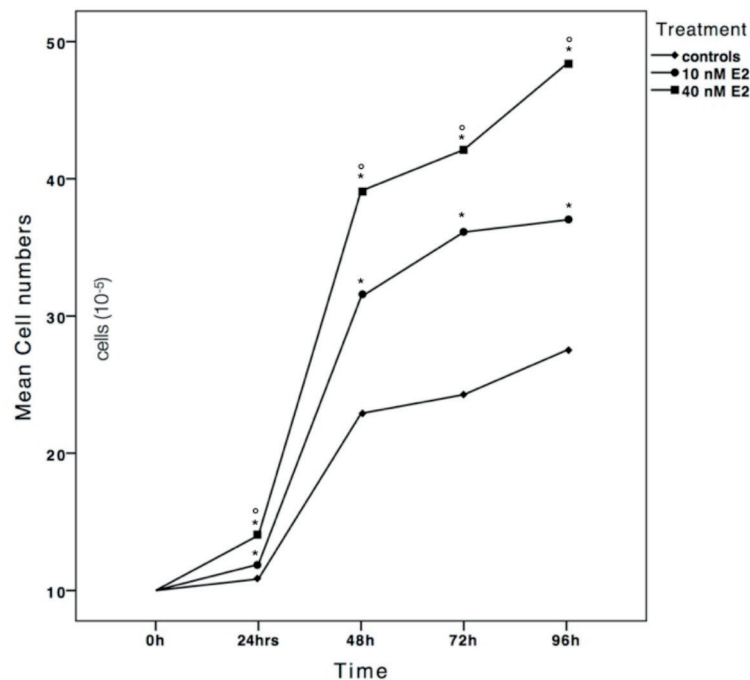
Figure 1. Growth curve of D-dSCs. D-dSCs were grown in absence (controls) and in presence of estradiol (E2) either 10 nM (10 nM E2) or 40 nM (40 nM E2). *$p<0.05$ *vs.* CTR; °$p<0.05$ *vs.* 10 nM E2.

n=0/11; squamous intraepithelial neoplasia, SIN I-III, n=0/35), and OSCC specimens (older-aged OSCC patients, n=0/46; young female OSCC patients n=0/7). So, they conclude that ER expression could be regarded as a seldom risk factor for OSCC, whereas PR expression seems to be not relevant for the development of OSCC.

The treatment with tamoxifen seems to significantly inhibit OSCC cell proliferation and invasion *in vitro*[1,7]; however, the activation status of ERα and the regulatory mechanism of ERα activation in OSCC cells are still discussed[27,28]. Aquino et al[29] tried to correlate the expression of these receptors to the histopathological degree of OSCC.

In particular, well-differentiated OSCC showed no significant difference between the expression of ERβ in tumor cells and the corresponding mucosa. In poorly-differentiated OSCC, the expression of ERβ was significantly higher in tumor cells than in the corresponding mucosa.

In patients without regular alcohol and/or nicotine abuse, there was no significant difference in ERβ expression in OSCC compared to the corresponding healthy mucosa in contrast to patients having these risk factors.

The Interplay Between Steroids and Stem Cells

Endothelial Growth Factor Receptor (EGFR) overexpression is a part of squamous cell carcinoma of head and neck (HNSCC) pathogenesis, and the role of EGFR signaling in HNSCC growth and invasion has been well reported[27,28] while the role of ERα and ERß is still discussed. Chang et al[28] suggest that ERα activity can be enhanced by focal adhesion kinase (FAK)/Protein kinase B (AKT) signaling, which is critical for promoting cell growth in OSCC cell lines. The aberrant expression or activation of FAK and ERα proteins are risk factors for promoting the malignant progression of various cancers. In the head and neck region, the increased expression of FAK or ER is found in malignant tissues, which is significantly correlated with reduced progression-free survival in HNSCC patients[29]. A cross-talk between ER and EGFR in HNSCC cell lines has been evaluated[30]; in fact, combined estrogen (E2) and Endothelial Growth Factor (EGF) treatment seem to increase phospho-MAP kinase (P-MAPK) levels.

Moreover, in HNSCC cells EGFR and nuclear ERα (ERα-nuc) levels seem to be increased while nuclear ERβ (ERβ-nuc) levels did not differ. In

this way, patients with high ERα-nuc and EGFR tumor levels could have reduced the progression-free survival compared to patients with low tumor ERα-nuc and EGFR levels[31]. These results suggest that the combined inhibition of these two pathways augment the inhibition of invasion compared with blockade of each pathway separately. Moreover, ERα and ERβ are expressed in HN-SCC, and the stimulation with ER ligands resulted in both cytoplasmic signal transduction and transcriptional activation (Figure 2).

Stem Cells in Oral Cancer

In the context of steroid hormones, stem cells may represent a powerful clinical tool for diseases of the steroidogenic organs. Gondo et al[32] demonstrated that adenovirus-mediated forced expression of Steroidogenic factor 1 (SF-1) could induce the long-term cultured mouse bone marrow cells (BMCs) to differentiate into steroidogenic cells. In particular, these cells, in response to adrenocorticotropic hormone (ACTH), make the *de novo* synthesis of multiple steroid hormones showing a mixed pattern of adrenal and gonadal phenotypes. Human studies of Yazawa et al[33] have demonstrated the generation of human steroidogenic cells, both *in vivo* and *in vitro*. Mesenchymal stem cells (MSCs) derived from human bone marrow expressing SF1 were able to produce adrenal and gonadal steroids after cAMP treatment. Another study on human steroidogenic-like cells was done by Wei et al[34], in which they showed that the umbilical cord Wharton's jelly-derived MSCs (UC-MSCs) became steroidogenic cells through retroviral or adenoviral overexpression of SF1.

Furthermore, they compared these cells to bone marrow-derived MSCs (BM-MSCs) and found that both cells expressed typical MSC markers and had the potential to differentiate into steroidogenic cells. Moreover, UC-MSCs had significantly higher proliferative potential than BM-MSCs, and differentiated UC-MSCs had significantly higher expression of all steroidogenic mRNAs[34]. These pathogenic models could represent a challenge to new targets and individual therapies in OSCC: therefore, it is essential to capitalize previously reported data, considering limits and pitfalls of the stem and undifferentiated cells (Figure 3).
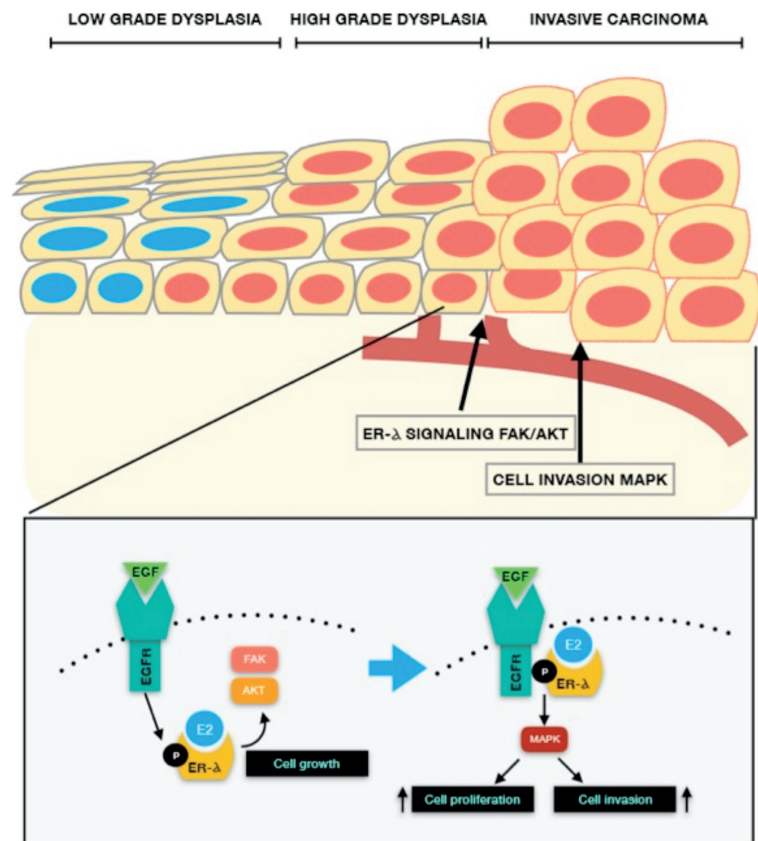

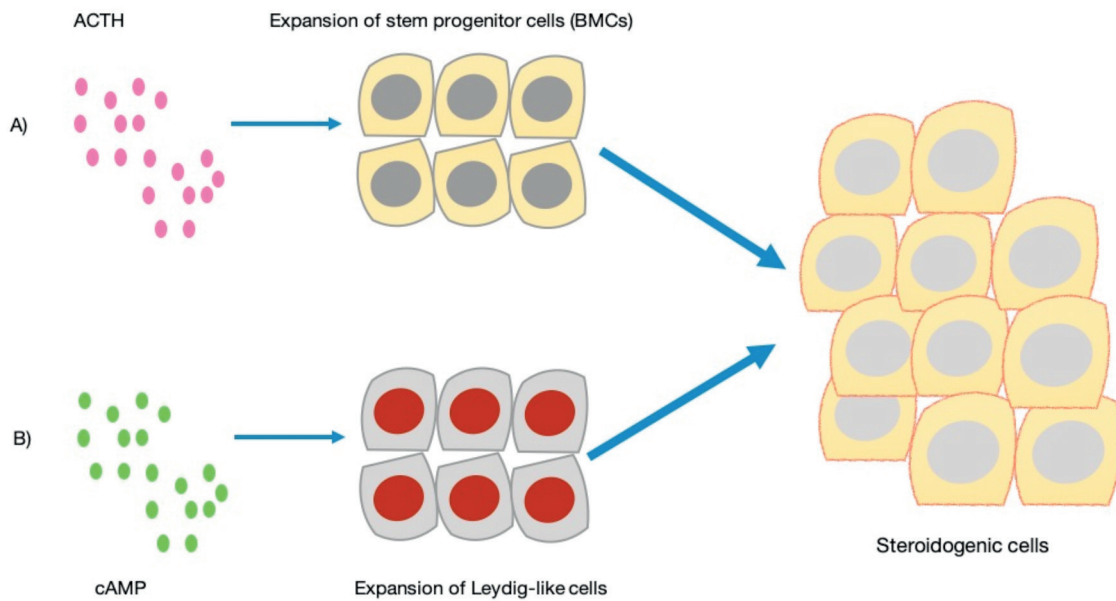**Figure 2.** Cross-talk between ERs and EGFR.

Figure 3. Characterization of the cell lineage of steroidogenic model. **A,** Schematic representation of model induced by ACTH33; **B,** Schematic representation of model induced by cAMP34.

Stem cells are characterized, in fact, by their ability to self-renew and differentiate into various organs. For example, the adult hematopoietic stem cells of the bone marrow can contribute to several lineages, as well as maintaining a niche of undifferentiated pluripotent cells. Although the differentiation potential of adult stem cells is limited, they play a crucial role in regulating tissue homeostasis, neural plasticity, and maintenance, as well as regeneration of organs after injury[19,34,35]. Somatic cells can become cancerous by activation of defined genetic elements, such as blockage of the p53/Rb pathway and activation of telomerase and several other well-described alterations to initiate metastasis and angiogenesis[36-39]. Furthermore, the tumor is composed of different types of cancer cells that contribute to tumor heterogeneity, within these populations, Cancer Stem Cells (CSCs) or Tumor-Initiating Cells (TICs) play an important role in cancer initiation and progression. Wang et al[40] proposed that cancers might arise from CSCs that replicate more extensively in the human body. Generally, they are defined as a subpopulation of cells within a tumor that can initiate, regenerate, and sustain tumors[40]. CSCs microenvironmental interactions may play a critical role in maintaining CSCs and their tumorigenicity[40]. Stem-cell populations are established in "niches" that constitute a basic unit of tissue physiology, integrating the signals that mediate the balanced response of stem cells to the needs of organisms. Additionally, the niche may also induce pathologies by imposing aberrant function on stem cells or other targets[41]. The implantation of human prostate cancer cells in an ectopic environment (subcutaneously) of nude mice does not permit the expression of metastatic potential, but the same cells injected into the orthotopic site (dorsal prostates) induced robust metastasis[42-47]. Thus, the outcome of cancer metastasis depends on multiple interactions of metastatic cells with a specific organ microenvironment[48-51]. Nakamura et al[52] by using gene expression profiles generated from microarray analysis, found that the differential gene expression profile in metastatic pancreatic cancer cells depends on growth in a biologically relevant orthotopic organ microenvironment. In particular, pancreatic cancer cells were injected orthotopically and ectopically, and only the cells injected orthotopically into the pancreas expressed metastatic potential. The orthotopic tumors expressed 226 unique genes, and the ectopic tumors expressed 98 genes. Many of the genes expressed in the metastatic cell line are involved in several phosphorylated signaling pathways and have been identified as metastasis-related genes, such as Tropomyosin-related kinase B, Axl receptor tyrosine kinase, phosphoglycerate kinase 1 (PGK1), mitochondrial ribosomal protein S6 (MRPS6)[53]. In adult human male germ

cell tumors the molecular mechanisms of germ cell transformation, differentiation, or sensitivity and resistance to chemotherapy sensitivity and resistance are linked to a very early overexpression of cyclin D2, loss of regulators of germ cell-totipotence and of embryonic development, genomic imprinting, and an apoptotic pathway altered p53-dependent[54,55]. MSCs are multipotent progenitor cells that exhibit a marked tropism for tumors, and they are non-hematopoietic stromal cells that are capable of differentiating and contribute to the regeneration of mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, and adipose. They are identified by the expression of many molecules, including CD105 (SH2) and CD73 (SH3/4), but not for the hematopoietic markers CD34, CD45, and CD14[56,57]. Many studies have shown that MSCs promote tumor progression and metastasis, while other studies report that MSCs suppress tumor growth. This difference could be attributed to multiple factors (differences in tumor models, heterogeneity of MSCs, the dose or timing of the MSCs injected, the animal host, or to other factors)[58,59]. In human renal cell carcinoma we identified a subset of cancer stem cells expressing the mesenchymal stem cell marker CD105 that displays stem cell properties, such as clonogenic ability, expression of Nestin, Nanog, and Oct3-4 stem cell markers, and lack of epithelial differentiation markers. This population, *in vivo*, can generate epithelial and endothelial cells and serially transplantable tumors[60-62]. Ghensi et al[63] studied the influence of Osteon Growth Induction (OGI) surface properties on the angiogenic and osteogenic behaviors of MSCs. They showed by analyzing gene expression profiler that MSCs on OGI surfaces are able to express endothelial and osteogenic markers such as vincula, FAK, and integrin, moreover they secreted typical osteoblastic factors, such as HGF and MCSF by the production of ALP and by the gene expression related to osteogenic commitment, such as osteopontin, osteonectin, and Runt-related transcription factor (RUNX)[63]. OSCC is a malignancy that arises in the squamous epithelium lining the oral cavity and includes the tumors found on the tongue, lip, gingiva, palate, the floor of the mouth, and buccal mucosa. However, the main negative prognostic factor is the presence of lymph node metastasis.

This subpopulation can invade the tumor's stroma, migrate, and reach the blood and lymphatic circulation. Once they arrive at a metastatic lymph node, they revert, through the reverse process of mesenchymal-to-epithelial transition (MET), to the proliferative non-EMT phenotype (CD44 high ESA high) to enable the formation of a metastatic tumor at that secondary site[64-69]. HNSCC is a heterogeneous disease that frequently shows local recurrence and metastasis after the initial treatment of the primary tumor. Several studies suggest that small populations of CSCs are responsible for initiation, tumorigenesis, progression, and metastasis. In fact, in HNSCC, a CD44+ subpopulation of cells with CSC properties has been identified to express a high level of the BMI1 gene that plays a role in self-renewal and tumorigenesis[70,71]. Han et al[72] have identified and characterized a distinct CD24+ subpopulation in the CD44+ population of HNSCC tumors. These CD24+/CD44+ cells displayed several features typically seen in cancer stem cells, including the ability to differentiate and self-renewal. Moreover, CD24+/CD44+ cells were more proliferative and invasive *in vitro* and more tumorigenic *in vivo*, forming larger tumors in athymic nude mice. Furthermore, they were slightly more resistant to chemotherapeutic agents compared to CD24-/CD44+ cells[72]. Oral tongue squamous cell carcinoma (OTSCC) is the most common oral cavity cancer. The current mainstay treatment for OTSCC is surgery, often with postoperative radiotherapy and sometimes chemotherapy. Baillie et al[73] have demonstrated the presence of two putative CSC subpopulations in moderately differentiated oral tongue squamous cell carcinoma (MDOTSCC): one within the TNs and the other within the peri-tumoral stroma. Also, they have demonstrated the expression of the components of RAS by these CSCs. The CSC subpopulation within the TNs expresses PRR, ATIIR1, and ATIIR2, while the CSC subpopulation within the peri-tumoral stroma expresses PRR, ACE, ATIIR1, and ATIIR2[73]. Itinteang et al[74] showed the expression and localization of cathepsins B, D, and G concerning the CSC subpopulations within MDOTSCC. Cathepsins B and D were localized to CSCs within the tumor nests, while cathepsin B was localized to the CSCs within the peri-tumoral stroma, and cathepsin G was localized to the tryptase+phenotypic mast cells within the peri-tumoral stroma[75]. For these reasons, it has been suggested that CSCs could be a potential therapeutic target for MDOTSCC, through modulation of cathepsin B and D, and potentially G, in addition to modulation of the classical RAS and RNA/microRNA (miRNA) molecular mechanisms[76,77].

Conclusions

This work underlines that D-dSCs represent a useful source for producing steroidogenic models that could provide a basis for their use in the target and gene therapies in translational medicine. Despite improvements in therapeutic and diagnostic techniques in recent years, OSCC remains a lethal disease with a five-year survival rate of approximately 50%, urging the need for novel treatment modalities. Therefore, the possible manipulation of steroids as treatments will be an intriguing perspective in clinical practice. Based on the principle of the epithelial-mesenchymal transition and, assuming that the OSCC cells present ERα and ERβ, D-dSCs could act as responsive hormone cells. This explorative study can be a starting point for a highly selective target therapy that intervenes in the processes of cell proliferation and death in the OSCC (mostly for OSCC refractory to any therapeutic approach), making the use of hormones a valid clinical perspective.

Authors' contributions

M.B. participated in the whole study design and supervised the experiments. D.D.S. participated in data analysis. A.B. and S.C. participated in patiens enrollment, stem cells isolation, and contributed to manuscript writing. L.Q. and E.B. drafted the manuscript. A.S., G.M., and E.X. contributed to reagents, materials, and analysis tools. P.A., G.D., M.C., M.N. and B.R. participated in bibliographic research. F.I. and M.D.D. supervised the manuscript and gave the final approval of the version to be published. All authors read and approved the final version of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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