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Oral Mucosa –Derived Stem Cells Relevant to their Therapeutic Potential (A Review)

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ABSTRACT

Regenerative medicine has paid attention to stem cell replacement therapy. This is in an attempt to construct artificial tissue for transplant, to correct specific systemic diseases and to create cell mediated wound healing therapies. A multitude of methods are being developed to obtain non-transformed adaptable cells, i.e. cells with functional plasticity. However, many ethical and scientific problems have made the establishment of a suitable technique to grow adult somatic cells with maximum plasticity, from human tissue, a difficult and a debated issue. Thus,oral mucosa has been focused as an attractive source of cells for regenerative therapy because it can be easily obtained without causing aesthetic issues or the need for tooth extraction. Also, it has strong tissue regeneration capabilities. Histologically, the oral mucosal tissue consists of surface epithelium and underlying connective tissue. Hence, this review aims to survey the current state of knowledge about isolation and characterization of both oral epithelial and mesenchymal –derived stem cells and their relevant therapeutic and regenerative potential.

Key words: Oral Mucosa, Epithelial, Mesenchymal-derived Stem Cells, Regeneration, Multipotency, Stem Cell Therapy.

Introduction

Cell- based therapies (Oliveira and Hodges,2005; Bavister *et al.,* 2005; Lei and Andreadis,2008; Hodgkinson *et al*.,2010; Mignone *et al*.,2010) have directed recent research work to investigate new techniques for stem cells isolation for construction of cell based wound healing and improvement of regenerative medicine. The establishment of a technique to grow adult somatic cells with maximum plasticity, from human tissue, has faced many of the well-known and currently debated ethical and scientific problems associated with the use of embryonic stem cells and induced pluripotent stem cells (Lister *et al*., 2011). In addition, complex isolation methods are not suitable for clinical application. Hence, the ease of isolation, accessible tissue source, and rapid *ex vivo* expansion, with maintenance of stable stem-cell like phenotypes, render oral mucosa- derived stem cells a promising alternative cell source for cell-based therapies. Oral mucosal tissue, which lines the inside of the oral cavity, consists of stratified oral squamous epithelial cells and an underlying connective tissue composed of the lamina propria and submucosal tissue. In previous studies, several researchers have focused their work on epithelial-derived stem cells supporting their efficiency in tissue regeneration and therapy (Pellegrini *et al*., 2001; Webb *et al*., 2004; Grinnell and, Bickenbach 2007; Aasen *et al.,* 2008; Marcelo *et al*., 2012). In addition , many authores have reported on mesenchymal stem cells (MSCs) derived from the gingiva or oral mucosa (Widera *et al*., 2009; Zhang *et al*.,2009; Davies *et al*.,2010; Fournier *et al*., 2010; Marynka-Kalmani *et al*.,2010; Wang *et al*.,2011; Zhang *et*

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al.,2012; Zhang *et al*.,2012; Fournier *et al*., 2013; Treves-Manusevitz *et al*., 2013; Matsumura *et al*.,2015) confirming their successful results in stem cell-based therapy. MSCs represent a heterogeneous population of non-hematopoietic stem cells, which were first characterized from bone marrow (Luria et al., 1971) and subsequently identified from various adult tissues, including oral tissues (Gronthos *et al*., 2000; Miura *et al*., 2003; Zhang *et al*., 2009). Originally, because of their multipotent capabilities, MSCs were regarded as the major source of reparative progenitor cells in tissue engineering to replace damaged tissues (Hermann *et al*., 2006; Kuroda *et al*., 2010). Thus, this review will cast light on the recent progress of research work for isolation and characterization of human adult oral epithelial and mesenchymal- derived stem cells and advances in designs of an adult stem cell-based therapy and their potential to be used in regenerative medicine.

Isolation and Characterization of Oral Mucosa-derived Epithelial Stem Cells

Epithelial cells, including those isolated from the epidermis and oral mucosa consist of a family of cells: stem cells, progenitor or transitional cells and differentiated cells. Stem cells can be defined as cells that have the potential to divide and to produce a replica cell as well as differentiated progeny and are thought to last the lifetime of the organism (Barrandon and Green,1985). In the interfollicular epidermis (non-hair follicle) and in the oral mucosa and other epithelia, these cells are reported to have specific expression patterns of several cell surface markers (Webb *et al*., 2004) , and in vitro are reported to be "small' in size (15–20 microns in diameter when attached to the growth surface) (Barrandon and Green,1985). Progenitor or transitional cells are dividing cells committed to differentiation, are larger (30–40 microns) and the differentiated cells are greater than 40 microns and have a distinctive "differentiated" appearance in culture.

In routine tissue culture of epithelia cells, it is thought that some stem cells are in the initial culture, along with progenitor or transitional cells but that the stem cells are "lost" during culture growth and passage, with progenitor cells forming the primary cultures, with limited life-span. The routine culture of primary keratinocytes from skin and other epithelia involves changing the spent medium over the cell monolayer every second day with standard amount of medium (T-25 flask uses 5 ml of medium; a T-75 flask holds 15 ml of medium, etc.). At about 70% confluence, the monolayer is passed or split, using trypsin or dispase and if treated with care, a primary culture can be passed 7–12 times. In this type of "routine" keratinocyte culture, monolayer confluence induces differentiation and eventual cell death if the cells are not passed soon after reaching 100%.

Recently, a technique has been developed that produced epithelial cell strains with a high percentage of small diameter cells (Marcelo *et al*., 2012). The cells have proliferative potential and grow in a coordinated monolayer/suspension. The cell suspension, called epithelial Pop Up Keratinocytes (ePUKs) were analyzed for culture expansion, cell size and glucose utilization, attachment to carrier beads, micro-spheroid formation, induction of keratinocyte differentiation, and characterized by immunohistochemistry. The technology has a number of unusual manipulations: the cells were fed once a day, with 2-3X the amount of medium, which is serum. The ePUKs were more fragile than their traditionally cultured counterparts, most notably being sensitive to centrifugation. It was thought that the cells might grow as spheroid cells (Lin and Chang,2008), since epithelial cells with enhanced growth potential can grow as bundles of cells in suspension, but no evidence of this type of cells growth was seen(Marcelo *et al*.,2012) . The ePUK producing monolayers appeared to have a high nutrient requirement, as indexed by high glucose utilization with subsequent rapid growth. This concept would suggest that constant feeding of the cells, as in a continuous feed bioreactor, may allow the cells to expand for a limitless number of passages allowing creation of master cell banks for use in cell based therapies. The importance of nutrient levels in ePUK proliferation implicates cell signaling pathways such as mTOR as control points in this type of in vitro epithelial cell growth (Zoncu *et al*., 2011). This pathway is also implicated by recent studies (Marcelo *et al*., 2012) demonstrating that rapamycin, an inhibitor of mTOR (C1) allows for greatly expanded growth of oral mucosal epithelial cells in monolayer cultures (Izumi *et al*., 2009).

Rapid expansion of high quality cells is essential for decreasing the culture period resulting in prompt treatment and, mitigation of labor and cost. Therefore, a successfully isolated stem/progenitor cell would provide a robust source of cells for use in regenerative medicine. This could be achieved through ePUK, which is human epithelial cells having high proliferative ability using a unique culture

system and expansion technique (Marcelo *et al*., 2012; Peramo *et al*., 2013). Further characterization of ePUKs is needed prior to their tissue engineering applications. ePUKs are floating, non-attached cells produced by large sized colonies in monolayer culture which are fed daily with 2-fold the usual amount of culture medium and the cell suspension containing ePUKs is poured into a new flask to form another monolayer without the use of enzymes to split the cultures (Marcelo *et al*.,2012). Small-sized cultured keratinocytes are reported as a progenitor/stem-cell-enriched population since they have a high ability of colony formation and long term proliferative capacity (Izumi *et al*., 2007; Fujimori *et al*., 2009). However, when investigating the ePUKs undifferentiated profile, it is important to eliminate the contamination of the large sized dead or aged cells which are also floating in the media over laying a confluent monolayer, that lose their adherent ability. In previous study, Gravity Assisted Cell Sorting (GACS) was reported as a unique method to separate cells by size (Izumi *et al*., 2013). Isolation of small sized cells in ePUKs using this method provides an enriched undifferentiated small-sized cell population. The authors hypothesized that a comparison of selected small-sized cells in ePUKs versus traditional monolayer cultured cells would assist in identifying specific markers, which would be useful to isolate them for clinical use. Thus, it was of great significance to characterize small-sized ePUKs comprehensively by a proteomics-based approach to define their usefulness in regenerative medicine.

Hence,Gravity Assisted Cell Sorting (GACS) has been used to isolate a small-sized population of undifferentiated cells enriched ePUKs. LC/MS/MS analysis was performed to define the cellular profile of ePUKs of primary human oral mucosa keratinocytes (Kato *et al*., 2015). Small sized ePUKs which showed increased expression of Dickkopf WNT signaling pathway inhibitor 1 (DKK1), serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1), follistatin and tenascin-C were verified by Western blots. These proteins are involved in the regulation of cellular movement, hair follicle development and the maintenance of its stem cell niche. The authors concluded that the fabrication of a tissue-engineered oral mucosa, ex vivo produced oral mucosa equivalent (EVPOME), using ePUKs showed increased abundance of these verified proteins. These findings indicate that the specific phenotype of ePUKs and their ability to influence wound healing promotion are implicated by highly expressed cellular movement regulatory proteins. Therefore,ePUKs proved to be a useful cell source for use in regenerative medicine.

Expression of MSCs-Related Genes in Oral Mucosa

Oral mucosa is similar to skin in histological structures and biological functions including oral defense and resistance to shear stress or friction (Stephens and Genever, 2007). However, gingiva has also been charecterized for its sensitivity to inflammation, fibrosis response, and proneness to drug-induced overgrowth (Nakasone *et al*., 2009; Garlet, 2010). So, MSCs derived from gingiva might possess some intrinsic properties distinct from those of oral mucosa-derived MSCs (Tang *et al*., 2011). Recent studies have shown that a population of clustered cells in the lamina propria of human gingiva displays positive signals for pluripotency-related markers, Oct-4, SSEA-4, and Stro-1(Zhang *et al*., 2009; Tang *et al*., 2011), with some co-expressing Oct-4/SSEA-4 or Oct4/Stro-1 (Zhang *et al*., 2009). In addition, the human oral mucosal/gingival lamina propria (OMLP) proved to harbor a population of cells positive for low affinity neurotrophin (p75), a marker of neural stem cells, organized in cord-like structures that are also positively stained for Oct-4 and Sox2 (Marynka-Kalmani *et al*., 2010). These findings suggest that human oral mucosa and gingival tissues harbor progenitors or adult stem cells; however, the potential biological differences between these 2 related populations of oral MSCs need more investigations.

Isolation of Oral Mucosa-derived MSCs

Although isolation of oral mucosal stromal stem cells has been previously reported, complex isolation methods are not suitable for clinical application. Gingiva- or oral mucosa-derived stem cells are multipotent MSCs that can differentiate into osteoblasts, chondrocytes, and adipocytes under appropriate conditions, with phenotypes similar to those of bone marrow MSCs (Fournier *et al*., 2010; Wang *et al*., 2011). In addition, oral mucosa-derived stem cells can differentiate into neural cells and have been proposed to have capabilities similar to neural crest stem cells (NCSCs) (Marynka-

Kalmani *et al.,* 2010;Davies et al.,2010; Widera *et al*., 2009; Fournier *et al*.,2013; Zhang *et al*., 2012; Matsumura *et al*.,2015).

The characteristics of mouse neural crest-derived cells in oral mucosa stem cells using Wnt1-Cre- R26R transgenic mice have been elucidated (Xu et al., 2013). Their data indicated that about 90% of colony-forming cells were derived from neural crest cells and that oral mucosa stromal stem cells contain cells from the neural crest .However, these experimental procedures are possible only in rodent models; it is not possible to use the same technique with human samples. NCSCs have been isolated from rodent or human tissues, including skin (Toma *et al*., 2005; Wong *et al*., 2006; Hunt *et al*.,2008; Hill *et al*.,2012), bone marrow (Nagoshi *et al*., 2008 ; Morikawa *et al*.,2009), and apical papilla (Abe *et al*., 2011; Abe *et al*.,2012), using a neurosphere formation technique, which enables the enrichment of stem/progenitor cells. The neurosphere culture technique is a convenient method for isolating NCSCs, and neurosphere generation is a phenotype of NCSCs (Toma *et al*., 2005; Hunt *et al*.,2008; Nagoshi., *et al* 2008; Abe *et al*.,2012). However, the process of isolating and characterizing human oral mucosa stromal cells (OMSCs) using the neurosphere culture system is not fully understood.

Recently, the molecular mechanisms underlying the application of this system to human OMSCs have been investigated (Abe *et al*., 2016). In this study, the authors attempted to isolate human NCSCs from OMSCs using the neurosphere technique. They eventually identified oral mucosa sphere-forming cells (OMSFCs). In addition, the human OMSCs formed sphere, exhibited self-renewal capabilities and multipotency. These cells were also enriched with populations of neural crest-derived cells. These results suggest that the neurosphere culture technique can be applied, without the need for complex isolation techniques, to produce multipotent spheres with the properties of NCSCs. Additionally, the hard tissue formation ability of OMSFCs was confirmed in vivo.Therefore, this study has demonstrated a convenient strategy for the isolation and culture of human OMSCs for clinical applications and tissue regeneration.

Characterization of Human Oral Mucosa-derived MSCs.

Although progenitor cells isolated from the lamina propria of oral mucosa and gingiva have been designated under different terms—*e.g*., gingiva-derived mesenchymal stem/stromal cells (GMSCs) (Zhang *et al*., 2009; Tang *et al*., 2011; Wang *et al*., 2011), gingival-tissue-derived stem cells (GT MSCs) (Tomar *et al*., 2010), gingival multipotent progenitor cells (GMPCs) (Fournier *et al*., 2010), human oral mucosa stem cells (hOMSCs) (Marynka-Kalmani *et al*., 2010), and oral mucosa lamina propria progenitor cells (OMLP-PCs) (Davies *et al*., 2010) ,yet, they are similar in MSC-associated properties.

Self-renewal

The self-renewal capabilities of human oral mucosa- and gingival propria-derived MSCs have been demonstrated by CFU-F assay (Zhang *et al*., 2009; Davies *et al*., 2010; Fournier *et al*., 2010; Marynka-Kalmani *et al*., 2010; Mitrano *et al*., 2010; Tomar *et al*., 2010; Tang *et al*., 2011; Wang *et al*., 2011). Moreover, human oral mucosa- and gingiva-derived MSCs invariably possess a higher proliferation rate than do bone marrow-derived stem cells (BMSCs) (Zhang *et al*., 2009; Davies *et al*., 2010; Marynka-Kalmani *et al*., 2010; Tomar *et al*., 2010; Tang *et al*., 2011).This was likely related to the constitutive expression of human reverse telomerase transcriptase (hTERT) (Zhang *et al*., 2009; Davies *et al*., 2010). Furthermore, the *in vivo* self-renewal capacity of gingiva-derived MSCs has been demonstrated by serial subcutaneous transplantation in immunocompromised mice (Zhang *et al*., 2009; Tang *et al*., 2011). These findings support that a population of MSCs with potent self-renewal and proliferative potentials can be readily isolated from human oral mucosa and gingival tissues and reliably expanded *ex vivo* for large-scale culture.

Multipotent Differentiation

Like BMSCs and adipose tissue-derived stem cells (ADSCs), human oral mucosa-/gingiva-derived MSCs can also differentiate into osteoblasts, adipocytes, and chondrocytes under specific *in*

vitro differentiating conditions (Zhang *et al*., 2009; Davies *et al*., 2010; Fournier *et al*., 2010;Marynka-Kalmani *et al*., 2010; Mitrano *et al*., 2010;Tomar *et al*., 2010; Tang *et al*., 2011; Wang *et al*., 2011). Additionally, oral mucosa-/gingiva-derived MSCs can differentiate into endodermal and ectodermal lineages, including various types of neural cells (Zhang *et al*., 2009; Davies *et al*., 2010; Marynka-Kalmani *et al*., 2010). As found *in vivo*, oral mucosa-/gingiva-derived MSCs embedded with carriers and subcutaneously transplanted into immunocompromised mice can generate connective tissue-like structures (Zhang *et al*., 2009; Tang *et al*., 2011), bone matrix (Fournier *et al*., 2010; Wang *et al*., 2011) and even 2 germ-layer-derived (teratoma-like) tissues (Marynka-Kalmani *et al*., 2010).

Expression of Cell-surface Markers on MSCs

Although, there is no a specific cell-surface marker for adult MSCs of distinct tissue origins (Nombela-Arrieta *et al*., 2011), they invariably express a panel of mesenchymal cell markers such as CD73, CD90, CD105, and CD44 but are negative for endothelial and hematopoietic markers such as CD31, CD34, and CD45 (Dominici *et al*., 2006). Similarly, human oral mucosa- and gingiva-derived MSCs consistently express CD29, CD44, CD73, and CD90 (> 80%) and are negative for CD34 and CD45, but are positive for CD105, CD146, and Stro-1 in variable population subsets . Collectively, these fundamental biological properties conferred by human oral mucosa-/gingiva derived progenitor cells fit the minimal criteria for human MSCs as proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici *et al*., 2006). Lately, several studies have suggested the potential neural crest origin of this unique population of MSCs (Zhang *et al*.,2009; Davies *et al*., 2010; Marynka-Kalmani *et al*., 2010); however, like other heterogeneous populations of tissue-resident MSCs, the *in vivo* identity and physiological functions of oral mucosa- and gingiva-derived MSCs remain largely unclear.

Effects of Human Oral Mucosa-Derived MSCs on the Immunity

Recently, serial *in vitro* and *in vivo* studies have been performed to investigate the immunomodulatory effects of human gingiva-derived MSCs (GMSCs) and their interplay with different types of innate and adaptive immune cells, as well as their potential clinical application in the treatment of several inflammation-related disease models in mice (Zhang *et al*.,2012).

Effects of GMSCs on T-cells

GMSCs revealed potent suppressive effects on the proliferation and activation of human peripheral blood mononuclear cells (PBMC) stimulated either by phytohemagglutinin (PHA) (Zhang *et al*., 2009) or allogenic lymphocytes in mixed lymphocyte reactions (MLRs) (Mitrano *et al*., 2010; Tang *et al*., 2011). GMSCs suppress PHA-stimulated T-lymphocyte proliferation and activation in a cell-cell contact-independent manner, apparently mediated *via* IDO (Zhang *et al*., 2009); whereas the inflammatory cytokine IFN-γ secreted by activated T-lymphocytes in the co-culture system serves as a feedback signal in the cross-talk between GMSCs and T-cells (Zhang *et al*., 2009) (Fig. 1). Davies *et al*., (2012) have recently reported that oral mucosa lamina-propria-derived progenitor cells induced inhibitory effects on activated T-lymphocytes independent of cell-cell contact, cell dose, or apoptosis, while IFN-γ or coculture with T-lymphocytes also led to the up-regulation of IDO expression (Davies *et al*., 2012). Similar immunomodulatory mechanisms mediated by elevated IDO have also been reported for other types of oral MSCs, particularly human periodontal ligament stem cells (Wada *et al*., 2009). Additionally, findings from both *in vitro* and *in vivo* studies have indicated that GMSCs could significantly inhibit Th17 cells and simultaneously promote the expansion of CD4+CD25+FoxP3+ regulatory T-cells (Tregs) (Zhang *et al*., 2009, 2010; Su *et al*., 2011; Tang *et al*., 2011). However, further studies are needed to elucidate the underlying mechanisms of interplay between oral mucosa and gingiva derived MSCs and specific types of T-helper cells.

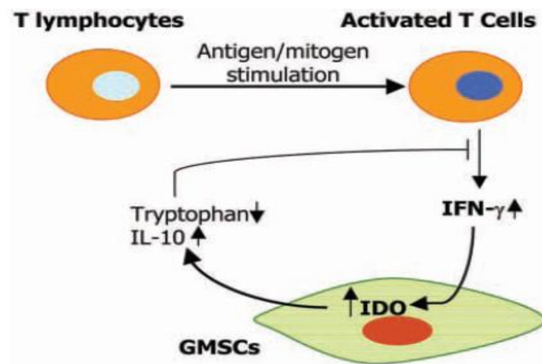

Fig. 1. Potential in teractions between activated T-lymphocytes and gingiva-derived MSCs. In response to antigen or mitogen stimulation, T-lymphocytes are activated and secrete the pro-inflammatory cytokine, interferon (IFN)-γ. Upon stimulation by IFN-γ, GMSCs express increased levels of IDO and IL-10, which subsequently dampen the proinflammatory function of activated T-cells. IDO, indoleamine 2, 3-dioxygenase.

Effects of GMSCs on Innate Immune Cells

Similar to BMSCs (English and Mahon, 2011; Lee *et al*., 2011), GMSCs exhibited potent immunomodulatory effects on several types of innate immune cells, particularly dendritic cells (DCs), macrophages, and mast cells (Zhang *et al*., 2010; Su *et al*., 2011)

Dendritic Cells

Previous studies revealed that MSCs possess profound capabilities to inhibit the maturation and activation of DCs under different settings (Spaggiari *et al*., 2009; Chiesa *et al*., 2011; Choi *et al*., 2012; Kapoor *et al*., 2012).Similarly, human GMSCs can significantly blunt the maturation and activation of DCs through the production of prostaglandin E2 (PGE2) (Su *et al*., 2011). This coincides with previous findings that MSC-derived PGE2 plays a central role in BMSC mediated inhibition of monocyte-derived DC maturation and functions (Spaggiari *et al*., 2009).

Macrophages

Macrophages constitute another essential cellular component of innate immune responses (Galli *et al*., 2011), which are generally categorized into M1 and M2 macrophages. Usually, M1 macrophages display pro-inflammatory properties, while M2 macrophages are considered to be anti-inflammatory because of their increased production of anti-inflammatory cytokines such as IL-10 and TGF-β (Laskin *et al*., 2011). Recent investigations have suggested an essential role of MSCs in modulating the phenotype and function of macrophages (Kim and Hematti, 2009; Nemeth *et al*., 2009; Bartosh *et al*., 2010; Maggini *et al*., 2010; Nakajima *et al*., 2012). Mice BMSCs proved to re-polarize macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype with enhanced interleukin-10 production (Nemeth *et al*., 2009), and co-culture with mouse BMSCs led to the conversion of activated macrophages to a regulatory-like profile (Maggini *et al*.,2010). In these studies, the secretion of PGE2 by MSCs was critical in the MSC-mediated phenotype conversion of macrophages (Nemeth *et al*., 2009; Maggini *et al*., 2010). Similarly, co-culture with human BMSCs triggers acquisition of M2 phenotype characterized by up-regulated expression of IL-10, increased phagocytic ability, and a decreased expression of pro-inflammatory cytokines (Kim and Hematti, 2009).Human BMSCs could also promote the alternative activation of infiltrated rat macrophage when they were locally transplanted at the injured spinal cord site (Nakajima *et al*., 2012).Additionally, MSC-mediated polarization of M2 macrophages displays increased phagocytic

and antimicrobial activities (Kim and Hematti, 2009; Nemeth *et al*., 2009; Maggini *et al*., 2010; Zhang *et al*., 2010), which may contribute to the emerging role of MSCs in host defense against infectious challenges (Auletta *et al*., 2012), as evidenced in a mouse model for sepsis (Nemeth *et al*., 2009; Krasnodembskaya *et al*., 2012) and zymozan-induced peritonitis (Bartosh *et al*., 2010; Choi *et al*., 2011). Likewise, GMSCs were shown to be capable of polarizing macrophages into the M2 phenotype *via* enhanced secretion of IL-6 and GM-CSF (Zhang *et al*., 2010) (Fig.2). Given the unique anatomic location of oral mucosa and gingival MSCs in the oral cavity, a complex ecosystem that contains a diverse assemblage of micro-organisms with different pathogenic potentials, it will need further studies to investigate whether GMSCs are capable of antimicrobial activity as compared with BMSCs.

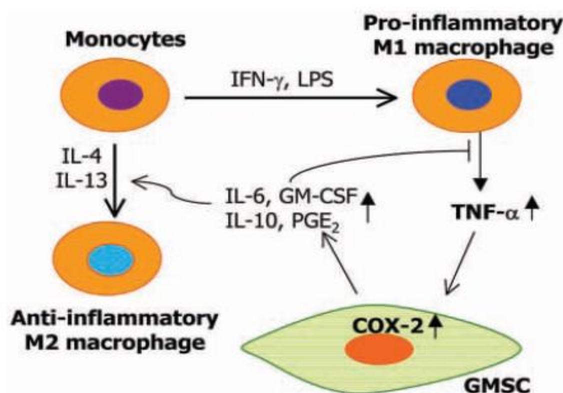

Fig. 2. Potential interactions between macrophages and gingiva derived MSCs. Activated by IFN-γ, TNF-α, or LPS, M1 macrophages produce TNF-α; which positively feeds back on MSCs to increase a variety of immunosuppressive or anti-inflammatory factors, some of which negatively regulate the M1 inflammatory responses. Other immunosuppressive factors produced by GMSCs promote the polarization of the M2 phenotype or the conversion of M1 to M2 macrophages. LPS, lipopolysaccharides; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2.

Mast Cells

Mast cells (MCs) are critical innate immune effector cells in allergic and inflammatory disorders (Sayed *et al*., 2008). It has been shown that mouse BMSCs and human GMSCs exhibited striking suppressive effects on specific functions of MCs *in vitro* and *in vivo* (Brown JM *et al*., 2011; Su *et al*., 2011). Human BMSCs and GMSCs suppressed *de novo* synthesis of the major pro-inflammtory cytokine, TNF-α, from activated human HMC-1 mast cells in a cell-cell contact-independent manner; however, it had no obvious inhibitory effects on their degranulation *in vitro* (Su *et al*., 2011). However, mouse BMSCs suppressed not only the production of pro-inflammatory cytokines by MCs, but also their degranulation, chemokinesis, and chemotaxis (Brown JM *et al*., 2011). Such discrepancies in MSC-mediated inhibitory effects on MCs may be due to the distinct cell contexts of both MSCs and MCs. However, in both studies, *in vivo* administration of BMSCs or GMSCs led to the suppression of MC degranulation in mouse skin and the peritoneal cavity (Brown JM *et al*., 2011; Su *et al*., 2011). The inhibitory effects of both human GMSCs and mouse BMSCs on MC functions were dependent on the COX2/PGE2 pathway (Brown JM *et al*., 2011; Su *et al*., 2011), and were facilitated through the activation of EP4 receptors in mouse MCs (Brown JM *et al*., 2011). These findings suggest that the TNF-α/COX2/PGE2 axis constitutes a negative feedback loop in the cross-talk between GMSCs and MCs (Su *et al*., 2011) (Fig. 3) and highlight the immunomodulatory functions of BMSCs and GMSCs on MCs and their potential application in cell-based therapy for MC-driven inflammatory diseases.

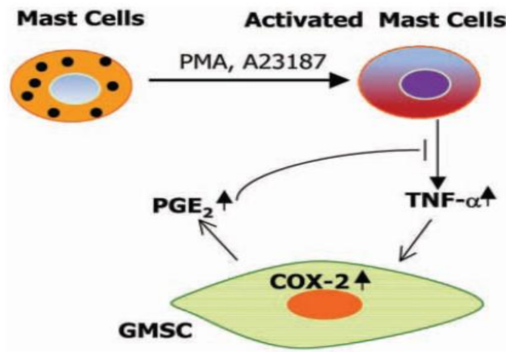

Fig. 3: Potential interactions between activated mast cells and gingiva-derived MSCs. In response to PMA stimulation, activated mast cells synthesize and secrete the pro-inflammatory cytokine, TNF-α, which acts on GMSCs to induce increased levels of COX-2 and PGE-2. These factors negatively feed back and dampen the pro-inflammatory activity of activated mast cells. PMA, phorbol 12-myristate 13-acetate; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2.

Therapeutic Potential of Human Oral mucosa- derived Epithelial Stem Cells

Oral mucosa keratinocytes are widely used for intra-oral and extra oral clinical applications including reconstructions of cornea, urethral/bladder and esophagus (Oliveira and Hodges 2005; Izumi *et al*, 2014). Induced pluripotent stem cells have been induced from human foreskin keratinocytes using specific transcription factors that were introduced using retroviral vectors indicating that the keratinocyte cell type can be reprogrammed (Lei and Andreadis 2008; Aasen *et al.*,2008). The large percentage of small diameter cells growing as ePUKs, in suspension, and lack of ePUK monolayer stratification and differentiation suggests that ePUK keratinocytes are more "plastic" in phenotype and that they may be a useful stem-cell like type for epigenetic manipulation with small molecules (Marcelo et al., 2012), as already been reported by other investigators using mouse keratinocyte cultures (Grinnell and Bickenbach 2007).

It has been shown that human epithelia keratinocytes in primary culture can be induced by tissue culture manipulation to produce, without the use of enzymes for passaging, large numbers of small cells in a combined suspension/monolayer culture. These small cells maintain their epithelial characteristics and will revert to epithelial morphology when grown at the airliquid interface on a skin equivalent model. For practical applications, the ePUK technology can be used to produce devices for wound healing and tissue engineering/ regenerative medicine possibly in higher numbers and faster than with other culture techniques (Marcelo *et al*., 2012). The ePUK keratinocyte phenotype resulted from absence of serum derived factors and manipulation of the calcium, essential fatty acid and metabolic nutritional status of the primary cultures. Thus keratinocytes in ePUK culture provide an additional controlled human primary cell system for investigation of the mechanisms regulating epithelia cell growth and differentiation. Additional studies are needed with this system because it provides an excellent possibility for the long-term maintenance of fresh, basal-like, non differentiated, proliferative epithelial populations.

The understanding of a cultured undifferentiated cell population might provide novel insights to tissue engineering where sustaining the cellular capacity of growth, proliferation and mobility is a key to the success of regenerative medicine (Markeson *et al*., 2015). It has been reported that the majority of the ePUKs are a small-sized population (Marcelo *et al.,* 2012), however, in culture supernatant, large-sized differentiated or dead cells are also floating where they have lost their adherent capacity secondary to their differentiation. Contamination of those large-sized cells cannot be ignored when we analyze the undifferentiated profiles. GACS was successfully used to enrich the population of small-sized undifferentiated cells (Izumi *et al*., 2007). In such results, proteins having higher expression in ePUKs small cells are involved in cellular movement, indicating they might have enhanced ability for wound healing and tissue regeneration. Furthermore, immunohistochemical results showed that the

increases in protein expression are seen in EVPOME devices, which requires culturing 11 days and induction of differentiation. Unlike other proteins, the intensity of tenascin-C expression in ePUKs after fabrication of EVPOME was weakened, indicating it might be affected by the change of culture conditions, which involves a differentiation process and being adherent to the scaffold. Application of ePUKs in regenerative medicine is suggested where expression of those molecules may be beneficial for wound healing after grafting. Further studies including in vivo transplantation are proposed for future tissue engineering use of ePUKs.

Given the finding among the four proteins validated by western blot, DKK1 specifically inhibits the Wnt/beta-catenin signaling cascade to bind to low-density lipoprotein receptor-related protein (LRP) 5/6 (Cruciat and Niehrs 2013). Wnt-regulated developmental processes including posterior axial patterning, somitogenesis, angiogenesis, vasculogenesis, and organ formation are implicated in pathological events, including cancer and bone disease (Cruciat and Niehrs 2013). It also regulates epithelial thickness and senescence in skin and oral mucosa (Yamaguchi *et al*., 2008; Zhao *et al*., 2009). Follistatin is an antagonist of activin and a subset of TGFβ super family molecules including myostatin and Bone Morphogenetic Proteins (deKretser *et al.,* 2012). Blocking activin action by pre-treatment with its binding protein, follistatin, modifies the inflammatory cytokine cascade, and reduces the severity of the subsequent inflammatory response and mortality (deKretser *et al*., 2012). Limited activation of activin by follistatin in keratinocytes is beneficial for the wound healing process to prevent fibrosis (Antsiferova *et al*., 2009).

SERPINE-1 modulates detachment/re-adhesion cycles involving cellular migration through cell surface receptors including integrin and laminin (Czekay *et al*., 2011). SERPINE-1 expression also correlates with tumor progression, where it is utilized as a cancer marker with poor prognosis (Providence *et al*., 2004; Hundsdorfer *et al*., 2005; Lee and Huang 2005; Cheng *et al*., 2008; Freytag *et al*., 2009). During epithelial wound healing, SERPINE-1 is expressed at the wound edge where cell migration is important to achieve wound closure (Czekay *et al*., 2011). Tenascin-C maintains the stem cell niche of the sub ventricular zone of the central nervous system, hematopoietic stem cell niches in bone marrow, corneal limbus and dental pulp (Chiquet-Ehrismann *et al.,* 2014). Regulation of cellular mobility and adherence to interact with fibronectin, integrins and heparin have an important role on wound healing (Aukhilt *et al*.,1993; Sriramarao *et al*.,1993; Yokosaki *et al*.,1994; Schnapp *et al*.,1995; Hauzenberger *et al*.,1999). Interestingly, tenascin-C, DKK1 and follistatin orchestrate the hair follicle development and maintenance of its stem cell niche (Nakamura *et al*., 2003; Chen *et al.,*2014;Hsu *et al*., 2014). High hierarchal progenitor population expressing these markers might be the reservoir of the high Hsu proliferative cells investigated in previous ePUKs study (Marcelo *et al*., 2012). As a similar concept of ePUKs, Chaffer *et al*.,2011 identified that populations of human mammary epithelial cells cultured in their normal mammary epithelial growth medium contained a small proportion of cells that grew as floating cells above the majority population of differentiated adherent cells which can revert to an undifferentiated state, indicating micro environmental signals to entering the stem cell state including epithelial-mesenchymal transition may be provoked in those culture conditions (Chaffer *et al*.,2011). Another explanation could be cellular competition, which is reported as a phenomenon to exclude different phenotype of the cells both in vivo and in vitro, i.e., transformed cells in early stage of carcinogenesis or to coordinate the patterning and growth of normal tissues during development (Hogan *et al*.,2011; Amoyel and Bach 2014; Yamauchi and Fujita 2012). In Marcelo study, a heterogeneous cell population of primary keratinocytes culture including differentiated cells, proliferating cells, or undifferentiated cells might cause cellular popping. Thus, under the environment of contact inhibition, differentiated keratinocytes may recognize undifferentiated cells as disparate neighbors and extrude undifferentiated cells from their society regulated by the specific signaling pathway, resulting in ePUKs (Zhao *et al*., 2011).

Therapeutic Potential of Human Oral mucosa- derived MSCs in Animal Models

The potent immunomodulatory and anti-inflammatory properties of human oral mucosa-/gingiva-derived MSCs position them as a promising cell source for MSC-based therapies for wound repair and a wide range of inflammation-related diseases.

Wound Healing

Wound healing is a complex process involving the participation of many types of immune and resident cells. Using a chemotherapy induced oral mucositis (OM) mouse model, a compromised wound model in oral mucosa, it was found that systemic infusion of human GMSCs could mitigate the pathology of OM, as evidenced by reversal of body weight loss and restoration of the disrupted epithelial lining and proliferative basal cells (Zhang *et al*., 2011). In addition, Wang *et al*. found that local application of human GMSCs could significantly promote the repair of mandibular wounds and calvarial defects in rats (Wang *et al*., 2011). In a murine excisional full-thickness skin wound model, systemic infusion of human GMSCs significantly accelerated the repair process, as evidenced by rapid re-epithelialization and increased angiogenesis (Zhang *et al*., 2010). Compared with normal skin, increased numbers of infused MSCs were detected at the wound bed, where they were close to and interacted with resident macrophages, potentially contributing to their conversion to an anti inflammatory M2 phenotype (Zhang *et al*., 2010). Meanwhile, systemic infusion of GMSCs significantly suppressed the local infiltration of inflammatory cells and proinflammatory cytokines such as TNF-α and IL-6, but simultaneously increased IL-10 (Zhang *et al*., 2010). These findings suggest that GMSCs enhance skin wound healing by promoting polarization of infiltrated monocytes or reprogramming resident macrophages into the M2 phenotype,thus preparing a special microinvronment for tissue repair and remodeling.

Murine Colitis

The immunomodulatory and anti-inflammatory effects of GMSCs were also tested in a dextran sulfate sodium (DSS)- induced murine colitis model, in which Th1 and Th17 cells play an essential role (Brown JB *et al*., 2012). Systemic administration of GMSCs could reverse body weight loss, improve the overall colitis score, and restore normal intestinal architecture (Zhang *et al*., 2009). At the cellular level, GMSC treatment strikingly reduced the infiltration of CD4+IFNγ+ (Th1) and CD4+IL-17+ (Th17) cells at the colitic sites, and increased the recruitment of Tregs. At the molecular level, GMSCs remarkably suppressed pro-inflammatory cytokines such as IL-6, IL-17, and IFN-γ and increased IL-10 (Zhang *et al*., 2009). These findings suggest that GMSCs ameliorate inflammation related tissue destruction caused by experimental acute colitis by suppressing the pro-inflammatory function of Th1 and Th17 cells and promoting the infiltration of Tregs.

Allergy-related Inflammatory Diseases

The pathological condition of allergic contact dermatitis (ACD) or contact hypersensitivity (CHS) is comprised of multiple overlapping stages characterized by a dynamic and complex cellular network, including dendritic cells,CD8+ T-cells, CD4+IFNγ+ (Th1), CD4+IL-17+ (Th17), mast cells, and Tregs, as well as their cytokines (Vocanson *et al*., 2009; Fonacier *et al*., 2010). Using a hapten (oxazolone)-induced murine CHS model, showed that both prophylctic and therapeutic dministration of GMSCs could mitigate clinical signs of CHS (Su *et al*., 2011). Following GMSC treatment, a reduced infiltration of dendritic cells (DCs), CD8+ T-cells, Th17, total and degranulated mast cells (MCs), a decreased level of a variety of inflammatory cytokines, and a reciprocal increased infiltration of Tregs and expression of IL-10 at regional lymph nodes and inflammatory areas were observed (Zhang *et al*., 2012). The underlying mechanism of GMSCmediated attenuation of CHS involves the COX2/PGE2 axis (Su *et al*., 2011). These findings suggest that GMSCs suppress CHS through targeting multiple types of innate and adaptive immune cells (Su *et al*., 2011), and the use of MSCs in cell-based therapy potentially contributes a novel modality for the treatment of allergic diseases.

Mouse Skin Allograft Model

Systemic infusion of GMSCs exhibited remarkable immune tolerance and promoted the survival of skin allografts,whereby the increased infiltration of Tregs may play a major role (Tang *et al*.,

2011). These immunosuppressant capabilities in the graft *vs*. host disease model further extend the clinical spectrum based on the unique immunomodulatory functions evidenced by GMSCs.

The Role of Human Oral Mucosa - Derived MSCs in Tissue Regeneration

It has been reported that MSCs could mediate tissue regeneration through their multipotent capabilities that enable them to replace damaged cells (Hermann *et al*., 2006; Kuroda *et al*., 2010). This could be achieved through interaction of MSCs with host/ resident cells and production of a large array of trophic factors, capable of immunomodulatory and anti-inflammatory functions (Prockop, 2009; Roddy *et al*., 2011; Prockop and Oh, 2012). Despite the reported multipotent capabilities of oral mucosa and gingiva-derived MSCs, both *in vitro* and *in vivo* (Zhang *et al*., 2009; Davies *et al*., 2010; Fournier *et al*., 2010; Marynka- Kalmani *et al*., 2010; Mitrano *et al*., 2010; Tomar *et al*., 2010; Tang *et al*., 2011; Wang *et al*., 2011), evidence supporting their direct role in tissue regeneration or replacement remains scanty. Using a chemotherapy-induced oral mucositis model, only a very few GMSCs were found to 'home' to the injured sites and transdifferentiate into epithelial-like cells (Zhang *et al*., 2011). The regenerative effects mediated by cultured GMSCs might be due to an increased expression of various chemokines and growth factors, as well as an increased resistance to oxidant stress-induced apoptosis (Zhang *et al*., 2011). In mouse models of skin wound and colitis, the mechanisms underlying GMSC-mediated acceleration of cutaneous and intestinal healing and regeneration might involve both pro-angiogenic and anti-inflammatory functions (Zhang *et al*., 2009, 2010). These findings further support that GMSCs, like other MSCs, may have promoted tissue regeneration *via* their trophic factors, not just their multipotent capabilities. Previous studies have implied that basal fibroblast growth factor (bFGF) can stimulate BMSCs to regenerate both bone and soft tissues, thus serving as an important growth factor for tissue regeneration (Sahoo *et al*., 2010; Tasso *et al*., 2012). However, its effect on GMSCs remains to be determined. Further research on this unique population of MSCs will undoubtedly contribute to a deeper understanding of the mechanisms underlying their immunomodulatory and tissue-regenerative functions under different pathophysiological settings. Further, this will enhance our understanding of the biological properties of oral mucosa-/gingiva-derived MSCs and their important roles in tissue regeneration and cell-based therapy of immune- and/or inflammation-related diseases.

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