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Dental pulp stem cells and osteogenesis: an update

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Abstract Dental pulp stem cells constitute an attractive source of multipotent mesenchymal stem cells owing to their high proliferation rate and multilineage differentiation potential. Osteogenesis is initiated by osteoblasts, which originate from mesenchymal stem cells. These cells express specific surface antigens that disappear gradually during osteodifferentiation. In parallel, the appearance of characteristic markers, including alkaline phosphatase, collagen type I, osteocalcin and osteopontin characterize the osteoblastic phenotype of dental pulp stem cells. This review will shed the light on the osteogenic differentiation potential of dental pulp stem cells and explore the culture medium components, and markers associated with osteodifferentiation of these cells.

Keywords Osteogenesis · Regeneration · Cytotechnology · Stem cells · Bioengineering · Medicine

Introduction

Dental pulp stem cells (DPSCs) are considered as undifferentiated cells that can, when placed under specific culture conditions, generate different cell lineages, among which osteoblast-like cells. They are mesenchymal stem cells (MSCs) exhibiting similar features to those derived from bone marrow including clonogenicity and capability of self renewal (Aghajani et al. 2016). Their multilineage differentiation potential gives them a privileged status and they are considered as a source of cells replacing lost clones in regenerative medicine. Their immunological privilege adds to their importance in cellular therapy. Their ability to be cryopreserved is another major advantage making them accessible in a time dependent manner (Takebe et al. 2017). The discovery of multilineage differentiation of DPSCs prompted researchers to characterize them as an emerging source of multipotent cells when compared to bone marrow-derived mesenchymal stem cells (BMMSCs). Furthermore, DPSCs proliferation rate was proven to be higher than that of BMMSCs (Shi et al. 2001; Mortada et al. 2017). This could be due to the developmental stage of the originating tissues, since the wisdom teeth from which DPSCs are usually extracted, are the last permanent teeth to develop which makes them less mature than the bone marrow. In this article, we are going to discuss the osteodifferentiation process of DPSCs and explore the culture medium components, and associated markers expressed.

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Osteogenic differentiation potential

DPSCs can undergo transcriptional changes giving precursor cells while preserving their self renewal capacity and phenotype (Liu et al. 2016b; Shen et al. 2016). This progression is thought to be the earliest step in stem cells commitment since precursor cells have a more restricted developmental program at this stage. The exit of DPSCs from the stem cell stage into the commitment stage happens when the precursor cell continues cell division and acquires specific properties of fully committed mature cells with characteristic phenotypes. The commitment and differentiation of DPSCs to specific mature cell types, such as osteocytes (Jaiswal et al. 1997; Heng et al. 2016), is a controlled process that involves influence of chemical stimuli and the activities of several growth factors, cytokines, and extracellular matrix (ECM) components (Doi et al. 2004). Studies have highlighted the capacities of DPSCs to differentiate into functional osteoblasts in vitro and they have also been found to produce the extracellular and mineralized matrix (Shi et al. 2001; Tabatabaei and Torshabi 2017) as evidenced by the presence of several osteogenic markers. Laino et al. demonstrated that DPSCs can differentiate into osteoblast precursors and then into osteoblasts, providing living autologous fibrous bone (LAB) tissue. This tissue reacted with bone antibodies and thus is a novel source of osteoblasts and mineralized tissue. After transplantation into immunocompromised rats, LAB formed lamellar bone-containing osteocytes (Laino et al. 2005). In another in vivo study, transplanted DPSCs could codifferentiate into osteoblasts and endotheliocytes thus giving rise to adult bone tissue (d'Aquino et al. 2007). Their osteogenic capacity was further proved by radiographic findings within three months of colonization (d'Aquino et al. 2009). In a study investigating the ability of human DPSCs to reconstruct large cranial defects in non-immunosuppressed rats, the authors reported the formation of a more mature bone in defects supplied with collagen membrane and DPSCs (de Mendonça Costa et al. 2008).

Culture medium components

Role of growth factors

Several studies attempted to characterize the effects of specific factors on the osteoinduction of DPSCs. Bone morphogenic proteins (BMPs) constitute the largest subgroup of the transforming growth factor beta (TGF-β) superfamily with pleiotropic functions during development, and many are involved in skeletal development, regeneration, and repair (Bragdon et al. 2011). Several BMP genes and their activities control key steps in tooth development in mice (Wang et al. 2012). Among the members of the BMP family, BMP-2 has an inductive effect on reparative dentinogenesis as shown in the amputated pulp of canines in vivo (Nakashima 1994). Several in vitro and in vivo studies evidenced that BMP-2 promotes mouse DPSCs differentiation into odontoblast lineages (Chen et al. 2008; Cho et al. 2010). In fact, SOX2 was capable of promoting the osteogenic differentiation of DPSCs by increasing the expression of the BMPs family (Yuan et al. 2017). On the other hand, BMP-2 knockout mice were generated by crossing Bmp2 floxed (Bmp2-fx/fx) with a 3.6Col1a1-Cre mouse (Singh et al. 2008). These mice showed a lack of odontoblast maturation leading to the formation of hypomineralized dentin and abnormal dentinal tubules (Yang et al. 2012a). Vascular endothelial growth factor (VEGF) was also found to facilitate neovascularization with increased microvessel density of severed human dental pulp in a tooth slice in vivo model (Mullane et al. 2008). Besides its angiogenic effects on endothelial cells, VEGF also has a direct effect on osteogenic cell migration and differentiation as well as osteoclastogenesis in the context of bone development, regeneration, and repair (Yang et al. 2012b). VEGF enhances osteoblast activity with increased mineral nodule formation and stimulation of bone-specific genes in preosteoblasts (Deckers et al. 2000). In terms of the effects on human DPSCs, in vitro studies showed that VEGF directly increases cell proliferation and osteodifferentiation (I et al. 2011). A recent in vitro study looking at the combined effects of BMP-2 and VEGF on DPSCs osteodifferentiation showed that the addition of VEGF in the early phase rather than a continuous presence of both VEGF and BMP-2, enhances the differentiation process (Aksel and Huang 2017). In another study evaluating the osteogenic

potential of epidermal growth factor (EGF) and fibroblast growth factor (bFGF), DPSCs were cultured in the presence of either growth factor. Results revealed that cells treated with EGF lost MSCs markers CD146 CD10, and produced calcium deposits. These cells also demonstrated increased mRNA expression of alkaline phosphatase (ALP) and osteocalcin (OCN) in relation to control groups. On the other hand, bFGF treatment had an inhibitory effect. The authors therefore concluded that DPSCs in combination with EGF can be an effective stem cell-based therapy for bone tissue engineering applications in periodontics and oral implantology (Del Angel-Mosqueda et al. 2015). The role of bFGF was further investigated in another study in which DPSCs were mixed with bFGF during the osteodifferentiation period or during the pretreatment period. The addition of bFGF in the osteodifferentiation period caused a decrease in the in vitro differentiation ability of DPSCs, while 1 week pre-treatment with bFGF increased the in vitro osteogenic differentiation ability of DPSCs, whereas 2 weeks pre-treatment decreased this process. The in vitro results were reproducible in vivo. bFGF was therefore reported to affect the osteodifferentiation of DPSCs in a treatment-dependent manner both in vitro and in vivo (Qian et al. 2015). A previous study had reported that bFGF inhibited ALP activity and mineralization of DPSCs in an osteogenic medium, while mediating neurogenesis in a neuroinductive medium (Osathanon et al. 2011). Additionally, insulin-like growth factor 1 (IGF-1), and tumor necrosis factor α (TNF-α) were found to promote osteodifferentiation of DPSCs through the mTOR and NF-κB signaling pathways, respectively (Feng et al. 2013, 2014; Xing et al. 2015). On the other hand, fibroblast growth factor 9 (FGF9) was reported to enhance the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) leading to the inhibition of DPSCs osteodifferentiation (Lu et al. 2015).

Role of chemicals

Maintenance of mesenchymal cultures in the presence of dexamethasone is known to enhance lineage progression along the osteogenic or adipogenic lineages, which may explain the relatively complex patterns of cell morphology and differentiation cultures (Ghali et al. 2015). Indeed, previous research has proven that dexamethasone either alone or in combination with ascorbate-2-phosphate (AsP), and BMPs are osteogenic inducers of MSCs (Jorgensen et al. 2004; Lim et al. 2016). A recent in vitro study investigated the osteoinductive effects of dexamethasone on DPSCs (da Cunha Moretti et al. 2017). Cells from the third molar pulp were divided into two experimental groups differentiated by the intake of dexamethasone in one of them. Following an assessment of proliferation, differentiation and viability through trypan blue, methylthiazol tetrazolium, and von Kossa and alizarin red assays, respectively, results showed that dexamethasone-treated cells exhibit earlier differentiation than the remaining cells, when observed in vitro. Moreover, hydroxyapatite/tricalcium phosphate powder was also found to induce the formation dentin-like structures upon DPSCs transplantation into immunocompromised mice. Markers of dentin matrix protein, including OCN and bone sialoprotein (BSP) were detected in the DPSCs transplants 6 weeks later (Gronthos et al. 2000).

Role of the extracellular matrix components

Differentiation is a continuously regulated process where interactions between the cell and its microenvironment play an important role in maintaining stable expression of differentiation-specific genes (Blau and Baltimore 1991; Ravindran et al. 2013). Co-culture of DPSCs with endothelial cells was demonstrated to enhance their osteogenicity due to physical interactions, such as heterotypic cellular contacts, as well as chemical interactions because of soluble factors that are known to play a critical role in the regulation of DPSCs (Dissanayaka et al. 2012). Several studies applied the mineral trioxide aggregate (MTA) to DPSCs under osteoinductive conditions (Seo et al. 2013; Wang et al. 2014a; Kulan et al. 2017; Varalakshmi et al. 2013), revealing that MTA consistently increased osteogenesis of DPSCs through the upregulation of osteoblastic markers. In a study involving inflammatory DPSCs, the authors reported that MTA enhanced DPSCs osteodifferetiation by activating the NF-κB pathway (Wang et al. 2014a). The combination of simvastatin and α-tricalcium phosphate (α-TCP) was also found to induce ostegenic differentiation of DPSCs, and its effect was actually superior to that of MTA (Varalakshmi et al. 2013). Moreover, the addition of enamel matrix derivative, or demineralized dentin matrix to DPSCs have been

shown to independently upregulate the expression of markers for odontoblast/osteoblast-like cells (Wang et al. 2014b; Liu et al. 2016a). The presence of calcium (Ca^{2+}) was also associated with greater osteodifferetiation potential of DPSCs. Sohn et al. studied the role of ORAI1, an essential pore subunit of store-operated Ca^{2+}entry (SOCE), on odontogenic differentiation of human DPSCs (Sohn et al. 2015). Results showed that ORAI1 plays critical roles in the odontogenic differentiation and mineralization of DPSCs via regulation of Ca^{2+} influx, highlighting a potential role for ORAI1 in enhancing reparative dentin formation. Oncostatin M (OSM), one of the interleukin 6 (IL-6) family cytokines has also been shown to enhance osteoblastic differentiation of DPSCs via the JAK3/STAT3 signaling pathway (Feng et al. 2016).

Markers of DPSCs osteodifferentiation

Although there is currently not a single marker to determine osteodifferentiation of DPSCs, several expression markers have been considered to study the differentiation process. Evaluating osteoblast proliferation and differentiation can be achieved by estimating the expression of few osteodifferentiation-specific genes (Kasperk et al. 1995; Su et al. 2014). DPSCs were reported to express the typical osteoblastic markers such as ALP, collagen type I (Col I), opsteopontin (OPN), and OCN and they could differentiate into osteoblast-like cells producing mineralized matrix (Mori et al. 2011; Kermani et al. 2014; Paduano et al. 2016). Other studies revealed an increase in ALP and Col I mRNA expression during initiation of bone formation (Jikko et al. 1999; Jaiswal et al. 1997).

Alkaline phosphatase

Although ALP is a ubiquitous protein, it has an influential role in osteogenesis and the mineralization of the ECM (Marom et al. 2005). Several studies reported it to be a marker for detecting osteodifferentiation of cells at early stage, where the gene expression and ALP protein levels are greatly enhanced during differentiation into osteogenic lineage and they are therefore correlated with secretory activity of the cell in a substantial way (Stucki et al. 2001; Chen et al. 2011). Two days following stimulation, the levels of ALP mRNA have been shown to increase in parallel to the osteodifferentiation process (Shui et al. 2003; Qi et al. 2003). Furthermore, ALP is an ectoenzyme implicated in the release of inorganic phosphate during the cytodifferentiation phase of mineralization and thus, it is a biological marker for bone turnover (Kulterer et al. 2007). ALP is also important for the formation of ECM and calcified tissue (Liu et al. 2008). Mori et al. (2011) cultured DPSCs in an osteogenic medium and reported a gruadual increase in ALP expression after 5 and 10 days.

Collagen type I

Col I is an important element of bone ECM which binds to other ECM proteins and cell surface integrins. However, it is not a bone specific protein and is present in several other cell types. Col I mediates cell adhesion, proliferation and differentiation of the osteoblast phenotype. It was found to be upregulated in response to several methods of in vitro force application (Klein-Nulend et al. 1997; Jagodzinski et al. 2004; Paduano et al. 2017) with its mRNA level increasing after 2 days of stimulation (Pavlin and Gluhak-Heinrich 2001; Pavlin et al. 2001). Col I can therefore be considered an early marker of osteodifferentiation.

Osteocalcin

OCN is a bone-specific protein which is considered as a suitable marker for osteogenic maturation. It regulates the mineralization of hard tissue and is usually found in bone and dentin. Its production is restricted to cells responsible for mineralization, such as osteoblasts, odontoblasts, and cementoblasts (Saygin et al. 2000; Sun et al. 2006). OCN expression regulates the mineralization ability of cells and the formation of mineral nodules (Nakamura et al. 2009; Khanna-Jain et al. 2012; Thomson et al. 2003). Nakamura et al. (2009) reported that OCN acts as an early osteoblastic differentiation marker in MSCs cultured under osteoinductive conditions. Other studies however characterize it as a late indicator of osteodifferentiation (Aubin 2001) and a terminal symbol in hard tissue regeneration (Zhang et al. 2008). Indeed, OCN expression was found to considerable increase in DPSCs undergoing osteoinduction (Bakopoulou et al.

2011; Khanna-Jain et al. 2012), especially at the fifth day mark (Mori et al. 2011).

Osteopontin

OPN is a secreted adhesive glycophosphoprotein which is present in many locations such as breast milk, kidneys, bone, teeth, epithelial lining tissues and blood plasma. Sodek et al. (2000) found that OPN, along with BSP, are essential for the process of mineralization, including the repair of mineralized tissue. In repairing dentine, OPN facilitates the early formation and mineralization of the tissue. It also mediates other steps including cell division, chemotaxis, cell migration, cell adhesion, cytodifferentiation (Smith et al. 2003) and intracellular signaling (Sodek et al. 2000) needed for the formation of a new generation of odontoblasts. Dysregulation in OPN expression was associated with tumor formation (Standal et al. 2004) and osteoporosis in postmenopausal women. Interestingly, high OPN expression may prevent osteogenesis, and counteracting OPN may be effective in activating osteoclasts (Yoshitake et al. 1999). In DPSCs osteoinduction, some studies consider OPN an early marker of differentiation (Jiang et al. 2009) while others regard it as a late osteogenic marker (Khanna-Jain et al. 2012). In an experiment studying OPN expression levels in bone development, Aubin et al. (2001) reported a peak in the proliferation phase (around day 4) as well as in the mineralization phase (around day 14–21).

Growth factors and signaling pathways

As noted above, activation of specific growth factors and signaling pathways is important during the process of osteogenesis. Feng et al. (2013) analyzed the function of TNF-α (10 ng/mL) on osteogenic differentiation of human DPSCs and the role of the NF-κB signaling pathway. TNF-α increased the mineralisation and the expression of BMP2, ALP, runt-related transcription factor 2 (RUNX2) and COL I during this process. PDTC, an NF-κB inhibitor, blocked the osteogenic differentiation induced by TNF-α. The authors also detected no effect of TNF-α on proliferation of DPSCs or cell cycle as evidenced by cell counting assays and methylthiazolyldiphenyl-tetrazolium bromide (MTT) analysis. Another study on the effects of IGF-1 showed a role for the mTOR pathway in promoting the proliferation and osteogenic differentiation of DPSCs (Feng et al. 2014). In a medium consisting of DPSCs supplemented with 0.1 μmol/L dexamethasone, 10 mmol/L b-glycerophosphate, 50 μg/mL ascorbic acid, and 100 ng/mL of IGF-1, the authors noted an increased expression of RUNX2, OCN, and COL1. IGF-1 was also found to increase DPSCs proliferation via the CCK-8 assay showing a higher proliferation rate in the IGF-1-treated group compared to that of the untreated group. Supeno et al. (2013) had testified that IGF-1 acts as controlling switch for long-term proliferation of neural stem cells, and IGF-1 can promote proliferation of placental mesenchymal stem cells (PMSCs) via distinct signaling pathways (Youssef and Han 2016; Youssef et al. 2014). Another study investigated the role of VEGF and associated signaling pathways in relation to DPSCs differentiation (Matsushita et al. 2000). Analyses by the reverse-transcription/polymerase-chain-reaction method and flow cytometry showed that the mRNAs of two VEGF receptors, *fms*-like tyrosine kinase and kinase insert domain-containing receptor, were expressed in DPSCs. VEGF induced the activation of activator protein I (AP-1) and c-fos mRNA expression in these cells. The AP-1 inhibitor as well as the VEGF antisense oligonucleotide strongly inhibited VEGF-induced ALP production in DPSCs. These results suggest that VEGF produced by human DPSCs acts directly upon human dental pulp cells in an autocrine manner, and may promote the chemotaxis, proliferation, and/or differentiation of human dental pulp cells via the utilization of kinase insert domain-containing receptor and in part through AP-1 by increasing c-fos.

Conclusion

Emerging evidence highlight an important role for DPSCs in osteogenesis and the advancement of regenerative medicine. The selection of appropriate healthy teeth extracted from young patients with a good medical history, the adoption of improved techniques of stem cells isolation and culture as well as the employment of the best osteoinductive protocols constitute the mainstay of a very promising revolution in this field. However, the long term side effects associated with the use of DPSCs and their potential to transform into tumors over time have not

been sufficiently studied thus far. More studies are warranted to clarify possible long term risks related to the use of these cells before DPSCs-based therapies find their way to the bedside.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest relevant to this manuscript.

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