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Odontoblast-like Cytodifferentiation of Dental Stem Cells: A Review

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ABSTRACT

Dental problems are common in human populations. Traditional treatments are focused on managing caries, soft tissue impairments, functional defects, poor aesthetics, digestive disorders and alveolar bone resorption. During the last two decades, basic and clinical researches on adult stem cells have established a potential therapeutic concept in tissue regeneration. Among major cells responsible for tooth development, odontoblasts play a key role in the formation of organic and inorganic constituents of dental tissue. A premier stride in the development of novel stem cell-based strategies for the treatment of reversible and irreversible pulpitis is odontoblast regeneration. Among different candidate cell sources for odontoblastic regeneration, use of dental adult stem cells is a preferred option because of their great ability to differentiate into odontoblasts and also their minimally invasive isolation procedure. This review emphasizes on articles that report successful odontoblast-like differentiation of dental mesenchymal stem cells which in turn provide a background for dentin-pulp complex cell therapies, using genetic or chemical manipulation. The series of experiments both *in vitro* and *in vivo* asserted that dental mesenchymal stem cells can efficiently differentiate into functional odontoblast-like cells. However, the review shows there are drawbacks in present methods. Future research should focus on optimizing protocols on odontoblast differentiation of dental stem cells by simultaneously introducing different genes with mutual synergy, combined with chemical or recombinant protein introduction.

Keywords: Dental Mesenchymal Stem Cells; Differentiation; Odontoblast

Introduction

Intense research on stem cells has made significant advancements in understanding their biology and their therapeutic potential, based on the possibility of differentiation into various cell types that could be used in regenerative medicine [1-3]. More particularly, the successful *ex vivo* and *in vivo* differentiation of adult stem cells using specific chemicals, growth factors, or genetic manipulation, into functional progeny followed by their transplantation have led to development of potential therapeutic approaches [4-6]. Stem cells are unspecialized cells that have multilineage differentiation potential. Mesenchymal stem cells (MSCs) have been identified within specific niches in most human tissues/organs including teeth [2, 7]. In human dental tissue, five different types of mesenchymal stem cells have been already isolated: dental follicle progenitor cells (DFPCs), stem cells from exfoliated deciduous teeth (SHEDs), dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs) and stem cells from apical papilla (SCAPs) [8, 9].

The dental pulp is a connective tissue containing different types of cells. Its primary function is to form dentin. Moreover, ameloblasts and odontoblasts form from the interactions between epithelial dental pulp cells. The inner layer of pulp chamber preserves a promising source of MSCs possessing self-renewal and proliferative capacity.

Table 1. Odontoblast-like cytodifferentiation of dental mesenchymal stem cells through genetic manipulation

Manipulated gene(s)	Cell type	Source	Vector	Experiment	ALP activity	Mineralization	Gene/Protein marker [1]	References
BBX	DPSC	Human	Electroporation (+)[2]	*In vitro*	+	+	*ALP, OPN, BSP, DMP1, and DSPP*	Choi *et al.*[10]
DELTA1	DPSC	Human	Lentivirus (-)[3]	*In vitro*	+	+	DSPP	Wang *et al.*[11]
CX43	DPSC	Human	Lentivirus (+)	*In vitro*	N	N	*DSPP*/DSPP **CX43* overexpression amplified the extracellular Ca-induced mRNA and protein levels of DSPP.	Li *et al.*[12]
TWIST1	DPSC	Human	Lentivirus (+)	*In vitro*	N	+	OCN, DMP1, DSP, and OPN **TWIST*1 overexpression stimulates *DMP1* and *DSPP* promoter activities.	Li *et al.*[13]
VEGF	DPSC	Human	Lentivirus (+)	*In vitro*	N	N	*ALP, OCN, DSPP, DMP1*/DMP1, and DSP	Zhang *et al.*[14]
ORAI1	DPSC	Human	Lentivirus (-), Retrovirus (-)	*In vitro, in vivo*	-	-	*OCN, DMP1*, and *BSP*	Sohn *et al.*[15]
INTEGRIN-α5	DPSC	Human	Lentivirus (-)	*In vitro*	N	+	*ALP, OCN, DMP1, ON, BSP*, and *DSPP*/DSPP	Cui *et al.*[16]
PIN1	DPSC	Human	Adenovirus (+)	*In vitro*	-	-	*ALP, OPN, OCN, DMP1, and* DSPP *The results were verified by PIN1 inhibitor (Juglone) treatment.	Lee *et al.*[17]
BCL2	DPSC	Human	Lentivirus (+)	*In vitro*	-	-	*ALP, OCN, DMP1, BSP, COL1*	Heng *et al.*[18]
NFIC	SCAP	Human	Lentivirus (+)	*In vitro*	+	+	*ALP, OCN, COL1*, and DSP	Zhang *et al.*[19]
NFIC	SCAP	Human	Lipofectamine (-)	*In vitro*	N	N	*DMP1* *DSPP* *Expression of *ALP, OCN* and *COL1* was not significantly changed	Gao *et al.*[20]
ZHX2	SCAP	Human	pcDNA3 (+)	*In vitro*	+	N	*RUNX2*/RUNX2, *OCN*/OCN, *BSP*/BSP, *and DSPP*/DSPP *The results were verified by knocking down the *ZHX2*.	Wan *et al.*[21]
BMP2	SCAP	Human	Lentivirus (+)	*In vitro*	+	+	*ALP, OCN, DSPP, and DMP1*	Zhang *et al.*[22]
BMP2* and/or *VEGF	SCAP	Human	Lentivirus (+)	*In vitro*	N	+	*ALP, OCN, DSPP, DMP1*, and DSP *Expression of *odontogenic* markers was significantly higher in co-transfected SCAPs	Zhang *et al.*[23]
KDM6B	SCAP	Human	Lentiviruses (-)	*In vitro*	-	-	*OSX, OPN, OCN*, and ALP *The results were verified by over expressing the *KDM6B*	Xu *et al.*[24]
BMI1	Senescent DPSC and SCAP	Human	Retrovirus (+)	*In vitro*	+	+	*OCN, DSPP, BSP, and DMP1* **Bmi1* transduction enhances odontogenic differentiation capacity in senescent dental MSCs	Mehrazarin *et al.*[25]
CREB	SCAP	Human	Lentivirus (+)	*In vitro*	N	+	*ALP, COL1, OCN, OSX, RUNX2*/RUNX2, and DSP *The results were verified by silencing the *CREB*	Su *et al.*[26]
Cav1.2	DPSC	Rat	Lentiviruses (-)	*In vitro*	-	-	*DSPP* *The results were verified by L-type calcium channel blocker (Nimodipine) treatment.	Ju *et al.*[27]
Human BMP2	DPSC	Rat	Adenovirus (+)	*In vitro*	+	+	*ALP, OCN, COL1, BSP, DSPP*, and *DMP1*	Yang *et al.*[28]
BMP9	iSCAP	Mouse	Adenovirus (+)	*In vitro, in vivo*	+	+	*OCN, OPN, BSP, DMP1, DSPP*, and *MEPE*	Wang *et al.*[29]

							*Ectopic mineralized tissue formation was seen	
***NFIC* cells were cultured in TGF-β1 treated medium**	SCAP	Mouse	pLenti6.3 (+)	*In vitro*	+	+	*ALP*, *OCN*, and *COL1* *Overexpression of NFIC antagonized the inhibition of odontogenic effects of TGF-β1 on SCAPs, while knockdown of NFIC enhanced these effects	He *et al.*[30]
β-Catenin	BMP9- and Wnt3A-induced SCAPs	Mouse	Transposon-based vector (-) (for β-Catenin)	*In vitro, in vivo*	-	-	*RUNX2/OPN* *Silencing β-Catenin expression significantly diminishes BMP9-induced odontoblast-like differentiation of iSCAP cells	Zhang *et al.*[31]

(**Genes and Proteins;** BBX: HMG box-containing protein 2, DELTA-1: Notch ligand Delta1, CX43: connexin 43, TWIST1: Twist-related protein 1, VEGF: vascular endothelial growth factor, ORAI1: Calcium release-activated calcium channel protein 1, PIN1: Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, BCL2: B-cell lymphoma 2, NFIC: Nuclear factor 1 C-type, ZHX2: Zinc fingers and homeoboxes protein 2, BMP: bone morphogenetic protein, KDM6B: Lysine demethylase 6B, BMI1: B cell-specific Moloney murine leukemia virus integration site 1, CREB: cAMP response element-binding protein, Cav1.2: L-type calcium channel a1C subunit, WNT5A: wingless-type MMTV integration site family member 5A, ALP: alkaline phosphatase, OPN: osteopontin, BSP: bone sialoprotein, DMP: Dentin matrix acidic phosphoprotein, DSPP: dentin sialophosphoprotein, OCN: osteocalcin, DSP: dentin sialoprotein, ON: osteonectin, COL1: Collagen type I, Runx2: Runt-related transcription factor 2, OSX: Transcription factor Sp7 which is also called Osterix, MEPE: Matrix extracellular phosphoglycoprotein. **Cells;** DPSCs: dental pulp stem cells, SCAP: stem cells from the apical papilla, iSCAP: immortalized mouse dental apical papilla progenitor cells. **Assays;** (+): significant increase compared to control group, (-): significant decrease compared to control group, N: not evaluated.) 1: Regular cells represent gene/protein marker upregulation, shaded cells represent gene/protein marker downregulation, 2: (+) overexpressed, 3: (-) knocked down

Dental pulp tissue of human exfoliated deciduous teeth contains undifferentiated mesenchymal cells, termed SHEDs. SHEDs are a source of stem cells capable of differentiating into odontoblast, osteoblast, adipocyte-, and neural cell-like cells [8, 9, 70]. The ability of periodontal ligament (PDL) to regenerate in response to mild trauma suggests that progenitor cells are present in fully developed PDL. Human PDLSCs have been isolated and characterized by single-colony selection and magnetic cell sorting, similar to those used to validate DPSCs and SHEDs [71]. The dental follicle (DF) is formed in the cap stage during tooth development by a progenitor cell population originating from the neural crest. The DF is critical for the coordination of periodontium development and tooth eruption. It is believed that this tissue includes mesenchymal stem cells. Suitable sources for stem cell isolation may be from impacted teeth extracted (to avoid inflammation) or orthodontic therapy extractions. These teeth are commonly discarded as medical waste; yet the dental follicle that might be isolated from them is an excellent source [7, 72]. In developing roots, the dental papilla shifts apical to the pulp tissue after the bell stage and forms apical papilla. Apical papilla appears to be histologically and morphologically distinct from pulp tissue complex and contains a unique population of dental mesenchymal stem cells, termed SCAPs. It is believed that one culture method and enzymatic dispersal method can be used for isolation and characterization of SCAPs. Application of SCAPs is thought to be crucial in root regeneration during treatment of all oral diseases [73, 74].

This review aims to describe the emerging concept of dentin-pulp complex regeneration with respect to odontoblast differentiation. In order to do this, we conducted an extensive review of the literature, focusing on the potential of dental MSCs to differentiate into odonto/osteoblast-like cells.

Biology and gene context of odontoblasts

In vertebrates, odontoblasts are tall columnar cells located along the interface between dental pulp and dentin. Odontoblasts biological function is dentinogenesis. They play a key role in secreting dentin extracellular matrix and contribute to matrix mineralization. Furthermore, these cells can trigger cytodifferetiation by paracrine function. Dentin consists of collagenous and non-collagenous organic matrix and apatite crystals of calcium and phosphate. During the early stages of craniofacial development, ectomesenchymal cells originating from the neural crest can be stimulated to proliferate and differentiate as odontoblasts [75-77].

Previous studies have demonstrated that dentin matrix protein-1 (DMP-1) expression is detectable during the development of odontoblasts. Moreover, Northern blot hybridization has revealed specific expression of DMP-1 in odontoblasts. Dentin sialophosphoprotein (DSPP) and Osteocalcin (OCN) are significantly expressed in mature odontoblasts throughout mineralization stage. *DSPP* over expression appears as a result of interaction failure between nuclear respiratory factor 1 (NRF1) and "CCAAT" enhancer binding protein beta (C/EBPbeta). Some researchers have suggested that transforming growth factor beta 1 (TGF-β1) transcript can only be detected in differentiated odontoblasts and not in odontoblast progenitors [75, 76, 78-81].

Table 2. Odontoblast-like cytodifferentiation of dental mesenchymal stem cells through chemical introduction

Introduced chemical(s)	Cell type	Source	Experiment	ALP activity	Mineralization	Gene/Protein marker [1]	References
Inflammatory Cytokines (TNFα and IL-1β)	DPSC	Human	*In vitro*	-	N	*ALP, NES, and DSPP* *The results were verified by NF-κB knock-down.	Hozhabri *et al.* [32]
Strontium	DPSC	Human	*In vitro*	+	+	DSPP, *RUNX2*, *OCN*, *MEPE*, *BMP2*, and *ON*	Huang *et al.* [33]
Ca^{2+}	DPSC	Human	*In vitro*	-	+	*BSP*/BSP, *OCN*/OCN, *DMP1*, *and DSPP* *BMP/Smad pathway is involved in the Ca^{2+}-induced odontoblast-like differentiation of DPSCs.	Li *et al.* [34]
LPS	DPSC	Human	*In vitro*	N	+	*OCN*, *DMP1*, *DSPP*, and *ALP* *LPS promotes odontoblast-like differentiation via TLR4, ERK, and P38 MAPK signaling pathways.	He *et al.* [35]
BMP2	DPSC	Human	*In vitro, in vivo*	N	+	*DSPP*, *DMP1*, *PHEX*, *MMP20* *Increased odontoblast-like cells, vascularization, and dentin matrix formation were observed at the areas, in which treated cells were transplanted.	Atalayin *et al.* [36]
MMP2-CLEAVAGE OF DMP1	DPSC	Human	*In vitro, in vivo*	N	N	*DSP*, *DMP1* *Implantation of this peptide in a rat injured pulp model resulted in dentin-like structure formation.	Chaussain *et al.* [37]
FGF2 and TGF-β	DPSC	Human	*In vitro*	+	+	*DSP*/DSP, *DMP1*/DMP1 * FGF and TGF-β co-treatment improves odontoblast-like differentiation inducing potential.	Unda *et al.* [97]
Insulin-like growth factor	DPSC	Human	*In vitro*	+	+	*OSX*/OSX, *RUNX2*/RUNX2, *OCN*/OCN, *DSPP*, DSP	LV *et al.* [38]
Platelet lysate	DPSC	Human	*In vitro, in vivo*	+	+	*N*	Chen *et al.* [39]
Semaphorin 4D	DPSC	Human	*In vitro*	-	-	*COL1/COL1, DSPP/DSPP, ALP, BSP, RUNX2*	Zou *et al.* [40]
Recombinant LOXL2	DPSC	Human	*In vitro*	-	-	*ALP, DMP1, and DSPP*	Kim *et al.* [41]
Trichostatin A (histone deacetylase inhibitor)	DPSC	Human	*In vitro, in vivo*	+	+	*DSPP, DMP1, BSP*	Jin *et al.* [42]
EDTA (cells were seeded on dentin slices)	DPSC	Human	*In vitro*	N	+	*DMP1* and *DSPP* *EDTA induced cell attachment.	Pang *et al.* [43]
$Ca(OH)_2$ and NaOCl &EDTA	DPSC	Human	*In vitro*	N	N	*DSPP* and *DMP1* *$Ca(OH)_2$ and EDTA promoted the differentiation of DPSCs after NaOCl treatment.	Park *et al.* [44]

(cells were seeded on dentin slices)							
Conditioned dentine slice and powder by EDTA, phosphoric acid, citric acid	DPSC	Human	*In vitro*	N	+	*ALP* and *OPN*	Sadaghiani *et al.* [45]
Ethanol	DPSC	Human	*In vitro, in vivo*	-	-	*BMP2*, *BMP4*, *OCN*, *OPN*, *ALP*, *DLX2* *Ethanol induced inhibition of KDM6B (a lysine demethylase). As a result, odontogenic differentiation is down regulated.	Hoang *et al.* [46]
Preameloblast-conditioned medium	DPSC	Human	*In vitro, in vivo*	N	+	*DSP, NES, COL1*	Lee *et al.* [47]
Human and swine tooth germ cell conditioned medium	DPSC	Human	*In vitro, in vivo*	+	+	*OPN*, *DSPP*, *DMP1*/DMP1, *OCN*, *BSP, DSP*	Wang *et al.* [48]
Ginsenoside Rg1	DPSC	Human	*In vitro*	N	N	*BMP2*/BMP2, *FGF2*/FGF2, *DSPP, ALP, OCN*	Wang *et al.* [49]
Zinc containing bioactive glasses	DPSC	Human	*In vitro*	+	+	*RUNX2*, *OCN*, *MEPE*, *BMP2*, *BSP*, *ON*, DSPP, DMP1	Huang *et al.* [50]
MTA	DPSC	Human	*In vitro*	N	N	*BSP*, *ALP*, *OCN*, *COL1*, *DSPP* *MTA can induce odontoblast-like differentiation via mitogen-activated protein kinase pathway.	Zhao *et al.* [51]
MTA	DPSC	Hunan	*In vitro*	N	+	*DMP1* and *DSPP* *Microarray analysis is done and list of differentially expressed genes which their differences are more than 4-fold are shown.	Seo *et al.* [52]
MTA and CEM	DPSC	Human	*In vitro*	N	+	*DSPP*, *DMP1*, *BMP2*, *TGF-β1*, *FGF4* *These chemicals stimulate different gene expression.	Asgary *et al.* [53]
MTA	SCAP	Human	*In vitro, in vivo*	+	+	*OCN*/OCN, *RNX2*/RUNX2, *ALP*, *DSPP, DSP* *MTA can induce odontoblast-like differentiation via Nuclear Factor Kappa B signaling pathway.	Yan *et al.* [54]
HEMA and TEDGMA	SCAP	Human	*In vitro*	-	-	*BSP*, *OCN*, *DSPP*	Bakopoulou *et al.* [55]
Basic FGF	SCAP	Human	*In vitro*	-	-	*BSP*, *ALP*, *OCN*, *OCN*, *DSPP* *This growth factor increases stemness of hSCAPs.	Wu *et al.* [56]
Insulin-like growth factor	SCAP	Human	*In vitro, in vivo*	+	+	*OCN*/OCN, *OSX*/OSX, *RUNX2*/RUNX2, *ALP*	*DSPP, DSP*	Wang *et al.* [57]

BMP2	SCAP	Human	*In vitro, in vivo*	+	+	*BSP, OCN, COL1, and DSPP* *Seeding cells on nanofibrous microspheres (NF-MS) in combination with controlled release of BMP-2 results in more mineralized tissue formation.		Wang *et al.* [58]
Recombinant human plasminogen activator inhibitor-1	SCAP	Human	*In vitro, in vivo*	+	+	*ALP, COL1, OPN, OCN, BSP, DMP1*		Jin *et al.* [59]
Conditioned medium from periapical follicle cells	SCAP	Human	*In vitro*	+	+	*DSPP, ALP, OCN*		Wu *et al.* [60]
Dexamethasone-releasing chitosan nanoparticle (Conditioned dentine)	SCAP	Human	*In vitro*	N	+	DMP1 and DSPP *Increasing dexamethasone releasing velocity results in odontogenic differentiation inducing potential.		Shrestha *et al.* [61]
Platelet lysate	DPSC, SCAP	Human	*In vitro*	+	+	*DSPP*, OPN *SCAPs show more potential for odontogenic differentiation.		Abuarqoub *et al.* [62]
Platelet-rich plasma	DPSC, PDLSC	Human	*In vitro*	+	+	*BSP, OPN, CEMP1, and DSPP*		Lee *et al.* [63]
BMP2 and dental slice scaffold	SHED	Human	*In vitro, in vivo*	N	N	*DSPP, DMP1, MEPE* *After BMP treatment, SHED express BMP receptors.		Casagrande *et al.* [64]
MEPE-derived ASARM (phosphorylated form)	SHED	Human	*In vitro, in vivo*	N	-	*ALP*, *MEPE*/MEPE	*DSPP*, DSP, *OCN*/OCN	Salmon *et al.* [65]
HEMA and TEDGMA	SHED	Human	*In vitro*	-	-	*BSP, ALP, OCN, DSPP*		Bakopoulou *et al.* [66]
EMD	TGSC	Human	*In vitro*	+	N	*DSPP*		Guven *et al.* [67]
MTA				-		*DSPP*		
Aminated mesoporous bioactive nanoparticles	DPSC	Rat	*In vitro*	+	+	*BSP, OCN, COL1, DMP1 and DSPP*		Lee *et al.* [68]
TNF-α	BMP9-Induced SCAPs	Rat	*In vitro, in vivo*	-	-	*OCN, OPN*		Wang *et al.* [69]

*(**Chemicals, Proteins, and Genes;** TNF-α: tumor necrosis factor alpha, IL-1β: Interleukin 1 beta, LPS: Lipopolysaccharide, BMP: bone morphogenetic protein, MMP: matrix metalloproteinase, DMP: Dentin matrix acidic phosphoprotein, FGF: fibroblast growth factor, TGF-β: transforming growth factor β, LOXL1: Lysyl oxidase homolog 1, EDTA: Ethylenediaminetetraacetic acid, MTA: mineral trioxide aggregate, HEMA: 2-hydroxyethyl methacrylate, TEDGMA: Tri-ethylene-glycol-dimethacrylate, MEPE: Matrix extracellular phosphoglycoprotein, ASARM: serine- and aspartic acid-rich motif, EMD: enamel matrix derivative, ALP: alkaline phosphatase, NES: Nestin, DSPP: dentin sialophosphoprotein, Runx2: Runt-related transcription factor 2, OCN: osteocalcin, ON: osteonectin, BSP: bone sialoprotein, PHEX: Phosphate-regulating neutral endopeptidase, X-linked, DSP: dentin sialoprotein, OSX: Transcription factor Sp7 which is also called Osterix, COL1: Collagen type I, OPN: osteopontin, DLX: Distalless, CEMP: cementum protein. **Cells;** DPSCs: dental pulp stem cells, SCAP: stem cells from the apical papilla, PDLSC: periodontal ligament stem cells, SHED: stem cells from exfoliated deciduous teeth, TGSC: tooth germ stem cells. **Assays;** (+): significant increase compared to control group, (-): significant decrease compared to control group, N: not evaluated.)*

1: Regular cells represent gene/protein marker upregulation, shaded cells represent gene/protein marker downregulation.

Odontoblast-like cytodifferentiation of dental mesenchymal stem cells

The concept of stem cell-based therapies for dental tissue engineering, using the body's own stem cells, would be a promising biological approach. In addition to easy and minimally invasive isolation method of dental stem cells, they display not only appropriate differentiation potential to odontoblast cells but also immunomodulatory functions. These characteristics make them candidate cell sources for regeneration of dentin-pulp complex and in stimulation of reparative dentinogenesis [2, 82]. Seemingly, odontoblast regeneration and overcoming restrictions associated with this process are primary steps in understanding the biological and clinical implications of tooth-resident stem cells for pulp/dentin regeneration. Among all these features, this review is mainly highlighting the *in vitro* and *in vivo* odontoblastic differentiation capacity of dental MSCs under various conditions and how genetic manipulation or chemical introduction can regulate this potential (Tables 1 and 2). This understanding would allow the introduction of more efficient protocols as well as the more in-depth address of the main issues in further studies. These include the objectives:

- How to control specific signal activation through consecutive gene expression during odontoblast-like cytodifferentiation
- Which type of dental MSCs demonstrate more promising capacity to differentiate into odontoblasts
- Identifying a possible combination of both chemical introduction and genetic manipulation which can modulate proliferation and differentiation of stem cells in the lab for regenerative purposes in the clinic

According to Tables 1 and 2, there are insufficient evidence or well-documented studies looking at the role of agents, proteins, and genes on odontoblast-like differentiation of dental mesenchymal stem cells. However, some of the most reported chemicals, proteins, and genes used in previous researches are explained.

Bone morphogenetic proteins (BMPs), belonging to TGF-β superfamily, control the proliferation, differentiation, and activity of various cell types, including dentin, bone and cartilage [83]. It has been shown that BMPs act through an autocrine or paracrine mechanism by binding to two different cell surface receptors (R: Type I and II) and triggering a sequence of downstream events [84]. They have been implicated in mesenchymal stem cell differentiation, particularly the up regulation of osteoblastic and odontoblastic features of MSCs during differentiation. Currently, the production of BMPs by genetic engineering has begun the process of elucidating the regenerative role of BMPs in clinical trials [84, 85]. According to Yang *et al.* [28], when DPSCs (to which *BMP2* gene was introduced) were encapsulated in ceramic scaffold and implanted into nude mice, they were observed to have over-expressed high amount of DSPP and DMP-1. Wang *et al.* [29] reported that expression of odontogenic differentiation markers, such as matrix extracellular phosphoglycoprotein (Mepe), DMP-1, and DSPP was promoted as a result of gene introduction of BMP9 in stem cells from mouse apical papilla (mSCAPs). Atalayin *et al.* [36] also reported that when DPSCs were cultured in the medium containing BMP2, their odontogenic differentiation potential was improved significantly. Similarly, Casagrande *et al.* [64] indicated that addition of neutralizing antibodies to the medium containing dentin-derived BMP2 resulted in down regulation of the expression of odontoblastic differentiation markers, as expected.

DMP is a highly phosphorylated protein that has been studied in great details due to its importance in proper mineralization of hard tissues such as bone and dentin. It is known to be a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family [86]. Based on recent studies, this protein functions as both intracellular and extracellular protein responsible for differentiation of dental mesenchymal stem cells. Thus, it is thought to be a good candidate for dentin tissue regeneration [78, 87, 88]. Almushayt *et al.* [89] study performed the implantation of the collagen matrix impregnated with recombinant DMP1 at the pulp exposure site in a rat model. Cytodifferentiation of dental pulp stem cells, as revealed by immunohistochemistry, was indicative of the importance of DMP1 in odontoblast-like cytodifferentiation in their investigation [89]. When the culture medium was supplemented with MMP2-cleavage of DMP1, expression of DMP1 and DSPP was increased indicating the differentiation towards odontoblast-like cells [37].

There are few scientific pieces of evidence demonstrating that direct contact of dental stem cells with mineral trioxide aggregate (MTA) can affect their differentiation into odontoblast-like cells. According to the researches of Paranjpe *et al.* [90, 91] when DPCs were cultured in direct contact with MTA, the expression of important odontoblastic genes like OCN and DSP was up-regulated. Zhao *et al.* [51] cultured human DPSCs in the medium containing MTA; the result of this experiment showed that application of MTA increased the expression of key markers for odontoblastic differentiation, such as alkaline phosphatase (ALP), DSPP, OCN, and bone sialoprotein (BSP). Yan *et al.* [54] claimed that MTA-treated SCAPs have shown odontoblast features through nuclear factor kappa B (NF-kB) pathway. In particular, when MTA is hydrated, a mixture of inorganic ions would be released, including Ca, Si, and inorganic phosphate. These ions are shown to have crucial role in activating the mitogen-activated protein kinase (MAPK) pathway. Subsequently, the expression of

ALP, DSP, DSPP, OCN and runt-related transcription factor 2 is promoted and the downstream effectors stimulate the odontoblastic differentiation [54, 92]. Similar scenario could be deducted from those studies elucidating the effect of calcium ion or calcium channel blockers on odontogenic differentiation potential of dental mesenchymal stem cells. Particularly, changes in intracellular calcium concentration can regulate extracellular signal-regulated kinase (ERK) and p38 MAPK pathways [27, 34].While the inorganic salts are responsible for differentiation inducing potential of MTA, Enamel Matrix Derivative is composed of a number of proteins, most of which are amelogenins and bone morphogenetic proteins. These proteins are thought to be the direct or indirect promoting factors during odontoblast development [67, 88, 93, 94].

Although the pool of growth factors in platelet lysate varies significantly from species to species, it contains elements such as TGF-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF) that can induce not only cell proliferation but cellular differentiation [39, 62]. More recent *in vitro* studies highlighted the functional role of growth factors in hard tissues formation through promoting specific gene expression and signaling pathways, notably MAPK pathway and BMP/Smad signaling pathway. To find novel molecular targets and develop therapeutic strategies, more studies should be conducted to elucidate the molecular mechanisms [95-98].

The role of nuclear factor 1 transcription factor has been highlighted by previous studies in root development. Accordingly, scientists hypothesized that nuclear factor 1-C (NF1C) might be a valuable target for regenerative approaches. NF1C over expression resulted in over expression of odontoblast gene markers. It is demonstrated that nuclear factor 1 transcription factor can regulate the expression of cyclins and cyclin inhibitors. Specifically, NF1C knock down in dental pulp cells increased the expression of p21 and p16. On the other hand, the expression of cyclins D1 and B1 was down regulated. This suppressed the odontogenic differentiation and proliferation of target cell sources [19, 20, 99].

The last few years has seen exponential progress in traditional dentin-pulp complex therapies in oro-dental research. Despite this positive growth in publication on cell-based therapies, many unresolved questions about cell sources and clinical approaches are still unanswered; and left for future studies. Recent results have shown that use of autologous dental stem cells with the potential to differentiate into several dental progenitor cells would be a beneficial medium for tooth tissue bioengineering; as it would avoid immunological rejection [100, 101]. Furthermore, it has been demonstrated that these cells can be cryopreserved for later applications in regenerative medicine [102-105]. *In vitro* or *in vivo* stem cell-based tissue regeneration using appropriate mesenchymal stem cells through genetic manipulation, applying recombinant proteins or synthetic chemicals would contribute to the development of strategies for pulp-dentin regeneration. In many cases, the studies highlighted some of the restrictions of recombinant proteins or synthetic chemicals, including time limit, requiring high concentrations, low efficiency, and cost [37, 55, 59, 64, 105, 106]. Although genetic manipulation overcomes these limitations relatively, there are drawbacks such as induced immune reaction and insertional mutagenesis associated with the use of such strategy [106-109]. Several studies have confirmed more efficiency in the odontoblast-like differentiation of dental mesenchymal stem cells in the case of simultaneous manipulating of different genes having mutual synergy combined with chemical or recombinant protein introduction [27, 32, 110].

Conclusion

Future research should preferably focus on optimizing odontoblast differentiation protocols of dental stem cells. Furthermore, there are unsolved questions and controversies about applications and clinical functions of these cells that should be considered; in particular, the following five issues:

- The potency of different dental stem cells to differentiate into odontoblasts
- The ability of transplanted stem cells or differentiated odontoblasts to find their optimum 'niche'
- Development of safe and minimally invasive delivery systems for depositing dental stem cells to recipient tissue
- The optimal time course for genetic manipulation and chemical or recombinant protein introduction
- The long-term survival of dental mesenchymal stem cells after transplantation into the recipient tissue

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