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Stem Cells from Human Exfoliated Deciduous Teeth: Biology and Therapeutic Potential

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Abstract

Stem cells isolated from human exfoliated deciduous teeth (SHEDs) are a type of mesenchymal stem cells (MSCs), widely investigated for regenerative treatment. They are isolated from dental pulp tissues remaining in physiologically shedding human deciduous teeth. Thus, SHEDs are easy to access and not required invasive procedure to obtain cells. SHEDs are multipotent mesenchymal stem cells; however, they possess distinct properties when compared to other MSCs. In this regard, SHEDs exhibit higher proliferative rate than bone marrow-derived MSCs and greater osteogenic differentiation potency than human dental pulp stem cells. This chapter reviews the isolation technique and basic characteristics of SHEDs. Moreover, the intracellular signalling involved in the stemness regulation and differentiation ability of SHEDs is discussed, particularly on fibroblast growth factor, Notch, and Wnt signalling. Finally, the potential regenerative therapeutic application of SHEDs is also described.

Keywords: stem cells, deciduous teeth, basic fibroblast growth factor, Wnt signalling, Notch signalling, mechanical stress

1. Introduction

Dental pulp is a loose connective tissue residing in pulp chamber inside both deciduous and permanent teeth. It surrounds by hard tissues called dentin. Nutrients and oxygen supply are acquired from blood vessels passing through apical and accessory foramen of the teeth's root. Dental pulp originates from cranial neural crest cells [1]. Dental pulp tissues are composed of extracellular matrix and various cell types, e.g. fibroblasts, odontoblasts, endothelial cells, pericytes, immune cells and stem cells. When injured, cells in dental pulp tissues are

capable of differentiating odontoblasts or odontoblast-like cells, leading to the promotion of tertiary dentin formation. The formation of tertiary dentin is a mechanism which can protect the tooth vitality. Dental pulp tissues remaining in physiological shedding of deciduous teeth are the alternative source of mesenchymal stem cells, due to the ease of accessibility and minimally invasive technique to obtain tissues [2]. Stem cells from human exfoliated deciduous teeth (SHEDs) are firstly identified by Miura et al. in 2003 [2]. SHEDs have high proliferation potency and are multipotent mesenchymal stem cells. These cells are able to differentiate into, not only, dental pulp-related cells, but also, other cell lineages, for example osteoblasts, adipocytes, neuronal-like cells and endothelial cells [2–8]. Taking these advantageous properties together, SHEDs are one of the candidate cell types for tissue regeneration study.

2. SHEDs' characteristics

SHEDs are heterogeneous population of cells isolated from dental pulp tissues remained in exfoliated deciduous teeth. Similar to those mesenchymal stem cells (MSCs), SHEDs exhibit fibroblast-like morphology, adhere on plastic tissue culture surface, express mesenchymal stem cell surface marker and have multipotential differentiation ability (**Figure 1**). SHEDs have higher proliferation rate compared to dental pulp stem cells (DPSCs) and bone marrow-derived mesenchymal stem cells (BMMSCs) [2, 9]. This could be due to the high expression of genes related to cell proliferation and extracellular matrix in SHEDs comparing with DPSCs [9]. First, a study by Miura et al. demonstrated that SHEDs express mesenchymal surface markers, STRO-1 and CD146 [2], though, the percentage of positive cells is low [2]. Later studies utilized various surface markers for SHEDs characterization protocol. SHEDs expressed CD44, CD73, CD90, CD105 and STRO-1 [6]. In addition, these cells lack of CD45 expression [6]. Besides these markers described above, SHEDs also express other surface markers for example, CD166 and SSEA4. Lack of CD34 is also reported [10]. There is no specific surface marker to precisely identify SHEDs population.

Up to date, MSCs can be isolated from many tissue types. Though, there is no specific marker to clearly identify these cells. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, the minimum criteria to identify MSCs are as follow [11]. First, the isolated MSCs should adhere to plastic tissue culture plate [11]. Second, MSCs must express several specific surface markers, namely CD105, CD73 and CD90 [11]. They also should not express CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR [11]. Finally, MSCs have to be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [11]. The following section describes general SHEDs' characteristics and addresses MSCs' characteristics of SHEDs according to these criteria.

2.1. Isolation technique

Two methods have been utilized for SHEDs isolation, namely an enzymatic digestion and a tissue explant. The enzymatic digestion is performed by digesting minced remaining pulp tissues from deciduous teeth, normally with type I collagenase and dispase mixed enzyme solution

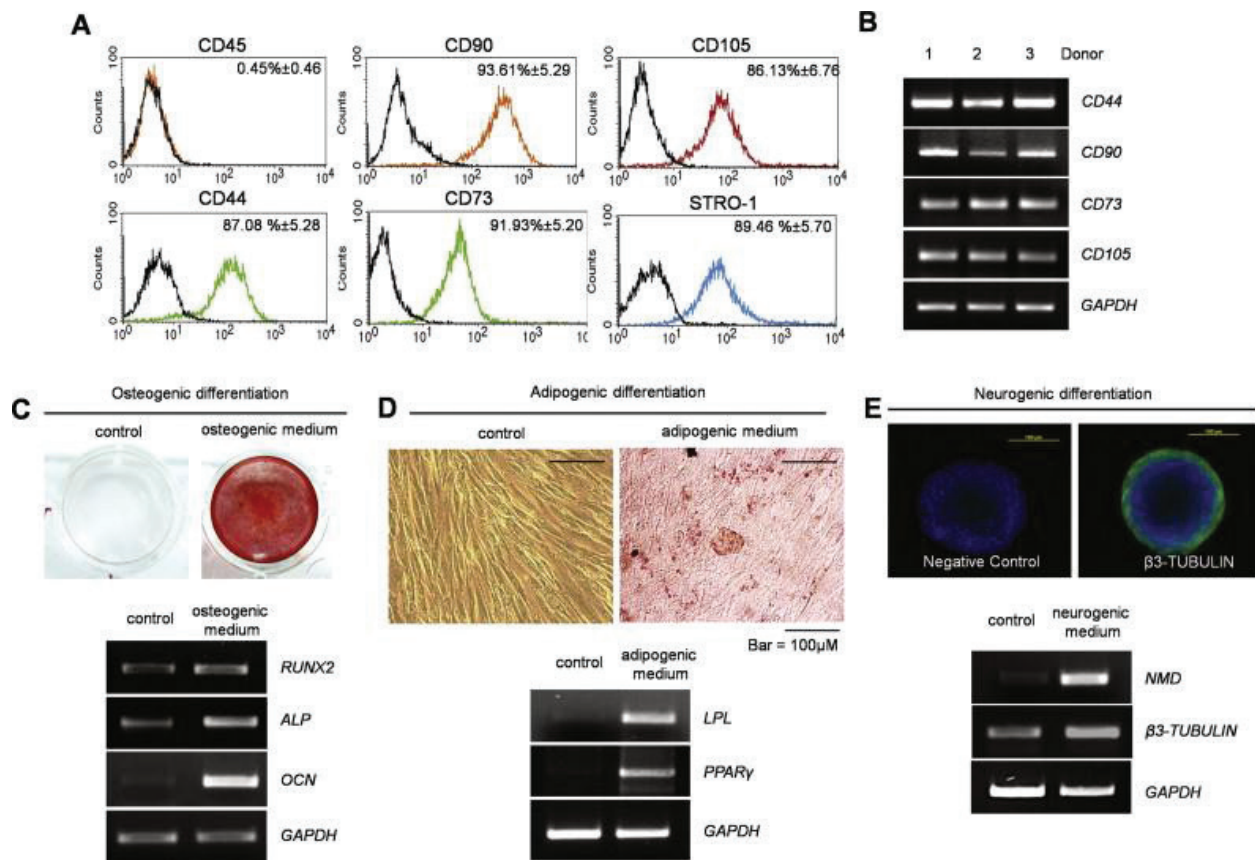


Figure 1. Characteristics of stem cells isolated from human exfoliated deciduous teeth. The expression of stem cells markers was evaluated using flow cytometry and conventional semi-quantitative PCR (A and B). The osteogenic, adipogenic and neurogenic differentiation were evaluated at day 14, 16 and 7 after induction, respectively (C–E). The mineral deposition and intracellular lipid accumulation were determined using alizarin red and oil red O staining, respectively (C and D). The β 3-tubulin protein expression was evaluated by immunocytochemistry staining (E). The expression of differentiation marker was examined using conventional semi-quantitative PCR. Reprinted from Archives of Oral Biology, 60(3), Nunthawan Nowwarote, Prasit Pavasant, Thanaphum Osathanon, Role of endogeneous basic fibroblast growth factor in stem cells isolated from human exfoliated deciduous teeth, 408–15, Copyright (2015), with permission from Elsevier [6].

[12–14]. For tissue explant, minced pulp tissues are placed on the tissue culture dishes, allowing the outgrowth of the cells from the tissues [12]. Enzymatic digestion technique leads to more heterogeneous population of isolated cells than those obtained from tissue outgrowth protocol [14]. A study illustrated that there is no significant difference regarding cell morphology and proliferation between cells isolated using enzymatic digestion and tissue outgrowth [14]. Enzymatic digestion-derived SHEDs had higher mineralization ability *in vitro* [14]. However, another study demonstrated that SHEDs isolated using enzymatic digestion exhibited higher cell proliferation and colony forming unit ability as well as adipogenic differentiation potency [13]. However, tissue explant-derived SHEDs had higher osteogenic differentiation ability than enzymatic digestion-derived SHEDs *in vitro* and *in vivo* [13]. The difference between these two studies, especially in osteogenic potential, may be due to the dissimilar osteogenic medium supplementation. The first study employed 0.01 μ M dexamethasone disodium phosphate, 1.8 mM monopotassium phosphate (KH_2PO_4) and 5 mM β -glycerophosphate [14]. However,

the osteogenic medium of later study was supplemented with 0.1 μ M dexamethasone, 2 mM β -glycerophosphate and 50 μ M ascorbic acid-2 phosphate [13]. Together, the different isolation technique resulted in the different population of SHEDs. Further comparison is needed to determine a suitable protocol for specific application of these cells.

2.2. Differentiation potential of SHEDs

Studies have shown that SHEDs possess multi-differentiation potency similar to MSCs. Those lineages include odontogenic/osteoblastic, adipogenic, neurogenic and angiogenic differentiation [2].

2.2.1. Odontogenic/osteoblastic differentiation potential

The ability of SHEDs to differentiate into odontoblastic lineage is widely known [2, 15, 16]. Primarily, SHEDs were characterized by their *in vivo* osteoinductivity [2] and follow by several *in vitro* studies to confirm their odontogenic/osteoblastic differentiation potential [5, 10]. SHEDs showed similar osteogenic potency when comparing with BMMSCs, exhibiting significantly elevated levels of ALP activity after 1 week of induction. In addition, several osteogenic markers such as RUNX2, DSP and OCN are also upregulated [10]. When cultured in an osteogenic medium, SHEDs formed mineralized nodules after 4 weeks of induction which indicate calcium deposition *in vitro* [2]. Transplantation of *ex vivo* expanded-SHEDs with hydroxyapatite/tricalcium phosphate (HA/TCP) into immunocompromised mice also induced mineralized tissue formation [2]. Recently, osteoinductivity of SHEDs has been shown in mice. SHEDs formed an osteoinductive template in immunocompromised mice and induced the recruitment of native osteogenic cells to repair calvarial defects [16]. The osteogenic potential of SHEDs in regenerating bone defects in maxillofacial region was also investigated by Zheng et al., the results found that autologous graft using stem cells from miniature pig primary teeth has the ability to regenerate and repair mandibular defects [15]. SHEDs were able to regenerate bone tissues with blood vessels around dental implants in dog model when mixed with platelet-rich plasma (PRP) [17].

Evidence suggested that SHEDs might have the preference towards the odontoblastic lineage due to its origin. SHEDs can be induced to become functional odontoblasts *in vitro* [2]. SHEDs can differentiate to become the odontoblast-like cells and regenerate the tissue with architecture and cellularity similar to the physiologic dental pulp when cultured in scaffolds prepared within human tooth slices and transplanted into immunodeficient mice [18]. It has been recently shown that SHEDs can generate functional dental pulp when injected with PuraMatrix or Collagen into root canals [19]. However, majority of the studies focusing on regenerating bone or dentin-pulp complex *in vivo* were performed in ectopic implantation models, mostly in skin or renal capsule, in mice or rats [20–22] which might not close to real clinical situation. More studies in the clinical relevance area such as tooth socket or jaw bone in larger animals such as pig or dog should be considered to make the results more valuable for application.

2.2.2. Neurogenic differentiation potential

Neurogenic potential of SHEDs is expected due to their neural crest embryonic origin. Several research studies focusing on differentiating dental stem cells to be used for neurodegenerative

disease therapy. These cells are prone to undergo neurogenic differentiation both *in vitro* and *in vivo*. Under the undifferentiating condition, SHEDs and other dental stem cells expressed the neural progenitor markers, nestin and the glial marker, glial fibrillary acidic protein (GFAP), at both the mRNA and protein levels [2, 23]. SHEDs can be induced to become a variety of specialized cells in neural lineage including dopaminergic neuron like cells and glial cells [24, 25]. When induced, SHEDs could form neural-like spheres *in vitro*. Further incubation with a combination of cytokines including sonic hedgehog, fibroblast growth factor 8, glial cell line-derived neurotrophic factor and forskolin can drive these neural spheres into the dopaminergic like neurons [25]. A similar trend was observed where SHEDs showed positive expression of both glial and neuronal markers after 21 days of neurogenic induction. Deposition of antemyelin basic protein was seen and the differentiated cells showed positive expression for neuronal markers such as β III-tubulin, apolipoprotein E (ApoE), intermediate filament peripherin and Brn3a [26]. SHEDs are able to differentiate into dopaminergic neuronal like cells *in vitro* [27]. However, SHEDs exhibited inferior differentiation ability towards dopaminergic neurons as compared with DPSCs [28]. In this regard, DPSCs upregulated dopaminergic neuron markers (Nurr1, Engrailed1 and Pitx3) higher than SHEDs after treated with sonic hedgehog, fibroblast growth factor 8 and basic fibroblast growth factor [28].

In vivo studies also show the promising results for generating the specialized cells in the neural system. Transplantation of neural-like spheres derived from SHEDs into the striatum of parkinsonian rats significantly improved the apomorphine-evoked rotation of behavioural impairment compared to transplantation of control SHEDs [25]. The results were in line with another study showing the partially recovery after inducing neural maturation of SHEDs into dopaminergic neuron-like cells and transplantation in parkinsonian rats [27]. Moreover, a complete recovery of hindlimb motor function was observed after implantation of neural-induced SHEDs in a rat spinal cord injury [29]. These results suggested that pre-induction of the undifferentiated SHEDs into the neural-like cells before implantation might improve the efficiency of SHEDs in regenerating specialized neural cells and potentially improve the treatment outcome.

2.2.3. Angiogenic differentiation potential

Angiogenic potential of SHEDs is another aspect of interest for the benefit of connective tissue regeneration. The rapid and effective induction of vasculature is required for sufficiently supply of oxygen and nutrients as well as removing the toxic waste from the newly synthesized tissues. Unstimulated SHEDs expressed VEGFR1 and NP-1, the known important receptors in angiogenesis and VEGFR1 signalling play an important role in VEGF-induced capillary tube formation by SHEDs as shown by VEGFR1 gene silencing [30]. SHEDs cultured in the tooth slice/scaffolds in combine with VEGF expressed several endothelial differentiation markers such as VEGFR1, VEGFR2, platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial cadherin (VE-Cadherin). When transplanted in immunodeficient mice, SHEDs actually lined the new blood vessels within the tooth slice/scaffolds close to the blood vessels of host [3]. Similar results were observed when SHEDs seeded in human tooth slice/scaffolds and transplanted into immunodeficient mice differentiate into human blood vessels that anastomosed with the mouse vasculature and VEGF induced the angiogenic

differentiation of SHEDs through Wnt/ β -catenin signalling [31]. Another study also showed that SHEDs can differentiate into VEGFR2-positive and CD31-positive endothelial cells *in vitro*. This phenomenon occurred via VEGF/MEK-1/ERK signalling pathway [30]. In addition to *in vitro* data, an *in vivo* study also showed that SHEDs differentiate into endothelial cells when seeded in biodegradable scaffolds and transplanted into immunodeficient mice [18], confirming the plasticity of SHEDs.

2.2.4. Adipogenic differentiation potential

Several studies have reported that SHEDs can be induced into adipogenic lineage [6, 32–34]. After cultured in an adipogenic medium, SHEDs' morphology changed from spindle-like to polygonal shapes and lipid vacuoles were observed, along with the increased in PPAR γ 2 and LPL mRNA [32]. However, the studies evaluated the adipogenic potential of SHEDs *in vivo* are sparse and the clinical application may not come in the near future.

2.3. Immunomodulatory property

Like other MSCs, SHEDs exhibit immunomodulatory properties. Though, the potency and mechanism are not exact the same to those of BMMSCs [10, 35]. SHEDs significantly reduced the percentage of IL17⁺IFN γ cells population in CD4⁺ T cells *in vitro* [10]. In addition, IL17 expression was decreased compared with the naïve T cell culture alone [10]. SHEDs were also able to rescue the systemic lupus erythematosus-associated symptoms in mice by increasing the ratio of regulatory T cells [10]. It has also been shown that acetylsalicylic acid treatment could improve the immunomodulation of SHEDs [36]. In this regard, acetylsalicylic acid-treated SHEDs enhanced apoptosis of T cells and reduction of IL17⁺IFN γ cells via TERT/FASL pathway [36]. SHEDs also modulate dendritic cell maturation. When co-culture with SHEDs, mature dendritic cells decreased CD40, CD80, CD83 and CD86 expression [37]. SHEDs treating monocyte-derived dendritic cells reduced CD4⁺ and CD8⁺ cell proliferation when co-culture with peripheral blood lymphocyte as compared to the control [37]. These immunomodulatory functions of SHEDs encourage them as an interesting MSCs source for regenerative therapy.

3. Basic fibroblast growth factor signalling in SHEDs

Basic fibroblast growth factor (bFGF) is a member in fibroblast growth factor family [38]. It binds to fibroblast growth factor receptors (FGFR) and further initiates intracellular signalling [39]. bFGF has been shown to participate in the regulation of stemness maintenance and cellular differentiation. In human DPSCs, bFGF promotes pluripotent stem cell marker expression, corresponding with the increase of colony-forming unit [40]. Furthermore, bFGF inhibits osteogenic differentiation by SHEDs, human DPSCs and human periodontal ligament stem cells (PDLSCs) when supplemented in osteogenic induction medium (**Figure 2**) [5, 40]. In this regard, alkaline phosphatase enzymatic activity and mineralization are markedly decreased under bFGF-treated condition compared with the control [5, 40]. On the contrary,

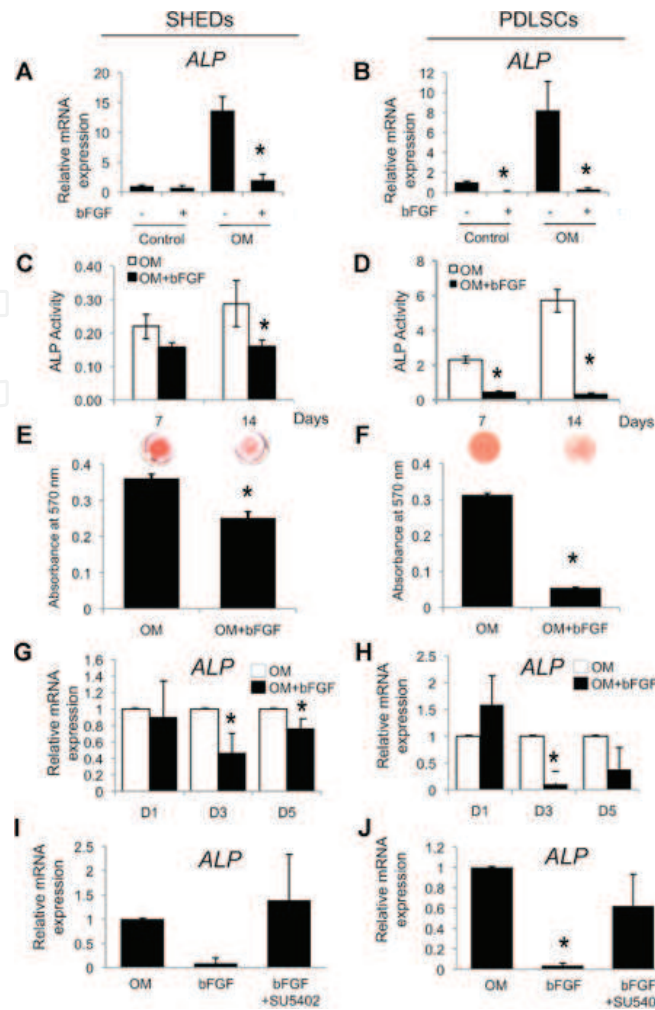


Figure 2. bFGF inhibited *ALP* expression and mineral deposition. The attenuation of *ALP* mRNA expression by bFGF at 7 days in normal and osteogenic medium was illustrated (A and B). The *ALP* enzymatic activity in osteogenic medium was shown at 7 and 14 days (C and D). Mineral deposition was determined at 14 days after maintaining in osteogenic medium (E and F). The time course experiments illustrated that bFGF attenuated *ALP* mRNA expression at 1, 3 and 5 days after cultured under osteogenic induction condition (G and H). The influence of bFGF was confirmed using FGFR inhibitor (SU5402). The *ALP* mRNA expression at 3 days was evaluated in osteogenic condition (OM), osteogenic condition supplemented with bFGF (bFGF) and osteogenic condition supplemented with bFGF and SU5402 (bFGF + SU5402) (I and J). The asterisk indicated the statistical significance compared to the control. Reprinted from Journal of Cellular Biochemistry, 114(11), Thanaphum Osathanon, Nunthawan Nowwarote, Jeeranan Manokawinchoke, Prasit Pavasant, bFGF and JAGGED1 regulated alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells, 2551–61, Copyright (2013), with permission from John Wiley & Sons Inc. [5].

bFGF enhances the expression of neurogenic marker, β III-tubulin, via FGFR and PLC γ when human DPSCs are cultured in a neurogenic induction medium supplemented with bFGF [40].

In SHEDs, long-term culture *in vitro* leads to the decrease of stemness as determined by the reduction of pluripotent stem cell markers, i.e. *OCT4*, *NANOG* and *REX1* [41]. In addition, the reduction of colony-forming unit ability is observed in high passage (passage 10) of cells [41]. Interestingly, bFGF enhanced *OCT4*, *NANOG* and *REX-1* mRNA levels in both short- and long-term maintaining *in vitro* [41]. bFGF also increased colony-forming unit in passage 10 [41]. Similarly, an attenuation of endogenous bFGF expression or blocking FGFR results in

the reduction of colony-forming number by SHEDs [6]. Further, bFGF promotes colony-forming unit ability in SHEDs isolated from inflamed dental pulp tissues [42]. For the regulatory mechanism, it has been demonstrated that bFGF regulated REX-1 expression in SHEDs via FGFR and Akt pathway [34]. IL-6 is also shown to involve in bFGF induced REX-1 expression as pre-treatment with antibody against IL-6 attenuates REX-1 expression [34].

Regarding osteogenic differentiation, bFGF attenuated osteogenic differentiation. In this regard, bFGF attenuated alkaline phosphatase enzymatic activity and mineralization in SHEDs after osteogenic induction [5, 43]. The inhibition of endogenous bFGF in SHEDs either by a chemical inhibitor for FGFR or lentiviral shRNA against bFGF resulted in the enhancement of osteogenic differentiation [6]. It was also demonstrated that bFGF attenuated alkaline phosphatase mRNA expression and mineral deposition via FGFR and MEK signalling pathway [5].

Several possible mechanisms were reported. Firstly, bFGF might attenuate osteogenic differentiation in SHEDs via decreasing Notch signalling [5]. Notch signalling activation led to the enhancement of mineralization in SHEDs [7]. Treatment with bFGF attenuated Notch receptor, ligand and target gene expression which may participate in bFGF attenuated osteogenic differentiation in SHEDs [5]. Secondly, bFGF inhibited matrix metalloproteinase (MMP) expression, for example *MMP-2*, *MMP-13* and *MT1-MMP* [5]. It has been demonstrated that MMP2 influenced odontogenic differentiation by DPSCs [44]. In this regard, MMP2 cleaved dentin matrix protein 1 (DMP1), resulting in the release of bioactive peptide that could promote odontogenic differentiation of DPSCs [44]. Finally, it has been shown that bFGF might inhibit canonical Wnt signalling pathway via the activation of ERK1/2 signalling [43]. ERK attenuation rescued bFGF inhibiting osteogenic differentiation by SHEDs both *in vitro* and *in vivo* [43]. In addition, ERK inhibitor increase β -catenin levels in bFGF-treated SHEDs [43]. Besides odonto/osteogenic differentiation, it has been shown that bFGF participated in angiogenesis induction properties of SHEDs. Priming with bFGF promoted SHEDs-induced angiogenesis *in vivo* [45]. This could be due to the increase production of VEGF and HGF by bFGF-treated SHEDs [45]. Together, these data denote the crucial influence of bFGF in the regulation of SHEDs stemness and differentiation mechanisms.

4. Wnt signalling in SHEDs

Canonical Wnt signalling also has a significant role in tooth development and stem cells self-renewal through β -catenin [46, 47]. Inactivation of β -catenin in the mesenchyme of developing tooth results in arrested tooth developmental at the bud stage [48]. Various studies established the influence of canonical Wnt signalling pathway to promote the osteogenic differentiation of dental stem cells, i.e. DPSCs, PDLSCs, stem cells from apical papilla (SCAPs) and dental follicle stem cells (DFSCs) [49–52]. However, the effect of the canonical Wnt/ β -catenin on SHEDs is very limited. The involvement of Wnt/ β -catenin on SHEDs-mediated mineralized tissue regeneration was investigated with the addition of basic fibroblast growth factor (bFGF) [43]. Treatment with bFGF attenuated SHEDs-mediated mineralized tissue

regeneration via activation of ERK 1/2 pathway and consequently inhibited Wnt/ β -catenin pathway, leading to osteogenic deficiency of SHEDs [43].

A recent *in vitro* and *in vivo* study reported that an activation of the canonical Wnt signalling pathway induced by Wnt3A can promote osteogenic differentiation of DPSCs [52]. Similar to previous study that activated Wnt signalling by using various concentrations of lithium chloride (LiCl), the result showed that Wnt/ β -catenin strongly upregulated expression of dentin sialophosphoprotein (DSPP), OCN and ALP in time- and dose-dependent manner [50, 51]. LiCl also upregulated protein expression of osteogenic transcription factors, including RUNX2, MSX2 and OSX. Whereas cells treated with Dickkopf-1 (DDK1), an inhibitor for canonical Wnt signalling, resulted in the inhibition of osteogenic mRNA expression and reduction the alkaline phosphatase enzymatic activity and matrix mineralization [50]. On the other hand, the contradictory evidence demonstrates that the canonical Wnt signalling can inhibit osteogenic differentiation, alkaline phosphatase enzymatic activity and formation of mineralized nodules in DPSCs [53]. Canonical Wnt signalling inhibited the odontoblast-like differentiation of DPSCs was first reported by Scheller et al. in 2008 [53]. This study showed that Wnt-1 inhibited alkaline phosphatase enzymatic activity and the formation of mineralized nodules in DPSCs when transduced with canonical Wnt-1 or the active form of β -catenin, with retrovirus-mediated infection. Moreover, overexpression of β -catenin was also sufficient to suppress the differentiation and mineralization of DPSCs [53]. Another study was established using Wnt3A and LiCl to examine the possible involvement of canonical Wnt signalling in regulating cementoblast behaviours. Activation of endogenous canonical Wnt signalling with both Wnt3A and LiCl suppressed alkaline phosphatase enzymatic activity and expression of genes associated with cementum function: *Alp*, *Bsp* and *Ocn*. This effect was accompanied by decreased gene expression of *Runx2* and *Osx* and by increased gene expression of *Lef-1*. In conclusion, these observations suggest that Wnt signalling inhibits cementoblast differentiation and promotes cell proliferation [54].

Activation of β -catenin by LiCl in SHEDs led to the significant decrease of colony formation by SHEDs [55]. In addition, LiCl enhanced subG0 population in SHEDs [55]. *OSX* and *DMP1* mRNA expression was markedly decreased in LiCl-treated SHEDs. These results imply the influence of Wnt signalling in SHED behaviours [55]. The canonical Wnt/ β -catenin pathway also implicates in angiogenic differentiation of SHEDs. Transplantation of SHEDs in human tooth slice/scaffolds into immunodeficient mice differentiates into new blood vessels that anastomose with the host vasculature. *In vitro* data showed that VEGF induced the vasculogenic differentiation of SHEDs via potent activation of Wnt/ β -catenin signalling while Wnt inhibition blocked this process [31]. Moreover, the study has been shown that the Wnt/ β -catenin pathway also participates in immunomodulatory properties of SHEDs [36]. Acetylsalicylic acid treatment enhances immunomodulatory properties of SHEDs as indicated by increased in SHED-mediated T-cell apoptosis and the decreased levels of T helper 17. Moreover, acetylsalicylic acid significantly improves SHED-based bone formation and these effects of acetylsalicylic acid treatment on SHEDs occurred via the regulation of the telomerase reverse transcriptase/Wnt and TERT/FASL pathways [36].

5. Notch signalling in SHEDs

Notch signalling controls various function of stem cells, ranging from stemness maintenance to cell-specific differentiation [56]. It is a highly conserved pathway, firstly identified in *Drosophila*. Notch signalling is initiated by the binding between membrane-bound Notch receptors and ligands of neighbouring cells [56–58]. Further, Notch receptors are cleaved by a γ -secretase enzyme, leading to the release of Notch intracellular domain (NICD) [56–58]. Subsequently, NICD translocates into nucleus and forms complex with other transcriptional molecules, resulting in the activation of Notch target genes [56–58]. Common Notch signalling target genes are *Hes* and *Hey* families [56–58]. In the canonical Notch signalling pathway, four receptors and five ligands are identified [56–58]. The four types of Notch receptors are Notch1, Notch2, Notch3 and Notch4. Five ligands are Delta-like-1 (Dll-1), Delta-like-3 (Dll-3), Delta-like-4 (Dll-4), Jagged1 and Jagged2 [56–58].

Notch signalling participates in odontogenesis, dental pulp repair and regeneration. Mice lacking of Jagged2 expression exhibited defective enamel formation of incisors and malformation of molars [59]. The expression of Notch receptors and ligands was upregulated in response to calcium hydroxide, a material for direct pulp capping treatment [60]. Human DPSCs over-expressing Jagged1 exhibited the reduction of osteogenic differentiation ability and mineralization *in vitro* and *in vivo* [61]. Dll-1 over-expressing human DPSCs had higher proliferative rate than the control and knock down Dll-1 expression in human DPSCs led to significantly enhancement of osteo/odontogenic differentiation [62, 63]. These evidence support the role of Notch signalling in the regulation of human DPSCs' behaviours and dental pulp tissue homeostasis of permanent teeth. Though, knowledge of Notch signalling in SHEDs is yet limited.

Studies illustrated that indirectly immobilized Notch ligands, Jagged1 or Dll-1, on tissue culture surface increased *HES1* and *HEY1* mRNA levels in SHEDs, implying the successful activation of intracellular Notch signalling [7]. The activation of Notch signalling in SHEDs led to the enhancement of osteogenic differentiation [5, 7]. However, Jagged1 exhibited higher potency to promote alkaline phosphatase enzymatic activity and mineralization than Dll-1 (**Figure 3**) [7]. Corresponding to study in primary human dental pulp cells isolated from deciduous teeth, Jagged1, but not Dll-1, attenuated cell proliferation [64]. The influence of Jagged1 on alkaline phosphatase enzymatic activity and mineralization *in vitro* could be attenuated by pretreatment with a γ -secretase inhibitor, DAPT, confirming the involvement of Notch signalling pathway [7]. Jagged1 significantly enhanced osteogenic marker gene expression, namely *ALP* and *COL1* [7]. In addition, Jagged1 downregulated a negative regulator of osteogenic differentiation, *TWIST2*, in SHEDs [7].

It has been shown that bFGF inhibited the mRNA expression of Notch signalling components. In this regard, bFGF significantly reduced the mRNA levels of *NOTCH1*, *NOTCH2*, *JAGGED1*, *DLL1* and *HES1* in SHEDs cultured in osteogenic induction medium [5]. In addition, bFGF was able to attenuate Jagged1-induced alkaline phosphatase mRNA expression and mineralization when SHEDs were maintained in osteogenic medium for 7 and 14 days, respectively [5]. bFGF significantly reduced alkaline phosphatase mRNA expression as early as 1 day in culture, corresponding to the significant reduction of *HES1* [5]. Taken all evidence together, bFGF and Notch signalling possibly interact and regulate mineralization process in SHEDs.

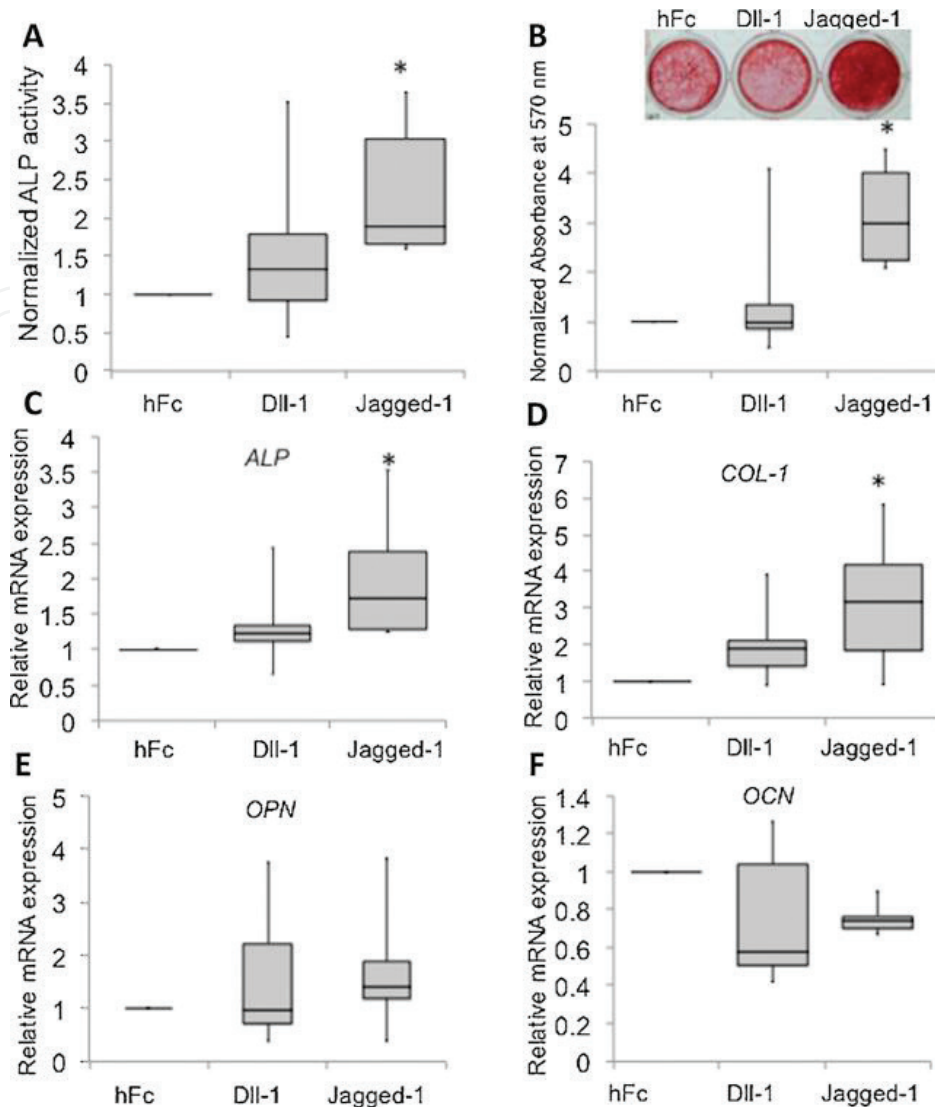


Figure 3. Effects of Dll-1 and Jagged1 on osteogenic differentiation. The alkaline phosphatase enzymatic activity (A) and mineralization (B) were evaluated at day 7 and 14 after osteogenic induction, respectively. For osteoblast marker gene expression, cells were cultured on Dll-1, Jagged1 or hFc treated surface for 7 days after osteogenic differentiation. The graphs demonstrated the relative mRNA expression of *ALP*, *COL1*, *OPN* and *OCN* upon seeding cells on Dll-1 or Jagged1 immobilized surface and normalized to the hFc control (D–F). Asterisks indicated statistical significance compared to the hFc control. Reprinted from Archives of Oral Biology, 65, Waleerat Sukarawan, Kannapas Peetiakarawach, Prasit Pavasant, Thanaphum Osathanon, Effect of Jagged1 and Dll-1 on osteogenic differentiation by stem cells from human exfoliated deciduous teeth, 1–8, Copyright (2016), with permission from Elsevier [7].

6. Mechanical stress influences SHEDs' behaviours

Dental pulp tissues are surrounded by hard tissues, namely dentin. During inflammation, an interstitial fluid pressure increases [65, 66], causing biological changes in local cells and tissues. In addition, fluid movement in dentin-pulp complex during normal occlusal force may expose cells to mechanical stimuli [67]. Mechanical forces are shown to regulate biological functions in many cell types, for example osteoblasts, osteocytes, periodontal ligament cells and dental pulp cells. Different types and magnitude of force lead to different cell responses.

In human DPSCs, uniaxial cycle stretching inhibited odonto/osteogenic differentiation but increased cell proliferation [68, 69], while cyclic hydrostatic pressure synergistically enhanced BMP-2-induced DSPP expression by human DPSCs *in vitro* and increased hard tissue formation *in vivo* [70]. Studies in SHEDs demonstrate that mechanical force may regulate stemness maintenance. In this respect, static compressive force upregulated pluripotent marker mRNA expression in SHEDs [8]. *REX-1*, *SOX2*, *OCT4* and *NANOG* mRNA levels increased in a magnitude-dependent manner [8]. Mechanical stress-induced *REX-1* expression is partly controlled via IL-6/JAK and ATP-P2Y1 signalling pathways [8, 71]. Though, the clinical significance of these phenomena requires further investigation.

7. Potential application of SHEDs in regenerative therapy

SHEDs are the good candidate for the stem cells used in regenerative therapy due to their high plasticity as well as ability to cross lineage boundaries and differentiate into several specialized cells. Current progresses have been made for tissue engineering-based therapies involving a large number of tissues. However, dentin-pulp complex and neuronal tissue seem to be the most promising aspects for the application of SHEDs in regenerative therapy.

The first evidence to show that SHEDs can differentiate to become the functional odontoblasts with the ability to generate the mineralized tissue resemble to dentin was shown in mice [3]. SHEDs were seeded within a scaffold in a tooth slice and implanted into the dorsum of mice. Dental pulp-like tissue was observed in the central area of the pulp chamber of the tooth slice [3]. The expression of odontoblastic differentiation markers such as DSPP and DMP-1 was detected [3]. Remarkably, the newly deposited dentin was observed and suggested that SHEDs can differentiate into fully functional odontoblasts *in vivo* [3]. Later in 2013, the regeneration of the dental pulp within the full length of the root canal was reported [19]. SHEDs were transplanted into the root canals with the scaffold and were observed for 28 days *in vitro*. The transplanted SHEDs were able to proliferate and inside the root canal [19]. The expression of odontoblastic differentiation marker such as DSPP, DMP-1 and MEPE was observed [19]. Interestingly, when the roots with SHEDs were implanted in the subcutaneous space of mice, a dental pulp-like tissue was formed in the majority of space in the root canal [19]. This *de novo* dental pulp-like tissue was capable of depositing new dentin [19]. However, this model is still considered as the ectopic transplantation model. Another concern for clinical translation is that most of the results interpretation was made from histological evaluation with the lack of functional testing. Therefore, the regeneration of dentin-pulp complex by SHEDs still needs further study in the more related oral environment and the additional functional of nerve innervation or vascularization should be performed before clinical application. Current possible experimental approaches for dentin pulp complex regeneration are summarized in **Figure 4**.

In addition to dentin-pulp complex regeneration, SHEDs also show the potential to be used in neuroregeneration. Stem cell therapy is the promising therapeutic options for treating the neurodegenerative diseases due to the limited regenerative capacity of the specialized cells in the nervous system. The neural crest cell in origin makes SHEDs the candidate cell model for neuron tissue regeneration. These cells are prone to undergo neurogenic differentiation

Dentin pulp complex regeneration strategies

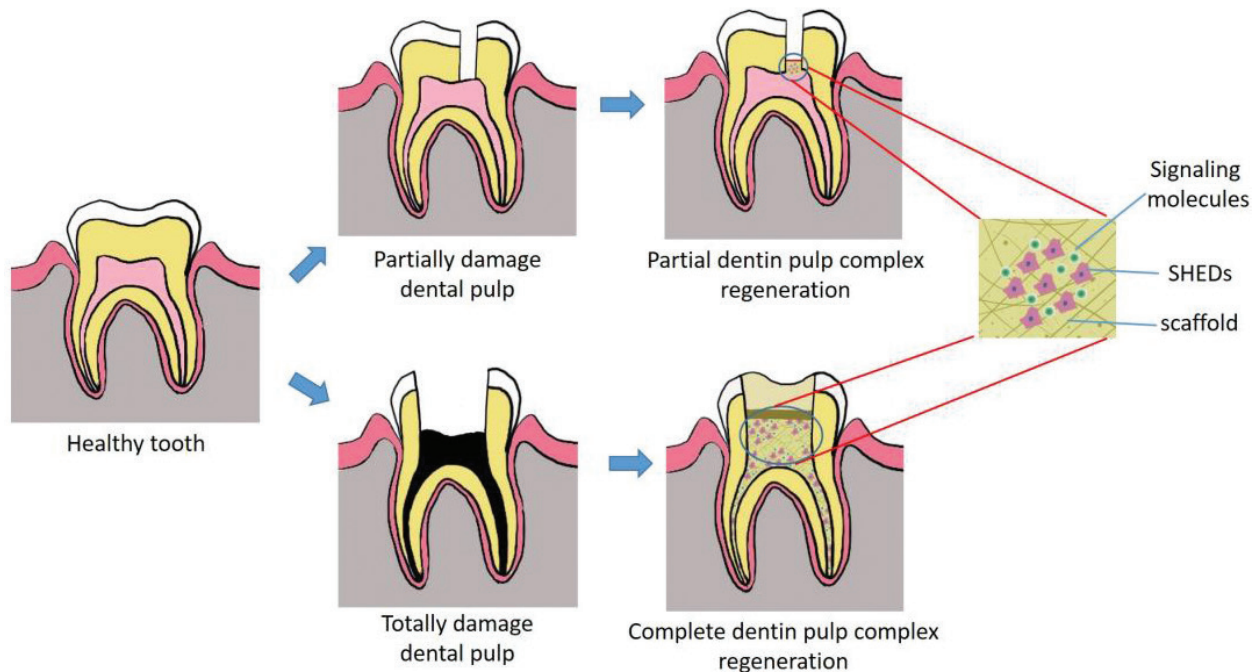


Figure 4. Current possible experimental approaches for dentin pulp complex regeneration using SHEDs.

both *in vitro* [2, 24–26] and *in vivo* [25, 27, 72]. Promising results from several *in vivo* studies lay the spotlight on SHEDs for their use as a stem cell source for treating neurodegenerative disease and other neuron-related conditions such as Parkinson disease, Alzheimer’s disease, focal cerebral ischemia and spinal cord injuries [27, 29, 72–74]. Transplantation of neurogenic induced SHEDs into the parkinsonian rat model significantly improved the recovery behavioural impairment compared to transplantation of control SHEDs [25, 27].

In a focal cerebral ischemia rat model induced by permanent middle cerebral artery occlusion, intranasal administration of supernatants from the medium used to culture SHEDs significantly decreased in the motor disability score and significantly reduced in the infarct volume [72]. Moreover, positive signals for neuronal nucleus, neurofilament H, doublecortin and rat endothelial cell antigen in the peri-infarct area were observed in the rats treated with SHEDs conditioned media compared to the DMEM control from approximately 140 mm³ in DMEM control to 50 mm³ in SHEDs conditioned medium [72]. These results suggest that SHEDs might secrete some compounds that positively influence the recovery of the brain lesion in focal cerebral ischemia [72].

Studies have shown that SHEDs have remarkable neuroregenerative activity and promote functional recovery in a spinal cord injury animal model [29, 75]. Rats that received SHEDs transplantation within the lesion created at the 9th–11th thoracic vertebral levels exhibited higher scores in the locomotor rating scale compared to the bone marrow stromal cells or fibroblasts transplantation control [75]. In addition, the rescue of hindlimb locomotor function was prominent in the rats that received SHEDs. These animals were able to move hindlimb coordinately and walk, while the bone marrow stromal cells transplantation exhibited only subtle movements

[75]. A similar trend was observed in another study, a complete recovery of hindlimb motor function was observed after implantation of neural-induced SHEDs in a rat spinal cord injury [29] which suggested that preinduction of the undifferentiated SHEDs into the neural-like cells before implantation might improve the efficiency of SHEDs in regenerating specialized neural cells. Taken together, these high neurogenic potential of SHEDs especially in animal models makes them the favourable source for stem cell regeneration treatment for neural diseases.

8. Conclusion

Dental stem cells, including SHEDs, have been extensively studied in the past decades leading to the better understanding in their unique biological properties and therapeutic potential. As SHEDs can be easily obtained with limited ethical concern, their multi-differentiation potentials have been demonstrated, which creates great opportunities for the application in the regenerative therapy. However, despite the intriguing results, we still need further study to deepen the understanding of the mechanisms underlying the differentiation processes to attain clinical reality. Also, the potential risks for the clinically use of SHEDs or other dental stem cells should be thoroughly studied for the safety of the patients who will greatly benefit from their regenerative ability.

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Abbreviations

Akt	Protein kinase B
ALP	Alkaline phosphatase
ApoE	apolipoprotein E
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BMMSCs	Bone marrow-derived mesenchymal stem cells
BSP	Bone sialoprotein
CD	Cluster of differentiation
COL1	Collagen type 1
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DDK	Dickkopf
DFSCs	Dental follicle stem cells

DII	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
DMP-1	Dentin matrix acidic phosphoprotein 1
DMP	Dentin matrix protein
DPSCs	Dental pulp stem cells
DSP	Dentin sialoprotein
DSPP	Dentin phosphoprotein
ERK	Extracellular signal-regulated kinase
FASL	Fas ligand
FGFR	Fibroblast growth factor receptor
GFAP	Glial fibrillary acidic protein
HA	Hydroxyapatite
Hes	Hairy and enhancer of split
Hey	Hairy and enhancer of split related with YRPW motif protein
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-antigen D related
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
LEF-1	Lymphoid enhancer binding factor 1
LiCl	Lithium chloride
LPL	Lipoprotein
MEK	Mitogen-activated protein kinase kinase
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
MSX2	Msh homeobox 2
MT1-MMP	Membrane type1- matrix metalloproteinase
NICD	Notch intracellular domain
Nurr1	Nuclear receptor related 1 protein
OCN	Osteocalcin
OCT4	Octamer-binding transcription factor 4
OPN	Osteopontin
OSX	Osterix
P2Y1	Purinergic receptor P2Y1

PCR	Polymerase chain reaction
PDLSCs	Periodontal ligament stem cells
PECAM-1	Platelet endothelial cell adhesion molecule 1
Pitx3	Paired like homeodomain 3
PLC γ	Phospholipase C gamma
PPAR γ 2	Peroxisome proliferator-activated receptor-gamma 2
PRP	Platelet-rich plasma
REX1	Reduced Expression Protein 1
RUNX2	Runt-related transcription factor 2
SCAPs	Stem cells from apical papilla
SHEDs	Stem cells isolated from human exfoliated deciduous teeth
shRNA	Short hairpin ribonucleic acid
SOX2	Sex determining region Y-box 2
SSEA4	Stage-specific embryonic antigen-4
TCP	Tricalcium phosphate
TERT	Telomerase reverse transcriptase
TWIST	Twist Family BHLH Transcription Factor
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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