# Transforming growth factor beta 3 induced odontoblast-like differentiation of stem cells from human exfoliated deciduous teeth

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**Abstract**

**BACKGROUND:** Studies have reported that the superfamily of transforming growth factors exert a role in the mineralization of various stem cells, but the combination effects of transforming growth factor β3 and heparin on proliferation and mineralization ability of stem cells from human exfoliated deciduous teeth remains to be studied.

**OBJECTIVE:** To explore the effect of transforming growth factor β3 on odontoblast-like differentiation of stem cells from human exfoliated deciduous teeth.

**METHODS:** Human deciduous teeth were collected using enzyme digestion. The 3rd passage dental pulp stem cells were incubated with 25 μg/L recombinant human transforming growth factor β3, 10 U/mL heparin or their combination. The dentin sialophosphoprotein mRNA and dentinsialoprotein expressions were detected by Q-PCR and western blot assay. Alkaline phosphatase activity was determined using alkaline phosphatase kit.

**RESULTS AND CONCLUSION:** Stem cells from human exfoliated deciduous teeth grew well after induction. The activity of alkaline phosphatase in the combination group was significantly higher than that in the transforming growth factor β3, heparin and control groups ($P < 0.01$). After combination induction, the cells were strongly positive for alizarin red staining. Results from α-PCR and western blot assay showed that the expressions of dentin sialophosphoprotein were both remarkably increased at mRNA and protein levels. In summary, stem cells from human exfoliated deciduous teeth can differentiate into odontoblast-like cells under the induction of transforming growth factor β3 plus heparin.

**Subject headings:** tooth, deciduous; dental pulp; stem cells; transforming growth factor beta 3; heparin; dentin
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## INTRODUCTION

Stem cells can be used for repair and reconstruction of damaged or lesioned tissues and organs, which provides a new idea for regenerative treatment of lesions and injuries in time. How to simply and non-invasively harvest seed cells has become a hot spot. Similar to human dental pulp stem cells, stem cells from human exfoliated deciduous teeth (SHED) can differentiate into nerve cells, adipocytes, and odontoblasts, and further form new bone and dentin[1-7]. By way of contrast, SHED cannot directly differentiate into osteoblasts and cannot build dentin-pulp-like complex, which is more immature. But SHED is superior to human dental pulp stem cells and bone marrow stromal stem cells in the proliferative rate and multipotent differentiation capacity. The most important is that the deciduous teeth originate from autologous tissue without immune rejection, but with a high safety and wide variety of sources, which cannot be involved in moral constraints. Therefore, SHED may be an ideal source for dental tissue repair. Under induction, dental pulp stem cells from human deciduous teeth can differentiate into odontoblasts and form the dentin. However, different induction methods means a great difference in the oriented differentiation of SHED into odontoblasts, and the common induction methods cannot be applied clinically.

Transforming growth factor β3 (TGF-β3) mainly exists in mesenchymal cells. In situ hybridization has shown that TGF-β1 and TGF-β3 express in mesenchymal cells and dental papilla cells at bud and cap stages. Our previous studies observed the effect of TGF-β3 on odontoblast-like differentiation of SHED. However, the local application of exogenous recombinant TGF-β3 was limited because of poor bioavailability, short biological half-life and other issues. Heparin, an acid mucopolysaccharide, has a special affinity with many important proteins and it can play an important role in the regulation of various biological signals. Studies have shown that heparin may provide multiple binding sites for TGF-β superfamily in intracellular matrix[8], prevent TGF-β from proteolytic hydrolysis and chemical inactivation, effectively protect the activity of TGF-β, and stabilize the molecular conformation[9]. Consequently, we explored the odontoblast-like differentiation of SHED induced by TGF-β3, heparin or their combination.

## MATERIALS AND METHODS

### Design

SHED was collected and cultured, and then divided into blank control group, heparin-treated group, TGF-β3-treated group, and combination group (heparin+TGF-β3). The osteogenic effects of different factors were analyzed at protein and mRNA levels, as well as pathological analysis and biochemical tests.

### Time and setting

The experiments were completed at the laboratory of Guangzhou Recomgen Biotech Co., Ltd. (Guangzhou, China) from November 2013 to May 2014.

### Materials

**Reagents and instruments for odontoblast-like differentiation of SHED induced by TGF-β3:**

| Reagents and instruments | Source |
|---|---|
| α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), 0.01 mol/L PBS, 0.25% pancreatic enzymes, collagen type I enzymes | GIBCO |
| Vimentin antibody, STRO-1 antibody, anti-DSPP antibody | Abcam |
| Microplate reader | Thermo Fisher Scientific |
| Alkaline phosphatase (AKP) kit | Nanjing Jiancheng Bioengineering Institute |
| Human recombinant TGF-β3, Trizol Reagent | Invitrogen |
| Heparin | Sigma |
| SYBR Green PCR Master Mix | TOYOBO |
| Quantitative PCR instrument (ABI PRISM® 7500 Sequence Detection System) | Applied Biosystems |

### Experimental procedures

#### *SHED isolation and culture*

Primary central incisors were collected from in-patient children aged 7–8 years, and cultured in α-MEM containing double antibodies. Within 4 hours, primary cells were collected according to the method of Gronthos *et al* [10]: the samples were placed in the mixture of 4 g/L protease and 3 g/L collagen type I at 1:1, incubated in a water bath at 37 ℃ for 1 hour, and then digested in 10 g/L DNAse digestive juice (50 μL) for 30 seconds. Thereafter, the cell suspension was rinsed in 10 mL Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS that was injected into a 15 mL tube and filtrated with a 200-mesh sieve to remove tissue blocks. The cell suspension was inoculated in the medium coated with gelatin and then placed into a 5% $CO_2$ incubator at 37 ℃. After cell adhesion, the cells were rinsed with PBS and cultured in α-MEM containing 10% FBS. Cell growth was observed and pictured under microscope day by day. Cells at 80%-90% confluence were digested using 0.25% pancreatic enzyme and passaged at 1:3.

#### *Immunocytochemistry staining*

Passage 3 SHED was collected and grew on the coverslip at a density of $1\times10^7$/L. Growing to 60%–80% confluence, cells were fixed using 40 g/L paraformaldehyde for 15 minutes. STRO-1 (1:300) and vimentin (1:200) immunochemical staining was carried. Subsequently, cells were incubated with the primary antibody overnight, and then incubated with secondary antibody (1:40) for 30 minutes. After that, cells were developed with 3,3'-diaminobenzidine, counterstained with hematoxylin, washed back to the blue and mounted with neutral gum.

#### *Induction experiment*

Passage 3 cells were taken and cultured in 6-well plates. After adhesion, cells were cultured with 25 μg/L human recombinant TGF-β3, 10 U/mL heparin, and their combination, respectively. The medium was changed to 10% FBS every 3 days. Cell differentiation into odontoblasts was observed.

#### *Alizarin red staining*

After 28 days of culture, cell supernatant was discarded in each group. Following washed twice in PBS, cells were fixed with 4% formaldehyde for 10 minutes and rinsed thrice with distilled water. 0.1% alizarin red-Tris-HCL solution (pH 8.3) was dropped to cover the cell surface, and cells were incubated at 37 ℃ for 30 minutes followed by distilled water washing, drying and taking pictures.

#### *AKP quantitative detection*

After cultured for 9 days, cells were rinsed twice with PBS and subjected to cell lysis at the same volume until clearly transparent. According to the instructions of AKP kit, the level of AKP in the cell lysate was detected.

#### *Real-time fluorescent quantitative PCR*

After 7 days of culture, the cell supernatant was discarded, and cells were washed with PBS twice. Total RNA was extracted using trizol and the purity of RNA samples at 260/280 nm was detected. Reverse transcription: 42 ℃ for 20 minutes, 99 ℃ for 5 minutes, 4 ℃ for 5 minutes,

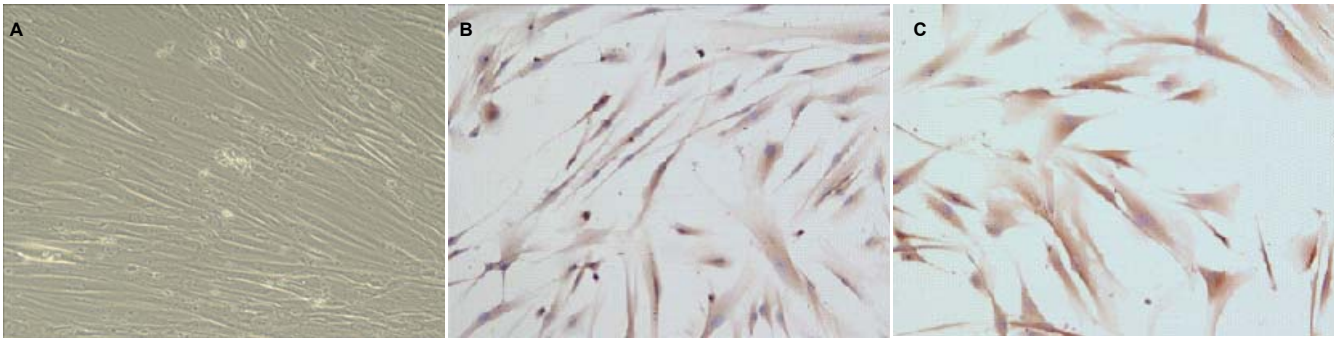



Figure 1 Morphology and immunohistochemical identification of stem cells from human exfoliated deciduous teeth
Note: A: After 7 days of primary culture, stem cells from human exfoliated deciduous teeth mostly covered the bottom of the bottle, and presented with a long spindle shape, with ovoid nuclei locating in the center of cytoplasm, showing a fibroblast-like growth (×100); B: Strongly positive for vimentin staining (×100); C: Strongly positive for STRO-1 staining (×100).

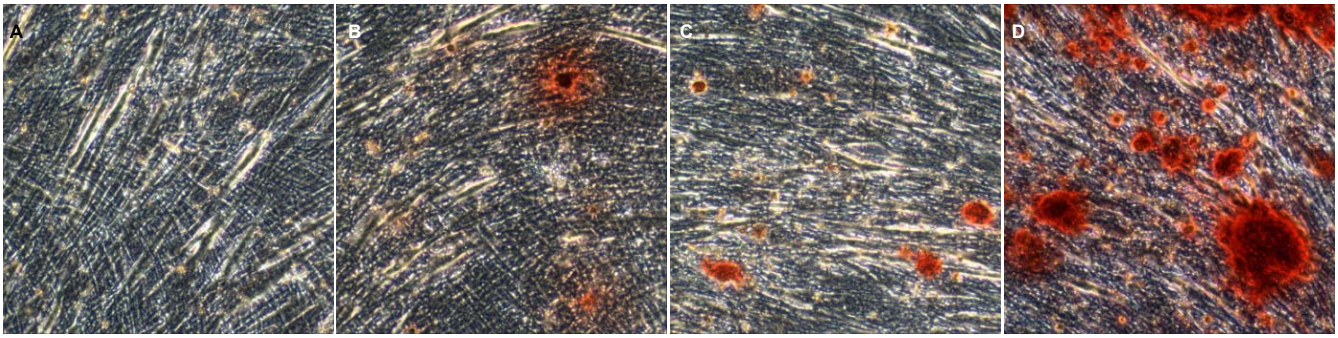



Figure 3 Alizarin red staining of passage 3 stem cells from human exfoliated deciduous teeth (×100)
Note: A: The control group was negative for alizarin red staining. B: A few of mineralized nodules positive for alizarin red staining were seen in the transforming growth factor-β3 (TGF-β3) group. C: Mineralized nodules positive for alizarin red staining were seen in the heparin-treated group. D: Mineralized nodules strongly positive for alizarin red staining were seen in the TGF-β3+heparin combination group.

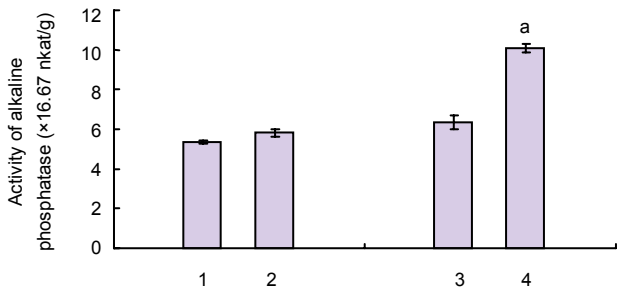



Figure 2 Effect of transforming growth factor-β3 (TGF-β3) on alkaline phosphatase (AKP) activity in stem cells from human exfoliated deciduous teeth
Note: 1, control group; 2, heparin-treated group; 3, TGF-β3 group; 4, combination group. [a]*P* < 0.01, *vs*. the other three groups.

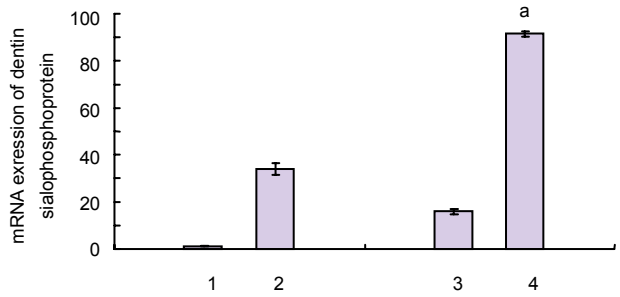
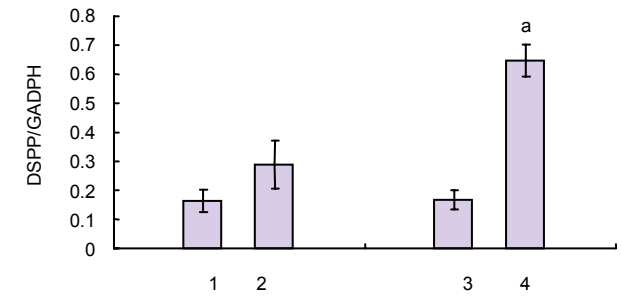



Figure 4 The mRNA expression of dentin sialophosphoprotein detected by real-time quantitative PCR
Note: 1, control group; 2, heparin-treated group; 3, TGF-β3 group; 4, combination group. [a]*P* < 0.01, *vs*. the other three groups.



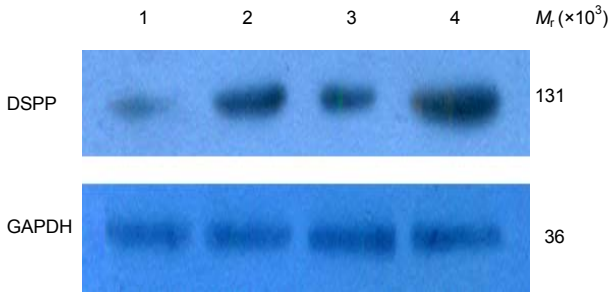




Figure 5 The protein expression of dentin sialophosphoprotein detected by western blot assay
Note: 1, control group; 2, heparin-treated group; 3, TGF-β3 group; 4, combination group. [a]*P* < 0.01, *vs*. the other three groups.

centrifuged instantaneously. cDNA product was subjected to real-time PCR reactions. Primers were designed as follows: DSPP upstream 5'-GAT CAT GAT AGT AGC ATA GGT C-3' and downstream 5'-CCA AGA TCA TTC CAT GTT GTC-3', the desired product was 361 bp; internal reference: 18SrRNA upstream 5'-CCT GGA TAC CGC AGC TAG GA-3' and downstream 5'-GCG GCG CAA TAC GAA TGC CCC-3', the expected product was 112 bp. Reaction conditions: 40 cycles of denaturation at 94 ℃ for 2 minutes, 94 ℃ for 15 seconds, 57 ℃ for 10 seconds, 72 ℃ for 30 seconds, with 0.2 second interval, reading once (Haugland, 1999), and then extension at 72 ℃ for 2 minutes.

***Western blot assay for detecting dentin sialophosphoprotein levels***

After culture for 14 days, cells were collected to extract total protein, and the protein concentration was determined by BCA method. The extracted protein samples were mixed with sample loading buffer at 2:1 and boiled for 5 minutes. The samples were subjected to 6% SDS-PAGE electrophoresis, and transferred to a membrane cryogenically for 60–120 minutes, sealed with 5% skim milk powder solution at room temperature for 1 hour, and diluted with primary antibody at 4 ℃ overnight. The membrane was washed with Tris-buffered saline containing Tween-20 (TBST) for 5 minutes (three times) and incubated with secondary antibody at 37 ℃ for 1 hour. After TBST rinsing, the membrane was placed onto a transparent plastic plate and luminescent substrates were uniformly added onto the surface of membrane with a pipette. The reaction was continued for 5 minutes. X-ray films were used for developing and fixation. The absorbance value was detected using a scanner and IPWIN60 software. The relative protein expression of dentin sialophosphoprotein=band absorbance value of dentin sialophosphoprotein/ GADPH band absorbance value.

## RESULTS

**Cell growth**

SHED adhered slowly. About 70% cells were adherent after 4 hours, and fundamentally spread after 48 hours. These cells were smaller and basically fusiform-shaped, possessing one or two processes. A small number of cells presented with irregular shape. Cells arranged tightly and some of them rapidly proliferated and appeared to have colony-like growth (**Figure 1A**). after 7 days, cells grew to 90% confluence.

**Immunocytochemical identification**

SHED was strongly positive for anti-Vimentin and STRO-1 staining in the cytoplasm (**Figure 1 B, C**).

**AKP quantitative detection**

The combination of 25 μg/L TGF-β3 and 10 U/mL heparin obviously promoted the activity of AKP in the SHED, significantly different from TGF-β3 and heparin alone as well as control gorup ($P < 0.01$; **Figure 2**).

**Odontoblast differentiation from SHED**

Passage 3 cells were *in vitro* cultured and induced for 14 days, and a small amount of brown crystals could be seen in all the groups except the control group under the microscope. At 28 days, mineralized nodules strongly positive for alizarin red staining were visible in the combination group, and bright red particles were significantly more and larger than those in the other groups. Positive cells accounted for over 90%. However, several small particles were seen in the TGF-β3 and heparin-treated groups, and the control group was negative for alizarin red staining (**Figure 3**).

**Real-time fluorescent PCR detection**

The combination of 25 μg/L TGF-β3 and 10 U/mL heparin obviously promoted the mRNA expression of dentin sialophosphoprotein in the SHED, significantly different from TGF-β3 and heparin alone as well as control group ($P < 0.01$; **Figure 4**).

**Western blot assay of the protein expression of dentin sialophosphoprotein**

Western blot assay showed that 25 μg/L TGF-β3 combined with 10 U/mL heparin obviously increased the protein expression of dentin sialophosphoprotein in the SHED, significantly different from TGF-β3 and heparin alone as well as control group ($P < 0.01$; **Figure 5**).

## DISCUSSION

Odontoblasts are the main cells for synthesis and secretion of dentin matrix. Because odontoblasts are difficult to obtain and easy to age and die after passage *in vitro*, current studies on the function of odontoblasts mainly focus on the application of differentiation characteristics of dental pulp cells and dental papilla cells[11-14]. SHED which is isolated by Miura *et al* using enzymatic digestion, has become a rookie in tissue engineering research because of their strong proliferation ability and multi-directional differentiation potential.

During tooth development , formation and repair after pulp damage, a variety of growth factors have been discovered. These growth factors provide a microenvironment for cell survival, proliferation and differentiation. The complex interactions between growth factors regulate various cell activities, and play an important role in the growth and differentiation of organs and tissues. TGF-β superfamily is a superfamily of regulating cell growth and differentiation, which is not only directly involved in the signal transduction between the mesenchyma and epithelium, and also control the formation of body's odontoblasts and osteoblasts in the embryonic stage, playing an important role in odontoblast growth, migration and differentiation[15-17]. In the TGF-β superfamily, TGF-β3 is mainly secreted by mesenchymal cells, and exerts a critical role in the differentiation and growth of mesenchymal cell lines. During tooth development, the dentin, dental pulp, and periodontal tissue are all derived from mesenchymal tissue. Therefore, this study investigated the odontoblast-like differentiation of SHED, so as to further explore the action mechanism and clinical value of SHED in the induction of dentin formation.

Studies have shown that TGF-β, human basic fibroblast growth factor, insulin-like growth factor, bone morphogenetic proteins and fibronectin can induce the *in vitro* differentiation of dental papilla cells or dental pulp cells *in vitro* into odontoblasts under tissue culture conditions[18-19]. Huojia *et al* [20] found that recombinant TGF-β3 can induce ectopic calcification of dental pulp cells from mouse dental germ, improve the levels of osteocalcin and collagen type I mRNA and protein in dental pulp cells, and induce the expressions of dentin sialoprotein, dentin matrix protein, osteocalcin, osteonectin, osteopontin in primary dental pulp cells, indicating that TGF-β3 can induce ectopic mineralization of dental pulp cells by upregulating osteocalcin and collagen type I, and thereby promoting the differentiation of dental pulp cells into odontoblasts.

However, a growth factor alone appears to have some shortcomings in cell induction, such as low efficiency and instable differentiation. Heparin is shown to have a special affinity with many biologically important proteins. Consequently, heparin is associated with cell adhesion, recognition, migration, and regulating the activity of various enzymes. There are a lot of affinity growth factors for heparin, including fibroblast growth factor, vascular endothelial growth factor, hepatocyte growth factor and TGF. These factors bind tightly to heparin PHS molecule in a specific extracellular matrix. Studies have shown that heparin can provide a plurality of cell binding sites for TGF-β superfamily in intracellular matrix[21], prevent TGF-β from proteolytic hydrolysis and chemical inactivation, effectively protect the activity of TGF-β, and stabilize the molecular conformation[22]. Begue-Kirm and co-workers reported in the experiments of culturing dental papilla tissue that TGF-β1 combined with heparin could promote the polarization of dental papilla cells, and these cells transcribed dentin sialophosphoprotein and secreted predentin, showing a differentiation state of odontoblasts[23].

Therefore, in this study, we isolated and purified SHED according to the Gronthos's method, and identified SHED using STRO-1 and vimentin staining. There were four groups: TGF-β3+heparin combination group, TGF-β3 alone group, heparin-treated group and control group. Passage 3 cells were collected and cultured with 25 μg/L recombinant TGF-β3, 10 U/mL heparin or their combination. Alizarin red staining, alkaline phosphatase detection, real-time quantitative PCR and western blot assay were used for observing the differentiation of SHED into odontoblasts. We found that SHED induced by TGF-β3 alone was only weakly positive for alizarin red staining, and the mRNA expression of dentin sialophosphoprotein in TGF-β3-induced SHED was slightly higher than that in the control group, indicating TGF-β3 alone cannot obviously accelerate the odontoblast-like differentiation of SHED. However, the combined use of TGF-β3 and heparin could significantly increase the AKP activity and expressions of dentin sialophosphoprotein at mRNA and protein levels as compared with the control group, moreover, cells in the combination group were strongly positive for alizarin red staining. Therefore, we believe that the combined use of TGF-β3 and heparin can remarkably facilitate the differentiation of SHED into odontoblasts, probably because heparin can protect TGF-β3 to form a stable complex, thereby preventing the enzyme hydrolysis or chemical inactivation of TGF-β3, enhancing the active role of TGF-β3 in the odontoblast-like differentiation of SHED.

Taken together, TGF-β3 is an important cytokine, which specifically binds to TGF-βR III (CD105), a cell surface receptor, and produces a major biological function. TGF-β3 can be stably combined with heparin to promote the growth, proliferation and odontoblast-like differentiation of dental pulp cells, thereby providing an important role in dental pulp repair.

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# 转化生长因子 β3 诱导人乳牙牙髓干细胞向成牙本质样细胞的分化

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**文章亮点：**

实验特征性的显示了在转化生长因子 β3 和肝素的联合作用下，乳牙牙髓干细胞的碱性磷酸酶活性、牙本质涎磷蛋白基因的 mRNA 水平以及蛋白表达水平均明显高于转化生长因子 β3 单独作用组、肝素单独作用组、对照组，且茜素红染色呈强阳性。说明在转化生长因子 β3 与肝素联合作用的诱导下，可明显促进乳牙牙髓干细胞向成牙本质样细胞分化。

**关键词：**

*干细胞；分化；人乳牙牙髓干细胞；转化生长因子β3；肝素；成牙本质样细胞*

**主题词：**

*牙，乳；牙髓；干细胞；转化生长因子β3；肝素；牙本质*

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**摘要**

**背景：**转化生长因子 β3 与肝素联合作用后对人乳牙牙髓干细胞的增殖和矿化能力的作用目前仍有待研究。

**目的：**探讨转化生长因子 β3 对人乳牙牙髓干细胞向成牙本质样细胞分化的作用。

**方法：**采用酶消化法将人乳牙牙髓分离培养，获得人乳牙牙髓干细胞；对体外培养的第 3 代人乳牙牙髓干细胞单独加入 25 μg/L 重组转化生长因子 β3、10 U/mL 肝素，或者二者联合进行培养；通过 Q-PCR 和 Western-blotting 方法，分别检测乳牙牙髓干细胞中牙本质涎磷蛋白基因及牙本质涎蛋白表达的情况；通过碱性磷酸酶试剂盒检测乳牙牙髓干细胞碱性磷酸酶活性的改变。

**结果与结论：**乳牙牙髓干细胞在诱导体系中生长状态良好。25 μg/L 转化生长因子 β3+10 U/mL 肝素联合作用组的碱性磷酸酶活性明显增强，与转化生长因子 β3 单独作用组、肝素单独作用组以及对照组相比差异有显著性意义($P < 0.01$)；转化生长因子 β3+肝素联合作用组的茜素红染色呈强阳性，Q-PCR 和 Western-blotting 结果均显示，转化生长因子 β3+肝素联合作用组的牙本质涎磷蛋白 mRNA 和蛋白表达均明显升高。结果可见在转化生长因子 β3 与肝素联合作用的诱导下，可促进乳牙牙髓干细胞分化为成牙本质样细胞。

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***利益冲突***：文章及内容不涉及相关利益冲突。

***伦理要求***：门诊收集 7-8 岁龄儿童丢弃的乳中切牙，研究过程不涉及道德制约。

***学术术语***：牙本质磷蛋白和牙本质涎蛋白是惟一由成牙本质细胞合成和分泌的两种牙本质特异性非胶原蛋白，起着启动牙本质矿化以及调节羟基磷灰石晶体大小和生长速度的作用。

***作者声明***：文章为原创作品，无抄袭剽窃，无泄密及署名和专利争议，内容及数据真实，文责自负。

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