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Insulin-like growth factor 1 can promote the osteogenic differentiation and osteogenesis of stem cells from apical papilla

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Abstract Insulin-like growth factor 1 (IGF-1) plays an important role in the regulation of tooth root development, and stem cells from apical papilla (SCAPs) are responsible for the formation of root pulp and dentin. To date, it remains unclear whether IGF-1 can regulate the function of SCAPs. In this study, SCAPs were isolated and purified from human immature root apex, and stimulated by 100 ng/mL exogenous IGF-1. The effects of IGF-1 on the proliferation and differentiation of SCAPs were subsequently investigated. IGF-1 treated SCAPs presented the morphological and ultrastructural changes. Cell proliferation, alkaline phosphatase (ALP) activity and mineralization capacity of SCAPs were increased by IGF-1. Western blot and quantitative RT-PCR analyses further demonstrated that the expression of osteogenic-related proteins and genes (e.g., alkaline phosphatase, runt-related transcription factor 2, osterix, and osteocalcin) was significantly up-regulated in IGF-1 treated SCAPs, whereas the expression of odontoblast-specific markers (e.g., dentin sialoprotein and dentin sialophosphoprotein) was down-

Abbreviations: ALP, alkaline phosphatase; α-MEM, alpha minimum essential medium; DSP, dentin sialoprotein; DSPP, dentin sialophosphoprotein; FBS, fetal bovine serum; FCM, flow cytometry; IGF-1, Insulin-like growth factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCN, osteocalcin; OSX, osterix; PVDF, polyvinylidene difluoride; RUNX2, runt-related transcription factor 2; SCAPs, stem cells from apical papilla

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regulated by IGF-1. *In vivo* results revealed that IGF-1 treated SCAPs mostly gave birth to bone-like tissues while untreated SCAPs mainly generated dentin-pulp complex-like structures after transplantation. The present study revealed that IGF-1 can promote the osteogenic differentiation and osteogenesis capacity of SCAPs, but weaken their odontogenic differentiation and dentinogenesis capability, indicating that IGF-1 treated SCAPs can be used as a potential candidate for bone tissue engineering.

Introduction

It is generally believed that root formation results from the interactions between Hertwig's epithelial root sheath (HERS) and adjacent undifferentiated mesenchymal cells. Stem cells from the apical papilla (SCAPs) represent a population of early mesenchymal stem/progenitor cells residing in the root apex of immature permanent teeth. These postnatal stem cells can generate the calcium nodules in the osteo/odontogenic medium and Oil red O-positive lipid clusters following the adipogenic induction *in vitro*(Sonoyama et al., 2006). Besides, they can bring about the formation of bone-like tissues and dentin-like tissues *in vivo* (Abe et al., 2008; Sonoyama et al., 2006; Sonoyama et al., 2008). In physiological conditions, SCAPs contribute to the formation of developing radicular pulp as well as odontoblasts that are responsible for the root dentinogenesis, and they also play a paramount role in the pulp healing and regeneration (Huang et al., 2008; Sonoyama et al., 2008). Recent clinical reports indicate that SCAPs are important to the apexogenesis of developing roots and continuous root maturation in teenagers suffering from the endodontic diseases (Banchs and Trope, 2004; Chueh and Huang, 2006). Moreover, SCAPs have been used as a competent candidate for dental tissue regeneration. Dentin-pulp-like tissues in the empty root canal space and bioengineered roots which can support a porcelain crown have been respectively generated by utilizing SCAPs recombined with biological scaffolds *in vivo* (Huang et al., 2009; Huang et al., 2010; Sonoyama et al., 2006).

During the tooth morphogenesis, growth factors and other paracrine signal molecules mediate the interactions both between and within the epithelial and mesenchymal tissues, such as the fibroblast growth factors (FGFs), transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), and insulin-like growth factors (IGFs) (Caton and Tucker, 2009; Torres et al., 2006, 2008). FGF signaling pathways are important to the evolution of mammalian dentition by giving rise to new cusps and crests (Charles et al., 2009). Among BMP family members, BMP2 has been extensively studied for its biological functions during root development, and it can promote dental pulp stem cell commitment to the odontoblast lineage (Yang et al., 2009). IGF-1 and IGF-2 play an important role in the regulation of tooth growth and differentiation. Tooth explants treated with IGF-1 show an increase in both dentin and enamel matrix, while IGF-2 appears to preferentially affect the enamel deposition (Caton et al., 2005). Following the odontoblastic differentiation, IGF-1 level is significantly up-regulated (Liu et al., 2009). There is a statistically significant difference of IGF-1 expression between immature and mature dental pulps (Caviedes-Bucheli et al., 2004, 2007), and exogenous IGF-1 can elongate the molar roots (Fujiwara et al., 2005). However, little knowledge is available about its effect on the differentiation of SCAPs.

In the present study, we hypothesize that IGF-1 plays an important role in the proliferation and differentiation of SCAPs. For this purpose, SCAPs were isolated from immature human third molar apex and treated with exogenous IGF-1. The effects of IGF-1 on SCAPs were subsequently evaluated both *in vitro* and *in vivo*. Our experimental data showed that the osteogenic differentiation and osteogenesis of SCAPs were enhanced by IGF-1, while their odontogenic differentiation and dentinogenesis were weakened, indicating that these IGF-1 treated SCAPs can be used as a potential candidate for bone tissue engineering.

Results

IGF-1 modified the morphology and ultrastructure of SCAPs

The effect of IGF-1 is mainly mediated through the type 1 IGF-1 receptor (IGF-1R). In this study, purified SCAPs expressed IGF-1R gene (Fig. 1A) and protein (Fig. 1B). These stem cells appeared microscopically as elongated and spindle-shaped cells (Fig. 2A). After the treatment with 100 ng/mL IGF-1 (Caton et al., 2005; Fujiwara et al., 2005; Li et al., 2009), SCAPs became flat at day 7 and many of them presented the typical osteoblast morphology, i.e., cuboidal or polygonal shape with several cytoplasmic processes (Fig. 2B). TEM analysis demonstrated that the untreated cells possessed the typical ultrastructural features of stem cells with a high nucleus/cytoplasm ratio and poorly developed cytoplasmic organelles (Figs. 2C and E). After the treatment with IGF-1, these stem cells contained more organelles in the cytoplasm including the rough endoplasmic reticulum and mitochondria (Figs. 2D and F).

IGF-1 promoted the proliferation of SCAPs

The cell-cycle analysis and MTT assay were performed to investigate whether IGF-1 can affect the proliferation of SCAPs *in vitro*. Flow cytometry was used to investigate the proliferation index, i.e., the percentage of cells in S and G_2M phases. As shown in Figs. 3A and B, the proliferation index in the IGF-1 treated group at day 3 ($S+G_2M=29.97\%$) was significantly higher than that in the untreated group ($S+G_2M=19.41\%$), indicating that IGF-1 can promote the proliferation of SCAPs. MTT assay revealed that the proliferation of IGF-1 treated SCAPs were more active than that of untreated ones (Fig. 3C). OD values significantly increased in the IGF-1 treated SCAPs compared with the untreated SCAPs from day 3 to day 6 ($P<0.01$). Moreover, SCAPs obtained from different donors exhibited a similar upward trend of proliferation rate when treated by IGF-1.

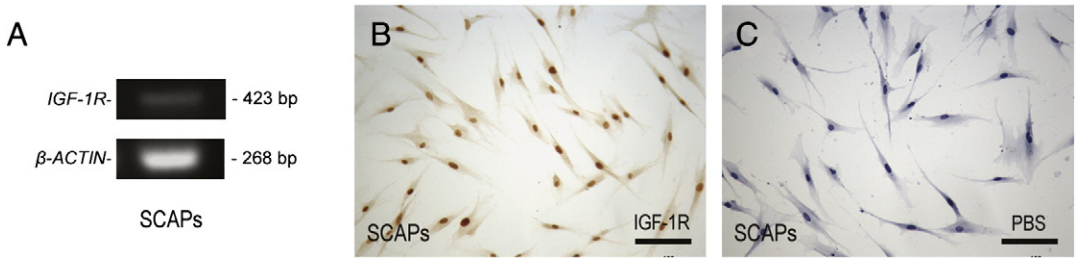

Figure 1 Expression of IGF-1R gene and protein in purified SCAPs. A: *IGF-1R* gene was detected in purified SCAPs by RT-PCR. *β-ACTIN* was used as a loading control. B: SCAPs were positive for IGF-1R by immunocytochemistry. C: Negative control using PBS instead of the primary antibody. Scale bars: B and C = 100 μm.

IGF-1 enhanced osteogenic differentiation but weakened odontogenic differentiation of SCAPs *in vitro*

To further evaluate the differentiation ability, ALP activity and matrix mineralization ability were analyzed at the defined time points. ALP activity of SCAPs was increased by IGF-1 at day 5 (Fig. 4A, $P<0.01$). Moreover, SCAPs treated with IGF-1 showed more mineralization nodules (Fig. 4B) and higher calcium concentrations (Fig. 4C, $P<0.01$) than untreated group by alizarin red staining and CPC assay after 14 days culure.

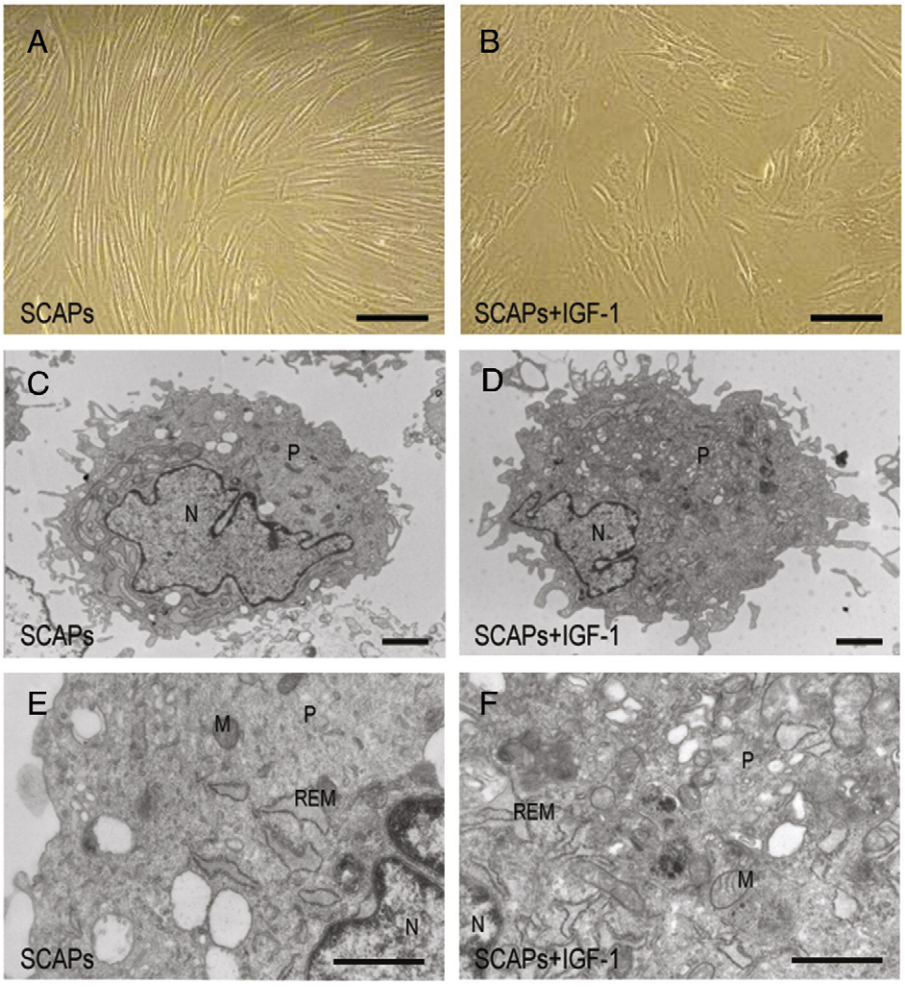

Figure 2 Morphological changes of SCAPs treated by IGF-1. A: Untreated SCAPs were elongated and spindle-shaped. B: IGF-1 treated SCAPs became flat at day 7 and some of them were cuboidal or polygonal containing multiple cytoplasmic processes. C and E: TEM analysis showed that untreated SCAPs have the typical ultrastructural features of stem cells, i.e., higher nucleus/cytoplasm ratio and immature cytoplasmic organelles. D and F: IGF-1 treated SCAPs contained more organelles in the cytoplasm including the rough endoplasmic reticulum and mitochondria. Scale bars: A and B = 50 μm; C and D = 2 μm; E and F = 1 μm. Abbreviations: M, mitochondria; N, nucleus; P, cytoplasm; RER, rough endoplasmic reticulum.

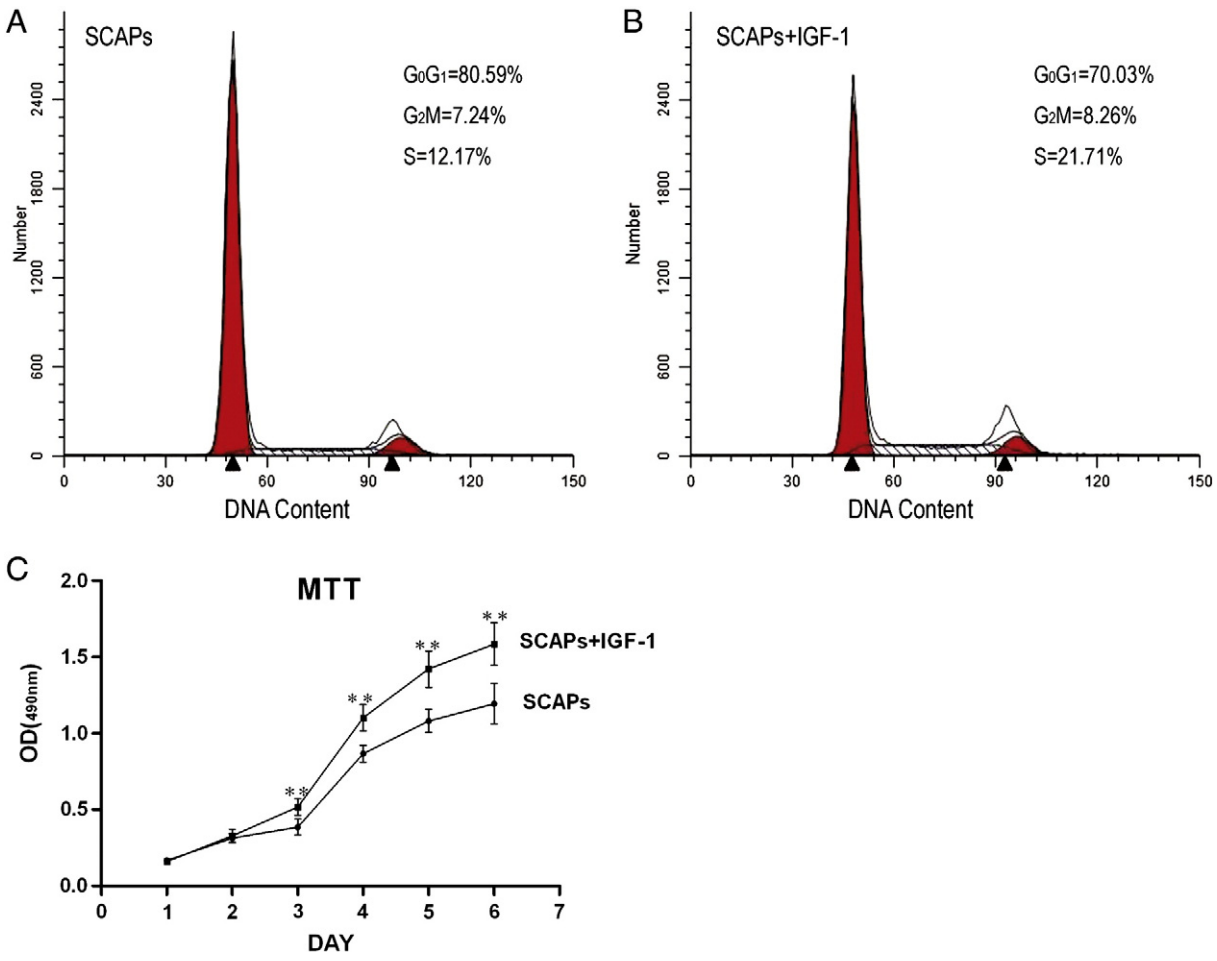

Figure 3 IGF-1 promoted the proliferation of SCAPs. A: Representative cell-cycle distribution profiles of untreated SCAPs. B: Representative cell-cycle distribution profiles of IGF-1 treated SCAPs. C: Growth curves of untreated and treated SCAPs plotted from MTT assay. The proliferation of IGF-1 treated SCAPs (days 3–6) was enhanced compared with untreated ones (**$P<0.01$).

Quantitative RT-PCR was performed to evaluate the expression levels of osteo/odontogenic genes including *ALP*, *RUNX2*, *OSX*, *OCN* and *DSPP*. Compared with the untreated group, there was a remarkable increase of the osteogenic markers (i.e., *ALP*, *RUNX2*, *OSX* and *OCN*) at days 7 and 14 in IGF-1 treated SCAPs, whereas *DSPP* mRNA level was significantly decreased at day 14 in treated SCAPs (Fig. 5A). Western blot analysis further verified these results. The protein expression of RUNX2 (at days 3, 7 and 14), OSX (at day 14) and OCN (at days 3, 7 and 14) in the IGF-1 treated group (Fig. 5B) was elevated while DSP was down-regulated at day 14, in comparison with the untreated group.

IGF-1 increased osteogenesis ability of SCAPs *in vivo*

At 6 weeks, all implants were harvested and analyzed. By visual inspection, all retrieved implants shared similar colors, sizes, and shapes. All implants exhibited mineralized tissues protruding from the surface of renal capsules (Figs. 6A and E). Radiographic analyses revealed that all implants contained radioopaque tissues, in which the IGF-1 treated implants presented more mineralized structures than the control group (inlays in Figs. 6A and6E). Histological examination indicated that untreated SCAPs mainly formed (18/20) dentin-pulp-like structures involving predentin, mature dentin, odontoblast-like cells and dentinal tubules (Figs. 6B and C) at week 6 post-transplantation. Polarized light showed that untreated SCAPs produced collagen fibers perpendicular to the matrix forming surface (Fig. 6D), which is similar in structure to the primary dentin. However, most IGF-1 treated SCAPs (14/20) generated the bone-like tissues in which osteoblast-like cells were arranged regularly along the surface of the calcified matrix and osteocyte-like cells were embedded in the lacuna structures (Figs. 6F and G). Collagen fibrils were deposited almost parallel to the forming surface or poorly ordered like woven bone (Fig. 6H).

The expression of mineralization-related proteins (DSP, OSX and OCN) was subsequently examined by immunohistochemical staining. Retrieved untreated implants displayed a positive expression of DSP (odontoblast marker; Fig. 7A) in odontoblast-like cell layer, predentin, and dentin structures, while OSX expression (osteoblast marker; Fig. 7B) was negative. However, OCN expression is positive in odontoblast-like cells, weaker positive in osteodentin-like tissues and negative in dentin (Fig. 7C). In IGF-1 treated group, weaker expression of DSP (Fig. 7E), positive expression of OSX/OCN (bone specific proteins, Figs. 7F and G)

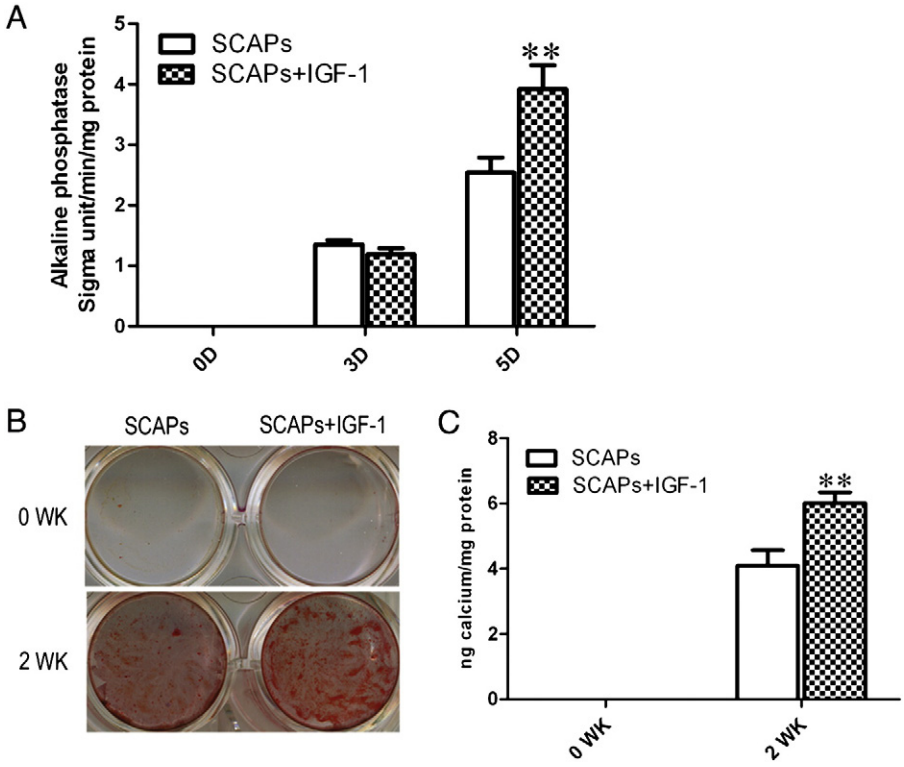

Figure 4 IGF-1 enhanced ALP activity and mineralization of SCAPs. A: Comparison of ALP activity between IGF-1 untreated and treated SCAPs. There was no significant difference of ALP levels at day 3 between two groups. Treated group presented a higher ALP activity at day 5 than control group. B: Comparison of the mineralized nodules between IGF-1 untreated and treated SCAPs by alizarin red staining. IGF-1 treated SCAPs generated more calcified nodules than untreated group after 14-day culture. C: Calcium concentrations in treated SCAPs were significantly higher than untreated ones (**$P<0.01$).

was respectively identified in bone-like tissues. These findings further confirmed that IGF-1 can promote the osteogenic differentiation as well as the osteogenesis of SCAPs, but diminish their odontogenic differentiation and dentinogenesis ability.

Discussion

There is a growing evidence that IGFs play an important role in the development of oro-dento-facial tissues and organs (Caton et al., 2005; Werner and Katz, 2004; Yamamoto et al., 2006), in which IGF-1 is involved in various processes including tooth development, growth, periodontal ligament homeostasis and different pathological circumstances (Caton et al., 2005; Fujiwara et al., 2005; Kheralla et al., 2010; Rath-Deschner et al., 2009). Diverse studies have revealed that IGF-1 can affect the odontogenic differentiation and dentin regeneration (Caton et al., 2007; Young, 1995). Moreover, IGF-1 can induce dental papilla cells to secrete the bone-like extracellular matrix (Caton et al., 2007). Previous studies have proved that SCAPs have a potent capacity to differentiate along the odontoblast lineages and perform the typical dentinogenesis (Abe et al., 2008; Lei et al., 2011; Sonoyama et al., 2006; Sonoyama et al., 2008). Therefore, it is reasonable that IGF-1 may have some distinguishable effects on the differentiation of SCAPs.

In this study, exogenous IGF-1 can stimulate the proliferation and modify the ultrastructural morphology of SCAPs *in vitro*. Besides, IGF-1 can up-regulate the ALP activity, the mineralization capacity, and expression of several osteoblast-related markers (*ALP*, *RUNX2*, *OSX*, *OCN*) in SCAPs. As a characteristic marker of osteoblast phenotype, *ALP* is an important marker during the early stage of osteogenic differentiation (Hanai et al., 2006; Hong et al., 2010; Park et al., 2009). The transcription factors *RUNX2* and *OSX* are also necessary for osteogenic differentiation (Baek et al., 2009; Kaback et al., 2008; Komori, 2006). Disruption of *RUNX2* and *OSX* can result in a complete lack of bone formation and tooth germ abnormality (Kobayashi et al., 2006). Moreover, RUNX2 can inhibit the terminal differentiation of odontoblasts, induce the transdifferentiation of odontoblasts into osteoblast lineages and form a bone structure (Miyazaki et al., 2008). *OCN* is thought to play an important role in the osteogenic differentiation and mineralization at the late stages of bone formation. *DSPP* gene and DSP protein are the putative differentiation markers of odontoblast lineages. They are involved in the nucleation and control of the hydroxyapatite mineral phase during dentin calcification (Lee et al., 2009; McKnight et al., 2008). Thus, the up-regulation of osteoblast markers (i.e., RUNX2, OSX, OCN, and ALP) and down-regulation of odontoblast markers (i.e., *DSPP* and DSP) in IGF-1 treated group suggests that IGF-1 can enhance the osteogenic differentiation but weaken the odontogenic differentiation of SCAPs. Moreover, the expression level of *DSPP* is quite low in both treated and untreated SCAPs. The plausible explanation for this phenomenon is that SCAPs from the

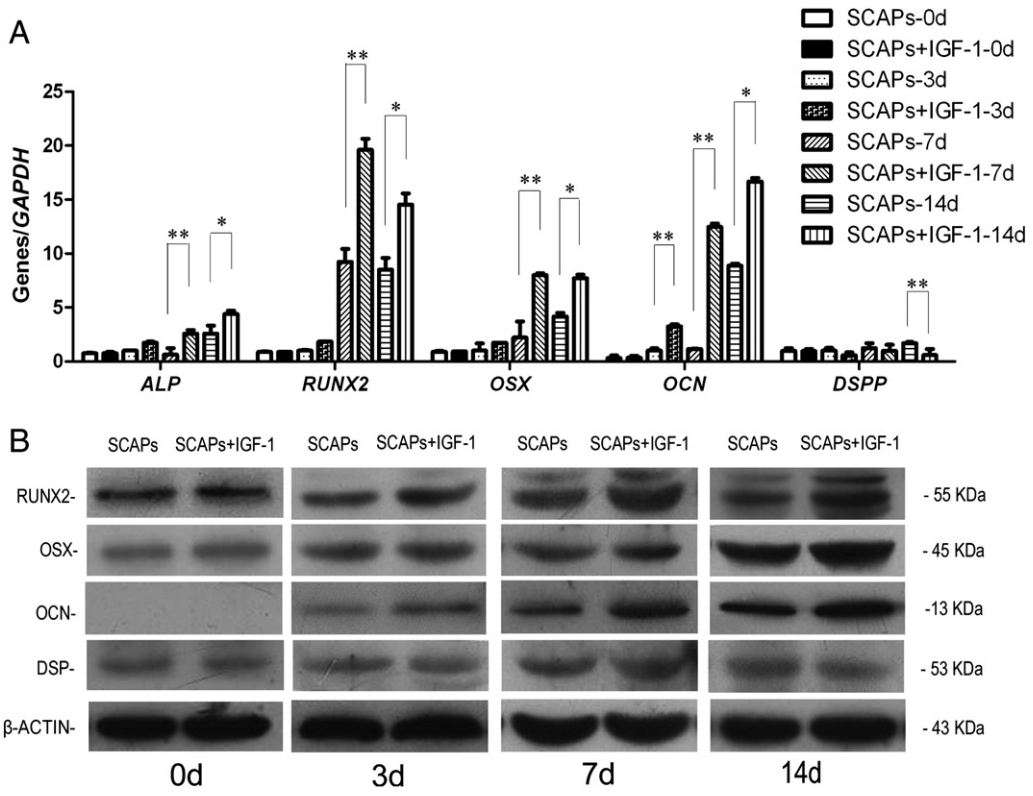

Figure 5 Effect of IGF-1 on osteo/odontogenic gene expression of SCAPs. A: QRT-PCR results of gene expression in untreated and treated samples for *ALP*, *RUNX2*, *OSX*, *OCN* and *DSPP*. *GAPDH* was used as an internal control. Gene expression was described as a fold change relative to the control group. Values are the means ± SD $n=4$ (**$2^{-\Delta\Delta Ct} \geq 2$, $P<0.01$; *$1<2^{-\Delta\Delta Ct}<2$, $P<0.01$). B: Western blot results of protein levels in untreated and treated SCAPs for RUNX2, OSX, OCN and DSP. β-ACTIN was used as a loading control.

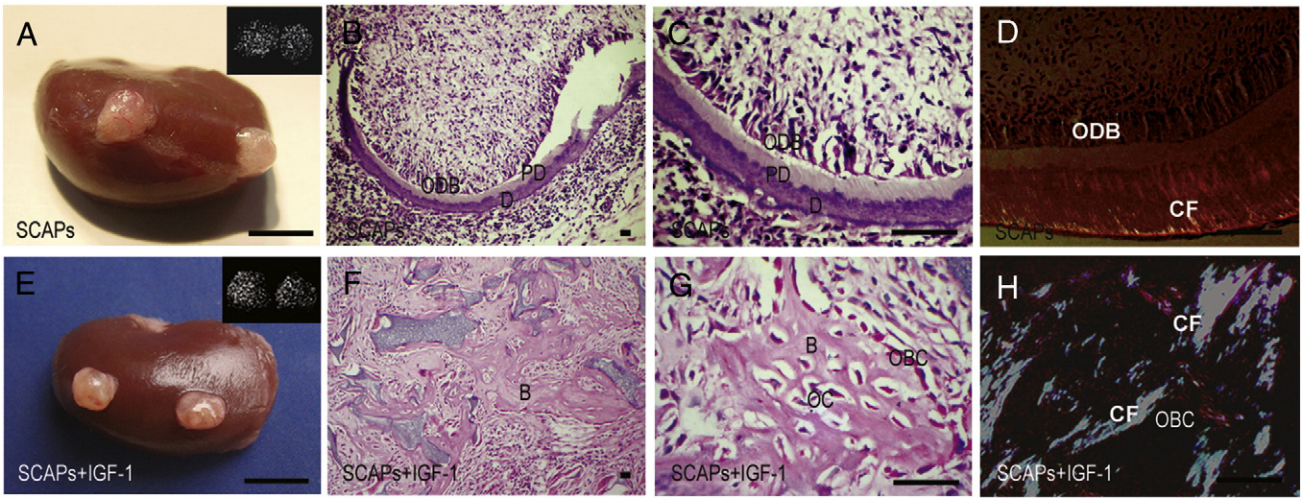

Figure 6 Histological evaluation of IGF-1 on SCAPs *in vivo*. A: Retrieved implants of untreated SCAPs after 6-week incubation *in vivo* observed by visual inspection and radiographic analyses. B and C: Histological examination of the retrieved implants of untreated SCAPs. Odontoblast-like cell, predentin, and dentin tissues appeared in the mineralized structures. D: Polarized light demonstrated the perpendicular alignment of collagen fibers to the dentin surface in untreated implants. E: Retrieved implants of IGF-1 treated SCAPs after 6-week incubation *in vivo* observed by visual inspection and radiographic analyses. F and G: Histological examination of the retrieved implants of IGF-1 treated SCAPs. Bone-like tissues were generated. Osteocytes within the osseous lacunae and osteoblast-like cells on the bone-forming surface can be observed in the mineralized tissue. H: Collagen fibrils under the polarized light were deposited almost parallel to the forming surface or poorly ordered like woven bone in IGF-1 treated implants. Scale bar: A and E = 1 cm. B, C, D, F, G and H = 50 μm. Abbreviations: B, bone-like tissue; CF, collagen fibers; D, dentin; OBC, osteoblast-like cell; OC, osteocyte-like cell; ODB, odontoblast-like cell; PD, predentin.

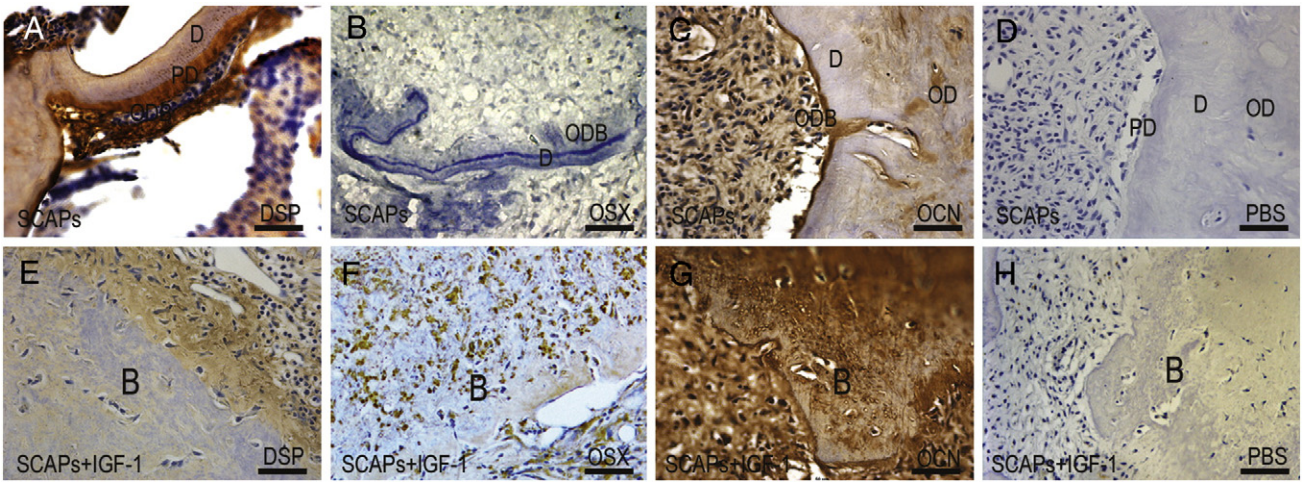

Figure 7 Immunohistological staining of osteo/odontogenic markers in IGF-1 treated SCAPs *in vivo*. A: Immunohistological positive staining for DSP in odontoblast-like cells, predentin, and dentin structures of untreated SCAPs pellets. B: Negative staining for OSX in odontoblastic layer and dentin of untreated SCAPs pellets. C: Positive staining for OCN in odontoblast-like cells and weaker positive in osteodentin-like tissues of untreated SCAPs pellets. Negative staining for OCN appeared in dentin layer. D: Negative control using PBS instead of primary antibodies in untreated SCAPs pellets. E: Weaker positive staining for DSP in bone-like structures generated by IGF-1 treated SCAPs. F: Positive staining for OSX in osteoblast-like cells of IGF-1 treated SCAPs pellets. G: Positive staining for OCN in bone-like structures generated by IGF-1 treated SCAPs. H: Negative control using PBS instead of primary antibodies in IGF-1 treated SCAPs implants. Scale bars = 50 μm. Abbreviations: B, bone-like tissue; D, dentin; OD, osteodentin-like tissues; ODB, odontoblast-like cell; PD, predentin.

developing dental tissues are more primitive and they cannot achieve full odontoblastic differentiation under *in vitro* culture conditions due to the loss of suitable microenvironment (Sonoyama et al., 2008).

Some studies have demonstrated that *RUNX2* and *OSX* are important for osteogenic and odontogenic differentiation, and both can regulate the expression of other osteo/odontogenic genes. In this paper, although *RUNX2* and *OSX* were both highly expressed in IGF-1 treated SCAPs, these stem cells mainly differentiate along the osteoblast lineages instead of odontoblast lineages both *in vitro* and *in vivo*, suggesting that *RUNX2* and *OSX* may act on different downstream target genes and affect different signaling pathways when SCAPs differentiate along different cell lineages, i.e., osteoblasts and odontoblasts (Chen et al., 2009; Chen et al., 2005; James et al., 2006).

The results of transplantation *in vivo* further confirmed this conclusion. Untreated SCAPs mainly generated dentin-pulp-like tissues, whereas IGF-1 treated SCAPs mostly brought about the formation of bone-like structures, indicating that IGF-1 can enhance the osteogenic potential but weaken their odontogenic capacity of SCAPs *in vivo*. Bone and dentin are mineralized tissues that closely resemble each other in composition and mechanism of formation. They are both formed by matrix-mediated mineralization mechanisms in which type I collagen generates the structural template for the epitaxial nucleation of hydroxyapatite. The specific mechanisms involved in controlling bone and dentin formation are still unknown. Non-collagenous proteins are generally believed to actively regulate the hydroxyapatite crystal nucleation, growth shape and orientation during the osteogenesis and dentinogenesis (Butler et al., 2002; Qin et al., 2004). These non-collagenous proteins are similar in odontoblasts and osteoblasts, and no definite protein is found specific. However, the expression of these molecules has the quantity differences between the two processes which induce the different final structures, i.e., bone and predentin. Our findings revealed that IGF-1 changed the collagen alignment and expression pattern of non-collagenous proteins, including the up-regulation of RUNX2/OSX/OCN and down-regulation of DSP. This may modify the cell polarization and mineralization pattern of IGF-1 treated SCAPs *in vivo*, which ultimately brings about the formation of bone-like tissues.

The effects of IGF-1 on bone formation have been extensively investigated, and many studies have demonstrated that IGF-1 is one of the key regulators of osteoblast differentiation/proliferation and exerts a beneficial effect on bone development/homeostasis (He et al., 2006; Kanbur et al., 2005; Tiago et al., 2010; Yao et al., 2008). IGF-1 triggers at least two signaling pathways, i.e., Ras/Raf-1/MAPK pathway and PI3K/PDK-1/Akt pathway (Grey et al., 2003; Hatakeyama et al., 2008; Laviola et al., 2007; Wu et al., 2008). The activation of PI3K/PDK-1/Akt pathway contributes to the osteoblast survival as well as cellular proliferation and differentiation. It is noted that p42/44 MAPK in Ras/Raf-1/MAPK pathway can converge with PI3K/PDK-1/Akt pathway on the downstream effector. IGF-1 can stimulate ALP activity via MAPK and PI3K pathways (Hanai et al., 2006; Noda et al., 2005), and regulate the activity of *RUNX2* and *OSX* via Akt-independent pathway and MAPK pathways (Celil and Campbell, 2005; Qiao et al., 2004). Furthermore, IGF-1 signals may modulate osteoblast mitogenesis and survival through parallel PI3K and MAPK pathways (Grey et al., 2003). Meanwhile, IGF-1 may favor the osteoblastogenesis by regulating β-catenin, a signaling molecule used by Wnt canonical signaling pathway, which is essential for the osteogenesis (Canalis, 2009; Krishnan et al., 2006; Wang et al., 2010). Since IGF-1 treated SCAPs mainly

differentiated along the osteoblast lineages and generated bone-like tissues, it is plausible that IGF-1 may regulate SCAPs in a way similar to the regulation of osteogenic differentiation.

Conclusion

The *in vitro* and *in vivo* evidence accumulated in the present study revealed for the first time that IGF-1 can promote the osteogenic differentiation and osteogenesis, but decrease the odontogenic differentiation and dentinogenesis capacity of SCAPs, suggesting that IGF-1 treated SCAPs can be used as a potential candidate for bone tissue engineering. Further studies are required to explore the potential signaling pathways regulating the osteogenic differentiation of IGF-1 treated SCAPs.

Materials and methods

Cell isolation and culture

Impacted third molars (n=24) were collected from young patients (17–20 years old) in Oral Surgery Department of Jiangsu Provincial Stomatological Hospital after the informed consent was obtained. The apical papillae were gently detached from the immature roots, minced and digested in a solution containing 3 mg/mL collagenase type I (Sigma, St. Louis, MO) and 4 mg/mL dispase (Sigma, St. Louis, MO) for 30 min at 37 °C. Then, these cells were purified by using rabbit anti-STRO-1 antibody (Santa Cruz, Delaware, CA) and sheep anti-rabbit IgG Dynabeads (Dynal Biotech, Oslo, Norway) according to the standard procedures for magnetic activated cell sorting (MACS). Purified stem cells from apical papilla (SCAPs) were seeded at 1×10^4 cells/mL into 10 cm culture dishes and cultured in alpha minimum essential medium (α-MEM, Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO2. The fresh medium was changed every 2 days. Cells were subcultured at the ratio of 1:3 when they reached 75~85% confluence. SCAPs at 1–3 passages were used for subsequent experiments. The expression of IGF-1R gene and protein in purified SCAPs was respectively detected by RT-PCR and immunocytochemistry methods (IGF-1R antibody was purchased from Bioworld Technology, USA). The treated cells were cultured in α-MEM containing 100 ng/mL IGF-1 (Peprotech, USA). Stem cells were routinely examined under the phase-contrast inverted microscope (Olympus). STRO-1^+ SCAPs were isolated from the same donor in some experiments, and SCAPs at the same passage were used in each experiment.

Cell morphology and ultrastructure

Morphological changes of IGF-1 untreated and treated SCAPs were evaluated at day 7 by a computer-control digit imaging system (Olympus, Japan). Meanwhile, 1×10^7 cells at day 7 were collected by centrifugation to deposit the cell pellets for ultrastructural analysis. The pellets were fixed in a 2.5% glutaraldehyde solution at 4 °C overnight, and then examined by transmission electron microscopy (TEM) (JEM-2000EX, Japan).

MTT assay for cell growth curve

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,5-tetrazolium bromide) assay was performed to investigate the cell metabolic activity. Briefly, SCAPs were seeded into 96-well plates (Costar) at a density of 5×10^3 cells/well, and divided into IGF-1 untreated and treated groups. After 1, 2, 3, 4, 5, and 6 days of culture, 20 μL fresh MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added into the wells and incubated for 4 h at 37 °C. MTT-formazan crystals were then solubilized with dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm using a microtiter plate reader (Titertek, Helsinki, Finland). Five independent experiments were performed in each group, and the graph was plotted according to the average value.

Flow cytometry for cell cycle

After 3-day culture, IGF-1 untreated and treated SCAPs (1×10^6) were respectively collected by exposure to trypsin/EDTA for 5 min and centrifuged at 1100 rpm for 3 min. Cell precipitates were washed twice with 0.01 mol/L PBS and resuspended in 1 mL physiologic saline, fixed in 2 mL cold dehydrated alcohol, and stored at 4 °C overnight. Then, each sample was washed again with PBS, and incubated with propidium iodide (100 mg/mL; Sigma) on ice for at least 30 min. Cell cycle fractions (G_0G_1-, S-, G_2M-phases) were then determined by flow cytometry. Three independent experiments from different donors were performed for each group.

Alkaline phosphatase (ALP) assay and alizarin red staining

SCAPs were grown in the α-MEM containing 100 ng/mL IGF-1. At days 3 and 5, ALP activity of IGF-1 untreated and treated SCAPs was performed as described previously (Fan et al., 2009; Lei et al., 2011) with an ALP kit (Sigma-Aldrich) and normalized on the basis of equivalent protein concentrations. At day 14, alizarin red staining was carried out and images were acquired using a scanner. Then, alizarin red was destained with 10% cetylpyridinium chloride (CPC) in 10 mmol/L sodium phosphate for 30 min at room temperature. The calcium concentrations were determined according to the absorbance at 562 nm using a standard calcium curve prepared in the same solution. The final calcium levels in each group were normalized with the total protein concentrations obtained from the duplicate plates.

Real-time RT-PCR

Total cellular RNA was obtained by adding TRIzol reagent (Invitrogen, Carlsbad, CA) to cell samples. Isolated RNA precipitates were completely dissolved in diethypyrocarbonate (DEPC) treated water (Ambion Inc., Austin, USA) and reversely transcribed using SuperScript® III cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed using

SYBR® Premix Ex Taq™ kit (TaKaRa, Bio, Otsu, Japan) and ABI 7300 real-time PCR system. Primer sets used for the detection of *ALP*, *RUNX*2, *OSX*, *OCN*, *DSPP* and *GAPDH* were listed in Table 1. Real-time RT-PCR reaction conditions were: 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s 60 °C for 31 s. The results were calculated from three independent experiments.

Western blot analysis

IGF-1 untreated and treated SCAPs were respectively collected after 3-day, 7-day and 14-day culture, washed twice with cold PBS and lysed in RIPA lysis buffer (Beyotime, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min. Protein concentrations were determined via Bio-Rad protein assay kit (Pierce, Rockford, IL). 30 μg proteins per lane were loaded on a SDS-PAGE gel for electrophoresis, and then transferred onto PVDF membranes (Millipore Co. Bedford, MA, USA) at 300 mA for 1 hour in a blotting apparatus (Bio-RAD, CA, USA). Membranes were blocked at room temperature for 2 h with blocking solution (5% w/v skim milk, 0.01 mol/L PBS, 0.1% Tween-20), and subsequently incubated overnight at 4 °C with primary polyclonal antibodies against RUNX2 (1:300; BOSTER, China), DSP (1:500; Santa Cruz), OSX (1:300; BOSTER, China), OCN (1:300; BOSTER, China) and monoclonal antibody against β-ACTIN (1:1,000; ABGENT, Flanders Count, CA, USA). Then, the membranes were rinsed with PBST (0.1% Tween-20 in 0.01 mol/L PBS), incubated with appropriate horseradish peroxidase conjugated secondary antibodies at 1:5000 (Santa Cruz) at room temperature for additional 1 h, visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo, Rockford, USA) and exposed to Kodak X-ray films. β-ACTIN served as an internal control in these experiments.

In vivo transplantation

All animal experiments were conducted in accordance with accepted standards of humane animal care and approved by Animal Care Committee of Nanjing Medical University (reference no. 200900128). 1×10^6 IGF-1 treated SCAPs at day 7 were collected by exposure to trypsin/EDTA for 5 minutes, spun down as cell pellets, maintained in α-MEM supplemented with 10% FBS, and incubated for another 3 h in tubes to make them well aggregated. IGF-1 treated cell pellets were seeded onto the AGS (absorbable gelatin sponges; Nanjing Pharmaceuticals) pre-immersed with 100 ng/mL IGF-1 solution and then transplanted into the renal capsules of immunocompromised mice. Untreated cell pellets on AGS materials served as a control. In the present study, AGS was used as a carrier which can facilitate the transfer of cell pellets from tubes to the renal capsules. All pellets (20 in IGF-1 treated group and 20 in untreated group) were retrieved at week 6 post-transplantation and radiographic analyses were performed by using FOCUS X-ray machine (Cone 50540, Tuusula, Finland) with Sopix-2 CCD sensors at 50 Kv and 7 mA for 0.63 s at a focal distance of 20 cm. After visual and radiographic inspections, these implants were fixed in 4% polyoxymethylene, decalcified, and processed for hematoxylin and eosin staining (H&E) and polarized light observation.

Immunohistochemistry

Immunocytochemical analyses of recovered implants were performed using the streptavidin-biotin complex method according to the manufacturer's recommended protocol. Briefly, tissue sections (5 μm) from representative paraffin blocks were deparaffinized in xylene and rehydrated through graded ethanol solutions. Endogenous peroxidases were blocked using 3% hydrogen peroxide. For the antigen retrieval, the sections were processed by the conventional microwave heating in 0.01 M sodium citrate retrieval buffer (0.01 M sodium citrate and 0.01 M citric acid, pH 6.0) for 5 min. Then, the sections were blocked by 10% normal swine serum for 20 min and incubated with primary antibodies (DSP, 1:200; OSX, 1:100; OCN, 1:00) overnight at 4 °C. Incubation with PBS instead of primary antibodies served as the negative controls. Finally, the sections were incubated with secondary

Table 1 Forward and reverse primers for reverse transcription-polymerase chain reaction.

Gene	GenBank No.	Sequences (5′−3′)	Size (bp)
ALP	NM_000478.4	Forward: GACCTCCTCGGAAGACACTC Reverse: TGAAGGGCTTCTTGTCTGTG	137
*RUNX*2	NM_001024630.3	Forward: TCTTAGAACAAATTCTGCCCTTT Reverse: TGCTTTGGTCTTGAAATCACA	136
OSX	NM_001173467.1	Forward: CCTCCTCAGCTCACCTTCTC Reverse: GTTGGGAGCCCAAATAGAAA	148
OCN	NM_001199662.1	Forward: AGCAAAGGTGCAGCCTTTGT Reverse: GCGCCTGGGTCTCTTCACT	63
DSPP	NM_014208.3	Forward: ATATTGAGGGCTGGAATGGGGA Reverse: TTTGTGGCTCCAGCATTGTCA	136
GAPDH	NM_002046.3	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAGATGGTGATGGGATTTC	225
IGF-1R	NM_000875.3	Forward: GCGCCTCCAACTTCGTCT Reverse: CGTACAGCATAATCACCAACCC	423
β-ACTIN	NM_001101.3	Forward: CTCCATCCTGGCCTCGCTGT Reverse: GCTGTCACCTTCACCGTTCC	268

antibodies for 45 min at room temperature. The reaction products were developed by 3, 3′-diaminobenzidine solution with hydrogen peroxide and counterstained with hematoxylin.

Statistics

The quantitative results were expressed as mean ± SD. Independent samples *t*-test and Chi-square test were performed with SPSS-Windows v.12.0 software. *P*-values less than 0.05 were considered to be statistically significant.

Conflict of interest statement

No competing financial interests exist in this manuscript.

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