Canonical Wnt signaling regulates branching morphogenesis of submandibular gland by modulating levels of lama5

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ABSTRACT Branching morphogenesis is a crucial developmental mechanism for the formation of the typical bush-like structure of the submandibular gland (SMG). However, the detailed mechanism underlying this process remains to be fully understood. Here, we have investigated whether cross-talk may exist between the Wnt/beta-catenin signaling pathway and lama5 during the branching process in SMG development. An embryonic mouse SMG organ culture model was established, and the validity of this model was confirmed. The roles and possible interactions of the Wnt/beta-catenin signaling pathway, FGF signaling, and lama5 in the branching process were investigated by morphogenesis assays and gene expression patterns. Here, we show that the E12 or E13 SMG organ culture model can be used as an ideal approach to study the process of branching morphogenesis. Our branching morphogenesis assay revealed that the epithelial branching process can be promoted when the canonical Wnt pathway is inhibited and significantly suppressed when the wnt pathway is over activated. Further experiments indicated that FGF signaling most likely acts upstream as a negative regulator of the canonical Wnt pathway during the branching process, whose effect could be partially reversed by Wnt3a. Finally, we show that Wnt/beta-catenin signaling regulates branching morphogenesis through Lama5. We conclude that the Wnt/beta-catenin signaling pathway acting downstream of FGF signaling can serve as a negative regulatory mechanism in the process of SMG branching morphogenesis through Lama5.

KEY WORDS: salivary gland, organ culture, Wnt/beta-catenin signal transduction pathway, laminin

Introduction

The typical bush-like structure of the submandibular gland (SMG) consists of a network of epithelial branches, which was formed by repeated furcation in the distal ends of the epithelial buds, a process termed branching morphogenesis (Borghese, 1950). This process is repeated in a reiterative manner and results in an exceedingly branched epithelial structure within a minimal packed volume in order to substantially expand the epithelial surface area for secretion or absorption (Gjorevski and Nelson, 2010). A similar process is exhibited as a crucial developmental mechanism for the formation of many other vertebrate organs (Lu and Werb, 2008).

The submandibular gland has been applied as a model to study the regulatory mechanisms of branching morphogenesis for decades. Extensive research has shown that the branching process of salivary gland epithelium is regulated by a complex network of parallel and broadly related signaling pathways involved in the regulation of collagen deposition, epithelium-mesenchyme interaction, and cell proliferation, migration and adhesion (Harunaga et al., 2011, Jaskoll et al., 2001, Sakai et al., 2005), which include EGF (Jaskoll et al., 2004b, Kashimata and Gresik, 1997, Morita and Nogawa, 1999), FGF (Hoffman et al., 2002, Jaskoll et al., 2005, Jaskoll et al., 2004b), Shh (Jaskoll et al., 2004a), etc.

The Wnt/beta-catenin signaling pathway which is also called the canonical Wnt signaling pathway is an ancient and evolutionarily
conserved pathway that regulates numerous processes through several distinct pathways in animal development (Nusse, 2005). It is now well established from a variety of studies, that this pathway is activated by the binding of Wnt ligands to a co-receptor complex consisting of frizzled and LRP5/6 proteins. Activating of the receptor complex inhibits the GSK-3 beta kinase, and finally leads to nucleus accumulation of the transcriptional coactivator beta-catenin which subsequently binds to members of the LEF/TCF transcription factor family. Eventually, this series of events will activate down-stream targets of Wnt signaling. And through this pathway, Wnt signaling regulates several key developmental processes (Bridgewater et al., 2008, Chu et al., 2004).

Rebustini et al. (Rebustini et al., 2007) demonstrated the influence of laminin-α5-integrin signaling pathway and the interaction between laminin-α5 (Lama5) and FGFR on SMG branching morphogenesis. And interactions between canonical Wnt signaling and Lama5 were found important for epithelial patterning during appendages development in zebrafish (Nagendran et al., 2015) and dermal papilla development during early hair morphogenesis in mouse (Gao et al., 2008). However, no similar study has been reported in SMG development.

The purpose of this study was to investigate whether a cross-talking may exist between Wnt/beta-catenin signaling pathway and Lama5 during the branching process in SMG development. Based on results obtained from morphogenesis assay, we found that Wnt/beta-catenin signaling pathway negatively regulates the branching process of SMG through Lama5, which may serve as a balance measure for FGF signaling in regulating branching process. Considering the previously reported conflicting results by other researchers, literatures focusing on the Wnt/beta-catenin signal transduction pathway and SMG branching morphogenesis were also discussed, and we rationally imply that more complicated mechanisms may be desirable for Wnt/beta-catenin signaling regulating the SMG branching morphogenesis.

Results

Establishment and assessment of SMG organ culture model

The mouse SMG can be excised from the embryo at E12 or E13 and it is because that this excised SMG grows and branches so beautifully in ex vivo culture, recapitulating many aspects of in vivo development, that it has been used frequently as an approach to mimic the in vivo process for the study of the general process of branching morphogenesis (Larsen et al., 2006, Molnick and Jaskoll, 2000). In this study we compared not only the end bud numbers as reported by most previous studies but the expression levels of several key regulating genes and the epithelial areas between the ex vivo model and in vivo gland.

To compare epithelial areas or end bud numbers, for ex vivo model the E12.5 SMG was obtained and cultured ex vivo for 48
hours, the epithelial ratios or spooner ratios were determined by dividing the epithelial area or bud number at 48h by that at 2h; For in vivo gland the epithelial ratios or spooner ratios were determined by dividing the epithelial area or bud number of the E14.5 gland by that of the E12.5 gland (Fig. 1A). The bud numbers were counted manually, and the epithelial areas was manually marked and calculated by ImageJ (Fig. 1B). Mean ratios were determined, data were arcsin transformed to insure normality and homoscedasticity, and compared by paired t test for all embryos studied(Jaskoll et al., 2004b, Spooner et al., 1989). No significant differences in epithelial ratios and spooner ratios were found between the ex vivo model and in vivo gland (Fig. 1C and 1D). Meanwhile, no statistically significant differences were found between the E14.5 glands and E12.5+48h explants in the expression levels of regulation gene Fgf10 and Egf, and marker gene Ck5, Ck14 and Aqp 5. The expression levels of Axin2 mRNA indicated that there are no differences in the activity of canonical Wnt signaling between the two groups. Taking together, these results demonstrated that there are no differences in both the phenotypes and gene expression levels between ex vivo models and in vivo glands, and that the cultured SMG would be applied as an ideal model for the research of branching morphogenesis.

**Branching morphogenesis assay reveals a negative regulating role of Wnt/beta-catenin signaling pathway in epithelial branching and that Wnt/beta-catenin signaling pathway is negatively regulated by FGF signaling**

A regulation role of canonical Wnt signaling in the development of SMG epithelium has been reported in previous studies but with confusing results(Häärä et al., 2011, Hai et al., 2010, Matsumoto et al., 2016, Pateli et al., 2011). We reexplored this question using the branching morphogenesis assay established as above.

We first tried to constitutively activate beta-catenin signaling by inhibiting GSK3-beta. LiCl was added in the culture media and four concentrations (5mM, 10mM, 20mM and 25mM, respectively) of LiCl were tested. While concentrations below 10mM had no apparent effect on the branching process and those above 25mM exhibited toxic effects, 20mM of LiCl kept the rudiments growing and had a negative effect on the branching process which significantly inhibited epithelial branching. This effect was fully recapitulated by addition of 100ng/ml Wnt3a instead of LiCl (Fig. 2A second and third columns). To further examine the hypothesis, we then used recombinant Dkk1 protein to down-regulate Wnt/beta-catenin signaling. Dkk1 specifically inhibits Wnt/beta-catenin signaling, by forming a complex with the Wnt co-receptor Lrp5/6(Bafico et al., 2001, Semënov et al., 2001) and Kremen transmembrane proteins(Mao et al., 2002) outside the cells, which promotes the internalization of LRP, making it becoming unavailable for Wnt reception. Three concentrations of Dkk-1 (150ng/ml, 200ng/ml and 250ng/ml, respectively) were tested. Enhanced branching morphogenesis was monitored at concentrations > 200ng/ml (Fig. 2A fourth column). These results indicated that the branching morphogenesis of the embryonic SMG was negatively regulated by the canonical Wnt signaling pathway.

The FGF families are found to positively regulate the branching morphogenesis of mouse salivary epithelium (Jaskoll et al., 2005, Jaskoll et al., 2004b, Morita and Nagawa, 1999). While it is reported that Wnt/beta-catenin signaling acts upstream of FGF signaling to regulate proximal-distal patterning in the lung (Shu et al., 2005), we conducted experiments to further our understanding of the interactions between FGF and Wnt/beta-catenin signaling during the regulation of the ex vivo branching process of SMG.

As reported previously, we found FGF10 induced a significant increase in the number of branching epithelial end bud in E12.5 + 2 SMGs (Fig. 3A second column). This effect could be reinforced by DKK1 (Fig. 3A third column) and reduced by Wnt3a (Fig. 3A fourth columns). These effects are statistically significant (Fig. 3B). These results suggested an interaction between FGF and Wnt/beta-catenin signaling during the branching process of SMG, which could be demonstrated by the qPCR results. As shown in Fig. 3C, FGF signaling significantly decreased the mRNA level of Axin2 after 48h cultivation, while treatment with SU5402 resulted in an almost 170% increase in Axin2 mRNA level. These data suggested that the Wnt/beta-catenin signaling pathway is negatively regulated by
FGF signaling in branching process.

However, we found in further study that the addition of 200ng/ml DKK1 induced an even greater decrease in the mRNA level of Axin2 than FGF10 did (Fig. 3C), which may imply that the Wnt/beta-catenin signaling was only partially suppressed by FGF signaling. Wnt/beta-catenin signaling regulates the branching morphogenesis through LAMA5

The branching morphogenesis assay was further applied to investigated whether a cross-talking between canonical Wnt signaling and LAMA5 exist during the branching process in SMG development as reported in epithelial patterning during appendages development in zebrafish(Nagendran et al., 2015) and dermal papilla development during early hair morphogenesis in mouse(Gao et al., 2008). As shown by branching morphogenesis assay, monoclonal antibody to LAMA5 (Antibody 4G6, at 20ug/ml or 40ug/ml) significantly blocked the inhibiting effect of canonical Wnt signaling in a dose dependent manner (Fig. 4 A,B), which implied that LAMA5 maybe a downstream target for Wnt signaling during SMG epithelial branching process. As LAMA5 is a subunit of laminin-511, -521 and -523, we investigated whether the expression of all these subunits were affected by canonical Wnt signaling. We found that while Dkk1 treatment only decreased the expression of LAMA5 to 89% of the control group level, Wnt3a increased the expression of LAMA5 to more than 1200% by activating Wnt signaling (Fig. 4C). However, the expression levels of Lamb1, Lamb2, Lamc1 and Lamc3 were not affected by the activities of wnt signaling. Taken together, these results suggested that LAMA5 acts downstream of canonical wnt signaling to regulate the branching morphogenesis of SMG.

Discussion

It has been recognized that, being one of the fundamental mechanisms that direct cell proliferation, cell polarity, and cell fate determination, signaling by the Wnt family of secreted lipoproteins has essential roles in multiple tissue morphogenesis during, but not limited to, mammalian animal development(Chu et al., 2004, Dean et al., 2005, Logan and Nusse, 2004, Maretto et al., 2003). We also note that though several studies have discovered the functions of wnt signaling pathway in embryonic SMG development, conflicting results exist in these studies and no research has focused on whether a cross-talking between canonical Wnt signaling and Lama5 during SMG development as in other tissues. Our aim here was to investigate whether a cross-talking may exist between Wnt/beta-catenin signaling pathway and Lama5 during the branching process in SMG development via ex vivo SMG organ culture system. First, to assess the validity of the culture model, we compared not only the bud numbers as in previous studies but also the expression of key regulating genes and epithelium areas of the explants with that of the in vivo gland, and no significant differences were found. After that, branching morphogenesis asssay was carried out, which revealed that the epithelium could not undergo normal branching process when canonical Wnt pathway was blocked by inhibitors or stimulated by activator. Then we found that wnt signaling is negatively regulated by FGF signaling and act upstream of lama5 based on the findings of rescue and neutralization experiments and confirmed by qPCR.

Branching morphogenesis is a key mechanism during develop-
ment to form a bush-like structure required by many organs for normal functions, such as secretion or absorption of substances (Gjorevski and Nelson, 2010), of which salivary gland is a classical research model. The development of mouse submandibular salivary gland (SMG) undergoes branching morphogenesis to turn a single epithelial bud into a functionally efficient, complex, but well-ordered tissue architecture which is comprised of an array of epithelial branches with substantially expanded epithelial surface area for secretion or absorption (Borghese, 1950): During the initial development stage, the mouse SMG first appears as a thickening of the epithelium projecting inwards from the mouth epithelium around the 11.5th day of embryonic development (E11.5), known as the pre-bud stage. The thickening protrudes into the underlying mesenchyme and as the epithelium invaginates it forms a bud linked to the oral surface by a duct from E12.5. This duct will go on to form the main duct of the salivary gland and deep 3D clefts will form in the surface of the primary bud to subdivide it into separate buds, which is followed by proliferative bud outgrowth. Alternating cleft formation and bud outgrowth will continue throughout E13-E15 (Hsu and Yamada, 2010) in a reiterative manner to give rise to a highly branched epithelial structure that maximizes epithelial surface area within a minimal packed volume (Gjorevski and Nelson, 2010). A similar process is conserved as a general developmental mechanism essential for the formation of many other vertebrate organs (Lu and Werb, 2008).

Morphogenesis of this bush-like highly branched epithelial structure in SMG is regulated by the functional integration of parallel and broadly related signaling pathways regulating cell proliferation, migration and adhesion (Jaskoll et al., 2001, Sakai et al., 2005), which include EGF (Jaskoll et al., 2004b, Kashimata and Gresik, 1997, Morita and Nogawa, 1999), FGF (Hoffman et al., 2002, Jaskoll et al., 2005, Jaskoll et al., 2004b), Shh (Jaskoll et al., 2004a), etc.

Since many components of the Wnt signaling pathway are found present in developing SMG as reported by Hoffman et al. (Hoffman et al., 2002), the activities of Wnt signaling during SMG development was investigated by several researchers. Using BATGAL Wnt reporter transgenic mice, Hai et al. (Hai et al., 2010) found that Wnt/beta-catenin signaling was involved in the postnatal development and regeneration process of SMG through regulating the activity of salivary gland stem/progenitor cells, and Wnt signaling was localized only in a few cells at the basal layer of intercalated ducts in the newborn SMGs. However, they also reported that no Wnt activation was observed in the parenchyma of salivary glands during the entire embryonic development, which is inconsistent with the findings reported by Häärä et al., (Häärä et al., 2011), who found that Wnt activity was active though entirely confined to the mesenchyme surrounding the branching epithelium at the early branching stage, then Wnt signaling could be detected in the ductal epithelium in addition to mesenchyme when the main salivary duct starts to differentiate, and from E16
onwards, the Wnt activity was exclusively epithelial and localized to the developing ducts. The latter results commonly accepted now are further supported by Patel et al., (Patel et al., 2011) who found that Wnt/beta-catenin signaling is active first in the mesenchyme and later in the ductal epithelium at the time of lumen formation. However, one thing should be noted is that a different Wnt reporter line, Axin2LacZ rather than BATGAL mice, was used by Häärä et al., and Patel et al., According to a systematic comparison of the expression patterns of three classical and commonly used canonical Wnt reporter lines, TOPGAL, BATGAL, and Axin2LacZ mice, contrasting expression patterns of Wnt signaling reporters was found (Al Alam et al., 2011). The same situation exists not only in developing lungs but in developing mammary glands (Jarde and Dale, 2012), and may have been widely ignored by many researchers as reviewed by Barolo (Barolo, 2006). So it would be rational to speculate that maybe an inappropriate reporter line was applied by Hai et al. Now that it is realized that the canonical Wnt pathway activity is required mainly in the mesenchyme when branching process dominates and is strictly limited to the duct epithelium and is much less active when the ducts begin to develop and the branching process to diminish, a regulatory role of Wnt signaling can be expected. In the same study performed by Häärä et al., mentioned above, it is reported that when the mesenchymal Wnt activity was inhibited by Wnt signaling inhibitors XAV939 or CKI-7 in E13 wild-type salivary gland, the epithelial branching would be significantly reduced simultaneously. They furtherly confirmed these results by mesenchymal conditional deletion of beta-catenin in vivo in the Dermo-Cre+; beta-catenin+/ mice (Häärä et al., 2011). However, opposite results are observed by our study and by Patel et al., (Patel et al., 2011): The epithelial branching process would be promoted when the canonical Wnt pathway was inhibited, and be significantly suppressed when Wnt pathway is over activated. This suppressing effect of canonical Wnt signaling during SMG branching process is consistent with those found in the lung and lacrimal gland (Dean et al., 2005). We first supposed that this suppressing effect of canonical Wnt pathway was a balance mechanism to the well-known FGF and EGF pathways in regulating the SMG branching process. The FGF families are found positively regulating the branching morphogenesis of SMG epithelium (Jaskoll et al., 2005, Jaskoll et al., 2004b). Further results obtained by qRT-PCR experiment in our study indicated that FGF signaling acts upstream of the Wnt/beta-catenin signaling pathway, and most likely acts as a negative regulator of the canonical Wnt pathway during the branching process. Moreover, we found that this effect of FGF signaling could be partially, but not all, reversed by Wnt3a. Considering that the interaction between FGF and canonical Wnt signaling is context dependent(Patel et al., 2011, Shu et al., 2005), further researches are needed to elucidate the details of this cross-talking.

Though conflicting results are found considering the expression manner and regulatory role of canonical Wnt signaling during SMG epithelial branching, several possible mechanisms of canonical Wnt signaling regulating SMG branching process are reported. Eda/Edar/NF-kB pathway is essential for embryonic SMG development (Jaskoll et al., 2003), and Häärä et al. (Häärä et al., 2011) found that canonical Wnt signaling can promote the branching process through Eda. However, Matsumoto et al. (Matsumoto et al., 2016) reported that canonical Wnt signaling restrained the branching process by suppressing Kit expression through up-regulation of Myb transcription factor. We think that a mesenchyme-epithelium cross-talking can be presumed for the mesenchymal canonical wnt signaling to regulate epithelial branching process. As interactions between canonical Wnt signaling and laminin α5 were found essential for epithelial patterning during appendages development in zebrafish (Nagendran et al., 2015) and dermal papilla development during early hair morphogenesis in mouse (Gao et al., 2008), we demonstrated in the present study that Lama5 might be the mediator of canonical Wnt signaling in regulating the branching process of SMG epithelium.

In conclusion, though several conflicting opinions have been reported as discussed above and more complicated mechanisms would be expected for the Wnt/beta-catenin signal transduction pathway to regulate submandibular gland branching morphogenesis, our present work demonstrated that Wnt/beta-catenin signaling pathway acting downstream of FGF signaling may serve as a negative regulatory mechanism in the process of SMG branching morphogenesis through Lama5.

Materials and Methods

**Mouse strain and tissue collection**

Adult mice of the ICR strain was used and raised at 25°C in a constant photoperiod (14L:10D). The guidelines for the Care and Use of Animals in Research were followed, and this research was approved by the Research Ethics Committee of West China Hospital of Stomatology of SiChuan University and Stomatological Hospital of Chongqing Medical University. Female mice were mated with fertile males of the same strain. The morning of finding a vaginal plug was designated as day 0 (EO). Pregnant females were anesthetized at 1130-1230h on day E12.5 of gestation with ether and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler (Theiler, 1989). Mandible sections were isolated, and submandibular glands with attached sublingual glands (hereafter referred to as SMG) were removed from these sections under a dissecting microscope.

**Culture system**

Rudiments of E12.5 SMG were cultured on membranes at the air/medium interface at 37°C in a humidified 5% CO2/95% air atmosphere for up to 48 hours. Six glands were placed on a Nuclepore Track-Etch Membrane (pore size 0.1 μm; diameter 13mm; Whatman) floating on 200μl DMEM/F12 (1:1) supplemented with vitamin C (150μg/ml), transferrin (50μg/ml), penicillin (100U/ml) and streptomycin (100μg/ml). The medium was replaced every 24 hours. In supplementation studies, Wnt3a (150ng/ml; #1324-WN/CF; R&D Systems) or LiCl and Dickkopf-1 (Dkk-1) (#5897-DK-010; R&D Systems) were used as the exogenous activator and specific inhibitor for Wnt/beta-catenin signaling pathway, respectively. Each was resuspended in PBS. For each experiment, at least six paired E12.5 SMGs were used for each treatment. Control glands were cultured with an equal volume of the vehicle.

As toxic effects were observed with LiCl, toxicity was recognized as an apparent darkening of the epithelial cells when imaged by bright field mi-

### Table 1

**LIST OF PRIMERS USED FOR qPCR ANALYSIS**

| Primer name | Forward | Reverse |
|-------------|---------|---------|
| Axin2 Mus   | 5′-CTCCCCACCTTTAGAAGAAGA-3′ | 3′-ACTGGTCGCTACCTTTGAATAA-5′ |
| Fgf10 Mus   | 5′-TGCGGACGCTACATCTCCTCCTTGTATC-3′ | 3′-GGATCTACAGGGACCTCATTAGC-5′ |
| Egf Mus     | 5′-TGACGAGATGAGGACGGTCTGCTG-3′ | 3′-AACGGTACACAGTAACAGGC-5′ |
| Cdk5 Mus    | 5′-TGACGAGATGAGGACGGTCTGCTG-3′ | 3′-GTCACGCTCGGATCAGTACC-5′ |
| Cx14 Mus    | 5′-GACCGAGGAGGAGGGTGGCG-3′ | 3′-GTACGCTCGGATCAGTACC-5′ |
| Aqap5 Mus   | 5′-GGCCATCTTGTTGGGATGATAC-3′ | 3′-CTTCCTGAGTACGTCGCTGCTG-5′ |
| Beta-actin Mus | 5′-CAGTGTCAAGTGCAGCAGTCTC-3′ | 3′-GTGGTCAAGGCGTACTCCACT-5′ |

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As toxic effects were observed with LiCl, toxicity was recognized as an apparent darkening of the epithelial cells when imaged by bright field mi-
croscopy and was defined operationally as a state at which the SMG would not resume proliferation after 24 h if the inhibitor or activator contained media were replaced with fresh media (Jaskoll et al., 2004b). Cultured rudiments were photographed at 2, 24, and 48 hours. The sparser ratios (end bud number/initial bud number) and epithelial ratios (epithelial areas at end time/epithelial areas at start time) were determined using ImageJ software for each group. Because a notable difference in SMG epithelial branch number is seen among littermates, we compared the number of terminal buds and epithelial areas in the left and right glands (treated and control) from each embryo for all embryos studied.

**Rescue and neutralization experiments**

Paired E12.5 SMG primordia were cultured in 25 μM SU5402 (#572630-500UGCN; MerckMillipore), a FGFR1 tyrosine kinase inhibitor that does not inhibit EGFR, PDGF or the insulin receptor, for at least 48 h, or in 200ng/ml recombinant mouse FGF10 (#8224-FG-025/CF; R&D Systems) for an initial period of 3 h and then each pair was cultured in FGF10, or FGF10 + 150 ng/ml Wnt3a, or FGF10 + 200 ng/ml DKK1 for at least 48 h. These Wnt3a and DKK1 concentrations were shown in previous supplementation studies to induce a significant effect on branching morphogenesis in E12.5 + 3 SMGs compared to controls.

For neutralization experiments, the monoclonal antibody to laminin α5 (Song et al., 2013, Sorokin et al., 1997) (Antibody 4G6, at 20 μg/ml or 40 μg/ml) or IgG were added into the culture mediums prior to adding Wnt3a.

For each experiment, at least six paired E12.5 SMGs were used for each treatment. Finally, the explants were collected, and the numbers of terminal buds were analyzed as described above.

**Analysis of RNA by qPCR**

Quantitative reverse transcription-PCR was carried out by real-time PCR with the SYBR Green reporter. Total RNA was isolated from freshly dissected or cultured SMGs using Rneasy Mini Kit (Qiagen) and was subsequently reverse transcribed to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). The quantitative real-time RT-PCR was performed using the SYBR premix Ex Tag II kit (Takara) according to the manufacturer’s instruction. The reaction mix was subjected to quantitative real-time PCR to detect levels of the corresponding target genes. The primer sequences used were shown in Table 1. The relative expression levels were quantified and analyzed using Bio-Rad Cycler IQ software. The comparative threshold cycle (CT) method was used to calculate amplification fold. Beta-actin gene was used as a reference control gene to normalize the expression value of each gene. Triplicate replicates were performed for each group of SMGs, and average expression value was computed for subsequent analysis. The relative expression level of the genes was calculated using the 2^ΔΔCt method (Livak and Schmittgen, 2001).

**Statistical analysis**

All quantitative assays were performed in triplicate and/or repeated three times. Analysis results were expressed as mean ± SD. Statistical significances were determined by student’s t test or the paired student’s t test. A value of p < 0.05 was considered statistically significant.

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**Conflict of interest**

The authors declare no competing conflicts of interests.

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