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Decreased BMP2 signal in GIT1 knockout mice slows bone healing

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Abstract

Endochondral ossification, an important stage of fracture healing, is regulated by a variety of signaling pathways. Transforming growth factor b (TGFb) superfamily plays important roles and comprises TGFbs, bone morphogenetic proteins (BMPs), and growth differentiation factors. TGFbs primarily regulate cartilage formation and endochondral ossification. BMP2 shows diverse efficacy, from the formation of skeleton and extracellular organs to the osteogenesis and remodeling of bone. G-protein-coupled receptor kinase 2-interacting protein-1 (GIT1), a shuttle protein in osteoblasts, facilitates fracture healing by promoting bone formation and increasing the secretion of vascular endothelial growth factor. Our study examined whether GIT1 regulates

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fracture healing through the BMP2 signaling pathway and/or through the TGFb signaling pathway. GIT1 knockout (KO) mice exhibited delayed fracture healing, chondrocyte accumulation in the fracture area, and reduced staining intensity of phosphorylated Smad1/5/8 (pSmad1/5/8) and Runx2. Endochondral mineralization diminished while the staining intensity of phosphorylated Smad2/3 (pSmad2/3) showed no significant change. Bone marrow mesenchymal stem cells extracted from GIT1 KO mice showed a decline of pSmad1/5/8 levels and of pSmad1/5/8 translocated into the cell nucleus after BMP2 stimulus. We detected no significant change in the pSmad2/3 level after TGFb1 stimulus. Data obtained from reporter gene analysis of C3H10T1/2 cells cultured in vitro confirmed these findings. GIT1-siRNA inhibited transcription in the cell nucleus via pSmad1/5/8 after BMP2 stimulus but had no significant effect on transcription via pSmad2/3 after TGFb1 stimulus. Our results indicate that GIT1 regulates Smad1/5/8 phosphorylation and mediates BMP2 regulation of Runx2 expression, thus affecting endochondral ossification at the fracture site.

Keywords

GIT1; TGFb; BMP2; Fracture healing

Introduction

Fracture healing is a complex process involving the growth and differentiation of bone marrow mesenchymal stem cells (BMMSCs) and the metabolism of extracellular matrices [1, 2]. After a bone fracture, the repair process initiates from hematoma formation and inflammatory reaction at the fracture site. In inflammatory phase, inflammatory cytokines induced the proliferation and differentiation of BMMSCs into chondrocytes and osteoblasts [3, 4]. In the following reparative phase, chondrocytes synthesize extracellular matrix to form cartilaginous callus [1]. In the modeling phase, new blood vessels grow into the cartilaginous callus and vascular osteogenic tissues gradually substitute avascular cartilaginous callus, leading to the formation of true bone tissues; eventually, osteoclasts remodel the fracture callus following the requirements of mechanics [5, 6]. Previous studies have proven that cartilaginous callus formation plays an important role in the development of bone tissues [7].

Chondrocyte differentiation and maturation are regulated by various cytokines, including transforming growth factor b (TGFb) superfamily members, which comprises TGFbs, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs). TGFb binds to two receptors with serine/threonine kinase activity, i.e., TGFbR1 and TGFbR2, further activating activin receptorlike kinases-5 (ALK5) and inducing Smad2/3 phosphorylation. The phosphorylated form of Smad2/3 (pSmad2/3) binds to Smad4 and then translocates into the cell nucleus, where it acts on the target genes, maintains the morphology of chondrocytes, and inhibits endochondral ossification [7].

More than 20 subtypes of BMPs are presently identified in the TGFb superfamily. Of these, BMP2 has the highest activity and is the only factor that can induce bone formation alone. Similar to TGFb, BMP2 also binds to two receptors with serine/threonine kinase activity,

i.e., BMPR1 and BMPR2, further inducing Smad1/5/8 phosphorylation. Then, the phosphorylated form of Smad1/5/8 (pSmad1/5/8) binds to Smad4, further regulating the target genes in the cell nucleus and inducing the differentiation of early cartilage-like tissues to osteogenic tissues during fracture healing [8, 9].

G-protein-coupled receptor (GPCR) kinase 2-interacting protein-1 (GIT1) is initially proven to bind to GRK2 and participate in the endocytosis of adrenergic receptors. GIT1 consists of five functional domains, including a zinc fingerlike domain related to ARF-GAP activity, three repetitive ankyrin domains, a Spa2 homology domain, a synaptic localization domain, and a carboxy-terminal paxillinbinding subdomain. Through the above domains, GIT1 binds to a variety of proteins such as ARF6, MEK1/2, phospholipase C-c(PLC-c), p-21-activated kinase-interacting exchange factor, and paxillin [10, 11]. As a result, GIT1 possesses diverse physiological functions. For example, GIT1/MEK1 binding causes sustained activation of extracellular-regulated protein kinase1/2 (ERK1/2) and affects cell function [12, 13], whereas GIT1/FAK binding regulates the migration of epithelial cells and osteoblasts [14]. Our study has shown that GIT1 plays an important role in regulating the function of osteoclasts [15]. More recently, our research has found that GIT1 is crucial to fracture healing and that many key amino acids affect fracture healing after GIT1 deletion [13]. However, the detailed mechanism by which GIT1 affects fracture healing is unclear.

Materials and methods

Establishment of a femoral fracture model for GIT1 KO mice

GIT1 KO mice (C57BL/6 background) were constructed in our laboratory (Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester, Rochester, NY 14642, USA) as previously described [16]. The operation and experiment on animals were approved by the Animal Committee at University of Rochester, Rochester, NY, USA. The femoral fracture model of GIT1 KO mice was established according to the method of Xie[17]. The mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. The skin tissues around the knee joint were disinfected, and a 25G injection needle was inserted into the femoral marrow cavity through the patellar tendon. Femoral fracture was made using a three-point bending method with Einhorn equipment and confirmed by X-ray radiography. Thereafter, each mouse was given subcutaneously 0.5 mg/kg buprenorphine (Abbott Labs, North Chicago, IL, USA) for 3 days to stop pain. X-ray radiographs were taken of anesthetized mice using a Faxitron system (Faxitron X-ray, Wheeling, IL, USA) on days 7, 14, 21, and 28 after fracture [17].

Cell culture experiment

C3H10T1/2 cells were cultured in a complete DMEM (10 % fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/L streptomycin) and incubated at 37 °C under 5 % (v/v) CO₂. To collect mouse BMMSCs, 2-week-old GIT1 WT and KO mice were immersed in 75 % (v/v) ethanol for 3–5 min. Cells were obtained from femoral and tibial marrow cavities, prepared as single-cell suspensions, and then inoculated into 25-cm² culture flasks (5 mL/flask). The cultures were incubated at 37° under 5 % (v/v) CO₂ for 72 h. Then, the medium was changed by half and substituted with DMEM containing 10 %

fetal bovine serum, further changed by half for every 48 h. When cell fusion reached 80 % and large colonies were formed, the culture was digested with 2.5 g/L trypsin, centrifuged, and passaged at a split ratio of 1:2 by resuspension and inoculation in fresh medium. Cells were treated with serum-free DMEM for 6-h starvation and then stimulated with 5 ng/mL TGFb1 or 10 ng/mL BMP2 (Peprotech, Rocky Hill, NJ, USA) for 0 and 5 min.

Real-time polymerase chain reaction (PCR) assay

Total RNA was extracted from each group of cells using Trizol reagent. The RNA templates were reverse-transcribed into cDNA using a commercial kit (Takara, Japan). Primer design and reaction conditions are listed in Table 1.

Immunohistochemical (IHC) staining

The specimens of bone tissue and callus were decalcified in 5 % EDTA-2Na, dehydrated in a graded ethanol series and sliced into 5- μ m sections. After hematoxylin/eosin (HE) staining, specific primary antibodies (anti-mouse Smad2/3, pSmad2/3, and Smad1/5/8 monoclonal antibodies, Cell Signaling Technology, Danvers, MA, USA; pSmad1/5/8, GIT1, and Runx2 monoclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied. The sections were examined under a light microscope, and four fields of view were randomly chosen for each section. Positive expression rates and target gray values were estimated at the same light intensity.

Western blot assay

Protein was extracted from cells and subjected to SDS-PAGE electrophoresis, then transferred to a PVDF membrane. The PVDF membrane was incubated with specific primary antibody at 4 ° overnight, followed by HRP-conjugated secondary antibody at 37 ° for 2 h. Protein bands were developed with a super ECL detection reagent and then detected using Quantity One (Bio-Rad, USA).

Functional identification of osteoblasts

For alkaline phosphatase (ALP) staining, BMMSCs were inoculated into 6-well plates containing a coverslip per well. When cells were spread evenly, the coverslips were taken out and fixed in 80 % (v/v) ethanol for 20 min. Then, the coverslips were incubated with ALP staining working solution (Pierce, USA) at room temperature for 4 h. After three rinses with distilled water, the coverslips were mounted and examined under a light microscope. For von Kossa staining, BMMSCs were inoculated into 6-well plates with one coverslip per well. When an opaque nodular area associated with cell aggregation was observed microscopically, the coverslips were taken out and fixed in 10 % paraformaldehyde for 10 min, followed by 0.1 % alizarin red staining at 37 °C for 30 min. Then, the coverslips were washed with distilled water, dehydrated, infiltrated, mounted, and examined under the light Microscope.

Luciferase reporter gene assay

C3H10T1/2 cells were inoculated into 6-well plates and incubated at 37 ° under 5 % CO₂. When 75–85 % fusion was reached, 1 μ g of pGL3 recombinant plasmid (Topflash, 4*SBE or

12*SBE) and 1 μ g of GIT1-siRNA (or control-siRNA) were co-transfected into cells using Lipofectinamine 2000. The cells were harvested after 24-h incubation, and the transcriptional activity of target genes was detected using a Luciferase Assay System (Promega Corporation). Luciferase reporter genes including pGLBasic-Topflash, pGL-Basic [4*SBE (smad binding element)], and pGL-Basic [12*SBE (smad binding element)] were obtained from our laboratory.

Immunofluorescence assay

Cells with 40–50 % fusion were washed with 0.2 % PBST, fixed in 4 % paraformaldehyde for 20 min, diluted with 0.1 % Triton X-100 in 0.1 % citrate, cooled on ice for 2 min, and then blocked with PBST-diluted rabbit serum for 1 h. The cells were incubated with anti-goat antiSmad1/5/8 antibody at 4° overnight and then incubated with rabbit anti-goat fluorescent secondary antibody for 1 h. The cells were washed with PBST several times followed by DAPI nuclear staining. The immunofluorescent density of cells was examined under the light microscope (Zeiss, Oberkochen, Germany) [15].

Statistical analysis

Data analysis was performed in SPSS13.0 software. The experimental data are presented as arithmetic mean values ($\pm$ standard deviation). All experiments were repeated three times independently. Difference between groups was examined by t test or single-factor analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Intracellular signal transduction of TGF β 1 was unaffected in GIT1 KO mice

As shown in Fig. 1a, TGF β 1 stimulus promoted Smad2/3 phosphorylation, but there was no statistically significant difference in the level of p-Smad2/3 between GIT1 WT and KO mice. Similarly, the IHC staining of p-Smad2/3 showed that there was no statistically significant difference in the fracture area between GIT1 WT and KO mice ($P>0.05$) (Fig. 1b, c). C3H10T1/2 cells were inoculated into 6-well plates and then co-transfected with 1 μ g of pGL3 recombinant plasmid (4*SBE) and 1 μ g of GIT1-siRNA (or control-siRNA) using Lipofectinamine 2000. After 24-h incubation, the cells were harvested for Smad activity assay using a Promega Luciferase Assay System. The results of luciferase reporter gene analysis showed that GIT1 protein levels had no significant effect on the amount of TGF β 1 transcription in the nuclei of BMMSCs through pSmad2/3 ($P>0.05$) (Fig. 1d).

Intracellular signal transduction of BMP2 was affected in GIT1 KO mice

The results of Western blot assay showed that BMP2 stimulus promoted Smad1/5/8 phosphorylation, and that the pSmad1/5/8 level was significantly higher in GIT1 WT mice than in GIT1 KO mice ($P<0.05$) (Fig. 2a). Similarly, the IHC staining of pSmad1/5/8 in the fracture area was significantly greater in GIT1 WT mice than in GIT1 KO mice ($P<0.05$) (Fig. 2b, c). The results of luciferase reporter gene assay showed that the level of intracellular GIT1 protein significantly affected the level of BMP2 transcription through pSmad1/5/8 ($P<0.05$) but not the levels of Wnt and TGF β 1 transcription in the nuclei of BMMSCs ($P>0.05$) (Fig. 2d). To further clarify the effect of GIT1 on the BMP2 signaling

pathway, we detected the capability of pSmad1/5/8 to be translocated into the nuclei of BMMSCs from GIT1 WT and KO mice after BMP2 stimulus for 0 and 5 min. For GIT1 WT mice, substantial pSmad1/5/8 was translocated into the nuclei of BMMSCs after BMP2 stimulus (arrow), whereas for GIT1 KO mice, the level of pSmad1/5/8 translocated into the nuclei of BMMSCs was significantly reduced after BMP2 stimulus (arrow, Fig. 2e, f).

Endochondral ossification at the fracture site was affected in GIT1 KO mice

Endochondral ossification significantly occurred in GIT1 WT mice, and the cartilage expansion area declined, which divided into multiple sub-areas (Fig. 3A-a). However, endochondral ossification was not significant in GIT1 KO mice and only occurred by surrounding the sheet-like cartilage expansion area; abnormal accumulation of chondrocytes was observed at the fracture site; the size of the cartilage area in the fractured bone was much greater than that in GIT1 WT mice (Fig. 3A-b). To investigate the effect of GIT1 on endochondral ossification, Runx2 staining was conducted at the fracture site. Runx2 expression at the fracture site was significantly higher in GIT1 WT mice (Fig. 3B-a) than in GIT1 KO mice (Fig. 3B-b) ($P < 0.05$) (Fig. 3C). We further tested the expression and activity of ALP, an important indicator for ossification in BMMSCs. The results of RT-PCR assay (Fig. 3D) and ALP staining showed that the level of ALP expression was higher in GIT1 WT mice (Fig. 3E-a) than in GIT1 KO mice (Fig. 3E-b). The osteogenic capability of BMMSCs from GIT1 WT and KO mice was determined by testing their mineralization capability, and the results showed that GIT1 WT mice had a higher capability than GIT1 KO mice (Fig. 3F).

BMP2 target gene detection in BMMSCs from GIT1 KO mice

To further determine whether GIT1 specifically affects the BMP2/Smad1/5/8 signaling pathway, we examined the expression of downstream genes of BMP2 in BMMSCs from GIT1 WT and KO mice [18]. The results showed that BMMSCs from GIT1 WT mice had higher ID1, Smad6, OC, DLX2, and JunB expression levels than GIT1 KO mice. Gene expression levels declined by more than 50 % in BMMSCs from GIT1 KO mice compared to those from GIT1 WT mice (Fig. 4).

Discussion

Chondrocyte differentiation and maturation are critical processes involved in the normal development of bone tissues, and the TGF β superfamily plays important roles in chondrocyte growth and maturation. The TGF β superfamily comprises more than 30 members, including TGF β s, BMPs, GDFs, Nodal, activin, and inhibin. These members can be classified into two sub-families, i.e., TGF β /activin/Nodal and BMP/GDF/muellerian inhibiting substance, which regulate cell proliferation, lineage differentiation, migration, adhesion, and apoptosis. These signaling molecules act on type-I and type-II transmembrane serine/threonine kinase receptors to activate different signal pathways: the classic Smads pathway and the nonclassical mitogen-activated protein kinase (MAPK) pathway. After binding to the ligand, the receptor forms heterodimers and causes phosphorylation of type-I receptor, further activating receptor-dependent Smads (R-Smads, i.e. Smad1,2/3,5,8) via phosphorylation. R-Smads recruits and binds to common Smads (Co-Smads, i.e. Smad4) to

form heterodimers, then enters the cell nucleus to regulate target gene transcription. Smad2 and Smad3 specifically transduce TGF β signaling and inhibit chondrocyte differentiation and maturation. For instance, addition of TGF β into both cultured chondrocytes and metatarsals inhibits hypertrophic differentiation of chondrocytes [19, 20]. In newborn Smad3 KO mice, accelerated hypertrophic differentiation of chondrocytes demonstrates that TGF β inhibits chondrocyte hypertrophy and mineralization [21]. BMP signaling tends to induce chondrocyte differentiation and maturation. Addition of BMP6 and BMP7 into cultured chondrocytes promotes the expression of type-X collagen (Col X), a molecular marker of maturation, while addition of BMP2 promotes cultured chondrocytes to express Col X, Ihh, parathyroid hormone/parathyroid hormone-related peptide receptor, and ALP. Expression of activated BMP receptor CA-Alk1.2/3.6 in chondrocytes contributes to chondrocyte maturation. As Smad1/5/8 specifically transduces BMP signal, overexpression of Smad1/5/8 in chondrocytes promotes chondrocyte maturation. On the contrary, Smad6 inhibits BMP signaling and thus prevents chondrocyte maturation. Maturation of chondrocytes is a key step in ossification. BMP2 promotes Smad1/5/8 phosphorylation and mediates Runx2 expression. Runx2 is the core cytokine promoting ossification [22, 23]. GIT1 is a shuttle protein widely distributed in cells, which binds to different proteins through its own domains and further regulates a variety of cell functions. Our previous work has shown that a disorder of vascular endothelial growth factor (VEGF) secretion causes the phenotype of pulmonary vascular dysplasia in GIT1 KO mice. More recently, our work has shown that through binding to FAK and ERK1/2, GIT1 regulates PDGF-induced angiogenesis in the fracture area, consequently promoting fracture healing [12, 13]. In the case of sufficient blood supply, BMP2 promotes the development of early cartilage-like tissues toward osteogenic tissues [4, 5]. Thus, GIT1 possibly increases angiogenesis at the fracture site and enhances BMP2 signal transduction, further promoting chondrocyte differentiation and maturation and contributing to fracture healing. In our previous study, we found that the GIT1/MEK1/ERK1/2 complex increases ERK1/2 activity after Ang II and EGF stimulation. We speculated that in addition to the BMP/Smad1/5/8 pathway, GIT1 might affect cartilage development and endochondral ossification through the parallel MAPK pathway. Alternatively, protein kinase c delta (PKC δ) might be involved in GIT1 regulation of pSmad1/5/8/Runx2, since our previous study had shown that PKC δ promotes GIT1 phosphorylation to increase its effect on cell function [16]. We propose that in addition to promoting Smad1/5/8 phosphorylation and increasing pSmad1/5/8, GIT1 might also regulate Runx2 expression through the angiogenesis and PKC δ pathways, thereby affecting chondrocyte differentiation and maturation.

In the present study, in vitro experimental results indicate that GIT1 protein levels did not significantly affect TGF β /Smad2/3 signal transduction in mouse BMMSCs; however, reduction of GIT1 protein expression decreased BMP2/pSmad1/5/8 signal transduction. The staining intensity of pSmad2/3 at the fracture site showed no substantial difference between GIT1 WT and KO mice, while that of pSmad1/5/8 significantly declined in GIT1 KO mice, leading to disorders of chondrocyte differentiation and maturation and substantial accumulation of chondrocytes, negatively affecting fracture healing. The data from Runx2 and ALP expression and the mineralization experiment further indicate that GIT1 affects chondrocyte maturation and bone mineralization. Together these findings reflect that GIT1

affects BMP2/pSmad1/5/8 signal transduction to regulate Runx2 expression, eventually affecting endochondral ossification. The results from our present study show that GIT1 did not affect pSmad2/3; however, this does not mean that GIT1 has no interaction with the TGF β pathway. In TGF β R2 KO mice, the expression levels of VEGF and platelet endothelial cell adhesion molecules both decline [24]; however, after binding to ERK1/2, GIT1 can promote VEGF mRNA expression [13]. Studies are still needed to investigate whether GIT1 and TGF β synergistically promote VEGF expression and increase angiogenesis at the fracture site, further increasing oxygen tension, promoting BMP2-induced ossification, and accelerating fracture healing.

Above all, our work demonstrates that GIT1 plays a role in cartilage development and endochondral ossification by regulating Smad1/5/8 phosphorylation, thereby affecting fracture healing. However, the exact mechanisms require further investigation.

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Fig. 1.

a Responses of mouse bone marrow mesenchymal stem cells cultured in vitro to TGFβ1 stimulus. The results of Western blot assay showed that TGFβ1 promoted Smad2/3 phosphorylation, but there was no significant difference in pSmad2/3 levels between GIT1 wild-type and knockout mice. b Immunohistochemical staining of pSmad2/3 at the fracture site 2 weeks after femoral fracture in GIT1 wildtype (a) mouse and GIT1 knockout mouse (b). c The result of statistical analysis of immune strength (P[0.05]). d GIT1-siRNA interference of C3H10T1/2 cells significantly inhibited endogenous GIT1 protein expression. Results showed that the intracellular GIT1 protein level had no significant effect on the level of TGFβ1 transcription through pSmad2/3 in the cell nucleus (P<0.05)

Fig. 2.

a BMP2 promoted Smad1/5/8 phosphorylation. And the phosphorylation level of Smad1/5/8 was higher in GIT1 wild-type mice than in GIT1 knockout mice. b Immunohistochemical staining of pSmad1/5/8 at the fracture site 2 weeks after femoral fracture was significantly greater in GIT1 WT mice (a) than in GIT1 KO mice (b) (Bar *200). c The result of statistical analysis of immune strength (P<0.05). d The intracellular GIT1 protein level significantly affected the level of BMP2 transcription through pSmad1/5/8 (P<0.05), but not the levels of Wnt and TGF β 1 transcription in the cell nucleus (P[0.05]). e In GIT1 WT mice, there was no evident pSmad1/5/8 in the cell nucleus of BMMSCs at 0 min (a, b); there was evident pSmad1/5/8 in the cell nucleus of BMMSCs at 5 min (arrow in c, d). f In GIT1 KO mice, there was no evident pSmad1/5/8 in the cell nucleus at 0 min (a, b) or 5 min (arrows in c, d).

Fig. 3.

a Immunohistochemical staining of anti-collagen type-II antibody showed that endochondral ossification significantly occurred in GIT1 WT mice (a), but not significant in GIT1 KO mice (b).

b Immunohistochemical staining of Runx2 was significantly higher in GIT1 WT mice (a) than in GIT1 KO mice (b) at the fracture site two weeks after femoral fracture. c The result of statistical analysis of immune strength ($P[0.05]$). d The results of real-time PCR assay showed that the alkaline phosphatase expression level of BMMSCs was significantly higher

in GIT1 wild-type mice than in GIT1 knockout mice. e Alkaline phosphatase staining of mouse bone marrow mesenchymal stem cells (BMMSCs) showed that alkaline phosphatase activity was significantly higher in GIT1 wild-type mice (a) than in GIT1 knockout mice (b). f Von Kossa staining showed that the mineralization capacity of BMMSCs was significantly higher in GIT1 wild-type mice (a) than in GIT1 knockout mice (b)

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Fig. 4. Total RNA was extracted from mouse bone marrow mesenchymal stem cells cultured in vitro. The results of real-time PCR assay showed that ID1, Smad6, OC, DLX2, and JunB expression levels were significantly higher in GIT1 wild-type mice than in GIT1 knockout mice; gene expression levels were reduced lower by more than 50 % in GIT1 knockout mice

Nucleotide sequences, Name Sequence Annealing temperature Length denaturation temperatures, and lengths of primers for ACP, ID1, Smad6, OC, DLX2, and JunB

Table 1

Name	Sequence	Annealing temperature	Length
ALP	F 5'-TGACCTTCTCTCTCCATCC-3'	55	110
	R 5'-CTTCTCTGGGAGTCTCATCT-3'		
ID1	F 5'-ACTGCGCCCTTAACTGCAT-3'	58	113
	R 5'-TGGAAATCCCAACCCCTAAA-3'		
SMAD6	F 5'-CGCCAAAGTGTCTAGACTGG-3'	58	88
	R 5'-CTCCTCAACGCACACTGAG-3'		
DLX2	F 5'-GCACATGGGTTCTCTACCACT-3'	60	153
	R 5'-TCCTTCTCAGGCTCGTTGTT-3'		
JUNB	F 5'-GAGCTCGTACCCGACGACCAC-3'	60	218
	R 5'-TTCCGACCCGCTTGGGCTCCAC-3'		