Loss of Smad3 gives rise to poor soft callus formation and accelerates early fracture healing

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A B S T R A C T
Smad3 is an intracellular signaling molecule in the transforming growth factor β (TGF-β) pathway that serves as a regulator of chondrogenesis and osteogenesis. To investigate the role of the TGF-β/Smad3 signaling in the process of fracture healing, an open fracture was introduced in mouse tibiae, and the histology of the healing process was compared between wild-type (WT) and Smad3-null (KO) mice. In KO mice, the bone union occurred more rapidly with less formation of cartilage in the callus and eventually the fracture was repaired more rapidly than in WT mice. Alkaline phosphatase staining showed that osteoblastic differentiation in the fracture callus was promoted in KO mice. Additionally, TRAP staining and the TUNEL assay revealed that the induction of osteoclasts and apoptotic cells was significantly promoted in the healing callus of KO mice. Sox9 expression clearly decreased at both mRNA and protein levels in the early stage of fracture in KO mice. In contrast, the expression of genes for osteogenesis and osteoclast formation increased from day 5 until day 14 post-fracture in KO mice compared to WT mice. From these results, we concluded that the loss of TGF-β/Smad3 signaling promoted callus formation by promoting osteogenesis and suppressing chondrogenesis, which resulted in faster fracture healing.

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Introduction
Open fractures often require a long period for convalescence because the damage to surrounding soft tissues delays bone healing and makes rehabilitation difficult (Clancey and Hansen, 1978). Although numerous clinicians and scientists have studied the various biochemical factors of fracture healing, the molecular mechanism by which fracture healing is regulated remains unclear.

The process of fracture healing consists of three phases: inflammation (hematoma); callus formation including cartilage, bone, and endochondral bone formation (repair); and remodeling. Biological studies on each phase of fracture healing have identified proteins that are required for these processes (Tsiridis et al., 2007; Bourque et al., 1993). The transforming growth factor β (TGF-β) superfamily includes multiple isoforms of TGF-β, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) (Cho et al., 2002). Several members of the subfamilies of these morphogens have been shown to promote the differentiation of mesenchymal stem cells into osteogenic and chondrogenic cells during fracture healing. TGF-β1, TGF-β2, and TGF-β3 play important roles in wound healing, development, induction of the epithelial–mesenchymal transition, the immunological system, and fracture healing (Bonewald and Dallas, 1994; Spagnoli et al., 1997; Yang et al., 1999). Although the role of TGF-β1 in fracture healing has been studied in vitro and in vivo (Balogh et al., 2005; Joyce et al., 1990b; Nielsen et al., 1994; Wrana et al., 1988), the exact function of TGF-β1 in the phases of fracture healing still remains unclear (Bonewald and Mundy, 1990; Bostrom and Asnis, 1998; Joyce et al., 1990a).

TGF-β3 receptors exert their effects mainly through the Smad-dependent signaling pathway. TGF-β3/Smad signaling begins when the TGF-β3 type I receptor transduces downstream signals via cytoplasmic latent transcription factors, i.e., Smad proteins. Smad2 and Smad3 are phosphorylated directly by the TGF-β3 type I receptor kinase, before they form a complex with Smad4 and translocate into the nucleus where they act as transcriptional regulators of target genes (Attisano and Wrana, 2000; Bonewald, 1999; Borton et al., 2001; Derynck and Zhang, 2003; Massague and Wotton, 2000; Roberts et al., 2003).

In this study, we addressed the functional role of TGF-β3/Smad3 signaling in the phases of fracture healing using Smad3-null mice (KO). Aschcroft et al. demonstrated that wound healing in skin was
accelerated in Smad3-null mice (Ashcroft et al., 1999). We examined whether fracture healing in KO mice was accelerated in the same manner as wound healing in skin.

**Materials and methods**

**Animals**

All experiments were performed according to the protocol approved by the Animal Care and Use Committee of Wakayama Medical University. Smad3-null (Smad3<sup>ex8/ex8</sup>) mice were generated as described previously (Yang et al., 1999). Female wild-type (WT) (average body weight: 23 g) and Smad3-null (KO) (average body weight: 18 g) mice were used at 10 weeks of age. Fifty mice of each genotype were used.

**Fracture model**

For general anesthesis, a ketamine (80 mg/kg) and xylazine (5 mg/kg) cocktail was administered intraperitoneally. The dorsal side of one leg was shaved and sterilized with an iodine solution. An open fracture was created in the tibia using a modified method described by Geris et al. (2006) and Maes et al. (2006). Briefly, a longitudinal incision of approximately 15 mm was made on the anterior side of the right lower leg to expose the patellar tendon and the tibial periosteum. A bone saw was used to introduce the fracture in the tibia approximately 7-mm distal from the proximal articular surface. The fracture was semi-stabilized with an intramedullary fixating pin using a thin-walled 27 gauge needle that was inserted into the tibia from the proximal articular surface and longitudinally spanned the main part of the bone through the marrow. To avoid gross displacement of the tibia halves, the fibula was cut at the mid-diaphysis. After irrigation, the wound was closed with 5–0 nylon sutures. All mice were permitted full weight bearing and were allowed full unrestricted cage activity following surgery. Two sets of 25 WT mice and 25 Smad3-null mice were sacrificed by cervical dislocation at days 5, 7, 10, 14, and 21 after the operation. Five mice of each genotype were used.

**Radiological analysis**

X-rays of the right tibiae from WT and Smad3-null mice were taken after the operation and before being sacrificed under anesthesia using a soft X-ray apparatus (CMB-2; Softex Co., Tokyo, Japan) and X-ray films (RX-U; FUJIFILM Co., Tokyo, Japan). To determine whether a bone union was formed, bony bridging on radiographs was evaluated by two blinded investigators.

**Histological analysis**

The harvested tibiae (n = 5) were fixed with 4% paraformaldehyde in a 0.1-M phosphate buffer at pH 7.4 and 4 °C for 24 h. After decalcification with 10% EDTA at 4 °C for 14 days, the tibiae were dehydrated with increasing concentrations of ethanol, embedded in paraffin, and cut into 4-μm-thick sagittal sections. The sections were individually stained with hematoxylin-eosin, alcin blue/sirius red, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), or processed for the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays.

**Histomorphometric measurements**

For histomorphometry, the center of the callus at the fracture line of the tibia was defined as the region of interest. To determine the cartilage and bone content in this region of interest, five slices of the center of the callus among 30 slices throughout the entire callus were stained with alcin blue/sirius red at days 5, 7, 10, 14, and 21 after the operation. Each of these sections was observed at ×40 magnification by a light microscope (Olympus BX51, Japan) and photographed using a digital camera (DP80 Olympus, Japan). The images were imported into a computer using Adobe Photoshop (Version 7), and the areas of cartilage and bone in each callus were selected. A single, blinded investigator performed the histomorphometric measurements using Downloaded Image J software. Each count was repeated at least three times.

To determine ALP and TRAP activity, five sections at days 5, 7, 10, 14, and 21 after the operation were used. ALP activity was detected by incubation with a mixture of 0.1-mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N.N-dimethylformamide, 2-mM MgCl2, and 0.6-mg/ml fast red TR salt in 0.1-M Tris-HCL (pH 8.5) at room temperature for 30 min. TRAP activity was detected by incubation with a mixture of 0.5-mg/ml naphthol AS-MX phosphate (Sigma) and 1.1-mg/ml fast red TR salt in 0.2-M acetate buffer solution (pH 5.0) with 50-mM L-(-)-tartaric acid at 37 °C for 60 min. The ALP-positive or TRAP-positive cells were counted in a fixed area (1 × 1 mm or 10 × 10 mm, respectively) after counting the total positive cells that were observed in the section of callus. Five fields were examined in a blinded fashion.

**TUNEL assay**

TUNEL assays were performed using an ApopTag Fluorescein in situ apoptosis detection kit (Chemicon, Temecula, CA) and the Ventana HX system (Ventana, Yokohama, Japan). The TUNEL-positive cells were counted and were recorded as a percentage of the total number of nuclei that were visualized in each section of callus at days 7, 10, 14, and 21 after the operation. Five fields from each section were selected in a blinded fashion and observed at >100 magnification at each time-point.

**RNA preparation**

Five specimens of callus (contralateral leg) were harvested and pooled at days 0, 5, 7, 10, 14, and 21 after the operation. Bone, cartilage, or granulation tissues 3-mm proximal and distal to the fracture site were included in the specimen. The harvested tissue was frozen in liquid nitrogen and stored at −80 °C until it was used for RNA isolation. The specimens were powdered in liquid nitrogen using a motor and pestle. Total RNA was extracted with Trizol (Invitrogen).

**Reverse transcription–polymerase chain reaction (RT–PCR)**

Total RNA (1 μg) was used for cDNA synthesis with the Superscript III system (Invitrogen) according to the manufacturer’s instructions. Amplification was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) using a GeneAmp PCR system 9600 (Perkin Elmer Life Science, Boston, MA). Specific primers were designed from the sequences available on GeneBank (Table 1). The PCR product of GAPDH cDNA was used to ascertain that an equivalent amount of cDNA was obtained from each sample.

**Immunohistochemistry**

Immunohistochemical localization of Sox9 in the callus at days 5 and 14 was examined using a rabbit anti-mouse Sox9 antibody (Santa Cruz Biotechnology). Five sections were selected from WT and KO mice based
on routine histology. Immunostaining was performed on paraffin sections using the dextran polymer conjugate two-step visualization system (Dako Envision System; DAKO, Carpinteria, CA). Briefly, the deparaffinized and rehydrated sections were preincubated with 10-mM citrate buffer at pH 6.0 for 10 min at 95 °C. After the pretreatment, the sections were incubated with 0.3% H2O2 for 10 min at room temperature to block endogenous peroxidase activity. The sections were washed with TBS before blocking with 5% skim milk powder in TBS for 60 min, followed by overnight incubation in a primary antibody solution at 4 °C. The primary antibody was diluted in TBS to a ratio of 1:100. The incubation was then washed with TBS and incubated with secondary antibody for 60 min at room temperature. After using the previously described visualization system, the sections were counterstained in Weigert’s hematoxylin, dehydrated, and mounted. Sections incubated without the primary antibody served as a negative control. The intensity of the immunoreaction was assessed by two blinded investigators.

Statistical analysis

Results are expressed as the mean ± S.E. Statistical analyses were performed with Student's unpaired t-test and Scheffe's method. Differences were considered significant (*) or highly significant (**) at p < 0.05 or p < 0.01, respectively.

Results

Radiological findings

Representative X-rays of the fracture model used in this study are shown in Fig. 1. In wild-type (WT) mice, callus formation was detected at day 7 and increased gradually afterward. Bony bridging at the fracture site appeared to be complete by day 21, although the fracture line, which is a radiolucent zone, was visible until day 21 on both the extensor and the flexor sides. On the other hand, in Smad3-null (KO) mice, callus formation was not detected until day 10 because the callus size was much smaller than that in the WT mice. Bony bridging at the fracture site appeared to be complete by day 14, and the fracture line was invisible at day 14 on both the extensor and the flexor sides (Fig. 1). After the completion of bony bridging, the callus gradually decreased in volume in WT and KO mice. Nonunion and atrophy were not observed in any WT or KO mice with fractures.

A hard callus was formed earlier in KO mice than in WT mice

To assess the phases of fracture healing, we first examined hematoxylin-eosin stained sections (data not shown). To monitor the development of cartilaginous and bony matrices in fracture healing, we assessed the fracture tissues using alcian blue and sirius red staining (Fig. 2A). We examined five sections of each fracture callus from five WT and KO mice; representative histological findings are shown in Fig. 2A. At day 5 after fracture, inflammatory cell infiltrate was observed at the fracture site in both WT and KO mice (Fig. 2A, a and f). Starting at day 7, cartilage formation and membranous ossification appeared with inflammatory cells and fibrous tissue at the center of the fracture gap in both WT and KO mice (Fig. 2A, b and g). The soft callus in KO mice was significantly smaller than in WT mice, except at day 5 after fracture. At day 10, the frame of cartilage for endochondral ossification was completely formed and was much smaller in KO mice than in WT mice (Fig. 2A, c and h). At day 14, unabsorbed cartilage still remained in the callus of WT mice, whereas hard callus formation due to endochondral ossification and membranous ossification was virtually complete in KO mice (Fig. 2A, d and i). At day 21, woven bone was
completely formed in WT mice (1 week later than KO mice). At this stage, the remodeling of bone was virtually complete in KO mice (Fig. 2A, e and j). These results demonstrate that hard callus formation followed by endochondral and membranous ossification was completed rapidly in KO mice compared to WT mice because the soft callus was poorly formed during the process of fracture healing in KO mice.

Histomorphometric measurements revealed a significant difference in the cartilaginous area as well as a difference in the whole callus between WT and KO mice, especially up to day 21 (Fig. 2B and C). The total callus area was approximately 3-fold higher in WT mice compared to KO mice (Fig. 2B). Additionally, the proportion of cartilage matrix to the total callus in KO mice was significantly lower than in WT mice at every stage (Fig. 2C). From these results, we concluded that cartilage formation was markedly suppressed in the callus by the loss of Smad3.

Differentiation of chondrocytes and osteoblasts was promoted in the KO callus

To investigate the maturation of chondrocytes and the activity of osteoblasts, sections of the fracture callus were analyzed by alkaline phosphatase (ALP) staining. The staining pattern showed a clear difference in the stage and area of the fracture callus between WT and KO mice (Fig. 3). At day 5, ALP-positive cells were detected inside the cartilage in KO mice, whereas no positive cells were detected in WT mice (Fig. 3A, a and f). At day 7, ALP-positive cells finally began to appear in WT mice as well (Fig. 3A, b and g). At day 10, the ALP-positive area increased in the cartilage and endochondral ossification areas in both WT and KO mice (Fig. 3A, c and h). In addition, statistical analysis showed that the number of ALP-positive cells in the total callus in KO mice gradually increased until day 10 but drastically decreased after day 14. In WT mice, the number of ALP-positive cells increased rapidly starting at day 10 and gradually decreased after day 21 (Fig. 3B).

Bone remodeling occurred more rapidly in KO mice than in WT mice

TRAP staining was performed to assess osteoclast and chondroclast activity (Fig. 4). Virtually no TRAP positive cells were detected until day 10 in WT and KO mice (Fig. 4A, a, b, f and g). At day 10, the area of TRAP-positive cells increased in the vicinity of the cartilage callus in both WT and KO mice (Fig. 4A, c and h). In addition, at day 14, the area of TRAP-positive cells had expanded into the endochondral and the membranous ossification areas in both WT and KO mice. However, the number of TRAP-positive cells was dramatically increased in the membranous ossification area in KO mice (Fig. 4A, d and i, B, and C). At day 21, TRAP-positive cells in KO mice had decreased below the levels observed at day 10, whereas TRAP-positive cells in WT mice were detected at nearly the same levels as observed at day 14 in the endochondral and membranous ossification areas (Fig. 4A, e and j, and C). From these results, we concluded that bone remodeling was achieved in a shorter period of time in KO mice than in WT mice.

Apoptosis proceeded one week faster in the fracture callus of KO mice

To examine whether the disruption of TGF-β/Smad3 signaling affected the apoptosis of chondrocytes and osteocytes in the remodeling process of the fracture callus, a TUNEL assay was performed (Fig. 5). Until day 14, few TUNEL-positive cells were detected in either WT or KO
mice (Fig. 5A, a, b, e and f). At day 14, the number of TUNEL-positive cells dramatically increased in the membranous ossification area in KO mice compared to WT mice (Fig. 5A, c and g, and B). At day 21, the TUNEL-positive cells decreased to baseline in KO mice, whereas WT mice had virtually the same number of TUNEL-positive cells in the endochondral ossification area (Fig. 5A, d and h, and C). Together with the remodeling results, these results suggest that the peak of apoptosis is 1 week earlier in the fracture callus of KO mice compared to WT mice.

Expressions of genes involved in chondrogenesis were markedly suppressed in KO mice

To investigate how the loss of Smad3 affected the expression of genes involved in callus formation, endochondral ossification, and remodeling of fracture healing, we examined gene expression levels in the fracture callus using RT–PCR. The expression of Sox9, aggrecan, Col2, and Col10 mRNA was examined for soft callus formation (which gives rise to endochondral ossification), while the expression of Runx2, alkaline phosphatase (ALP), and Col1 mRNA was examined for hard callus formation (which gives rise to membranous ossification). The level of Sox9 expression, which is critical for chondrogenesis, was low from day 5 through day 14, with a peak at day 7 in KO mice (Fig. 6A); in WT mice, high expression was observed from day 5 through day 7. The relative intensity of aggrecan expression showed a similar pattern to that of Sox9 (Fig. 6A). Runx2 expression appeared at day 5 and continued until day 14 in KO mice, while it decreased after the maximum expression at day 7 in WT mice (Fig. 6A). The relative intensity of ALP expression seemed identical to that of Runx2 (Fig. 6A).

Sox9 expression was markedly suppressed in KO mice

Immunohistochemical analysis showed that Sox9 was more weakly stained in the callus of KO mice compared to WT mice at day 5 (Fig. 6B, a and c). Virtually no staining was detected in KO mice, whereas there was some positive staining in WT mice at day 14 (Fig. 6B, b and d). These results suggest that the loss of Smad3 represses primary chondrogenesis via Sox9.

Discussion

In this study, we demonstrated that fracture healing, which is a series of processes consisting of inflammation, callus formation involving cartilage and bone formation, and remodeling, appeared to be accelerated in Smad3-null (KO) mice compared with wild-type (WT) mice. In KO mice, the number of apoptotic cells, chondroclasts and osteoclasts increased and peaked at day 14 after fracture; endochondral ossification was completed and remodeling started one week faster than WT mice. These results suggest that the loss of Smad3 results in more rapid healing of bone fractures.

This is the first report to address the role of Smad3 in fracture healing, although a previous study investigated temporal protein expression of Smads, TGF-β, and BMPs in rat fracture healing (Yu et al., 2002). There are some reports that exogenous TGF-β can induce osteogenesis and chondrogenesis; TGF-β1 and TGF-β2 injections around the rat femoral bone result in increased formation of both

Fig. 3. Histological analysis of alkaline phosphatase (ALP)-positive cells of the fracture callus in wild-type (WT) mice and Smad3-null (KO) mice. (A) Representative histological sections of calluses in the time course after fracture in WT mice and KO mice. ALP-positive cells are stained bright red. Bar: 200 μm. (B) Comparison of the number of ALP-positive cells per unit area of callus tissue between WT and KO mice.
cartilage and bone (Joyce et al., 1990b; Wrana et al., 1988); TGF-β1 induced rapid bone closure of skull defects (Beck et al., 1993); and local injection of TGF-β increased the strength of tibial fractures in the rat (Nielsen et al., 1994). These reports indicate that TGF-β may promote the repair of fracture healing. Our data showed that fracture healing was promoted, although the disruption of Smad3 decreased the volume of both cartilage and bone. It is possible that TGF-β may stimulate the proliferation of mesenchymal cells and differentiation of these cells into chondrocytes and osteoblasts without accelerating the whole process of fracture healing.

It has been reported that Smad3-mediated TGF-β signaling inhibits the maturation of chondrocytes (Ferguson et al., 2000) and negatively regulates alkaline phosphatase activity in osteoblastic cells (Sowa et al., 2002). In addition, in KO mice, the maturation of chondrocytes was accelerated (Li et al., 2006), and the osteocyte fate of osteoblasts was promoted (Borton et al., 2001). These findings are consistent with our results for fracture healing, although the previous studies were performed in vitro. Although it has been previously reported that the loss of Smad3 resulted in less bone formation and osteopenia (Borton et al., 2001), X-ray findings in our experiment did not show any signs of osteopenia at the region of the bone union.

We also demonstrated that osteoclasts and apoptotic cells rapidly increased during the time between endochondral ossification and remodeling in KO mice. This is consistent with the studies showing that TGF-β inhibited bone resorption, osteoclast formation, and osteoclast activation (Bonevaid and Mundy, 1990) and that the loss of Smad3 promoted apoptosis of osteoblasts and osteocytes (Borton et al., 2001). It is known that bone formation and resorption are closely associated

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**Fig. 4.** Histological analysis of tartrate-resistant acid phosphatase (TRAP)-positive cells in the fracture callus of wild-type (WT) and Smad3-null (KO) mice. (A) Representative histological sections of the callus in the time course (at days 5, 7, 10, 14, and 21) after fracture in WT and KO mice. TRAP-positive cells are stained bright red. Bar: 200 μm. (B) Representative pictures at higher magnification at day 14 after fracture in WT and KO mice. Bar: 200 μm. (C) Comparison of the number of TRAP-positive cells per unit area of callus tissue between WT and KO mice.

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with proliferation and apoptosis of osteoblasts and osteoclasts during fracture repair (Li et al., 2002).

Among the various signaling molecules during the phases of fracture healing, it should be noted that the Smad3 pathway represents a key player in Sox9-dependent transcriptional activation in primary chondrogenesis in vitro (Furumatsu et al., 2005). In the present study, we found that Sox9 expression in the callus was dramatically repressed at the early stages after fracture in KO mice when compared to WT mice. It is possible that because the loss of Smad3 represses primary chondrogenesis and stimulates the maturation of chondrocytes, the ratio of the soft callus to the whole callus was lower in KO mice during the entire process of fracture healing. On the other hand, it has been previously demonstrated that TGF-β/Smad3 signaling negatively regulates Runx2 transcriptional activity in osteoblast MC3T3-E1 cells (Sowa et al., 2004). However, in our study, the loss of Smad3 did not clearly influence Runx2 transcriptional activity at the early stages of fracture healing. While BMP/Smad signaling acts on Runx2 (Gazzerro and Canalis, 2006), TGF-β/Smad3 signaling also acts on late stage differentiation of osteoblasts. Because BMP-2 and TGF-β1 exert opposite effects on osteoblast differentiation and maturation (Spinella-Jaegle et al., 2001), the loss of Smad3 may not influence Runx2 transcriptional activity to the same degree as Sox9. However, it is still possible that Runx2 transcriptional activity may be accelerated at the later stages of fracture healing in KO mice.

It is possible that various mechanical environments, especially mechanical stability at fracture sites, influence fracture healing and can decrease cartilage formation (Ashhurst, 1986). However, stability does not always accelerate fracture healing because some movements can be useful for fracture healing (Buckwalter, 1996). In the results of our study of fracture healing, the smaller callus formation in KO mice could be due

Fig. 5. Histological analysis of the TUNEL assay of the fracture callus in wild-type (WT) and Smad3-null (KO) mice. (A) Representative histological sections of calluses in the time course (at days 7, 10, 14, and 21) after fracture in WT and KO mice. TUNEL-positive cells are stained dark brown. Bar: 200 μm. (B) Representative pictures for TUNEL assay at higher magnification at day 14 after fracture in WT and KO mice. Bar: 200 μm. (C) Comparison of the TUNEL-positive cells to the total number of nuclei in the fracture callus between WT and KO mice.
to our sample size because it is known that body weights are different between WT and KO mice. However, in X-rays, the size of the tibia fixed with a pin seemed to be virtually equal in WT and KO mice. In addition, our modified transverse fracture model, WT mice may have obtained more stability at fracture sites with a longitudinal strength of body weight than KO mice (Geris et al., 2006).

In our study, we did not examine bone strength during fracture healing. Therefore, the point at which the original strength of the tibia was regained was not determined. The large cartilaginous callus in the WT mice may have provided a more mechanically stable environment, while the smaller KO callus was less stable, leading to continued periosteal bone formation and bone remodeling. However, the size of the callus would not be the only factor promoting healing. It would be more important that the healing process progresses without any obstacles and that the fractured bone quickly regains the matrix and strength of the original bone.

In conclusion, we showed in this study that the loss of Smad3 repressed Sox9 expression and stimulated ALP activity at the early stages, and promoted TRAP activity and apoptosis at the later stages of fracture healing. These aspects eventually facilitated the establishment of soft callus formation and accelerated early fracture healing in Smad3-null mice. Further studies will be needed to define the relationship between TGF-β/Smad3 signaling and osteogenic factors such as Runx2 or BMPs during fracture healing.

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References


Fig. 6. (A) Semi-quantitative RT-PCR for gene expression in the callus harvested at various time points (at days 0, 5, 7, 10, 14, and 21) after fracture in wild-type (WT) and Smad3 null (KO) mice. (B) Immunohistochemical localization of Sox9 in the callus at days 5 and day 14 after fracture in wild-type (WT) and Smad3 null (KO) mice. Bar: 200 μm.


