CHAPTER II

MATERIALS AND METHODS

Animals and anesthesia

A total of 51 male Wistar rats weighing 350-400 g were obtained for the experiments. Two rats were housed in each standard laboratory plastic cage with free access to normal laboratory diet and tap water *ad libitum* in a room with ambient temperature of 21 °C and a 12 h day/night cycle. After 7 days of acclimatization, the animals were anesthetized intramuscularly with sodium pentobarbital anesthesia (Nembutal sodium solution, Abbott Laboratories, North Chicago, IL) at a dosage of 75 mg/ kg of body weight. The area of surgery was shaved and then disinfected using 90% ethyl alcohol.

Experimental outlines

In study I, bone regeneration and growth factors expression were studied at the healing periods of 3, 7, 21, 28 days. Rats were divided into 4 groups (N=3 for each group) for 4 different time point. Each rat underwent a surgical procedure to put a standardized titanium chamber on the left femoral bone. The chamber served as a rigid and fixed-geometry cell exclusion barrier. One hour before sacrificed at the appropriate periods, the animals were injected with 5-Bromo-2'deoxy-uridine (BrdU) in order to characterize cellular proliferative activity. After sacrifice, left femoral bones were collected and processed for decalcification and routine paraffin embedding. 5 μm thickness sections were prepared and stained with hematoxylin and

eosin and used for morphometric analysis. Some sections were used for immunohistochemistry staining for BrdU expression, as well as IGF, PDGF-B, COX-2 and BMP-6.

In study II, five animals were used in the experiment of bone regeneration with COX-2 inhibitor. Three rats were treated with NS-398 to determine the effects of COX-2 inhibitor on growth factor expression and bone regeneration. After sacrifice at 14 days, some femoral bones were collected for RNA isolation and then RT-PCR for evaluation of BMP-2, BMP-6, PDGF-A, PDGF-B and β-actin mRNA. The others were processed for decalcification and routine paraffin embedding. Five μm thickness sections were prepared and stained with hematoxylin and eosin and used for morphometric analysis. Some sections were used for immunohistochemistry staining for IGF, PDGF-B, COX-2 and BMP-6 expression.

In study III, the effect of Δ^{12}-PGJ_2 on bone regeneration and on PDGF, IGF, BMP-2 and -6 expression was examined using the described rat femoral transcortical defects with titanium chambers in 28 rats. Collagen sponge containing a given concentration of Δ^{12}-PGJ_2 was filled in the titanium chamber and then placed on top of the defect. BrdU was administered one hour before the animals were sacrificed to measure the mitotic rate activity. The animals were sacrificed 10 days after surgery. Bone sections were prepared for immunohistological staining of PDGF-A, PDGF-B, BMP-2, BMP-6 and IGF expression and then quantification by morphometric analyses at the defect area.

In study IV, screw-shaped titanium implants (1.35x1.5 mm) were randomly inserted to both femoral bones (2 implants for each femur) in 6 rats. Five μl of carboxymethylcellulose, as carrier, alone (for control) or with Δ^{12}-PGJ_2 ($1x10^{-5}$ and $1x10^{-3}$ M) were injected into surgically prepared holes before the insertion of implants. Rats were sacrificed at 3 and 8 weeks after surgery. The femoral bones with implants were embedded in methyl methacrylate resin and were prepared to make undecalcified sections. Histomorphometric analysis was performed at new bone regeneration around the implants.

Titanium chamber and titanium implant preparation

Titanium chambers were prepared by trimming and curving 0.2 mm thickness titanium plate to half-cylindrical shape measuring 8x1.5x2 mm in length, height and width respectively. These chambers were sealed at both ends with a surgical grade self-curing acrylic resin in order to form a closed half cylinder which is open only in the sagital direction preventing an ingrowth of connective tissues. Prior to use, the chambers were cleaned in ultrasonic bath and autoclaved to sterilize them and to create a titanium oxide surface layer.

Screw-shaped pure titanium implants with an outer diameter of 1.35 mm and a total length of 1.5 mm was machined from commercially pure titanium at the Department of Physic, University of North Carolina at Chapel Hill. They were cleaned in an ultrasonic bath and sterilized in autoclave prior to use.

Surgical procedures

In study I and II, after pupillary reflex was inhibited, a 3-cm incision was made through skin and muscle along the long axis over the dorsal aspect of left femoral bone. Then the periosteum was incised and removed from the surface of the bone. Subsequently, a 5x1.5 mm transcortical defect was made through the cortical bone into the bone marrow at the midshaft region using round and cylindrical steel burs with low speed micromotor. Sufficient sterile 0.9% saline solution has been used as a coolant to avoid heat-induced necrosis of surrounding bone. Blood was collected from the defect and was used to fill up the volume inside the half-cylindrical titanium chamber. The chamber was placed over the defect and was stabilized by wiring to the femoral bone using # 010 orthodontic wire to ensure a stable anchorage and a reliable peripheral sealing of the chamber (**Fig. 1 and 2**). The muscle and skin were then repositioned and sutured in two layers using bioresorbable suturing material (Chromic gut; Ethicon®, Johnson & Johnson company, Somerville, NJ) and stainless steel wound clips (Autoclip®; Becton Dickinson and company, Parsippany, NJ). The animals were monitored every day after surgeries to assure that the animals were ambulatory, eating, drinking and non-febrile

In study III, after transcortical defect was made as described above at both femoral bones, one side of the animal was randomly chosen for Δ^{12}-PGJ_2 treatment, while the contralateral side was used as a vehicle control. Δ^{12}-PGJ_2 (Cayman Chemicals, Ann Arbor, MI) was diluted in 0.9% NaCl, pH 7.2, to the desired concentrations (10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9} M). Resorbable collagen sponges (Colla-Tec Inc., Plainsboro, NJ) 5x1.5 mm in size were used as carrier and reservoirs for the

drug. The collagen sponges were imbibed with 10 µl of either saline or Δ^{12}-PGJ_2 solution, then they were packed inside the half cylindrical titanium chambers. This provided an approximate dosage of $3x10^{-6}$, $3x10^{-8}$, $3x10^{-10}$ and $3x10^{-12}$ g of Δ^{12}-PGJ_2 in each standardized defect. The remaining volume inside each chamber was filled with blood collected from the defect. The chamber were inverted, placed over the defects and stabilized by circumosseous wiring using # 010 orthodontic wire.and sutured back as described above.

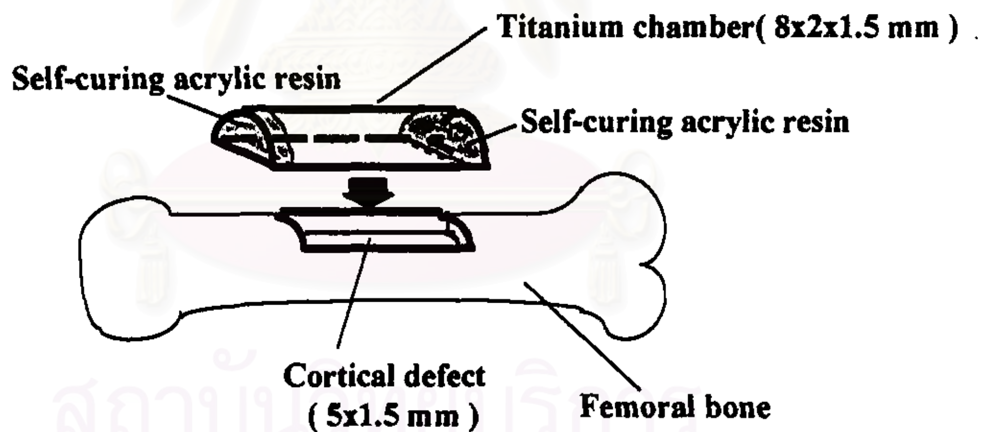

Figure 1 Illustration of the cortical defect on femoral bone and titanium chamber sealed with self-curing acrylic resin at both ends.

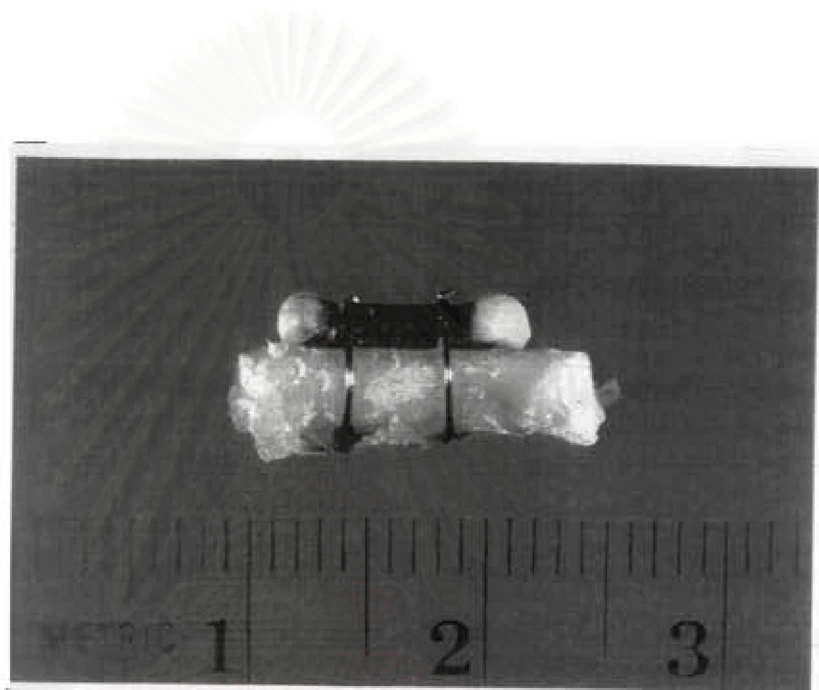

Figure 2 the picture shows the titanium chamber stabilized with femoral bone by #010 orthodontic wire.

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In study IV, a 2-cm incision was made through skin and muscle along the long axis over the dorsal aspect of both femoral bones. Then the periosteum was incised and removed from the surface of the bone. Two holes (1.35 mm. in diameter) were prepared in each femoral bone using low speed micromotor with round steel dental bur and sufficient sterile 0.9% NaCl as a coolant. Four % (w/v) methylcellulose gel (Methocel®, Dow Chemical Company, Midland, MI) used as a carrier was prepared by mixing 20 μg of methylcellulose powder with 500 μl of 1×10^{-5} or 1×10^{-3} M Δ^{12}-PGJ_2 in 0.9% NaCl following manufacturer's instruction. Gel was loaded in tuberculin syringe with gauge 21 needle. Approximately 5 μl of prepared methylcellulose gel were injected into each hole. Then the titanium implant was immediately press fit into the hole until the top surface of the implant was at the same level as the cortical surface of the femoral bone. The control sites were injected with methylcellulose gel without Δ^{12}-PGJ_2.

After the appropriate healing periods, the animals were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbital. The thoracic cavity was exposed and transcardial perfusion with 0.1% heparinized (heparin sodium 1000 units/ml) 0.1 M phosphate buffer (pH 7.4) was performed. Then 400 ml of 4% para-formaldehyde (Fisher Scientific company, Fair Lawn, NJ) in 0.1 M phosphate buffer (pH 7.4) was perfused as a fixative. After fixation was completed, both femoral bones were dissected, removed and postfixed in the same fixative for 48 hours.

In study I-III, the chambers were carefully removed and the samples were then decalcified using 10% ethylenediamine tetraacetic acid (EDTA), pH 7.4 at 4 °C. The decalcification endpoint was determined by radiography (approximately 10-14

days). The EDTA solution was changed every 3 days. The tissues were then rinsed under running tap water for at least 2 hours and dehydrated for embedding in paraffin wax according to a routine procedure. The embedded specimens were cut in a plane perpendicular to the long axis of the femoral bone into 5 μm thickness sections using microtome (Model 820, American Optical Corporation, Buffalo, NY). The sections were floated in the water bath, and mounted onto slides coated with 0.5% gelatin and 0.05 % chrome alum (chromium potassium sulfate). The sections were dried overnight at 37 ° C in an oven to enhance adhesiveness, then stained with hematoxylin and eosin (H&E) and Masson's Trichrome.

In study IV, the femoral bones with titanium implants were embedded in methyl methacrylate resin and were prepared to make undecalcified sections (approximately 100 μm in thickness) using a low speed saw (Isomet®, Buehler, Lake Bluff, IL). Sections were stained with toluidine blue. The central section that cut through the middle of the implant was used for histomorphometric analysis for each sample. and the extent and amount of new bone regeneration on endosteal surface were determined.

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COX-2 inhibitor administration

Experiment of bone regeneration with COX-2 inhibitor was performed on 5 male Wistar rats. All animals underwent the surgical procedure described above. Three rats were injected (IP) with COX-2 inhibitor (NS-398) at a dose of 3 mg/kg/day one day before starting of the surgery and continued daily until sacrifice at 14 days. Daily dose was divided into two doses and injected twice a day. NS-398 was

dissolved in DMSO (10 mg/ml) and further diluted with 0.9% normal saline (1:1) to the final volume of 500 μl before intraperitoneal injection. Two rats were injected with 500 μl of DMSO and 0.9% normal saline (1:1) alone for control. After sacrifice, samples from right femoral bone were collected for RNA isolation and the others from the left were collected and processed for immunohistochemical staining.

BrdU uptake as indicator of mitotic activity

All rats in study I and eight of the rats treated with 10^{-3}, 10^{-5} or 10^{-7} M of Δ^{12}-PGJ_2 in study III were used for this experiment. One hour before sacrifice, 5-Bromo-2'deoxy-uridine (BrdU, Boehringer Mannheim GmbH, Mannheim, Germany: 100 mg/kg) was dissolved in 2 ml of sterile 0.9% NaCl to reach a concentration of 40 mg/ml. It was administered by intra-peritoneal injection. BrdU is an analog of thymidine which is incorporated into cells during DNA synthesis. In order to detect BrdU expression representing cellular mitotic activity, sections were exposed to a monoclonal anti-BrdU antibody, and revealed by using a highly sensitive streptavidin-biotin staining system. Diaminobenzidine (DAB) in the presence of hydrogen peroxide was used as a chromogen, staining BrdU-incorporated nuclei a dark brown. A BrdU staining kit (Zymed Laboratories Inc., South San Francisco, CA) was used following procedures the manufacturer's instructions. Briefly, slides were deparaffinized with Hemo-De® (Fisher Scientific, Pittsburgh, PA) and rehydrated through a graded series of ethanol. Then 0.125% trypsin solution was used and the samples were incubated in a moist chamber at 37 °C for antigen recovery. Then denaturing solution (4 N HCl) and blocking solution (3% H_2O_2 in methanol) were applied to denature the DNA and block endogenous peroxidase respectively.

Biotinylated mouse anti-BrdU was then applied for 40 minutes at room temperature. Streptavidin-peroxidase and Diaminobenzidine tetrahydrochloride (DAB) mixture were applied respectively. After each step, the sections were rinsed in 0.1 M PBS twice for 5 minutes. The slides were counterstained lightly with hematoxylin. Nuclei that had incorporated BrdU were identified by the presence of a brownish reaction product.

Two types of negative controls were performed. In one case, the complete immunohistochemistry procedure was performed on bones from rats which had not received BrdU administration, while in the other case the complete procedure was performed while omitting the use of primary anti-BrdU antibody. A positive control was obtained by performing the procedure on sections of small intestine from BrdU labeled rats. In this case, nuclei of crypt epithelial cells were BrdU labeled.

Immunohistochemistry

In study I and II, the immunohistochemical staining for COX-2, PBGF-B, BMP-6, and IGF were performed. In study III, the immunohistochemical staining for PDGF-A, PDGF-B, BMP-2, BMP-6, and IGF were performed. From each sample, three sections from different areas of the defect were used. After deparaffinization with Hemo-De® (Fisher Scientific, Pittsburgh, PA), the slides were rehydrated by addition of a gradient series of ethanol and incubated for 10 minutes in 3% hydrogen peroxide in methanol to quench endogenous peroxide activity. The sections were then washed 3 x 5 minutes in phosphate buffer, blocked for 20 minutes with 10% normal rabbit serum (for COX-2, BMP-2, and BMP-6 staining) or 10% normal goat serum

(for PDGF-A, PDGF-B, and IGF staining). In study I and II, they were then incubated for 1 hour at room temperature with diluted (1:30) rabbit anti-human PDGF-BB antibodies, diluted (1:25) mouse anti-human IGF-I antibodies, diluted (1:40) goat anti-COX-2 antibodies or diluted (1:20) goat anti-BMP-6 antibodies (all from Research Diagnostic Inc., Flanders, NJ; dilution). In study III, they were then incubated at 4 °C overnight with diluted (1:20) rabbit anti-human PDGF-A antibodies, diluted (1:30) rabbit anti-human PDGF-BB antibodies or diluted (1:25) mouse anti-human IGF-I antibodies (all from Research Diagnostic Inc., Flanders, NJ) which react with rat PDGF-A chain, PDGF-B chain and rat IGF-I and -II respectively. For BMP-2 and -6 staining, goat anti-human BMP-2 and -6 (Research Diagnostic Inc., Flanders, NJ; dilution 1:20) were used overnight at 4 °C and 1 hour at room temperature respectively. These antibodies have previously been demonstrated to cross-react with rat to permit detection. After extensive washing, secondary antibody (Goat anti-rabbit or rabbit anti-goat immunoglobulin G conjugated with biotin (Zymed Laboratories Inc., South San Francisco, CA) were applied for 30 minutes. The sections were washed again and streptavidin-biotin peroxidase complexes were applied for 30 minutes. Finally, the sections were incubated in a solution containing of 3-amino-9-ethylcarbazole (AEC) and 0.02% hydrogen peroxide for 5 minutes. Counterstaining was done in some sections with hematoxylin for 1 minute. Negative control was performed by complete immunohistochemistry procedure omitting primary antibody.

Quantitative evaluation of bone regeneration, growth factor expression and mitotic activity

For the quantification of bone regeneration, the amount of new bone formed within the intracortical defect and supracortical defect area (in study I and II) and only the intracortical defect area (in study III) were measured using computerized image analysis system. Sections from different areas of each defect (at 0.5-1 mm intervals) were evaluated for histomorphometric quantification. It is necessary to analyze multiple sections of different regions within each defect since the difference in the amount of new bone formed among the different regions is statistically significant (Wong and Rabie, 1999). Measurements were made using a 40x magnification using a light microscope (Model BH-2, Olympus Optical corporation, Japan) connected to a computerized image analysis system (JAVA video analysis software, Jandel Scientific, Corte Madera, CA). The image analysis software allowed the calculation of the areas of different color intensity. The amount of new bone was expressed as the percentage of total defect area. The morphometrical measurements of the crossectional area of mineralized bone were expressed as the percentage of the total generated bone area between the two cut cortical edges and as a percentage of the supraosteal chamber filled as shown in **Figure 3**. The cortical defect area was defined by drawing an imaginary line connecting the endosteal surfaces and the periosteal surface. The supracortical bone fill zone was from the periosteal line to the inside edge of the titanium chamber. The percentage of bone fill in these two compartments was used to express bone regeneration. Three to five sections were analyzed for each femur and the results were pooled to reflect a mean value for each bone defect in each animal. Animals within treatment groups were combined to form a group mean and pooled estimate of standard error.

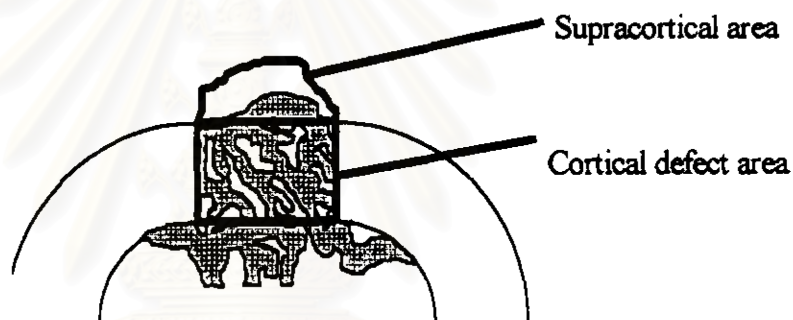

Figure 3 Schematic illustration of the area of bone regeneration which was used by histomorphometric analysis.

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In study IV, histomorphometric analysis was performed at new bone regeneration around the implants. Vertical and horizontal distances of new bone regeneration surround both sides of the implant at the endosteal surface were analyzed. Vertical distance of new bone regeneration around titanium implants were measured (μm) from endosteal surface to the most apical part of the new bone regeneration. Horizontal distance was measured from the thickness of new bone regeneration at endosteal surface, 130 μm and 260 μm below the endosteal surface. Data from both side of the implant were averaged and expressed as mean $\pm$ SEM (μ m). Average bone area in every subcortical thread on both side of the implant were also investigated and expressed as total area of bone in the thread per total area bounded by thread.

For the quantification of growth factor expression, sections were viewed on an Olympus BX70 microscope at 100x magnification, and measurements of the immuno-positive areas were made using JAVA video analysis software (Jandel Scientific, Corte Madera, CA). Three to five sections were analyzed for each femur and the results were pooled to reflect a mean value for each bone defect. Animals within each treatment group were combined to form a group mean and pooled estimate of standard error. Results were expressed as percentage of positive immunostaining area per total cortical defect area.

For mitotic activity, the same image analysis software was used to count the number and observe localization of BrdU-labeled cells in the entire cortical bone defect area at 100x magnification. The BrdU-labeled cells were identified by BrdU-incorporated nuclei that presence a brownish color. Two fields were counted per

defect area and the results were expressed as the number of replicating cells per total defect area.

To ensure stability of the illumination source, it was allowed to warm up for at least one hour before each examination sessions. Stability of the entire imaging setup was checked regularly during examination by recording the same standard section and comparing the result (Willemse et al., 1993).

RNA isolation and reverse transcriptase-polyerase chain reaction (RT-PCR)

The healing tissue samples were obtained separately from the supracortical region and cortical region of bone regeneration by dissection and immediately used to RNA isolation. Tissue was homogenized in guanidium-thiocyanate (Trireagent®, Sigma, St.Louis, MO). Total cellular RNA was extracted using a single step method by acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The final RNA precipitate was stored in diethyl pyrocarbonate (DEPC) water at –70° C. For the RT-PCR, total RNA was reverse-transcribed with the SuperScript™ preamplification system (Life Technologies, Inc., Gaithersburg, MD). Briefly, 1 μg of total RNA was reversed-transcribed by incubating samples at 42° C for 50 min in 20 μl final volume of a 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) containing 0.5 μg of oligo(dT), 2.5 mM of $MgCl_2$, 0.5 mM of each dNTP mix, 10 mM dithiothreitol and 200 units or Superscript II reverse transcriptase. The solutions were incubated, then heated at 70° C for 15 min, and cooled rapidly at 4 °C.

RT reaction mixture (2 μl) was added to 48 μl of 1x PCR buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.2 mM of each primer and 2.5 units of Taq DNA polymerase (Boehringer Mannheim Gmbh, Germany). Amplification was performed in a DNA thermal cycler (Thermolyne, Dubuque, IA). The program was set at the following conditions: denaturation at 95 °C for 45 sec; annealing at 60 °C for 45 sec; and extension at 72 °C for 2 min in each cycle for 30-40 cycles (the cycle number was chosen to be in the exponential range of the amplification). The primers for amplification were designed referring to the sequences of cDNA that had reported and published primer sequences with some modification (**Table 2**). Negative results were controlled by PCR without the prior RT step. No products were yielded by the RNA sample.

PCR products were analyzed by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. The differences between the PCR products were quantitated by the luminescence values of the DNA bands. The relative intensities were measured by an image analyzer (Lumi-Imager^{TM}, Boehringer Mannheim Gmbh, Germany). Results were presented as the ratio of each growth factor mRNA level to the β-actin mRNA level.

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Table 2 Sequences of the PCR primers for amplification and predicted size of products

Primer	Sequence	Size	Reference
BMP-2	5'-GAG TTT GAG TTG AGG CTG CTC- 3'	440 bp	(Bodamyali et al., 1998)
	5'-TGA GTC ACT AAC CTG GTG TCC- 3'		
BMP-6	5'-GCA GAA GGA GAT CTT GTC GG- 3'	612 bp	(Knittel et al., 1997)
	5'-GTC TCT GTG CTG ATG CTC CT- 3'		
PDGF-A	5'-CTC GGC TGC GGA TAC CTC GC- 3'	400 bp	(Dai et al., 1998;
	5'-GAG GGC TGG CAC TTG ACG CTG C- 3'		Katayose et al., 1993)
PDGF-B	5'-CCG CTC CTT TGA TGA CCT TCA- 3'	284 bp	(Dai et al., 1998;
	5'-CAG CCC GAG CAG CGC TGC ACC TC- 3'		Herren et al., 1993)
b-Actin	5'-TGG CAC CAC ACC TTC TAC AAT GAG- 3'	838 bp	+
	5'-TAC TCC TGC TTG CTG ATC CAC ATC- 3'		

+ Clontech Laboratories, Inc., Palo Alto, CA

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM) from the mean of at least 3 sections from each defect in each group. A statistical comparison between the control and test group was preformed using the unpaired Student's *t-test.* The significant level was set at $p < 0.05$.