CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Chemicals and materials used in this study are described in Appendix.

2.2 Methods

2.2.1 Generation and preparation methods

2.2.1.1 Generation of conditional *Vcan* knockout mice

To generate mice in which *Vcan* gene is deleted in early limb mesenchyme, the conditional knockout mice were generated by combining Cre/*loxP* with a Flp/FRT system as follows. A targeting vector harboring $Vcan^{flox}$ allele was constructed by flanking exon 2 of the mouse *Vcan* gene with *loxP* sites in the combination of PGK-neo^R cassette flanked by the FRT sequence. Then, mouse embryonic stem (ES) cells were electroporated with linearized targeting construct and cultured with G418 to positively select for clones that had integrated the targeting construct. Clones were screened by genomic PCR, and positive clones were confirmed by southern blotting. After blastocyst injection and homologous recombination, chimeric mice whose genomic DNA contained $Vcan^{flox}$ allele were obtained. Germline transmission was attained by crossing these chimeric mice with C57BL/6 mice. Then, by crossing with CAG-flippase transgenic (Tg) mice, $Vcan^{+/flox}$ mice whose genomic DNA lacks the PGK-neo^R fragment were obtained. We crossed these mice with C57BL/6 to segregate the CAG-flippase transgene and backcrossed to C57BL/6 at least four times ($N \geq 4$). Next, by crossing $Vcan^{+/flox}$ mice with *Prx1*-Cre Tg mice with

background of C57BL/6, *Prx1*-Cre/*Vcan*$^{+/flox}$ mice were obtained. *Prx1*-Cre/*Vcan*$^{+/flox}$ male and female mice were crossed to obtain *Prx1*-Cre /*Vcan*$^{flox/flox}$ mice whose *Vcan* gene was removed by Cre-mediated excision.

2.2.1.2 Preparation of *Alpinia galanga* hexane extracts

Galanga powder (from fresh rhizomes of *A. galanga* Linn.) was obtained from The Common Life of Love and Unity of the Mountain People Foundation (CLUMP), specimen BKF no. 102287, from the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resourse and Environment, Bangkok, Thailand. Dried powder of *A. galanga* rhizomes was extracted with hexane with constant stirring for 7 days. To ensure complete extraction, 5 kg of powder was extracted with 17 L of hexane and the process was repeated 4 times. The filtrates from the extractions were mixed and dried in a rotary evaporator under reduced pressure at 40 °C. The yield of the extract was determined. Dried residue was weighed and stored at -20 °C.

The hexane extract was further separated by column chromatography over silica gel (Merck No. 7734, 500 g). Elution started with hexane and was gradually enriched with ethyl acetate in hexane up to 20 % ethyl acetate in hexane. Fractions were collected, monitored by TLC and combined. The solvents were evaporated to dryness to yield four fractions (F1-F4). All 4 fractions were used as plant materials. The phytochemical profiles of the hexane fractions were determined by HPLC.

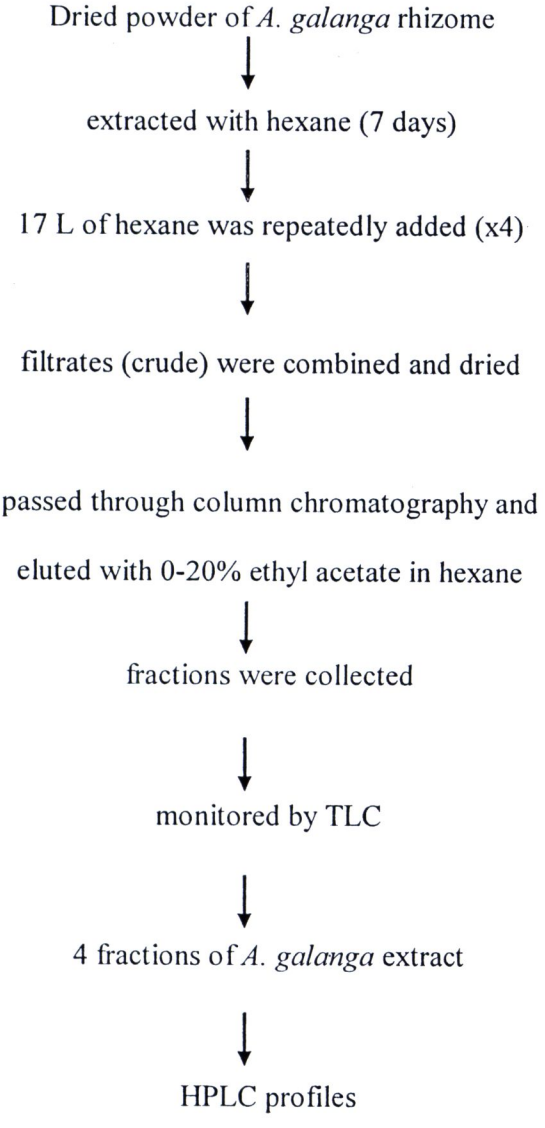

Figure 2.1 Diagram of *Alpinia galanga* hexane extract preparation.

2.2.2 Identification methods

2.2.2.1 DNA extraction

DNA template was obtained from tail biopsy. Tail was cut at the length of 0.3-0.5 cm and put into microcentrifuge tube. Next, 200 µl and 5 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 20 mM EDTA, 1% SDS) and proteinase K (10 mg/ml) was added, respectively. The tube was then incubated at 50°C for overnight, centrifuged at 15,000 rpm for 1 min, and 100 µl of the lysate was transferred to a new tube. Then, 60 µl of extraction buffer (130 mM Tris-HCl pH 8.0, 65 mM EDTA, 2.5% sodium N-lauroylsarcosine) was added. After mixing, 200 µl of 7.5 M NaI was added. Then, the tube was mixed and incubated at 60°C for 10 min. Next, 400 µl of cold isopropanol was filled, the tube was mixed by inversion and centrifuged at 4°C for 20 min. Supernatant was then removed, and DNA was rinsed with 1 ml of cold 70% ethanol. After removing the residual ethanol, 20 µl of TE buffer was added, the tube was then incubated at 65°C for 15-30 min. Genomic DNA was used for polymerase chain reaction (PCR) or kept at 4°C.

2.2.2.2 Genotyping of versican transgenic mice

The genotyping of transgenic mice was performed by PCR using DNA template from proteinase K digestion of tail biopsy as described above. Wild-type (WT) and floxed alleles were identified by PCR using a forward primer of Int1-1 sequence 5'-TGAGCTCTAGCCATATAGGAAGGC-3' and a reverse primer of Kpn-1 sequence 5'-CGCATGCAGACGACATGAAGCAGGAGC-3', generating a PCR product of 3kb in wild-type and 2 kb in floxed alleles. PCR condition was performed for 25 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation for

5 min at 72°C in reaction buffer containing 2 mM $MgCl_2$, 1× *Ex Taq* buffer (Takara, Japan), 0.25 mM dNTPs (Takara, Japan), 0.2 μM of each primer, and 1 μl of prepared genomic DNA. PCR product was analyzed on 0.7% agarose gel electrophoresis, bands were visualized by ethidium bromide, and photographed by gel documentator. Floxed alleles were then further identified by DNA sequencing.

2.2.2.3 Genotyping of *Prx1*-Cre mice

DNA template was prepared by tail biopsy. PCR for Cre transgene was performed using the following primer set of *Prx1*-Cre forward 5'-CCTGGAAAATG CTTCTGTCCGTTTGCC-3' and *Prx1*-Cre reverse 5'GAGTTGATAGCTGGCTGGT GGCAGATG- 3' which generated a PCR product of 620 bp. The PCR reaction was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation for 1 min at 72°C in reaction buffer as described in previous section. PCR product was analyzed on 2% agarose gel electrophoresis, bands were visualized by ethidium bromide, and photographed by gel documentator.

2.2.2.4 Genotyping of ROSA26 mice

ROSA26 allele was identified by PCR using a forward primer of HR113 sequence 5'-GGCTTAAAGGCTAACCTGATGTG-3'and a reverse primer of HR114 sequence 5'-GCGAAGAGTTTGTCCTCAACC-3', generating a PCR product of 1.1 kb. PCR reaction was amplified for 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 1 min, and elongation for 1 min at 72°C in reaction buffer as previously described. PCR product was analyzed on 1% agarose gel, bands were visualized by ethidium bromide, and photographed by gel documentator.

2.2.2.5 DNA sequencing

After genotyping of versican transgenic mice was examined by PCR using a forward primer of Int1-1 and a reverse primer of Kpn-1 as described above, samples which contained floxed alleles were used to enlarge scale by PCR with the same primer set for sequencing. Then, PCR product was analyzed on 0.7% agarose gel, bands were visualized by ethidium bromide, and the floxed band was cut under UV light. The DNA was extracted by gel extraction kit (Qiakit, Japan), and DNA mass was calculated by comparative relation with Lambda DNA-Hind III. Next, PCR was performed using forward primers of 001 sequence 5'-TCTGTGATAACTAATACGA G-3' and a reverse primer of 002 sequence 5'-AACCAATGTATCCTAAAGAG-3', with big dye terminator, then the PCR or cycle sequencing products were purified by ethanol-EDTA precipitation method. Briefly, the cycle sequencing products were transferred to new tubes, then, 5 μl of 125 mM EDTA and 60 μl of 100% ethanol was added, respectively. They were subsequently mixed by inversion, incubated for 15 min at room temperature (RT), centrifuged at 1,400 rpm at 4°C for 20 min. The supernatant was removed, added with 60 μl of 100% ethanol, centrifuged at 4°C for 10 min, and dried for additional 10 min without heat. Then, 12.5 μl of Hi-Di formamide was added, boiled for 5 min, transferred to the new tubes, and sequenced by ABI PRISM 3100 Genetic Analyzer. Blast basic local alignment search tool was applied for query identification using NCBI mouse DNA database.

2.2.3 Analytic methods

2.2.3.1 Analysis of *Prx1*-Cre activity

ROSA26 reporter mice were first crossed with *Prx1*-Cre transgenic line to obtain the *Prx1*-Cre/R26R founders. Then, *Prx1*-Cre/R26R were bred together to generate *Prx1*-Cre/R26R embryos at several stages. These embryos were then used for analysis.

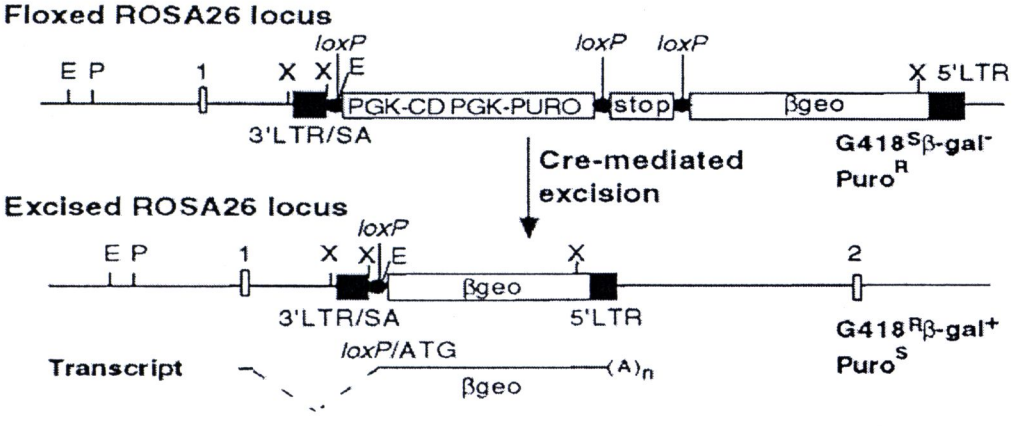

Figure 2.2 Genomic mapping for conditional deletion of the floxed stopper sequence of ROSA26 reporter mice. When *Prx1*-Cre is active, then the stopper cassette will be pruned out by site specific recombination, and lead to transcription of β-galactosidase (β-gal) (228).

To examine β-gal activity, X-gal staining was performed as described below. X-gal or an indole derivative (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is a common substrate for the β-gal. When β-gal activity is present, the insoluble blue compound will be visualized.

2.2.3.2 X-ray and histological analysis

X-ray analysis was performed using a soft X-ray apparatus. Newborn mice or embryos from several embryonic stages were fixed in 10% neutral buffered formalin. Hindlimbs were dissected and embedded in paraffin, 4 or 5 μm thick-paraffin sections were cut and mounted on Superfrost Mascot slides, sections were put in 37°C incubator for overnight and stored at RT, then hematoxylin and eosin staining was performed. Slides were deparaffinized in xylene for 4 min, four times. Then, they were rehydrated in anhydrous ethanol for 5 min, 100% ethanol for 5 min, 90%, 80%, and 70% ethanol for 3 min each. They were then stained with hematoxylin for 3-5 min, washed with tap water and dipped in warm water for 5 min. Next, they were stained with eosin for 3-5 min, dehydrated in 100% ethanol for 5 min four times, and anhydrous ethanol for 5 min twice, and dipped in xylene for 3 min five times. The slides were mounted with mounting solution and enclosed with cover glass slips.

2.2.3.3 Cryosectioning and X-gal staining

To examine Cre activity, hindlimbs from various embryonic stages were cut and put into cold 1x phosphate-buffered saline (PBS) containing 2mM $MgCl_2$, and then fixed in 4% paraformaldehyde (PFA) at 4°C for 2-3 h. They were then washed with PBS twice, soaked in the gradient of sucrose solution at the concentrations of 15% and 30% at 4°C overnight or until they sank. They were briefly rinsed with PBS, and then embedded in OCT compound and kept at -80°C. Cryosections were cut at a thickness of 15 μm. The slides were fixed in 4% PFA at 4°C for 10-15 min, then washed twice with PBS at 4°C. Slides were stained in X-Gal solution (1 mg/ml X-Gal, 5 mM $K3Fe(CN)_6$, 5 mM $K4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% deoxycholate, 0.02%

Nonidet P-40) at 37°C overnight. After staining, they were washed in PBS and counterstained with eosin, dehydrated in ethanol, xylene, and mounted.

After 3 days of micromass culture (as described below), cultures were washed three times with PBS, fixed with 2% formaldehyde for 5 min, washed twice with PBS and stained in staining solution (0.1 M Citric acid, 0.2 M sodium phosphate pH 6.0, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 150 mM NaCl, 2 mM $MgCl_2$) at 37°C overnight.

2.2.3.4 Immunostaining and hyaluronan detection

Sections were deparaffinized in xylene, rehydrated in a gradient of ethanol, briefly washed with PBS. Then, endogenous peroxidase was inactivated through incubation in 3% H_2O_2 in methanol for 20 min. Pre-treatment with chondroitinase ABC (5mU/μl) 30-45 min was required for versican and aggrecan. Antigen retrieval with citrate buffer pH 6.0 by autoclave for 20 min was also essential for CD44, β-catenin, and Cre immunostaining. Then, they were blocked with blocking solution at room temperature for 1 h and incubated with primary antibodies, including anti-versican GAG β domain at 1:1000, anti-aggrecan clone 1C6 at 1:20, anti-link protein clone 8A4 at 1:100, anti-CD44 at 1:250, anti-β-catenin at 1:500, anti-phosphoERK1/2 at 1:1000, anti-Cre at 1:2000, anti-TGF-β at 1:20, anti-TβRII at 1:250, and anti-phosphoSmad2/3 at 1:50. After washing, they were incubated with secondary antibodies consisting of LSAB2 kits for colorimetric reaction, or Alexa Fluor 594 streptavidin and Alexa Fluor 594 anti-rabbit IgG at 1:1000 for fluorescent detection. Images were photographed using an Olympus BX50 microscope and Olympus DP71 digital camera. For detection of HA, sections were incubated with biotinylated hyaluronan binding protein (HABP) at 1:500 at 4°C overnight, followed by

incubation with biotinylated-linked streptavidin for colorimetric reaction or Alexa Fluor 594 streptavidin at 1:1000 for fluorescent detection. Hyaluronan binding protein (HABP) is used to detect HA. As HA is masked by a large amount of chondrotin sulfate of aggrecan, pretreatment with chondroitinase ABC is usually necessary to detect HA in cartilage. Without the pretreatment, HABP mainly binds to HA which is less-incorporated or not incorporated into the matrix. To detect HA in the joint interzone, chondoritinase ABC pretreatment was not performed.

2.2.3.5 Micromass culture

To generate cultures missing *Vcan*, embryos (N=13) at E10.5 or E11.5 obtained from crossing *Prx1*-Cre/*Vcan*$^{flox/flox}$ x *Vcan*$^{flox/flox}$ were used. Similarly, embryos (N≥33) at E10.5 or E11.5 obtained from crossing *Prx1*-Cre/*Vcan*$^{+/+}$ x *Prx1*-Cre/*Vcan*$^{+/+}$ were used as a control, and to investigate *Prx1* activity, embryos obtained from crossing *Prx1*-Cre/R26R x *Prx1*-Cre/R26R were used. Limb micromass culture was carried out as described previously (229) with some modifications. Briefly, limb buds were removed and digested with 0.1% trypsin-0.1% collagenase at 37°C for 20 min to dissociate mesenchymal cells into a single cell suspension. Then, their cell density was adjusted to $2x10^7$ cells/ml, and 10 µl of the cell suspension was plated onto LabTek-II chamber slides or 35 mm culture dishes. After cells were allowed to attach at 37°C for 1 h, cultures were then flooded with DMEM F-12 HAM containing 10% FBS. Cultures were incubated at 37°C in a CO_2 incubator for 6 days with fresh conditioned medium added daily. Following incubation, cultures were processed for alcian blue staining, HA staining, or immunostaining. For double immunofluorescent staining, the sample was co-incubated with rabbit anti-versican GAG β domain at

1:1000, and mouse anti-TGF-β at 1:20 for 4°C overnight, and after washing with PBS, with a mixture of Alexa Fluor 594 anti-rabbit IgG at 1:400 and Alexa Fluor 488 anti-mouse IgG at 1:400. The photos of micromass culture were taken by a confocal laser scanning microscope. To precisely evaluate the localizations of molecules, the Z-stack program was applied. Immunostaining for CS chains was performed using anti-CS LY111 at 1: 200 and Alexa Fluor 488 anti-mouse IgM at 1: 400, as primary and secondary antibody, respectively.

2.2.3.6 Western blot analysis

Three micromasses at day 6 were collected, and the sample was subjected to 15% SDS-PAGE under a non-reducing condition. The proteins were electrotransferred to a polyvinylidene difluoride membrane, and the membrane was soaked in 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T) for blocking. The membrane was treated with mouse anti-TGF-β at 1:250 for 1 h RT. After washing three times with TBS-T, the membrane was treated with peroxidase-conjugated goat anti-mouse IgG at 1:1,000 for 1 h RT. After washing three times as above, the signal was detected with Western Lightning™ Plus-ECL. The data were analyzed using LAS 4000 mini, luminescent image analyzer. For dilution of antibodies, Can Get Signal™ was used. The membrane was treated with Restore ™ western blot stripping buffer, and used for immunoblot analysis of actin. The band density was analyzed using Image J.

2.2.3.7 Alcian blue staining

Whole limbs from embryos at E13.5 and E15.5 days of gestation were fixed in 96% ethanol overnight. Then, they were placed in alcian blue staining solution (0.1% alcian blue, 70% ethanol, 1% HCl) for 2-3 days, dehydrated in 100% ethanol for 5 days, followed by maceration in 1% KOH for 3-4 days for close observation. After that, they were cleared in 25, 50 and 80% glycerol in distilled water for 1 day for each step, and stored in 100% glycerol.

Alcian blue staining was carried out on day 3, 6, and 9 of micromass culture as follows. Cultures were washed twice with cold PBS, fixed in 100% ethanol for 5 min, and incubated with alcian blue solution overnight. Excess stain was washed off with de-ionized distilled water and photos were taken using a Zeiss Stemi SV11 microscope with a Nikon CoolPix 995 digital camera or Olympus SZX12 microscope with an Olympus DP12 camera.

2.2.3.8 Phytochemical analysis by HPLC

The characterization of four fractions of *A. galanga* hexane extract was carried out using a Shimazu CLASS-VP V5.02 with column ODS C18 (4.6 i.d x 250 mm) and UV-Vis Diode array as the detector, controlled by computer using the software provided by the manufacturer. Twenty microliters of each fraction were injected into the column using CH_3CN: H_2O (20:80) as mobile phase. The flow rate and retention times were 1 ml/min and 30 min.

2.2.3.9 Human synovial fibroblast and treatments

Non-inflammatory human synovium were acquired from the arthoscopic diagnosis of a flat pad syndrome patient at Maharaj Nakorn, Chiang Mai Hospital, (Department of Orthopedics, Faculty of Medicine, Chiang Mai University, Thailand). Synovial fibroblasts were dissociated from synovium by trypsin at 4°C overnight, followed by collagenase (Sigma type IA) at 37°C for 3 h. The cells were washed with PBS and grown in DMEM containing 10% FCS as high density primary monolayer cultures until confluent growth. The synovial fibroblasts at passage 4 were used in this study. Cells were distributed in 25 cm^3 culture flasks, grown to confluence, washed with PBS and starved in serum-free DMEM for 24 h. Then, the cells were treated with various concentrations of *A. galanga* hexane extract (0.1,1,10,20,50 μg/ml) from each fraction in the presence of 10 ng/ml human recombinant IL-1β for 24 h. After that, the cells and supernatants were collected for further experiments.

2.2.3.10 Viability assay

Cells were cultured at 37°C, 5%CO_2 overnight in flat-bottomed, 96-well tissue culture plates. The cells were treated with various concentrations of *A. galanga* hexane extract (0.1,1,10,20,50 μg/ml) from each fraction, and incubated for 24 h. Then, tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added into the wells and incubated for further 4 h at 37°C. Media was discarded, and the cells were incubated with DMSO for 10 min. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. Samples were read directly in the wells at wavelengths between 540 and 630 nm.

2.2.3.11 Gelatin zymography

MMPs in the conditioned medium were detected by gelatin zymography as previously described (230). The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel containing 0.1 mg/ml of gelatin (Sigma-Aldrich) under non-reducing conditions at 4°C. After electrophoresis, SDS in the gel was removed by rinsing with 2.5% Triton-X 100 pH 7.5. The gel was then incubated at 37°C in the incubating buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3) for 18 hr. After incubation, the gel was stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA) in 50% methanol /10% acetic acid, and destained with 10% acetic acid /50% methanol. The gelatinolytic activity was analyzed using Scion image software.

2.2.3.12 Gene expression analysis

RNA was extracted from monolayer cells using an Aurum total RNA purification kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacture's guidelines. 500 ng total RNA of each sample were reverse transcribed into complementary DNA (cDNA) using a RevertAid™ First Strand cDNA synthesis kit (MBI Fermentus, Germany). Primers and probe sets were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet Taqman® requirements and were designed to bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. The primer nucleotide sequences are shown in table 2.1.

The amplified products were electrophoresed on 2% (w/v) agarose gels, stained with ethidium bromide, imaged using a Bio-Rad Gel-Doc fluorescent image analyzer,

and integrated densities calculated using Scion image analysis software. The gene products were normalized to the house-keeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) to permit semi-quantitative comparisons in mRNA levels as previously described (231, 232).

Table 2.1 Primers for semi-quantitative RT-PCR.

Gene	Annealing temperature (°C)	Product size (base pairs)	Sequences (5'to 3')	GenBank accession number
MMP1	68	84	Forward: CTGTTCAGGGACAGAATGTGCT Reverse: TCGATATGCTTCACAGTTCTAGGG	NM_002421
MMP3	65	138	Forward: TTTTGGCCATCTCTTCCTTCA Reverse: TGTGGATGCCTCTTGGGTATC	NM_002422
MMP13	65	96	Forward: TCCTCTTCTTGAGCTGGACTCATT Reverse: CGCTCTGCAAACTGGAGGTC	NM_002427
MMP2	56	100	Forward: TCAAGTTCCCCGGCGAT Reverse: TGTTCAGGTATTGCACTGCCA	NM_004530.4
MMP9	56	100	Forward: TGAGAACCAATCTCACCGACAG Reverse: TGCCACCCGAGTGTAACCAT	NM_004994.2
COX2	55	424	Forward: TTCAAATGAGATTGTGGGAAAAT Reverse: AGATC-ATCTCTGCCTGAGTATCTT	NM_000963
GAPDH	60	225	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTTC	NM_002046