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# Epigenetic characterization of the PBEF and TIMP-2 genes in the developing placentae of normal mice

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**Reprogramming errors, which appear frequently in cloned animals, are reflected by aberrant gene expression. We previously reported the aberrant expression of TIMP-2 and PBEF in cloned placenta and differential expression of PBEF genes during pregnancy. To examine the epigenetic modifications that regulate dynamic gene expression in developing placentae, we herein analyzed the mRNA and protein expression levels of PBEF and TIMP-2 in the placentae of normal mice during pregnancy and then examined potential correlations with epigenetic modifications. DNA methylation pattern analysis revealed no difference, but ChIP assays using antibodies against H3-K9/K14 and H4-K5 histone acetylation revealed that the H3-K9/K14 acetylation levels, but not the H4-K5 acetylation levels, of the TIMP-2 and PBEF loci were significantly correlated with their gene expression levels during placentation in normal mice. These results suggest that epigenetic changes may regulate gene expression level in the developing placentae of normal mice and that inappropriate epigenetic reprogramming might be one cause of the abnormal placentae seen in cloned animals. [BMB reports 2011; 44(8): 535-540]**

## INTRODUCTION

Successful placental development is essential for the survival of the developing conceptus. The study of placental development is important for understanding the pathogenesis of diseases and the pregnancy failures seen among cloned animals (1, 2).

A major cause of pregnancy loss in cloned animals is placental abnormality; this may include significantly decreases in the placentome, placental overgrowth, accumulation of allantoic fluid, hydroplasia of the trophoblastic epithelium and reduced placental vascularization (2). In cloned mice, placentae tend to show placentomegaly, which appears to be caused by expansion of the spongiotrophoblast-layer, increases in the numbers of glycogen cells and the enlargement of trophoblastic cells (3). Researchers have speculated that placentomegaly is likely to reflect epigenetic abnormalities that may arise at least in part from inadequate nuclear reprogramming. Consistent with this hypothesis, recent studies have shown that errors in the epigenetic reprogramming of the somatic cell genome can result in the dysregulated expression of developmentally imprinted genes in cloned embryos, fetuses and placentae, leading to abnormalities in the resulting cloned animals (4-6). In this context, numerous groups have reported abnormal gene expression patterns related to somatic cell nuclear transfer (7-10). However, the existence, importance and regulation of abnormal gene expression through epigenetic modification have not yet been fully elucidated in the context of placentation.

Historically, the term "epigenetic" has been used to reflect changes in gene expression during development (11). Epigenetic modifications of the genome support a number of processes, including: accurate gene activation during development; the configuration of histones and histone variants into nucleosomes; and the remodeling of other chromatin-associated proteins, such as linker histones, polycomb group members, nuclear scaffold proteins and transcription factors (12). Changes in chromatin configuration, which are primarily determined by the acetylation/methylation status of histones and methylation of the genomic DNA, are very important for normal development. In particular, successful placental development depends on the precisely regulated expression of many genes and may be negatively influenced by the abnormal expression of developmentally significant genes (13). Abnormal gene expression patterns in the placenta, perhaps reflecting epigenetic errors, may lead to altered phenotypes of the placenta and potentially even the conceptus.

We previously reported the aberrant expression of TIMP-2 and PBEF in cloned bovine and mouse placentae, particularly at the end of gestation (8-10). TIMP-2 was related to trophoblastic invasion and extracellular matrix (ECM) remolding during pregnancy (14). High-levels of TIMP-2 secretion from binucleate giant cells in bovine placentae were found to inhibit the proteolytic activity of MMP-2 (matrix metalloproteinase-2),

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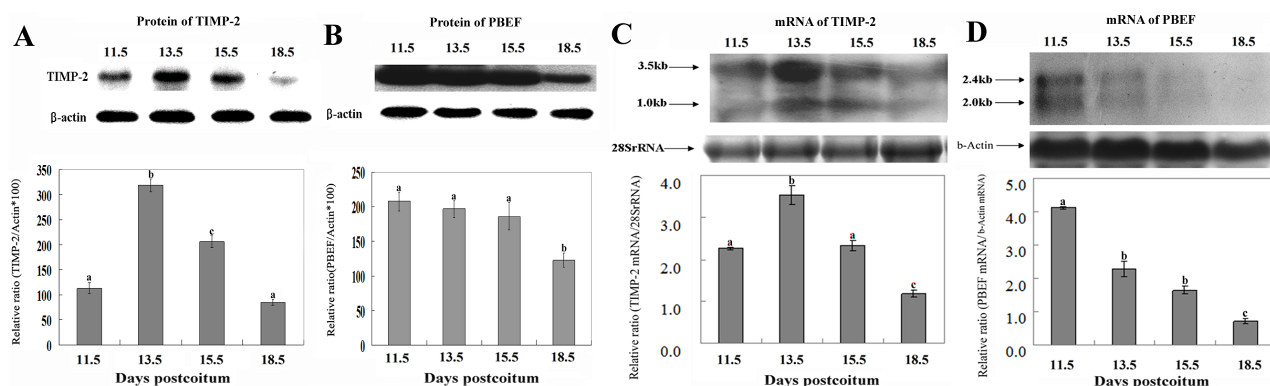


**Fig. 1.** Western and Northern blot analysis of TIMP-2 and PBEF gene expression in placentae during pregnancy. The 11.5, 13.5, 15.5 and 18.5 dpc were chosen in this experiment due to their physiological significances during mouse pregnancy such as rapid placenta growing, a massive increase of glycogen cells, Reichert's membrane breakdown and preparation of labor, respectively. (A) This figure shows protein expression with anti-TIMP-2 and β-actin antibodies from samples taken during placentation. (B) Representative bands detected by the anti-PBEF and -β-actin antibodies. TIMP-2 and PBEF protein levels are presented as a ratio of band intensity (C) TIMP-2 mRNA expressions. (D) PBEF mRNA expression. The results were normalized with respect to the levels of 28SrRNA and β-actin mRNA. Abbreviations: a, b, and c indicate $P < 0.05$. Error bars show the standard error (S.E.).

leading to decreased ECM degradation during the prepartum period (15). At the end of pregnancy, the number of binucleate giant cells decreases, as does TIMP-2 protein production (15). At the same time, enzymatic ECM degradation increases, the placenta detaches and fetal membranes are released (16). PBEF, which is believed to function at the proximal end of the labor-initiation pathway (17), was previously shown to be up-regulated following distension of human amniotic epithelial cells (18). These results indicate TIMP-2 and PBEF are probably important genes for implantation, placentation and labor. To examine the potential involvement of epigenetic modification in this process, we herein assessed the mRNA and protein levels of TIMP-2 and PBEF in normal mouse placentae during pregnancy and sought to correlate the observed changes with alterations in the DNA methylation and histone acetylation patterns at the promoter regions of these genes. The novel identification of these differentially expressed genes in placenta and their epigenetic regulation provide important new insights into the molecular mechanisms underlying placental development and may help researchers improve cloning efficiency in the future.

## RESULTS

### Protein and mRNA expression of TIMP-2 and PBEF in developing placenta

Western blotting was performed to examine the protein expression patterns of TIMP-2 and PBEF in samples of normal mouse placenta taken during various stages of pregnancy (11.5, 13.5, 15.5, and 18.5 days postcoitum [dpc]). A TIMP-2-immunoreactive band of the appropriate size (~24 kD) was found in all tested samples; TIMP-2 protein expression appeared to be highest at mid-gestation (13.5 dpc) and was significantly lower during late gestation (Fig. 1A). PBEF was detected at appropriately ~56 kDa in all tested samples; its expression levels gradually decreased from 11.5 to 18.5 dpc, but remained detectable throughout (Fig. 1B). Three placenta samples at each stage were analyzed with duplication of each sample in this experiment.

The relevant mRNA sequences were obtained from the NCBI database (NM_011594.3, TIMP-2; AF234625.1, PBEF) and Northern blot analysis was used to examine the mRNA expression patterns of TIMP-2 and PBEF during placentation. Northern blot analysis using a partial cDNA of TIMP-2 for the probe yielded positive signals from total RNA samples from 11.5 to 18.5 dpc placentae (Fig. 1C). The TIMP-2 mRNA expression levels appeared to change during this period, peaking at 13.5 dpc and decreasing thereafter. This pattern is consistent with that seen for TIMP-2 protein expression (Fig. 1A), indicating that TIMP-2 expression appears to be regulated at the transcriptional level in developing mouse placentae. Similarly, the mRNA expression levels of PBEF gradually decreased from 11.5 to 18.5 dpc (Fig. 1B), in a pattern that was consistent with the observed changes in PBEF protein expression (Fig. 1D).

### Epigenetic modification of the TIMP-2 and PBEF promoter regions during placentation

To examine whether the observed changes in TIMP-2 and PBEF expression could be associated with epigenetic changes, we examined the DNA methylation and histone acetylation levels of their encoding genes. We first used bisulfite sequencing to examine the DNA methylation levels in the gene promoters. Our results revealed that the TIMP-2 gene promoter region, which encompassed 641 bp and included 47 CpG sites (Fig. 2A), was largely unmethylated and did not show any significant change in methylation from 11.5 to 18.5 dpc (Fig. 2B). Similarly, we did not detect any significant change in the

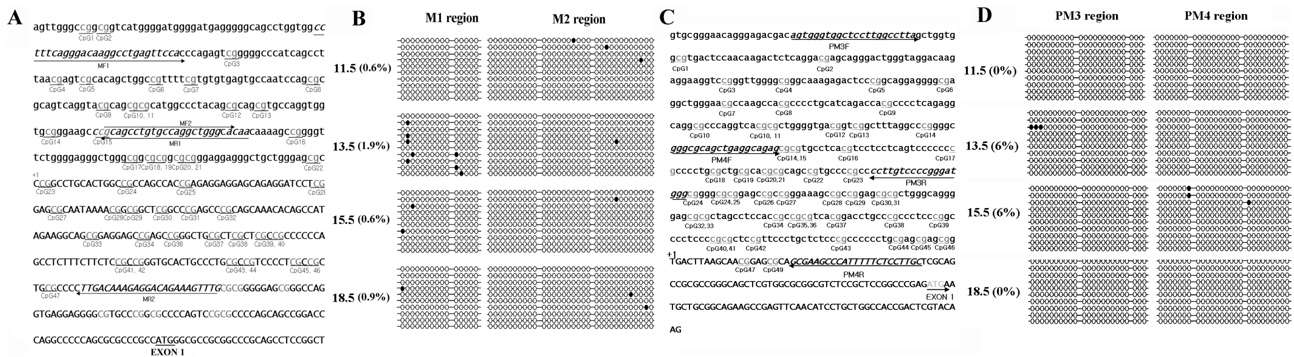


**Fig. 2.** Methylation patterns at the TIMP-2 and PBEF promoter region. (A) Positions of the 47 CpG dinucleotides within the 641-bp region upstream of the TIMP-2 transcription initiation site. (B) The methylation status of these CpG sites in mouse placentae at 11.5, 13.5, 15.5 and 18.5 dpc, as determined by bisulfite sequencing of at least ten independent PCR clones. (C) 49 CpG dinucleotides within the 652-bp region upstream of PBEF. (D) The methylation status of PBEF. Open circle: unmethylated; closed circle: methylated. Percentages indicate the proportion of the total available CpG sites that are methylated.

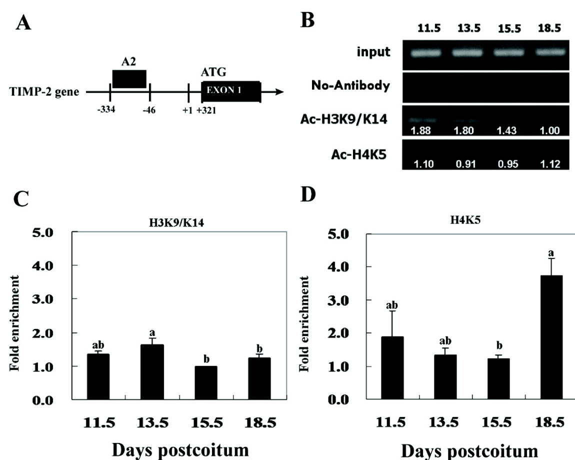


**Fig. 3.** Histone modification status at the TIMP-2 promoter region. (A) Schematic presentation of the TIMP-2 promoter region. Abbreviations: +1, the transcription start site; A2, analytic site for histone acetylation. (B) ChIP assays were performed for each stage of placentation using PCR and it is repeated for 3 times. (C) H3- K9/K14 acetylation of TIMP-2 from 11.5 dpc to 18.5 dpc, as assessed by real-time PCR. (D) The graph shows the H4-K5 acetylation status of TIMP-2. The minimum value was standardized to 1. Abbreviations: a, b, and ab indicate P < 0.05. Error bars show the standard error (S.E.).

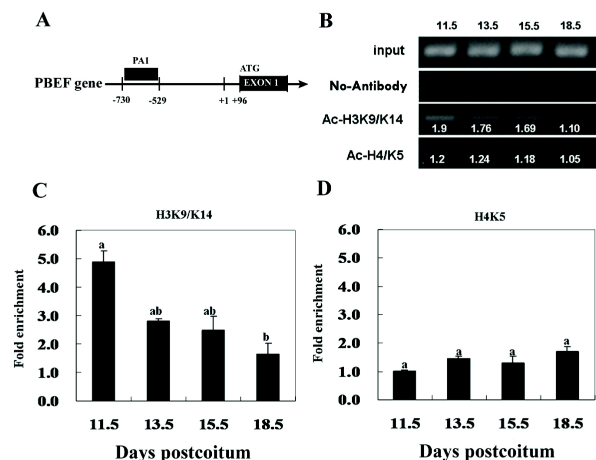


**Fig. 4.** Histone modification status at the PBEF promoter region. (A) Schematic presentation of the PBEF promoter region. Abbreviations: +1, the transcription start site; PA1, analytic site for histone acetylation. (B) ChIP assays shows for each stage of placentation using PCR and it is repeated for 3 times. (C) H3-K9/K14 acetylation of PBEF was assessed by real-time PCR. (D) The graph shows the H4-K5 acetylation status of the PBEF. The minimum value was standardized to 1. Abbreviations: a, b, and ab indicate P < 0.05. Error bars show the standard error (S.E.).

methylation status of the PBEF promoter during placentation (Fig. 2C, D). Also, no sequence mutations were identified in regulatory regions of TIMP-2 and PBEF genes.

Next, we used ChIP to examine the histone acetylation of the TIMP-2 and PBEF genes during normal gestation (Fig. 3, 4). Chromatin fragments were immunoprecipitated using antibodies against acetylated histone H3 (acetyl-lysines 9 and 14) and H4 (acetyl-lysine 5) and followed by PCR amplification of fragments representing the promoter regions of TIMP-2 or PBEF. The input means the fraction of total histone (modified and unmodified) in regulatory regions as a loading control. Our results revealed that the acetylation levels of H3-K9/K14 in the TIMP-2 gene were highest at 13.5 dpc and decreased thereafter (Fig. 3B-D), paralleling our findings for the mRNA and protein expression levels. In contrast, the H4-K5 acetylation of the TIMP-2 gene was significantly higher at 18.5 dpc than at 15.5 dpc (Fig. 3D), providing no evidence for a significant correlation. Similarly, the H3-K9/K14 acetylation levels for PBEF gradually decreased from 11.5 to 18.5 dpc in a manner consistent with our observations of the mRNA and protein levels (Fig. 4B, C). In contrast, no significant difference was found for the H4-K5 acetylation of PBEF during placentation (Fig. 4D).

These findings indicate that H3-K9/K14 acetylation, but not H4-K5 acetylation, appear to be important for TIMP-2 and PBEF gene activation in developing mouse placentae.

## DISCUSSION

We quantitatively determined the mRNA and protein expression patterns for TIMP-2 and PBEF in normal mouse placentae during development. Proper control of TIMP-2 secretion may be important for successful placentation and parturition. In cloned animals, we speculate that TIMP-2 overexpression at the end of gestation would lead to decreases in proteolytic activity and MMP-induced ECM degradation, perhaps explaining the dysfunctional and enlarged placentae seen in some cloned animals (19, 20). Here, we showed that TIMP-2 mRNA and protein expression was highest at mid-gestation (13.5 dpc). This might be due to the high number of giant cells found in the junctional zone between 12.5 dpc and 14.5 dpc (21). We further found that TIMP-2 expression decreased significantly from 13.5 dpc to 18.5 dpc, perhaps due to the gradual loss of binucleate cells that occurs toward the end of pregnancy, in preparation for parturition and placental release (21).

Here, we showed that the protein and mRNA expression levels of PBEF in normal mouse placentae gradually decreased from 11.5 to 18.5 dpc, but remained detectable throughout this period. Even though mRNA level at 18.5 dpc was below the detection level of Northern blotting RT-PCR amplified PBEF mRNA (data not shown). Similarly, a previous study found that PBEF is constitutively expressed in placentae and may play a role during normal pregnancy (22). These results suggest that PBEF is probably significant for proper placental function (17). Furthermore, the expression levels of PBEF increase when the fetal membranes are transiently distended (18). PBEF has been suggested to act as a growth regulator, facilitating the accommodation of the placenta tissue, thereby preventing it from rupturing prematurely when advancing gestation increasingly distends the membranes (16). In addition, PBEF was recently reported to have a local protective anti-apoptotic effect in fetal membranes during placentation (23, 24). Since distension of fetal membranes *in vivo* may increase apoptosis in the placenta (16), our present results and the previous reports may collectively suggest a model wherein PBEF expression in the developing mouse placenta could protect against the fetal membrane distension-induced apoptosis of placental cells.

Following cloning, the transferred nucleus must undergo epigenetic reprogramming in order to support successful development. Previous studies have shown that epigenetic reprogramming defects often occur in cloned embryos, as reflected by aberrant gene expression (6) and abnormal DNA methylation patterns (4-6). Furthermore, tissue-dependent differentially methylated regions (T-DMRs) within *Spalt-like gene 3* (Sall3) locus at the telomeric E3 subregion of mouse chromosome 18 was reported to be an epigenetic hotspot for aberrant DNA methylation associated with placentomegaly among cloned mice (25). Together, these previous findings indicate that aberrant gene expression in placentae may be due in part to DNA methylation abnormalities. Accordingly, we herein examined whether the changes we observed in TIMP-2 and PBEF expression across 11.5, 13.5, 15.5 and 18.5 dpc placentae could occur through epigenetic programming (i.e., changes in DNA methylation and/or histone acetylation in the promoter regions of the encoding genes). We did not observe any significant pregnancy-stage-related difference in the methylation statuses of the TIMP-2 and PBEF genes, indicating that the DNA methylation status does not appear to crucially influence TIMP-2 and PBEF gene expression in the developing mouse placenta. In contrast, ChIP assays revealed that the H3-K9/K14 acetylation levels of TIMP-2 and PBEF varied across the stages of placentation in patterns consistent with those of their protein and mRNA expression levels. This suggests that H3-K9/K14 histone acetylation may be important for regulation of TIMP-2 and PBEF gene expression in the developing mouse placenta. Thus, our present results support the notion that cloned placentae probably appear to suffer from failed reprogramming of histone modifications in developmentally important genes, leading to aberrant expression of their protein products and changes in TIMP-2 and PBEF expression are likely to be involved in the placental abnormalities seen among cloned mice and cows (e.g., enlargement and/or improper functionality) (8-10).

Although future work will be required to determine whether the differential expressions of TIMP-2 and PBEF in cloned placentae are regulated by epigenetic modification, the present study provides the first *in vivo* evidence that TIMP-2 and PBEF are regulated through epigenetic modification during mouse placentation.

## MATERIALS AND METHODS

### Placental samples

For isolation of mouse placentae, 6- to 8-week-old female ICR mice were housed with adult male mice of the same strain and examined daily for vaginal plugs. We designated noon of the day on which a vaginal plug was found as being 0.5 days post-coitum (dpc). Cesarean sections were performed at 11.5, 13.5, 15.5 and 18.5 dpc. Animals were treated according to a pre-approved protocol. All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University.

### Protein extraction and Western blot analysis

Each placental sample was mixed with an equal volume of lysis buffer A containing 1% SDS, 1 mM PMSF, protease inhibitor (Roche Diagnostics) and 100 mM Tris-HCl. The samples were sonicated for 15 sec according to the manufacturer's instructions. The resulting protein samples were treated with 100 U/ml endonuclease (Sigma) and the solubilized protein

extracts were quantified using a Bradford assay kit (Bio-Rad). Protein lysates (30 μg) were resolved by 12% SDS-PAGE, electro-transferred to PVDF membranes (Bio-Rad). Anti-mouse TIMP-2 (diluted 1 : 1,000; Abcam), anti-rabbit β-actin (diluted 1 : 5,000; Abcam) and anti-rabbit PBEF (diluted 1 : 10,000; Bethyl) anitibodies were used to measure the expression level of each protein.

### RNA extraction and Northern blot analysis

Total RNA was extract from placenta using the AccuZol$^{Tm}$ reagent (Bioneer) and 10 μg samples were resolved by electrophoresis on a 1% agarose/formaldehyde gel. The RNA was transferred to a nylon membrane and then fixed to the blot by UV-cross linking (a total of 1.5 J/cm$^2$). The RNA probes for TIMP-2, PBEF and β-actin were labeled with digoxigenin (DIG)-UTP using a DIG Northern kit (Roche diagnostics). Prehybridization and hybridization were carried out at 68°C using the DIG Easy Hyb hybridization reagent (Roche Diagnostics). Each blot was washed twice for 5 min in low-stringency buffer (2X SSC and 0.1% SDS) at room temperature and then twice for 15 min in high stringency buffer (0.1X SSC and 0.1% SDS) at 68°C. The mRNA species were then detected by the alkaline phosphatase reaction (Roche Diagnostics) and exposure to X-ray film (AGFA). The values given represent the ratio of the RNA-integrated optical density of TIMP-2 or PBEF versus that of β-actin.

### Isolation and bisulfite treatment of genomic DNA

Genomic DNA was isolated from normal mouse placentae using phenol/chloroform extraction and ethanol precipitation. Sodium bisulfate treatment of the purified genomic DNA (1 μg) was performed using MSP kit (In2Gen). The treated DNA was purified according to the protocol provided with the MSP kit, precipitated with ethanol and resuspended in 20 μl of distilled water. The TIMP-2 and PBEF promoter regions were PCR amplified using primers designed to convert the cytosines to uracils, as follows: for TIMP-2, MF1(F)5′-TTTTTTAGGGATAA-GGTTTGAGTTTTAT-3′ and MR1(R)5′-TTATACCCAACCTAAC-ACAAACTAC-3′; and MF2(F)5′-GTAGTTTGTGTAGGTTGGG-TTT-3′ and MR2(R)5′-CAAACTTTATATCCTCTTTATCAAAA-3′; for PBEF, PM3(F)5′-AGTGGGTGGTTTTTTGGTTTTA-3′ and PM3(R)5′-CCCATCCCRAAAACAAAA-3′; and PM4(F)5′-GGG-YGTAGTTGAGGTAGAG-3′ and PM4(R)5′-ACAAAAAAAAAA-ATAAACTTCRC-3′. Amplification was performed using an ExTaq Hot Start kit (Takara). The resulting PCR products were ligated into T-plasmids using the pGEMP®P-T Easy Vector System (Promega). Ten subclones were randomly picked and sequenced.

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as previously described (26) with the acetylated histone H3 (acetyl-lysines 9 and 14) and H4 (acetyl-lysine 5), since these modifications have been correlated with gene activation. The anti-acetyl-histone H3-K9/K14 and anti-acetyl-H4-K5 antibodies were obtained from Upstate. Tissues samples (20-40 mg/ml) were frozen, homogenized and then centrifuged. The pellets were resuspended in 1 ml of 4% formaldehyde in PBS, cross-linked at 37°C for 30 min and then centrifuged at 2,000 g for 5 min at 4°C. The resulting pellets were resuspended in SDS lysis buffer (150 mM NaCl, 25 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate; Upstate) per the manufacturer's instructions. The mixtures were sonicated four times for 10 sec each at 13% maximal power (Hielscher) to generate fragments of $<$ 500 bp in length, as determined by agarose gel electrophoresis. To perform ChIP, sonicated chromatin (150 μl) was diluted 10-fold, cleared with salmon sperm DNA/protein A-agarose (80 μl) and purified with specific antiserum (2-5 μl) and protein A-agarose (60 μl). The DNA from the bound chromatin after cross-linking reversal and proteinase K treatment was precipitated and diluted in 100 μl of low-TE buffer (1 mM Tris, 0.1 mM EDTA). Real-time PCR amplification was performed in triplicate using SYBR green on a Rotor Gene 2000 PCR machine (Cobett research), with the following primers: for TIMP-2(F)5′-TGTGTGGCTGCTTAGATTGC-3′ and TIMP-2(R)5′-CAG-TCTCACCTGCTGAGTGC-3′; for PBEF(F)5′-CTTTCCCTAAG-ACGCAAAGG-3′ and PBEF(R)5′-ACGATGGATGGAATCTTT-GG-3. The fold induction over input was calculated using the $2^{-\Delta\Delta C}$ T method (27).

### Statistical analysis

Significant differences among samples were determined by Duncan's multiple range tests following ANOVA analysis using the GLM found in the SAS package (SAS Institute Inc.). P values less than 0.05 were considered statistically significant.

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