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TITLE: MMP-8, a Breast Cancer Bone Metastasis Suppressor Gene

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| <b>14. ABSTRACT:</b><br><br>In order to study the role of MMP-8 on inhibition of cancer growth and progression, we initiated our work to clone the human MMP-8 cDNA and express it <i>in vitro</i> . The MMP-8 cDNA with a V5 epitope tag was cloned downstream into the CMV promoter vector. The construct was verified by sequencing. But the expression level of MMP-8 was not detected by Western blot analysis. The molecular mechanisms of how TGF-β1 mediates stimulation of invasion and formation of bone metastasis have yet to be completely determined. ATF-3 (activating transcription factor-3) was strongly stimulated and its level was sustained by TGF-β1 in highly invasive and bone metastatic breast cancer cells. We have identified for the first time that cyclin A1 and MMP-13 are ATF-3 target genes. ATF-3 also regulates Runx2 (a bone specific transcription factor) in human breast cancer cells and that may provide a molecular phenotype for ATF-3 to regulate its target genes associated with bone metastasis. |                         |                                |                                   |                                                                  |                                                  |
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## Introduction:

**TGF- $\beta$  Signaling** TGF- $\beta$  (transforming growth factor-beta), a multipotent cytokine has a wide range of physiological and pathological effects (1, 2). TGF- $\beta$  is the most potent known growth inhibitor for epithelial cells (3). Mice with targeted disruption of the *Tgfb1* gene develop carcinomas (4). In the breast, loss of TGF- $\beta$  antiproliferative and apoptotic responses may compromise the turnover of the mammary epithelium, thus favoring tumor formation (5-7). TGF- $\beta$  signaling involves the type I receptor T $\beta$ R-I, the type II receptor T $\beta$ R-II, the regulatory Smads (Smad2 and Smad3), and Smad4 (8). Most of these components have mutations in several human cancers. But, mutations in TGF- $\beta$  receptors or Smads are rare in breast cancer (9, 10). Moreover, for breast cancer cells, TGF- $\beta$ 1 is a crucial molecule in metastatic breast cancer stimulating invasion (11, 12) and formation of TGF- $\beta$ -dependent bone metastases in model systems (13).

Advances have been made in understanding how TGF- $\beta$  signals inhibit cell division in normal epithelial cells. The progress of the cell cycle is regulated by the sequential expression of cyclins, followed by the activation of their associated cyclin-dependent kinases (cdks). A number of specific cyclins have been isolated and characterized in mammalian cells, and their temporal patterns of expression have been mapped to specific phases of the cell cycle.

**ATF-3** Independent observations over the years have defined a small group of immediate TGF- $\beta$  target genes that contribute to the effect of TGF- $\beta$  on epithelial cell homeostasis and the importance that its disruption has in cancer (5, 13). Aberrant expression of the AP-2 transcription factor has been linked to the progression of human breast cancer (14). A selective loss of c-myc transcription factor repression by TGF- $\beta$ 1 has been shown in a highly invasive and bone metastatic human breast cancer cell line (MDA-MB231) (15). There is growing evidence indicating that transcription factors such as GADD153, Twist, Runx2, Stat3, NRIF3, TBX3, NF kappaB, DEC1 (16-22) have the ability to alter the progression of breast cancer growth and metastasis and thus, transcription factors are major targets for cancer therapy (23).

ATF-3 (activating transcription factor-3), a member of the ATF/CREB subfamily is a bZip transcription factor (24-27). ATF-3 is expressed at very low levels in normal, quiescent cells but can be rapidly and highly induced in different cell types by multiple and diverse extracellular signals (28-30, 31). ATF-3 is a common target of TGF- $\beta$ 1 and stress signals and serves to inhibit cell growth in normal epithelial cells (24). Oligonucleotide microarray analysis showed that TGF- $\beta$ 1 prolonged expression of ATF-3 in breast cancer cells (15). Although the biological functions of ATF-3 have yet to be completely elucidated, there is strong circumstantial evidence that this transcription factor plays an important role in the regulation of normal and neoplastic growth responses. To date, only a few target promoters for ATF-3 (*gadd153/CHOP10*, *cyclin D1* and ATF-3 itself) (30, 32-34) have been identified. The presence of potential ATF-3 binding sites in the promoter regions of other cyclins (35) and of Rb itself (36, 37) suggests that several additional cell cycle-related genes may be subject to regulation by ATF-3.

**MMP-13** The matrix metalloproteinases (MMPs) are a family of enzymes that are important for tissue remodeling. MMPs, however, also contribute to pathological conditions including rheumatoid arthritis, coronary artery disease, and cancer (38-42). Tumor cells utilize the matrix degrading capability of these enzymes to spread to distant sites. In addition, MMPs promote the growth of these tumor cells once they have metastasized such as lung, brain, and bone. TGF- $\beta$ 1 stimulates MMP-13 (collagenase-3; an invasive and metastasis gene) expression in MDA-MB231 cells and these cells are known to form bone metastases (13, 43, 44). MMP-13 is over-expressed in a variety of malignant tumors (45-49). MMP-13-driven extracellular matrix (ECM) proteolysis may support cancer cell growth both biochemically, by exposing mitogenic factors, and physically, by providing space for the proliferating cells. A greater understanding of the regulatory mechanisms of MMP expression is necessary and will provide several new avenues for therapeutic intervention in controlling breast cancer cell growth, invasion, and metastasis.

In order to investigate the role played by ATF-3 in breast cancer cell growth and metastasis, we first examined TGF- $\beta$ 1 regulation of ATF-3 in MCF-10A (normal human mammary epithelial cells) and MDA-MB-231 cells (invasive and bone metastatic human breast cancer cells). TGF- $\beta$ 1 stimulated expression of ATF-3, c-Jun, and JunB in both MCF-10A and MDA-MB231 cells but ATF-3 and c-Jun levels were sustained in MDA-MB231 (Fig. 1). There was no significant change in the level of JunD expression by TGF- $\beta$ 1 after normalization with  $\alpha$ -tubulin expression in MCF-10A and MDA-MB231 cells.

### Body:

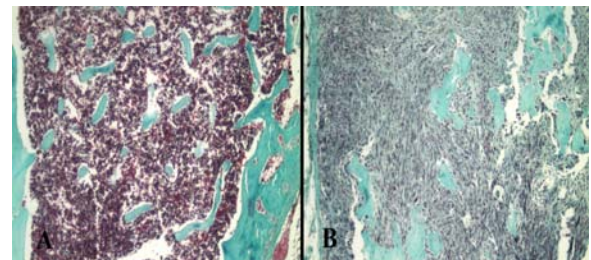
The specific aim of this proposal was to test if overexpression of MMP-8 in breast cancer cells will contribute to a less aggressive phenotype in breast cancer cells which have metastasized to bone. In order to study the role of MMP-8 on inhibition of cancer growth and progression, we proposed to utilize a transgenic mouse model to overexpress MMP-8 under the control of the bone specific osteocalcin promoter. The osteocalcin promoter has been shown to confer differentiated osteoblast- and post-specific expression to a reporter gene *in vivo*. To generate transgenic mice overexpressing MMP-8, we first initiated our work to clone the human MMP-8 cDNA (1.4 kbps) and express it *in vitro*. We used pcDNA3.1 Directional TOP Expression construct (Invitrogen) for this purpose. The pcDNA3.1 contains the following elements: human cytomegalovirus (CMV) immediate-early promoter/enhancer that permits efficient, high-level expression of recombinant protein and V5 epitope that allows detection of recombinant protein with anti-V5 antibody. The MMP-8 cDNA with a V5 epitope tag was cloned downstream into the CMV promoter sequence. The construct was sequenced to verify cloning of the MMP-8 cDNA insert in frame. The construct was transfected into COS-7 cells using the GeneJammer according to the guidelines provided by the company. Cells were lysed and subjected to Western blot analysis. The expression of V5-epitope tagged MMP-8 protein was not detected by the V5-epitope antibody.

One of our collaborators (Dr. Susan Rittling) generated a series of metastatic murine mammary epithelial cell lines using normal mice rather than using nude mice. Cardiac injection of mouse mammary pad tumor cell line r3T into 129 strain female mice leads to development of bone metastases (50). To get expertise in the techniques of cardiac injection of cancer cells into the mice and tumor analysis, we utilized those cancer cells with normal mice.

**Fig 1. Osteolytic bone metastases.** Female mice were sacrificed three weeks after left ventricle injection of medium alone or medium containing breast cancer cells (r3T). Bones were dissected and cleaned of soft tissues, and visualized by X-ray. Arrows indicate regions of bone loss.

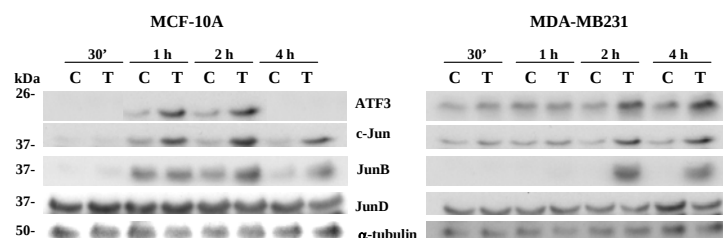


**Fig 2. Histological appearance of metastatic tumor cells.** Bones were decalcified in EDTA, embedded in paraffin and stained with Gomori trichrome. (A) distal femur, control mouse. (B) distal femur, mouse with osteolytic metastasis. Note replacement of entire marrow with tumor cells.



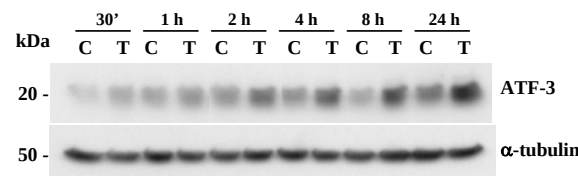
TGF- $\beta$ 1 is the most potent known growth inhibitor for epithelial cells. In breast tissue, loss of TGF- $\beta$ 1 anti-proliferative response favors tumor formation. Moreover, in breast cancer cells, TGF- $\beta$ 1 is a crucial molecule for stimulation of invasion and formation of bone metastases. The molecular mechanisms of how TGF- $\beta$ 1 mediates these effects have yet to be completely determined. In my laboratory, we have found a defect in repression of ATF-3 (activating transcription factor-3) expression by TGF- $\beta$ 1 in bone metastatic human breast cancer (MDA-MB231) and mouse mammary pad tumor (r3T) cells.

**Fig 3. TGF- $\beta$ 1 stimulates expression of ATF-3, c-Jun, and JunB.** MCF-10A (normal human mammary cells) and MDA-MB231 cells were treated with control or TGF- $\beta$ 1 (1 ng/ml)-containing media for the indicated times. Total lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure.  $\alpha$ -tubulin represents the loading control.



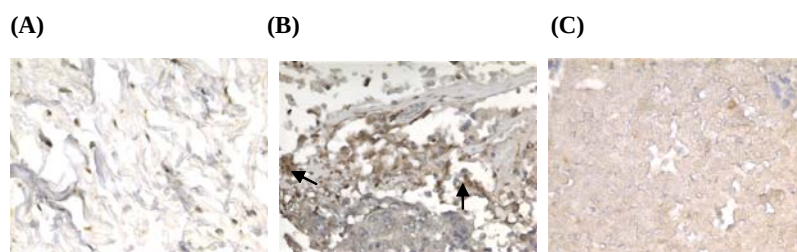
TGF- $\beta$ 1 stimulated expression of ATF-3 and its level was sustained even at 24 h in r3T cells (Fig. 4). In contrast, in normal murine mammary glandular epithelial cells (NMuMG), ATF-3 expression peaked at 2 h after TGF- $\beta$ 1 and then declined (data not shown). *In normal mammary epithelial cells, TGF- $\beta$ 1 stimulates a transient expression of ATF-3; whereas in metastatic mammary epithelial cells, TGF- $\beta$ 1 stimulates a sustained expression of ATF-3. The sustained and prolonged expression of ATF-3 may lead to alterations in homo- and heterodimerization of AP-1 family members and other proteins, which could activate the genes that participate in breast cancer progression.*

**Fig 4. TGF- $\beta$ 1 stimulates ATF-3 expression.** Mouse mammary pad tumor cells (r3T) were treated with control or TGF- $\beta$ 1 (1 ng/ml)-containing media for the indicated times. Total lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure.  $\alpha$ -tubulin represents the loading control.



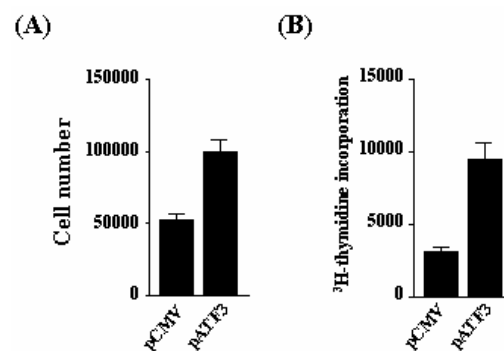
We wanted to detect the level of ATF-3 protein in breast tissues obtained from normal and cancer patients. Tissue microarray slides containing normal human breast tissues and human primary breast cancer tissues (Imgenex, CA) were processed for immunohistochemical staining (a kit from LabVision, CA) with ATF-3 antibody. The results (Fig. 5) clearly indicated that ATF-3 expression is very low in normal breast tissue and is high in breast cancer tissues.

**Fig 5. ATF-3 expression in human primary breast carcinomas.** Histospot staining in tissue microarrays include low level staining in normal breast tissue (A) and strong staining in breast cancer tissues (B and C) of two different patients (40X magnification). ATF-3 protein is represented by brown color staining.



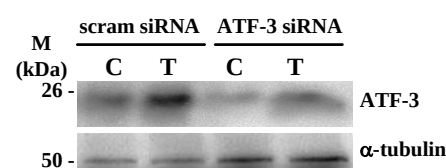
Since ATF-3 is highly expressed in human breast cancer cells (Fig. 1) and mouse mammary pad tumor cells (Fig. 2) and human primary tumors (Fig. 3), we wanted to first determine whether overexpression of ATF-3 is sufficient to induce cellular proliferation *in vitro*. We transiently transfected the ATF-3 eukaryotic expression plasmid (pCMV-ATF-3) into normal human mammary epithelial cells (MCF-10A). The empty eukaryotic expression plasmid (pCMV) was also transfected as a control for transfection effects. The cells were counted using a haemocytometer after 6 days (~80% confluency). In parallel, cells were also pulsed with 0.5  $\mu$ Ci/ml [ $^3$ H]thymidine for 4 h before harvesting and assessing radioactivity. The results indicated that overexpression of ATF-3 increases normal human mammary epithelial cell number (Fig. 6A) and DNA synthesis (Fig. 6B) over control (empty vector).

**Fig 6. ATF-3 increases normal human mammary epithelial cell growth.** (A) Cells were trypsinized, harvested, and counted by haemocytometer. (B) Cell proliferation in terms of DNA synthesis was assessed by measuring the incorporation of  $^3\text{H}$ -thymidine into cells for a 4 h period. Cells were harvested onto glass fiber filters using an automated cell harvester, and counted in a Packard liquid scintillation counter. Data represent mean  $\pm$  S.E. of three experiments.



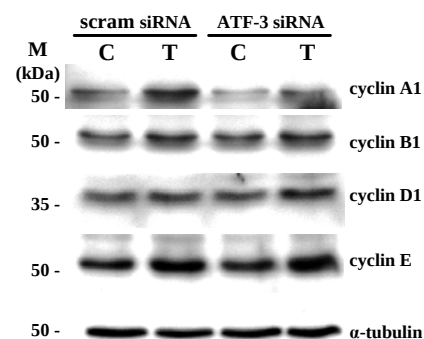
To determine the functional role of ATF-3 in breast cancer progression, we used the RNA interference technique for *in vivo* depletion of a gene product. The hairpin oligonucleotides that target ATF-3 (ATF-3 siRNA) or nonspecific sequences (scrambled siRNA) were cloned into the psiSTRIKE U6 hairpin vector (Promega). Transient transfection of MDA-MB231 cells with the psiSTRIKE vector that contained hairpin oligonucleotides with a human ATF-3 target sequence decreased both the basal and TGF- $\beta$ 1-stimulated ATF-3 expression, compared with the nonspecific target sequences (Fig. 7).

**Fig 7. ATF-3 siRNA reduces both basal and TGF- $\beta$ 1-stimulated ATF-3 expression.** MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA vectors for 24 h and then treated with control or TGF- $\beta$ 1 (1 ng/ml)-containing media for 4 h. Total lysates were prepared and subjected to Western blot analysis using the ATF-3 and  $\alpha$ -tubulin (loading control) antibodies.



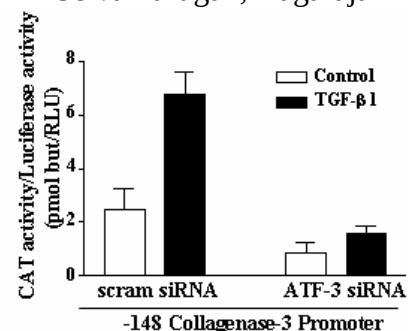
Since TGF- $\beta$  loses its antiproliferative activity in breast cancer cells, we determined whether knockdown of ATF-3 expression has any effect on expression of the cell cycle genes. As shown in Fig. 8, TGF- $\beta$ 1 stimulated expression of cyclin A1, -B1, -D1, and -E in these cells while ATF-3 siRNA only decreased expression of cyclin A1 in both control and TGF- $\beta$ 1-stimulated MDA-MB231 cells. Thus, ATF-3 must be the mediator of TGF- $\beta$ 1-stimulation of cyclin A1 and cyclin A1 is likely to be an ATF-3 target gene. Cyclin A1, an alternative A-type cyclin that is essential for spermatogenesis contributes cell cycle progression from G1 to S phase (51) and is also expressed in hematopoietic progenitor cells and in acute myeloid leukemia.

**Fig 8. ATF-3 siRNA reduces both basal and TGF- $\beta$ 1-stimulated cyclin A1 expression.** MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA plasmids for 24 h and then treated with control or TGF- $\beta$ 1 (1 ng/ml)-containing media for 24 h. Total lysates were prepared and subjected to Western blot analysis using the antibodies as indicated.  $\alpha$ -tubulin was a loading control.



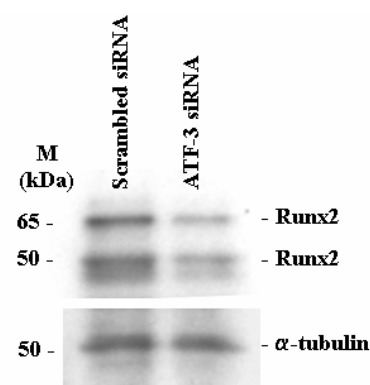
Since MDA-MB231 cells are highly invasive and bone metastatic in nature and TGF- $\beta$ 1 stimulates MMP-13 (an invasive and metastatic gene) (43) and ATF-3 expression in these cells, we next determined whether MMP-13 is a target gene for ATF-3. The -148 MMP-13 promoter that contains 148 base pairs upstream of the transcription initiation site retains the TGF- $\beta$ -responsive region (44). The -148 MMP-13 promoter fused with a reporter gene, chloramphenicol acetyl transferase (CAT) was transiently transfected with either scrambled siRNA or ATF siRNA constructs into MDA-MB231 cells. As shown in Fig. 9, TGF- $\beta$ 1 stimulated MMP-13 promoter activity and ATF-3 siRNA reduced both the control and TGF- $\beta$ 1-stimulated MMP-13 promoter activity in these cells. Hence, the MMP-13 gene (another potential ATF-3 target gene) is regulated by TGF- $\beta$ 1 via ATF-3.

**Fig 9. ATF-3 siRNA reduces both the basal and TGF- $\beta$ 1-stimulated MMP-13 promoter activity.** The wild type MMP-13 promoter construct (-148) was transiently cotransfected with either scrambled siRNA or ATF-3 siRNA plasmids into MDA-MB231 cells for 24 h and then treated with control or TGF- $\beta$ 1 (1 ng/ml)-containing media for 24 h. Lysates were prepared and assayed for CAT activity. Renella luciferase was used to normalize the transfection efficiency. Data represent mean  $\pm$  S.E. of three experiments.



MDA-MB231 cells are highly bone metastatic in nature. Runx2, a bone specific transcription factor responsible for expression of bone marker genes including MMP-13 is regulated by TGF- $\beta$ 1 in these cells (44). Hence, we next determined whether Runx2 could be a target gene for ATF-3. MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA vectors and lysates were prepared and subjected to Western blot analysis. As shown in Fig. 10, ATF-3 siRNA decreased expression of Runx2 proteins (65 kDa and 50 kDa) in human breast cancer cells, indicating that Runx2 may act as a direct ATF-3 target gene.

**Fig 10. ATF-3 regulation of Runx2.** MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA plasmids for 24 h. Total lysates were prepared and subjected to Western blot analysis using the Runx2 antibody.



### Key Research Accomplishments:

- The construct pCMV-V5-MMP-8 was made.
- Cardiac injection of tumor cells into mice and histology of bone metastasized cells were standardized.
- TGF- $\beta$ 1 stimulated prolonged and sustained expression of ATF-3 protein in the human breast cancer cell line MDA-MB231 and in the mouse mammary gland cancer cell line r3T.
- TGF- $\beta$ 1 stimulated expression of cyclin A1, B1, D1 and E in MDA-MB231 cells while ATF-3 siRNA decreased only expression of cyclin A1 in both control and TGF- $\beta$ 1-stimulated MDA-MB231 cells.
- ATF-3 must be the mediator of TGF- $\beta$ 1-stimulation of cyclin A1 and Cyclin A1 is likely to be an ATF-3 target gene.
- TGF- $\beta$ 1 stimulated MMP-13 promoter activity and ATF-3 siRNA reduced both the control and TGF- $\beta$ 1-stimulated MMP-13 promoter activity in MDA-MB231 cells.
- MMP-13 is another potential ATF-3 target gene and regulated by TGF- $\beta$ 1 via ATF-3.
- ATF-3 siRNA decreased expression of Runx2 proteins, suggesting that Runx2 is another target gene for ATF-3.

### Reportable Outcomes:

### Manuscript:

1. Parathyroid hormone stimulation and PKA signaling of latent transforming growth factor-beta binding protein-1 (LTBP-1) mRNA expression in osteoblastic cells.

S. Kwok, L. Qin, N. C. Partridge, and N. Selvamurugan (2005) \* corresponding author

Journal of Cellular Biochemistry **95**:1002-11



2. Overexpression of Runx2 directed by the matrix metalloproteinase-13 promoter containing the AP-1 and Runx/RD/Cbfa sites alters bone remodeling in vivo.

**N. Selvamurugan**, Jefcoat, S. Jr., S. Kwok, Y. Yang, R. Kowalewski, J. Tamasi, and N. C. Partridge (2006) Journal of Cellular Biochemistry, April 25 (Epub ahead of print)

### Abstract:

TGF- $\beta$ 1 Regulation of ATF-3 and its Target Genes in Bone Metastasizing Breast Cancer Cells.

Presented at the 27<sup>th</sup> Annual meeting of American Society for Bone and Mineral Research, Nashville, TN, on September 23-27, 2005.

### Conclusions:

1. The application of a transgenic mouse model will contribute greatly to the understanding of the pathogenesis of bone metastasis. Identification of the exact nature of these tumor-bone interactions may not only generate valuable information on underlysing regulatory mechanisms in invasion and bone metastasis but can also be of value in the development of therapeutic strategies.
2. We are the first to identify the TGF- $\beta$ 1-regulation of ATF-3 and its target genes, cyclin A1, MMP-13, and Runx2 in bone metastasizing breast cancer cells. The dysregulation of ATF-3 by TGF- $\beta$ 1 in breast cancer cells may be key to the subsequent metastasis of these cells to bone.

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## Appendices:

1. Parathyroid hormone stimulation and PKA signaling of latent transforming growth factor-beta binding protein-1 (LTBP-1) mRNA expression in osteoblastic cells.

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2. Overexpression of Runx2 directed by the matrix metalloproteinase-13 promoter containing the AP-1 and Runx/RD/Cbfa sites alters bone remodeling in vivo.

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# Parathyroid Hormone Stimulation and PKA Signaling of Latent Transforming Growth Factor- $\beta$ Binding Protein-1 (LTBP-1) mRNA Expression in Osteoblastic Cells

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**Abstract** Parathyroid hormone (PTH) regulates bone remodeling and calcium homeostasis by acting on osteoblasts. Recently, the gene expression profile changes in the rat PTH (1–34,  $10^{-8}$ M)-treated rat osteoblastic osteosarcoma cell line, UMR 106-01, using DNA microarray analysis showed that mRNA for LTBP-1, a latent transforming growth factor (TGF- $\beta$ )-binding protein is stimulated by PTH. Latent TGF- $\beta$  binding proteins (LTBPs) are required for the proper folding and secretion of TGF- $\beta$ , thus modifying the activity of TGF- $\beta$ , which is a local factor necessary for bone remodeling. We show here by real time RT-PCR that PTH-stimulated LTBP-1 mRNA expression in rat and mouse preosteoblastic cells. PTH also stimulated LTBP-1 mRNA expression in all stages of rat primary osteoblastic cells but extended expression was found in differentiating osteoblasts. PTH also stimulated TGF- $\beta$ 1 mRNA expression in rat primary osteoblastic cells, indicating a link between systemic and local factors for intracellular signaling in osteoblasts. An additive effect on LTBP-1 mRNA expression was found when UMR 106-01 cells were treated with PTH and TGF- $\beta$ 1 together. We further examined the signaling pathways responsible for PTH-stimulated LTBP-1 and TGF- $\beta$ 1 mRNA expression in UMR 106-01 cells. The PTH stimulation of LTBP-1 and TGF- $\beta$ 1 mRNA expression was dependent on the PKA and the MAPK (MEK and p38 MAPK) pathways, respectively in these cells, suggesting that PTH mediates its effects on osteoblasts by several intracellular signaling pathways. Overall, we demonstrate here that PTH stimulates LTBP-1 mRNA expression in osteoblastic cells and this is PKA-dependent. This event may be important for PTH action via TGF- $\beta$  in bone remodeling. *J. Cell. Biochem.* 95: 1002–1011, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** PTH; TGF- $\beta$ ; LTBP-1; osteoblast; PKA signaling

Abbreviations used: PTH, parathyroid hormone; LTBP-1, latent transforming growth factor-beta binding protein-1; TGF- $\beta$ , transforming growth factor-beta; ECM, extracellular matrix; SLC, small latent TGF- $\beta$  complex; LLC, large latent TGF- $\beta$  complex; LAP, latency associated protein; FBS, fetal bovine serum; RT-PCR; reverse transcriptase polymerase chain reaction; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C.

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Parathyroid hormone (PTH) is one of the major calcitropic hormones affecting serum calcium levels and bone remodeling [Tam et al., 1982; Dobnig and Turner, 1997; Swarthout et al., 2002]. PTH acts by binding to the PTH1R, a G-protein-coupled receptor on osteoblasts, resulting in functional changes in the actions of both osteoblasts [Bellows et al., 1990] and osteoclasts [Kanzawa et al., 2000; Qin et al., 2004]. The molecular mechanisms regulating the activities of both cells by PTH are still not completely known.

There is growing evidence that growth factors and cytokines released from bone matrix play important roles in the coupling of bone resorption to bone formation and in repair processes such as fracture healing. Bone extracellular matrix (ECM) is the major storage site in the body for transforming growth factor-beta (TGF- $\beta$ ), which is a multipotent cytokine [Seyedin et al., 1985; Hauschka et al., 1986]. TGF- $\beta$  is

synthesized as a homodimeric pro-protein and the dimeric pro-peptide is cleaved intracellularly from the growth factor [Taipale et al., 1994]. The TGF- $\beta$  propeptide binds to TGF- $\beta$ , and the proteins are secreted as a complex [Annes et al., 2003]. In this small latent complex (SLC), TGF- $\beta$  cannot bind to its surface receptors. Therefore, the propeptide is called the latency associated protein (LAP). The SLC is secreted by bone cells [Bonewald et al., 1991], chondrocytes [Pedrozo et al., 1999], kidney cells [Marra et al., 1996], and prostate cells [Dallas et al., 2005]. The dissociation or activation of TGF- $\beta$  from LAP is a critical regulatory event as all TGF- $\beta$  is secreted in a latent form. The LAP dimer is usually disulfide bonded to a second gene product, latent TGF- $\beta$  binding protein (LTBP), and the trimolecular aggregate is called the large latent complex (LLC) [Rifkin, 2005].

A major mechanism for storage of secreted latent TGF- $\beta$  in bone matrix is via its association with the latent TGF- $\beta$  binding protein-1 (LTBP-1) [Taipale et al., 1994; Dallas et al., 1995; Dallas et al., 2000]. LTBP is a member of the LTBP/fibrillin protein family, which comprises fibrillin-1, fibrillin-2 and fibrillin-3, and LTBP-1, LTBP-2, LTBP-3, and LTBP-4 [Ramirez and Pereira, 1999; Oklu and Hesketh, 2000]. LTBPs are required for the proper folding and secretion of TGF- $\beta$ , thus modifying the activity of TGF- $\beta$  [Miyazono et al., 1991]. In human and rats, *LTBP-1* appears as two mRNA species, which encode for two different NH<sub>2</sub>-terminal variants, the longer LTBP-1L having a 346 amino acid extension not present in the shorter LTBP-1S isoform [Kanzaki et al., 1990; Saharinen et al., 1999]. Both isoforms possess their own, independent promoter regions, capable of regulating the tissue type specific expression of *LTBP-1* isoforms [Koski et al., 1999]. The LLC-containing the LTBP-1L is found in ECM [Kanzaki et al., 1990]; whereas the LLC-containing the LTBP-1S is found in platelets [Wakefield et al., 1988]. This ECM-bound TGF- $\beta$  stored in a latent form can be released and activated by resorbing osteoclasts [Oreffo et al., 1989; Oursler, 1994]. Once released from the matrix and activated, TGF- $\beta$  can influence inhibition of osteoclast activity, osteoblast proliferation, and stimulation of production of bone ECM proteins [Hughes et al., 1996; Roberts, 1998; Bonewald, 1999]. TGF- $\beta$  has therefore been implicated as a coupling factor that coordinates the processes of bone resorption and subsequent bone formation.

The rat osteoblastic cell line, UMR 106-01 is a useful model system for studying the effects of PTH on osteoblastic cells in vitro. Recently, the gene expression profile changes in these cells treated with rat PTH (1–34,  $10^{-8}$ M) using DNA microarray analysis have been published [Qin et al., 2003]. LTBP-1 expression was stimulated by PTH in these cells. Since PTH stimulates LTBP-1 mRNA expression and that controls the activity of TGF- $\beta$ , LTBP-1 seems to be a mediator in controlling PTH action on osteoblasts via TGF- $\beta$ . In this study, we show PTH stimulation of LTBP-1 mRNA expression in the mouse and rat osteoblastic cell lines and in proliferating, differentiating, and mineralizing rat primary osteoblasts. We have also identified the signaling pathways used by PTH in stimulation of LTBP-1 mRNA and TGF- $\beta$ 1 mRNA expression in rat osteoblastic cells.

## MATERIALS AND METHODS

### Materials

Rat PTH (1–34) and human TGF- $\beta$ 1 were purchased from Sigma, St. Louis, MO and Promega, Madison, WI, respectively. Synthetic oligonucleotides were synthesized by Invitrogen, Carlsbad, CA. Tissue culture medium and reagents were also obtained from Invitrogen. The MEK1/2, p38 MAPK, JNK, PKA, and PKC inhibitors were purchased from Calbiochem, San Diego, CA. All other chemicals were obtained from Sigma.

### METHODS

#### Cell Culture

The rat osteoblastic cells (UMR 106–01) and the mouse preosteoblastic cells (MC3T3) were maintained in monolayer in Eagle's minimal essential medium (with Earle's salts; EMEM) supplemented with nonessential amino acids, 25 mM HEPES (pH 7.3), 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

#### Rat Primary Osteoblastic Cells

Rat primary osteoblasts were isolated by the method of Shalhoub et al. [1992]. Osteoblasts were derived from postnatal day 1 rat calvariae by sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A, 0.25% trypsin. Cells from digests one and two were discarded.

Cells from the third digest were plated at  $6.4 \times 10^3$  cells/cm<sup>2</sup> and grown in minimal essential medium (MEM) supplemented with 10% FBS. After reaching confluence (day 7), the medium was switched to BGJ<sub>b</sub> with 10% FBS containing 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate to allow for initiation of differentiation and mineralization. Medium changes were performed every 2 days. The determination of proliferating, differentiating, and mineralizing stages of osteoblasts has been established by demonstration of alkaline phosphatase activity, osteocalcin production, alizarin red staining, and a sensitive adenyl cyclase response to PTH [Shalhoub et al., 1992; Winchester et al., 1999].

#### Total RNA Isolation and Real Time Reverse Transcriptase-PCR

The rat osteoblastic cells and the mouse preosteoblastic cells were treated with either rat PTH (1–34,  $10^{-8}$ M) or human TGF-β1 (1 ng/ml) or both together for different time periods. To determine de novo protein synthesis, cells were pretreated with cycloheximide (30 µg/ml) for 1 h before PTH treatment. To determine the signaling pathways, cells were pretreated with DMSO, PD98059, SB203580, SP600125, H89, or GF109203X for 20 min before PTH treatment. Cells were rinsed once with 10 ml of cold (4°C) PBS, pH 7.4, and harvested. Total RNA was isolated using the QIAGEN RNeasy Mini kit. Reverse transcription was carried out using TaqMan reverse transcription reagents (Roche Applied Science, Indianapolis, IN). PCRs were performed using a real time PCR DNA Opticon Engine (MJ Research, Inc., Watertown, MA) according to the manufacturer's instructions, which allow real time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. Each analysis was performed three to four times with independent sets of cells. The data are represented as mean ± SEM. Statistical analysis was performed by Student's *t*-test. Primers for rat LTBP-1, TGF-β1, MMP-13, and β-actin were designed using Primer Express software (PerkinElmer Life Sciences). The sequences of the above primers were as follows: *LTBP-1*: Forward, 5'-CGTGGCTGGAATGGACAATG, Reverse, 5'-TGGTCTGGTGTGGGGCTGTA; *TGF-β1*: Forward, 5'-TTAGGAAGGACCTGGGTTGGA, Reverse, 5'-ACTGTGTGTCCAGGCTCCA-

AAT; *MMP-13*: Forward, 5'-GCCCTATCCCTTGATGCCATT, Reverse, 5'-ACAGTTCAGGCTCAACCTG; *β-actin*: Forward, 5'-TCCTGAGCGCAAGTACTCTGTG, Reverse, 5'-CGGACTCATCGTACTCCTGCTT.

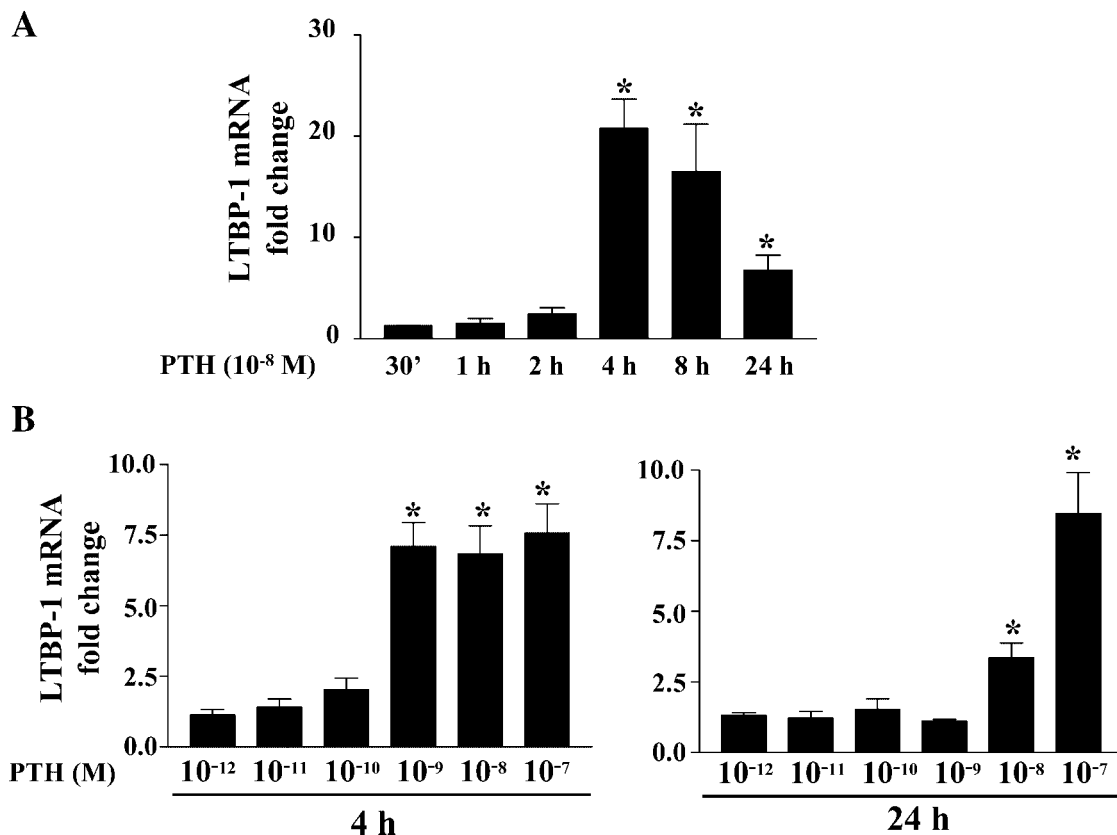
## RESULTS

### PTH Stimulates LTBP-1 mRNA Expression

To study the effect of PTH on expression of LTBP-1 in the rat osteoblastic osteosarcoma line UMR 106-01, cells were treated with rat PTH (1–34) either for different time periods with  $10^{-8}$ M concentration (Fig. 1A) or at different concentrations for 4 and 24 h (Fig. 1B). Total cellular RNAs were purified and analyzed by real time RT-PCR using specific primers for rat LTBP-1 and β-actins. As shown in Figure 1A, LTBP-1 mRNA expression was maximally stimulated (20-fold) by  $10^{-8}$ M PTH at 4 h in UMR 106-01 cells and was still significant at 24 h. A wide range of PTH concentrations from  $10^{-9}$  to  $10^{-7}$ M stimulated LTBP-1 mRNA expression at 4 h in these cells but with  $10^{-7}$  and  $10^{-8}$ M PTH concentration, the fold stimulation of LTBP-1 mRNA expression was maintained out to 24 h (Fig. 1B).

### PTH Stimulates LTBP-1 mRNA Expression in Mouse Preosteoblastic and Rat Primary Osteoblastic Cells

We next determined PTH-stimulated LTBP-1 mRNA expression in other osteoblastic cells. The MC3T3 mouse preosteoblastic cells were treated with control or rat PTH (1–34,  $10^{-8}$ M)-containing media for 1, 4, 12, and 24 h. Total RNA was isolated and examined for LTBP-1 mRNA expression by real time RT-PCR analysis. PTH significantly stimulated LTBP-1 mRNA expression at 4 h in these cells (Fig. 2A) but the fold stimulation was less than that seen in UMR 106-01 cells (Fig. 1A). To determine expression of PTH-regulated LTBP-1 in rat primary osteoblastic cells, proliferating, differentiating, and mineralizing cells were treated with either control or rat PTH (1–34,  $10^{-8}$ M)-containing media for different times. Total RNA was isolated and subjected to examination for LTBP-1 mRNA expression by real time RT-PCR analysis. Our results (Fig. 2B) show that LTBP-1 mRNA expression was stimulated by PTH in proliferating (1 h) and mineralizing (4 h) osteoblasts. In differentiating osteoblasts, LTBP-1 mRNA expression was



**Fig. 1.** Effect of PTH on expression of *LTBP-1* mRNA levels in the rat osteoblastic cell line, UMR 106-01. **A:** Time course of the PTH stimulation of *LTBP-1*. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10<sup>-8</sup>M) for different times as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. **B:** Concentration-dependence of the PTH stimulation of *LTBP-1*

mRNA. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing PTH at different concentrations as indicated for 4 or 24 h. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.

found at 1 h and its level persisted up to 4 h with PTH-treatment.

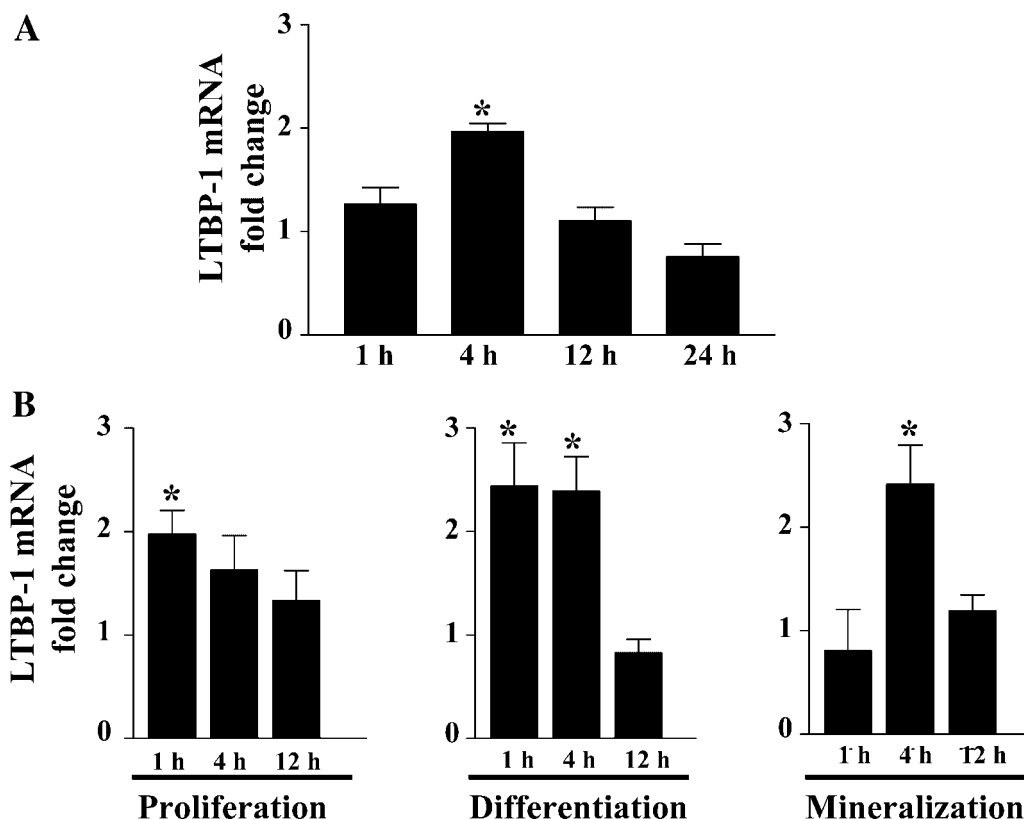
#### PTH Stimulation of *LTBP-1* mRNA Expression Is a Primary Effect

To determine whether the PTH-mediated increase in *LTBP-1* mRNA is a primary response, UMR 106-01 cells were treated with control medium or medium containing rat PTH (1–34, 10<sup>-8</sup>M) for 4 h in the presence or absence of 30  $\mu$ g/ml cycloheximide added 1 h before treatment. Total RNA was subjected to real time RT-PCR analysis using specific primers for rat *LTBP-1* and  $\beta$ -actin. As shown in Figure 3A, cycloheximide did not inhibit PTH induction of *LTBP-1* mRNA expression, indicating that the PTH stimulation of *LTBP-1* expression is a primary effect and de novo protein synthesis is not required for this purpose. In fact, cyclohex-

imide increased the PTH-response, indicating the inhibition of de novo synthesis of repressor proteins for this effect. As a positive control for cycloheximide treatment, we analyzed mRNA expression of MMP-13 (matrix metalloproteinase-13; collagenase-3) in UMR 106-01 cells (Fig. 3B). We have previously shown that the PTH stimulation of MMP-13 expression is a secondary effect in these cells [Scott et al., 1992].

#### PTH Stimulates TGF- $\beta$ 1 mRNA Expression and Both PTH and TGF- $\beta$ 1 Have an Additive Effect on *LTBP-1* mRNA Expression

Since LTBP-1 is required for the proper folding and secretion of TGF- $\beta$  [Miyazono et al., 1991], the increased *LTBP-1* expression caused by PTH may be correlated with increased expression of TGF- $\beta$ . Hence, we determined if



**Fig. 2.** Effect of PTH on expression of *LTBP-1* mRNA levels in mouse preosteoblastic cells and in rat primary osteoblastic cells. **A:** MC3T3 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for different time periods as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. **B:** Osteoblasts derived from postnatal day 1, rat calvariae were grown in 6-well plates in MEM, 10% FBS to confluence (day 7), after which the cells were switched to differentiation and mineralizing medium (BGJb, 10% FBS, 50  $\mu$ g/

ml ascorbic acid, and 10 mM  $\beta$ -glycerophosphate). Proliferating, differentiating, and mineralizing osteoblasts were treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 1, 4, and 12 h at days 7, 14, and 21 of culture. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.

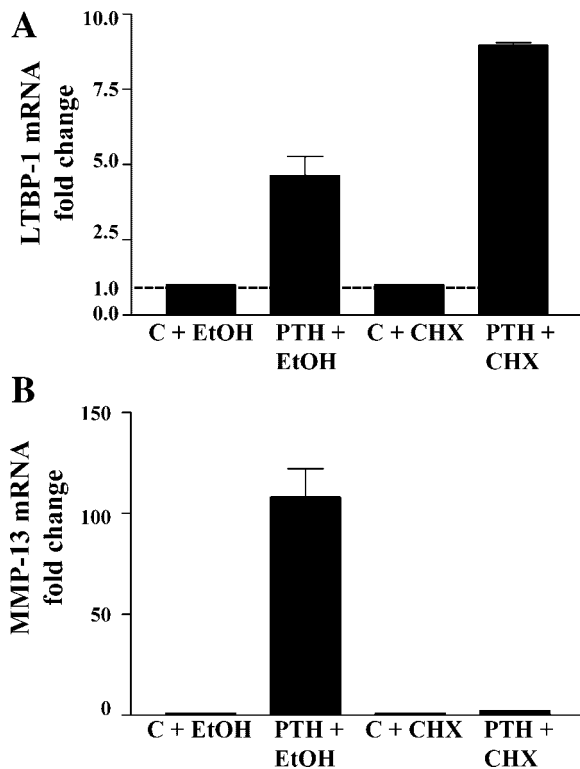
PTH stimulated TGF- $\beta$ 1 mRNA expression in rat primary osteoblastic cells treated with rat PTH (1–34,  $10^{-8}$ M) for different times during the three stages of differentiation. The results (Fig. 4) indicate that PTH stimulated TGF- $\beta$ 1 mRNA expression in differentiating and mineralizing osteoblasts at 4 h treatment indicating that osteoblasts respond to PTH and synthesize TGF- $\beta$ 1 only at these stages. Since PTH stimulates mRNA expression of both *LTBP-1* and TGF- $\beta$ 1 mRNAs, we wanted to determine whether there is a synergistic effect with combined treatment with PTH and TGF- $\beta$ 1 on *LTBP-1* expression in rat osteoblastic cells. UMR 106-01 cells were treated with human TGF- $\beta$ 1 (1 ng/ml), rat PTH (1–34,  $10^{-8}$ M) or both together at different time periods. Total RNA was isolated and subjected to real time RT-PCR analysis.

TGF- $\beta$ 1 and PTH stimulated *LTBP-1* mRNA expression to  $3.5 \pm 1.1$ -fold and  $11.7 \pm 2.1$ -fold, respectively at 4 h and to  $1.4 \pm 0.2$ -fold and  $7.3 \pm 2.4$ -fold, respectively at 24 h in UMR 106-01 cells. When cells were treated with TGF- $\beta$ 1 and PTH together, an additive effect was observed at both 4 h ( $17.1 \pm 1.6$ -fold) and 24 h ( $9.5 \pm 2.7$ -fold) in these cells (Fig. 5).

#### PTH Stimulation of *LTBP-1* mRNA Expression Is Dependent on the PKA Signaling Pathway

To identify the signaling pathways in PTH-stimulated *LTBP-1* mRNA expression, we used MAPK, PKA, and PKC pathway inhibitors. UMR 106-01 cells were pretreated with DMSO, PD98059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNKII inhibitor), H89 (PKA inhibitor), or GF109203X (PKC





**Fig. 3.** PTH-stimulated *LTBP-1* mRNA expression is a primary effect. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 4 h in the presence or absence of 30  $\mu$ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. **B:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 24 h in the presence or absence of 30  $\mu$ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat *MMP-13* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls.

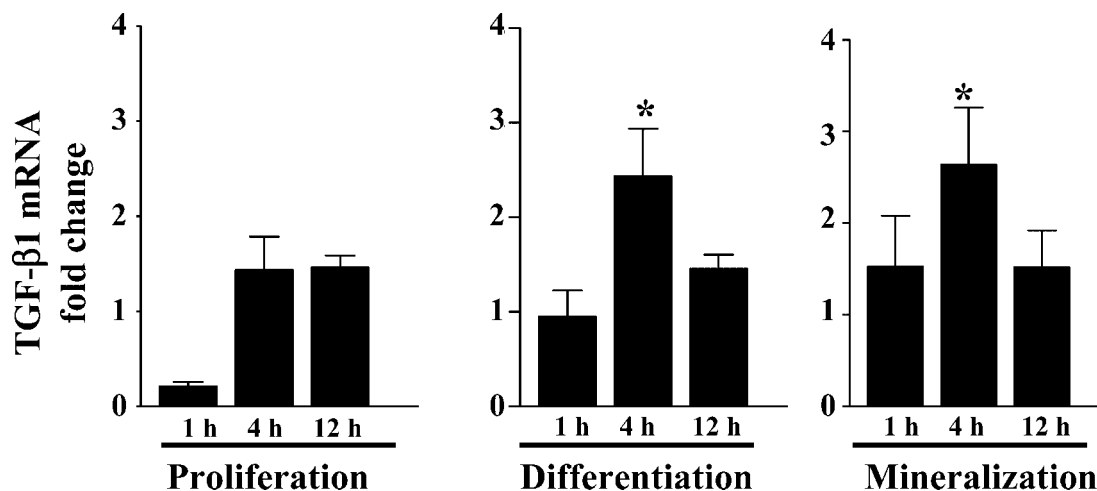
inhibitor) for 20 min, then treated with or without rat PTH (1–34,  $10^{-8}$ M) for 4 h. Total RNA was isolated and real time RT-PCR was performed. The PTH-stimulated *LTBP-1* mRNA expression was not significantly decreased by MAPK and PKC inhibitors, suggesting that the MEK, p38 MAPK, JNK, and PKC signaling pathways are not involved in PTH stimulation of *LTBP-1* mRNA expression in UMR 106-01 cells. The PKA inhibitor, H89 inhibited PTH-stimulated *LTBP-1* mRNA expression in these cells (Fig. 6). The effective concentrations and specificity of these inhibitors have been previously determined [Selvamurugan et al., 2002; Selvamurugan et al., 2004].

#### PTH Stimulation of TGF- $\beta$ 1 mRNA Expression Is a Secondary Effect and Is Dependent on the MAPK Signaling Pathway

Since PTH stimulated TGF- $\beta$ 1 mRNA expression in rat osteoblastic cells (Fig. 4), we wanted to determine whether this stimulation is a primary effect and if it requires the PKA signaling pathway as we found for PTH stimulation of *LTBP-1* mRNA expression in UMR 106-01 cells (Figs. 3A and 6). UMR 106-01 cells were treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 4 h in the presence or absence of 30  $\mu$ g/ml cycloheximide added 1 h before treatment. Total RNA was subjected to real time RT-PCR analysis using specific primers for rat TGF- $\beta$ 1 and  $\beta$ -actin. As shown in Figure 7A, cycloheximide inhibited PTH induction of TGF- $\beta$ 1 mRNA expression, indicating that PTH stimulation of TGF- $\beta$ 1 expression is a secondary effect and de novo protein synthesis is required for this purpose. To identify the signaling pathways in PTH-stimulated TGF- $\beta$ 1 mRNA expression, we used MAPK, PKA, and PKC pathway inhibitors. Similar to Figure 6, UMR 106-01 cells were pretreated with DMSO, PD98059, SB203580, SP600125, H89, or GF109203X for 20 min, then treated with or without rat PTH (1–34,  $10^{-8}$ M) for 4 h. Total RNA was isolated and real time RT-PCR was performed. The PTH stimulation of TGF- $\beta$ 1 mRNA expression was not significantly decreased by JNK, PKA, and PKC inhibitors; whereas MEK and p38 MAPK inhibitors inhibited PTH-stimulated TGF- $\beta$ 1 mRNA expression in rat osteoblastic cells (Fig. 7).

#### DISCUSSION

The gene expression profile changes in UMR 106-01 cells treated with rat PTH (1–34,  $10^{-8}$ M) using DNA microarray analysis showed that *LTBP-1* is one of the genes stimulated by this hormone [Qin et al., 2003]. We report here that PTH stimulates *LTBP-1* mRNA expression in rat osteoblastic and mouse preosteoblastic cells (Figs. 1 and 2). *LTBP-1* is required for the proper folding and secretion of TGF- $\beta$  [Miyazono et al., 1991], which is a local factor produced by both osteoblasts and osteoclasts [Pfeilschifter and Mundy, 1987; Bonewald and Dallas, 1994]. Bone ECM is the major storage site in the body for TGF- $\beta$  [Seyedin et al., 1985; Hauschka et al., 1986]. This ECM-bound TGF- $\beta$ ,



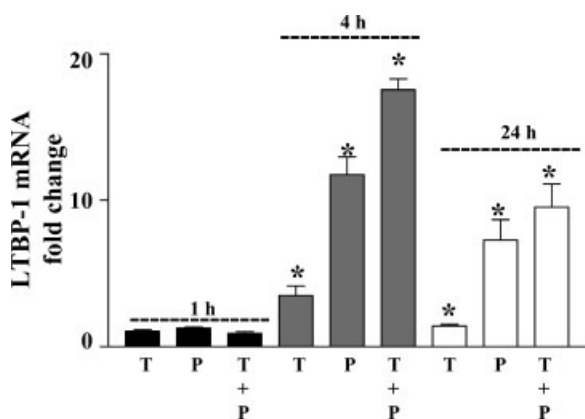
**Fig. 4.** Effect of PTH on expression of *TGF-β1* mRNA levels in rat primary osteoblastic cells. Proliferating, differentiating, and mineralizing rat primary osteoblasts were treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 1, 4, and 12 h at days 7, 14, and 21 of culture. Total RNA was isolated

and subjected to real time RT-PCR using specific primers for rat *TGF-β1* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.

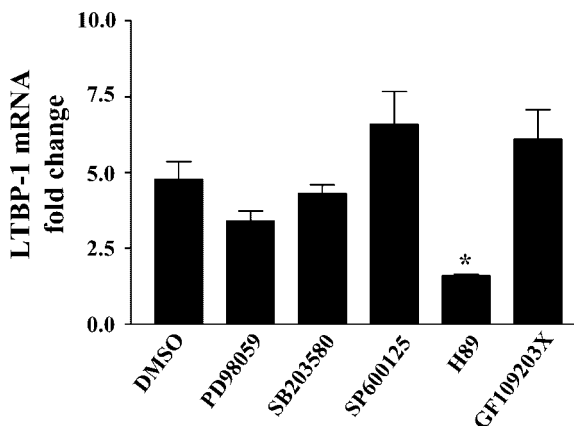
which is predominantly the *TGF-β1* isoform, is stored in a latent form and can be released and activated by resorbing osteoclasts [Oreffo et al., 1989; Oursler, 1994]. Several mechanisms for the activation of latent *TGF-β* complexes have been well documented [Munger et al., 1997; Koli et al., 2001].

*TGF-β* can influence many of the steps in the bone remodeling pathway. It can both inhibit [Hughes et al., 1996] and stimulate osteoclast

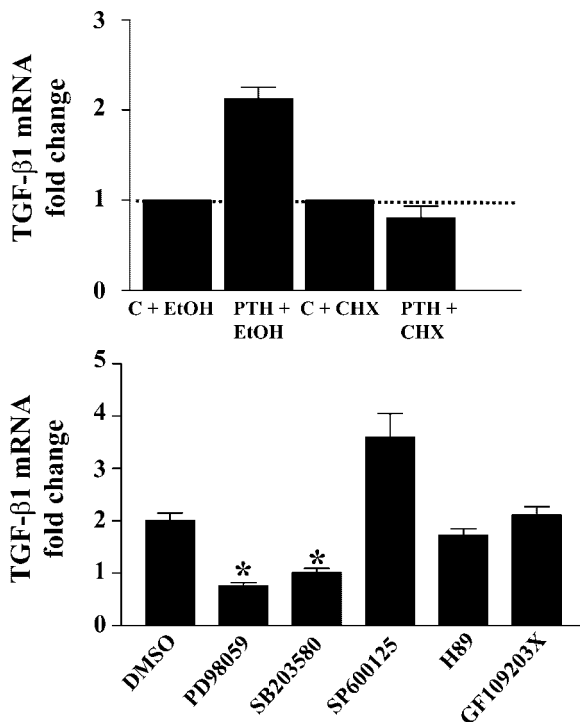
activity [Horwood et al., 1999; Sells Galvin et al., 1999] depending on conditions. *TGF-β* inhibits osteoclast activity, both by stimulating osteoclasts to undergo apoptosis and by inhibiting formation of osteoclasts from their precursors. *TGF-β* is also a powerful chemoattractant and mitogen for osteoblast precursors [Bonewald, 1999]. The effect of *TGF-β* on mature osteoblasts is then to inhibit proliferation and stimulate production of bone ECM proteins, including



**Fig. 5.** Effect of PTH and *TGF-β1* on expression of *LTBP-1* mRNA levels in rat osteoblastic cells. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) (P), human *TGF-β1* (1 ng/ml) (T), or both together for 1, 4, and 24 h. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.



**Fig. 6.** PTH-stimulated *LTBP-1* mRNA expression depends on the PKA signaling pathway. UMR 106-01 cells were serum-starved for 24 h and then treated with control or rat PTH (1–34,  $10^{-8}$ M)-containing medium for 4 h in the presence or absence of inhibitors PD98059, SB203580, SP600125, H89, and GF109203X (added 20 min before PTH). Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.



**Fig. 7.** PTH-stimulated TGF- $\beta$ 1 mRNA expression requires de novo protein synthesis and depends on the MEK and p38 MAPK signaling pathways. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34,  $10^{-8}$ M) for 4 h in the presence or absence of 30  $\mu$ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat TGF- $\beta$ 1 and  $\beta$ -actin. **B:** UMR 106-01 cells were serum-starved for 24 h and then treated with control or rat PTH (1–34,  $10^{-8}$ M)-containing medium for 4 h in the presence or absence of inhibitors PD98059, SB203580, SP600125, H89, and GF109203X (added 20 min before PTH). Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat TGF- $\beta$ 1 and  $\beta$ -actin. The relative levels of mRNAs were normalized to  $\beta$ -actin, and the PTH-fold changes were calculated over controls. The asterisks represent  $P < 0.05$  compared with control.

type I collagen, fibronectin, and osteocalcin [Roberts, 1998]. TGF- $\beta$  has therefore been implicated as a coupling factor that coordinates the processes of bone resorption and subsequent bone formation. A functional role for LTBP in regulating the local activity of TGF- $\beta$  has emerged using antibodies to LTBP-1 [Miyazono et al., 1991]. TGF- $\beta$ 1 also induces expression of its own mRNA as well as expression of LTBP-1 [Dallas et al., 1994; Roberts, 1998]. This is consistent with our results that TGF- $\beta$ 1 stimulates LTBP-1 mRNA expression in rat primary osteoblastic cells (Fig. 4). We report here that PTH stimulated expression of both LTBP-1 and TGF- $\beta$ 1 in rat osteoblastic cells and this effect may be required to maintain the level of LTBP

and its bound latent TGF- $\beta$  in bone matrix. It is most likely that the PTH stimulation of LTBP-1 expression in osteoblasts has an effect on osteoclasts via TGF- $\beta$ . It is possible that LTBP-1 may play an important role to link signaling between the systemic (PTH) and local (TGF- $\beta$ ) factors. This may be one of the PTH regulatory mechanisms that is necessary for maintaining the balance between osteoblastic and osteoclastic activity.

The PTH effect on LTBP-1 expression is a primary effect thus, not requiring de novo protein synthesis (Fig. 3A). This result supports the fact that, by association of TGF- $\beta$  with the ECM, it is stored in a readily mobilized form, which could allow extracellular signaling to proceed rapidly in the absence of new protein synthesis. This event is particularly important in situations such as tissue repair following injury. PTH and TGF- $\beta$ 1 stimulated LTBP-1 mRNA expression (Fig. 5) and both together had an additive effect on LTBP-1 mRNA expression, indicating that PTH and TGF- $\beta$  may have separate intracellular components to activate *LTBP-1* gene expression. It is well documented that PTH mediates its effects by the PTH1R, and TGF- $\beta$  mediates its effects by TGF- $\beta$  type II and type I receptors [Attisano and Wrana, 1998; Massague and Wotton, 2000; Swarthout et al., 2002; Derynck and Zhang, 2003; Qin et al., 2004]. Even though PTH activates both PKA and PKC signaling pathways [Swarthout et al., 2002; Qin et al., 2004], we identified that the PKA signaling pathway is responsible for PTH-stimulated LTBP-1 mRNA expression in UMR 106-01 cells (Fig. 6). The requirement of the PKA signaling pathway for PTH stimulation of LTBP-1 mRNA expression (Fig. 6) and the requirement of de novo protein synthesis and the MEK and p38 MAPK signaling pathways for PTH-stimulated TGF- $\beta$ 1 mRNA expression (Fig. 7) suggest that PTH mediates its effects on osteoblasts by several intracellular signaling components in rat osteoblastic cells. In summary, we provide evidence for PTH stimulated and PKA-dependent LTBP-1 mRNA expression in osteoblastic cells, which appears to be important for PTH regulation of the TGF- $\beta$  system to mediate bone remodeling activities.

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# Overexpression of Runx2 Directed by the Matrix Metalloproteinase-13 Promoter Containing the AP-1 and Runx/RD/Cbfa Sites Alters Bone Remodeling In Vivo

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**Abstract** The activator protein-1 (AP-1) and runt domain binding (Runx/RD/Cbfa) sites and their respective binding proteins, c-Fos/c-Jun and Runx2 (Cbfa1), regulate the rat matrix metalloproteinase-13 (MMP-13) promoter in both parathyroid hormone (PTH)-treated and differentiating osteoblastic cells in culture. To determine the importance of these regulatory sites in the expression of MMP-13 in vivo, transgenic mice containing either wild-type (–456 or –148) or AP-1 and Runx/RD/Cbfa sites mutated (–148A<sub>3</sub>R<sub>3</sub>) MMP-13 promoters fused with the *E. coli lacZ* reporter were generated. The wild-type transgenic lines expressed higher levels of bacterial  $\beta$ -galactosidase in bone, teeth, and skin compared to the mutant and non-transgenic lines. Next, we investigated if overexpression of Runx2 directed by the MMP-13 promoter regulated expression of bone specific genes in vivo, and whether this causes morphological changes in these animals. Real time RT-PCR experiments identified increased mRNA expression of bone forming genes and decreased MMP-13 in the tibiae of transgenic mice (14 days and 6 weeks old). Histomorphometric analyses of the proximal tibiae showed increased bone mineralization surface, mineral apposition rate, and bone formation rate in the transgenic mice which appears to be due to decreased osteoclast number. Since MMP-13 is likely to play a role in recruiting osteoclasts to the bone surface, decreased expression of MMP-13 may cause reduced osteoclast-mediated bone resorption, resulting in greater bone formation in transgenic mice. In summary, we show here that the 148 bp upstream of the MMP-13 transcriptional start site is sufficient and necessary for gene expression in bone, teeth, and skin in vivo and the AP-1 and Runx/RD/Cbfa sites are likely to regulate this. Overexpression of Runx2 by these regulatory elements appears to alter the balance between the bone formation-bone resorption processes in vivo. *J. Cell. Biochem.* 9999: 1–12, 2006. © 2006 Wiley-Liss, Inc.

**Key words:** bone formation; bone remodeling; bone resorption; Runx2; collagenase-3; matrix metalloproteinase-13

Bone is the body's main reservoir of calcium and phosphate ions, and is constantly regenerated through continuous formation and resorption in the process of bone remodeling. This physiological process occurs throughout adult

life to maintain a constant bone mass. In several pathological conditions, the tight balance between bone formation and resorption is not preserved [Giusti and Krane, 1998]. Bone formation in vivo is a complex phenomenon

Abbreviations used: MMPs, matrix metalloproteinases; AP-1, activator protein-1; RD, runt domain binding site; Cbfa, core binding factor alpha; AML, acute myeloid leukemia; ECM, extracellular matrix; PTH, parathyroid hormone; RT-PCR, reverse transcriptase-polymerase chain reaction; BSP, bone sialoprotein; OC, osteoclast; ALP, alkaline phosphatase; OPN, osteopontin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappaB ligand.

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whereby recruitment and replication of mesenchymal precursors of osteoblasts, differentiation into preosteoblasts, osteoblasts, and mature osteoblasts ultimately results in the accumulation and mineralization of the extracellular matrix [Aubin, 1998]. Osteoclasts responsible for bone resorption are derived from hematopoietic precursor cells belonging to the monocyte/macrophage lineage. Osteoclast differentiation is a multistep process that leads eventually to multinucleated bone-resorbing osteoclasts [Roodman, 1999].

Runx2, also called Cbfa1 or Pebp2αA, is a transcription factor that belongs to the runt-domain gene family [Komori and Kishimoto, 1998]. Runt proteins are a group of transcription factors conserved from *C. elegans* to humans. They share a typical 128-amino acid DNA-binding domain called the Runt domain. Runx2 acts as an inducer of osteoblast differentiation and it has all the characteristics of a differentiation regulator in the osteoblast lineage [Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Karsenty, 1999]. Bone formation is not solely controlled at the level of osteoblast differentiation and Runx2 is also required for osteoblast function [Ducy et al., 1999]. Runx2 is able to induce both early and late markers for osteoblast differentiation, including alkaline phosphatase (ALP), type I collagen, osteopontin (OPN), bone sialoprotein, and osteocalcin (OC) in several cell lines [Harada et al., 1999]. Genes involved in the bone resorption process, such as RANKL, OPG, and MMP-13 are also regulated by Runx2 [Geoffroy et al., 2002].

MMP-13, a matrix metalloproteinase, is expressed as a late-differentiation gene in osteoblasts, and is primarily responsible for the degradation of extracellular bone matrix components (type I, II, and III fibrillar collagens). MMP-13 gene expression is regulated by bone-resorbing agents, such as PTH, cytokines such as interleukin-1 and -6, and growth factors that promote bone turnover [Scott et al., 1992; Varghese et al., 1995, 1996, 2000; Kusano et al., 1998]. In vivo, MMP-13 has been shown to be expressed in ossifying centers during bone development [Schorpp et al., 1995] and is detectable by immunohistochemistry in rat calvariae 14 days after birth [Partridge et al., 1995]. The regulation of this gene is likely to have important consequences for both normal and pathological remodeling of bone where the balance

between bone resorption and bone formation is disrupted. Using mutant mice homozygous for a targeted mutation in *Col1a1* that are resistant to collagenase cleavage of type I collagen, Zhao et al. [1999] showed that PTH-induced bone resorption and calcemic responses were markedly diminished. The number of osteoclasts was also reduced and the animals had thicker than normal bones [Zhao et al., 2000]. Studies with a null mutation of the MMP-13 gene in mice showed that in *Mmp13*<sup>-/-</sup> embryos, the growth plates were strikingly lengthened, a defect ascribable predominantly to a delay in terminal events in the growth plates, with failure to resorb collagens, as well as a delay in ossification at the primary centers [Inada et al., 2004].

Previous work in our laboratory has determined that the activator protein-1 (AP-1) and runt domain binding (Runx/RD/Cbfa) sites and their respective binding proteins, c-Fos/c-Jun and Runx2 (Cbfa1), regulate the MMP-13 promoter in both PTH-treated and differentiating osteoblastic cells in culture [Selvamurugan et al., 1998; Winchester et al., 2000]. Furthermore, protein-protein interaction studies indicate that Runx2 and the runt domain of Runx2 alone interact with c-Fos and c-Jun [D'Alonzo et al., 2002]. Also, co-transfection of Runx2 with the MMP-13 promoter in UMR 106-01 cells has been shown to enhance transactivation of the MMP-13 promoter [D'Alonzo et al., 2002]. In the present study, we wished to investigate the importance of the AP-1 and Runx/RD/Cbfa sites and Runx2 in the expression of MMP-13 and bone specific genes in vivo. To determine if the regulatory elements responsible for MMP-13 expression in vitro operated in vivo, transgenic mice containing either wild-type (−456 or −148) or AP-1 and Runx/RD/Cbfa site mutant (−148A<sub>3</sub>R<sub>3</sub>) MMP-13 promoters fused with the *E. coli lacZ* (β-galactosidase) reporter were generated. Since Runx2 is involved in bone development and bone remodeling, we also investigated if overexpression of Runx2 under the control of the MMP-13 promoter in vivo regulates bone specific genes, and whether this causes changes in the bone phenotype of these animals.

## MATERIALS AND METHODS

### Transgenic Mice Generation

The rat MMP-13 promoter fragments were generated by PCR, with SalI linkers engineered

onto the 5' and 3' ends. The fragments were subcloned into pSV0CAT (Promega, Madison, WI) vectors and the AP-1 and RD mutations were generated using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The fragments were released by Sall digestion, ligated to bacterial  $\beta$ -galactosidase and mouse protamine 1 which is a protein necessary for haploid DNA packaging and paternal procreation. These transgenes were then gel purified and used for injection into mouse blastocysts. The pCMV-c-myc mammalian expression vector expressing the N-terminal c-myc epitope tag was used to clone the rat MMP-13 promoter (–148 containing the AP-1 and RD sites) driving Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by a SphI and PvuII digestion and used for injection into mouse blastocysts. The Institutional Animal Care and Use Committee at Saint Louis University approved all procedures for the generation of the mice and collection of tissues from the mice bearing the MMP-13 promoter/reporter genes and the MMP-13 promoter/Runx2 genes. The Institutional Animal Care and Use Committee of UMDNJ-Robert Wood Johnson Medical School approved all treatments and procedures for collection of tissues from both sets of mice.

#### RNA Isolation and Real Time RT-PCR

Bone samples were ground with the grinding mill in a TRIZOL (Invitrogen) solution. RNA extraction was performed according to the instructions given by the company. Reverse transcriptase (RT) reaction was carried out using the TaqMan Reverse Transcription reagents (Roche). PCR reactions were performed according to the real-time thermocycler machine manufacturer's instructions (DNA Engine Opticon, MJ Research, MA), which allow real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCR reactions was purchased from Perkin Elmer Applied Biosystems. Primers used in this study were designed using the PrimerExpress software (Perkin Elmer Applied Biosystems). For PCR amplification, the following sets of primers were used: OC, 5' AAGCAGGAGGG-CAATAAGGT 3' and 5' AGCTGCTGTGACAT-CCCATAC 3'; MMP-13, 5' GCCACCTTCTT-

CTTGTTGAGCTG 3' and 5' ATCAAGGGATA-GGGCTGGGTCAC 3'; ALP, 5' AGGCAGGATT-GACCACGG 3' and 5' TGTAGTTCTGCTCAT-GGA 3'; OPN, 5' CCAATGAAAGCCATGAC-CACA 3' and 5' CGTCAGATTCATCCGAGTC-CAC 3'; Osteoprotegerin (OPG), 5' CGAGGA-CCACAATGAACAAG 3' and 5' TCTCGGCATT-CACCTTTGGTC 3'; RANKL, 5' CAGAAGACAG-CACTCACTGC 3' and 5' ATGGGAACCCGAT-GGGATGC 3'.

#### Immunohistochemistry

Soft tissue samples were harvested and fixed in fresh 4% paraformaldehyde in PBS at 4°C for 24 h. Bone tissue was harvested and immediately placed into fresh 4% paraformaldehyde and incubated at 4°C for 1 h. The bones were demineralized in 0.1 M Tris-HCl, 0.3 M EDTA, pH 7.4 at 4°C for 4 days with daily changes. Fixed samples were embedded in cryopreservative (O.C.T. Compound), frozen in nitrogen-cooled isopentane and stored at –70°C until sectioning. Five-micrometer frozen sections were thawed at room temperature, and washed in PBS twice, 5 min each. The sections were postfixed in 4% paraformaldehyde for 15 min, and after rinsing with PBS/BSA (1 mg/ml), were kept for 30 min in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase activity. After brief rinsing, non-specific antibody binding was suppressed by normal rabbit serum diluted 1:70 in PBS/BSA for 30 min. After a brief rinse, the sections were incubated with polyclonal rabbit anti-bacterial  $\beta$ -galactosidase antibody (Rockland, Inc., Gilbertsville, PA) at 2  $\mu$ g/ml (1:500 dilution) in PBS/BSA at 4°C overnight. After rinsing, the sections were incubated with a biotinylated anti-rabbit antibody. The bound antibody complex was visualized by the avidin-biotin-peroxidase procedure using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethyl-carbazole (AEC) as chromogen. Control sections incubated with biotinylated rabbit IgG or sections from non-transgenic animals showed very low background and no specific cell staining. Photomicrographs were taken with the use of a Spot insight color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Nikon Microphot-FXL microscope (Nikon, Melville, NY), and Spot Advanced imaging software (Diagnostic Instruments, Inc., Sterling Heights).



### Histology

Bones from the wild-type mice and transgenic mice were decalcified and fixed in 4% paraformaldehyde/0.1 M phosphate buffer. They were then paraffin-embedded, sectioned (5  $\mu$ m thick), and stained with hematoxylin and eosin.

### $\mu$ CT (Microcomputed Tomography)

Measurements of trabecular architecture were done on the proximal tibiae cleared of all soft tissue using a  $\mu$ CT 20 (Scanco Medical AG, Bassersdorf, Switzerland). After an initial scout scan, a total of 100 slices with an increment of 22  $\mu$ m were obtained on each bone sample, starting 1.5 mm below the growth plate in the area of the secondary spongiosa. The area for analysis was outlined within the trabecular compartment, excluding the cortical and subcortical bone. Every 10 sections were outlined, and the intermediate sections were interpolated with the contouring algorithm to create a volume of interest. Segmentation values used for analysis were sigma 1.2, support 2, and threshold 286. A three-dimensional (3-D) analysis was done to determine bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and connectivity density (Conn.D). Cross-sectional area was determined by outlining the periosteal surface and performing a two-dimensional analysis.

Cortical bone was measured on the tibia 2 mm below the tibia-fibula junction, where the diaphysis is most uniform in shape. Ten slices of the diaphysis were made, and the same segmentation parameters were used for analysis. The periosteal surface was outlined, and a two-dimensional analysis was done to determine cross-sectional area, bone volume, and periosteal perimeter (Ps.Pm). The endocortical surface was outlined, and the analysis repeated to determine endocortical perimeter (Ec.Pm). The mean cortical thickness (Ct.Th) was determined by distance measurements at eight different points on the cortical slice.

### Histomorphometric Analyses

For the assessment of dynamic histomorphometric indices, mice were injected twice with calcein at a dose of 16  $\mu$ g per g body weight and analyzed at 14 days or 6 weeks of age. The 14 days group received dual injections at 7 and 2 days before sacrifice, and the 6-week group received them at 12 and 2 days before

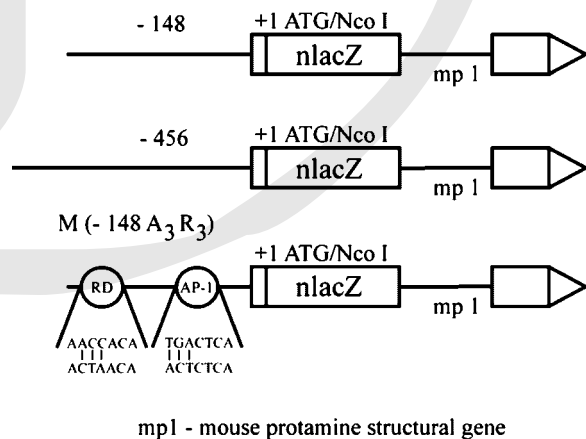
sacrifice. Tibiae were fixed with ethanol, and the calcified bones were embedded in glycol-methacrylate. Three-micrometer longitudinal sections from the proximal parts of tibiae were stained with toluidine blue and analyzed using a semiautomated system (Osteoplan II; ZEISS). Nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research [Parfitt et al., 1987]. The histomorphometric service was provided by the Center for Metabolic Bone Disease, University of Alabama, Birmingham, AL.

### Statistical Analysis

The results are expressed as means  $\pm$  standard errors of the means (SEM). Statistical analyses were carried out using Student's *t* test (Microsoft Excel 97). All statistical tests were two tailed and unpaired.

## RESULTS

We have previously shown that 148 bp upstream of the transcriptional start site of the rat MMP-13 promoter retains all PTH-responsiveness and differentiating elements in osteoblastic cells [Selvamurugan et al., 1998; Winchester et al., 2000]. Three transgenic mouse lines were generated which carried the *E. coli*  $\beta$ -galactosidase reporter (marker) gene attached to 148 bp sequence upstream of the MMP-13 gene (Fig. 1). Transgenic founder mice were identified by PCR and Southern blot analyses of DNA isolated from tail snips. The bone and soft tissues



**Fig. 1.** Generation of transgenic mice overexpressing *E. coli* *lacZ* ( $\beta$ -galactosidase). The structure of the rat MMP-13 promoter fragments and *nlacZ* (nuclear *lacZ*) hybrid genes that were microinjected into mouse blastocysts.

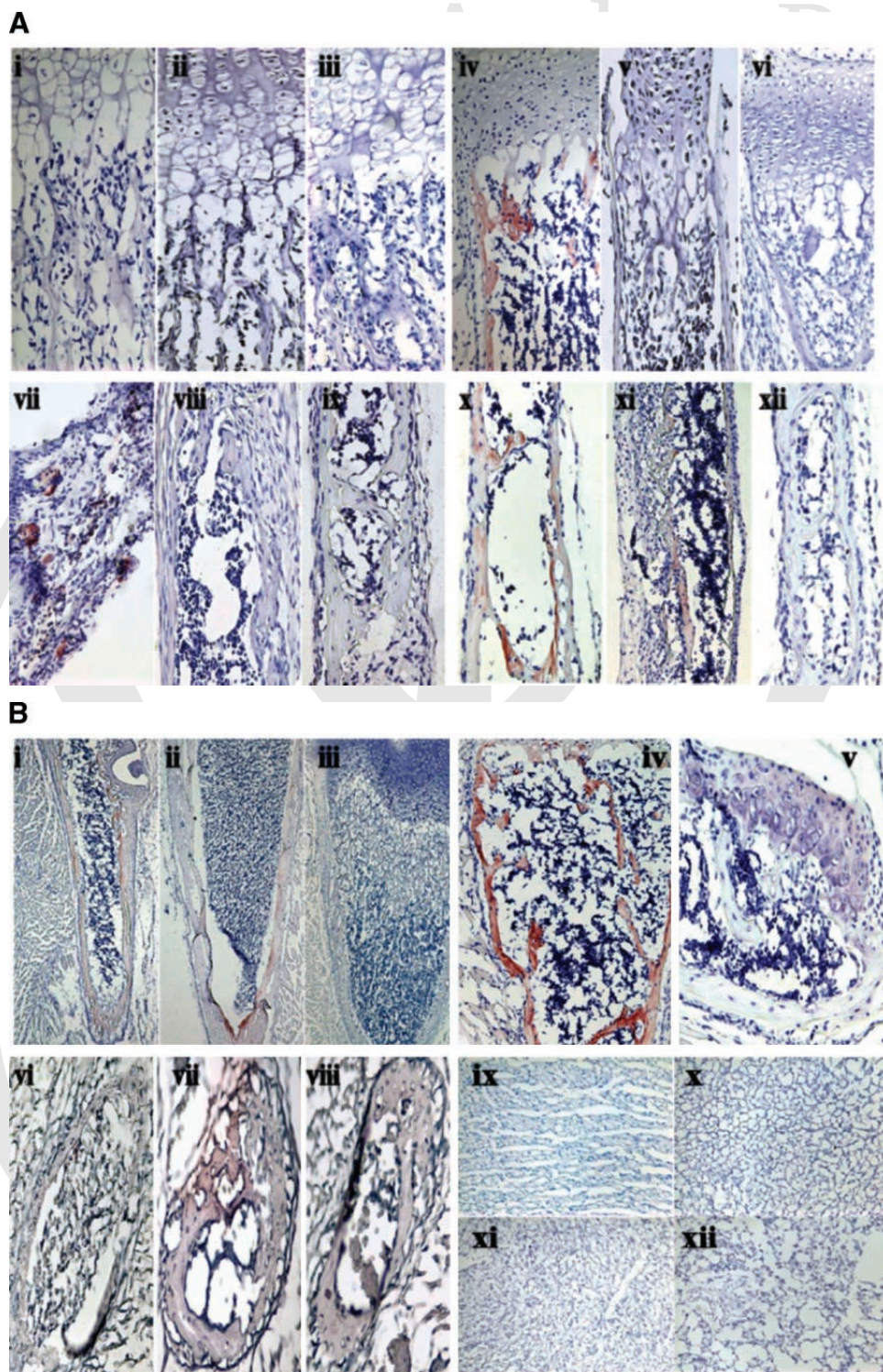
from transgenic mice and non-transgenic siblings at various ages were analyzed for expression of  $\beta$ -galactosidase by immunohistochemical staining using anti- $\beta$ -galactosidase antibody. Expression could be detected in all of the lines at 14–17 days postnatally in bone (Fig. 2) and teeth (data not shown). Some signal was also seen in skin (data not shown). No signal was seen in other tissues. Faint staining from expression of the transgene (–148 promoter/ $\beta$ -galactosidase) can be observed in developing cartilage in the 14.5-day embryo, which is more evident in the cartilage of the spine in 17.5-day embryos. There is also some expression of this transgene in the skin of postnatal animals (data not shown). Thus, it appears that expression of MMP-13 gene in bone, teeth, and skin is regulated by elements in the 148 bp upstream of the transcription start site.

To be sure that no other elements are involved in tissue-specific expression in vivo, transgenic mice harboring –456 bp upstream sequence of the rat MMP-13 gene attached to the *E. coli*  $\beta$ -galactosidase marker gene were also generated (Fig. 1). Two transgenic founders were identified by PCR of genomic DNA obtained from tail biopsies. Similar experiments were conducted as for the –148/ $\beta$ -galactosidase construct. We have found that the transgene is expressed in bone at 14–17 days postnatally. The level of expression of the reporter gene seems greater with the –456 bp promoter compared with the –148 bp promoter. This may be due to other elements such as Smad Binding Elements (SBEs) in the –456 to –148 region. Tissues such as heart, liver, and lung show no expression of either of these constructs (Fig. 2). We have also generated three lines of mice with the Runx/RD/Cbfa and AP-1 sites mutated (Fig. 1) similar to our in vitro experiments [Selvamurugan et al., 1998; Winchester et al., 2000; Inada et al., 2004]. The –148 construct with the RD and AP-1 site mutated (–148A<sub>3</sub>R<sub>3</sub>) showed expression but it is far less than the wild-type –148 or –456 constructs at 14 days postnatal in calvariae (Fig. 2). It appears that 148 bp upstream of the transcriptional start site of the rat MMP-13 promoter is sufficient to confer gene expression in bone, teeth, and skin.

Runx2 is a bone specific transcription factor and is able to induce expression of genes that are involved in bone formation and bone resorption [Ducy et al., 1997; Harada et al., 1999; Liu et al., 2001; Geoffroy et al., 2002]. We wanted to

investigate whether overexpression of Runx2 by the MMP-13 promoter regulates expression of bone specific and bone related genes (MMP-13) in vivo, and whether this causes morphological changes in these animals. The pCMV-myc mammalian expression vector (Clontech) expressing the N-terminal c-myc epitope tag was used to clone both the rat MMP-13 promoter (–148 containing the AP-1 and RD sites) and Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by SphI and PvuII digestion (Fig. 3A) and used for injection into mice blastocysts for the transgenic work. To determine that the transgene is expressed and is regulated by PTH, the transgenic DNA construct (–148/c-myc/Runx2), the negative control constructs (pSV0/-148, pCMV/c-myc), and the positive control (pCMV/c-myc/Runx2) were transiently transfected into COS-7 cells for 48 h and then treated with or without 8-bromo-cAMP (8BrcAMP) for 24 h. The cells were then lysed and the c-myc-tagged Runx2 (Cbfa1) was identified by Western blot using the c-myc antibody (Fig. 3B). The result indicated that the transgene (c-myc-tagged Runx2) is expressed and its level was increased in response to 8BrcAMP treatment in COS-7 cells. Five lines of mice were generated that carry the rat MMP-13 promoter (–148) driving expression of the c-myc tagged Runx2 gene. None of the transgenic mice lines had significant visual phenotypic changes compared to the wild-type mice.

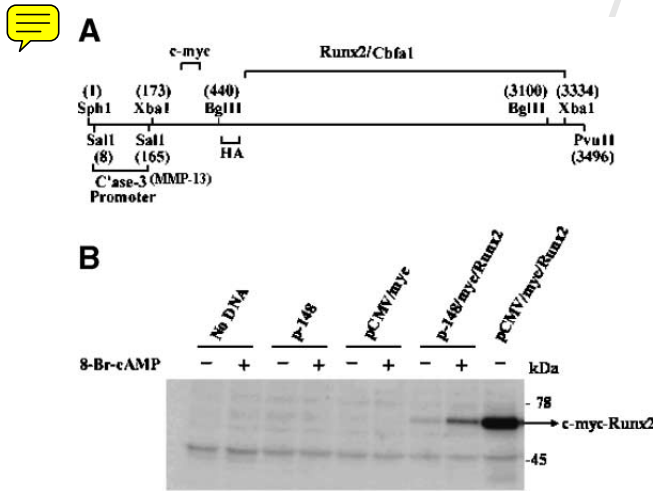
In order to determine tissue specific expression of c-myc tagged Runx2, total RNA was isolated from the bones and soft tissue of the 14 days and 6 weeks old wild-type and transgenic mice. Semi quantitative RT-PCR was carried out to determine the level of expression of the transgene (Fig. 4). In 14 days and 6 weeks old transgenic mice, MMP-13 promoter-directed expression of c-myc-Runx2 was seen in highly mineralized tissues such as tibiae, calvariae and teeth, whereas in soft tissue (liver) there appears to be no expression of c-myc-Runx2. There was also expression of the transgene in skin of the 6 weeks old mice. Expression of the transgene was not detected in either the bone or soft tissues of wild-type mice. Thus, these results indicate that c-myc-Runx2 is expressed only in transgenic mice and its expression under the control of the MMP-13 promoter seems to be restricted to bone, teeth, and skin.



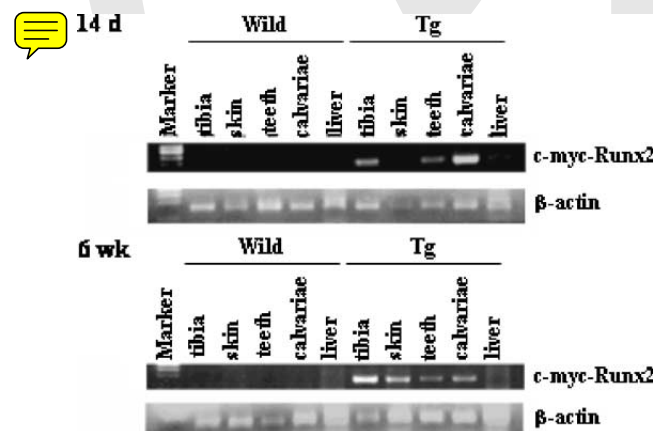
**Fig. 2.** Photomicrographs of  $-456/lacZ$ ,  $-148/lacZ$ ,  $-148(A_3R_3)/lacZ$  transgenic mice, and wild-type mice. Bones and tissues from 14, 16, and 17 day postnatal mice, and 17.5 day prenatal mouse embryos were analyzed immunohistochemically for *E. coli*  $\beta$ -galactosidase. **A:** 14 day postnatal  $-148/lacZ$  mouse femur (i), 14 day postnatal  $-148(A_3R_3)/lacZ$  mouse femur (ii), and 14 day postnatal wild-type mouse femur (iii), 14 day postnatal  $-456/lacZ$  mouse tail (iv), 14 day postnatal  $-148(A_3R_3)/lacZ$  mouse tail (v), and 14 day postnatal wild-type mouse tail (vi), 16 day postnatal  $-148/lacZ$  mouse calvaria (vii), 16 day postnatal  $-148(A_3R_3)/lacZ$  mouse tail (viii), and 16 day postnatal wild-type mouse tail (ix),

17 day postnatal  $-456/lacZ$  mouse calvaria (x), 17 day postnatal  $-148/lacZ$  mouse calvaria (xi), and 17 day postnatal  $-148(A_3R_3)/lacZ$  mouse calvaria (xii). **B:** 17 day postnatal  $-148/lacZ$  mouse femur (i), 17 day postnatal  $-456/lacZ$  mouse femur (ii), and 17 day postnatal  $-148(A_3R_3)/lacZ$  mouse femur (iii), 17 day postnatal  $-456/lacZ$  mouse rib (iv), and 17 day postnatal  $-148(A_3R_3)/lacZ$  mouse rib (v), E17.5 day wild-type mouse vertebra (vi), E17.5 day  $-148/lacZ$  mouse vertebra (vii), and E17.5 day  $-148(A_3R_3)/lacZ$  mouse vertebra (viii), 14 day postnatal  $-148/lacZ$  mouse heart (ix), kidney (x), liver (xi), and lung (xii).





**Fig. 3.** Generation of transgenic mice overexpressing Runx2. **A:** Structure and restriction map analysis of the  $-148/c\text{-myc}/\text{Runx2}$ . The pCMV-myc mammalian expression vector expressing the N-terminal c-myc epitope tag was used to clone the rat MMP-13 promoter ( $-148$  containing the AP-1 and RD sites) and Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by a SphI and PvuII digestion and used for injection into mouse blastocysts. **B:** Expression of the transgene in vitro. The transgenic DNA construct (p-148/c-myc/Runx2) as well as control constructs (p-148 and pCMV/c-myc) were transiently transfected into COS-7 cells using Lipofectamine reagent for 48 h and then treated with or without 8Br-cAMP ( $10^{-3}$  M) for 24 h. The cells were then lysed and the c-myc-tagged Runx2 was identified by Western blot using the monoclonal antibody to the c-myc epitope tag.



**Fig. 4.** Semiquantitative RT-PCR analysis of the temporal expression of the transgene in transgenic mice. Total RNA was extracted from tibiae, skin, teeth, calvariae, and liver of wild-type and transgenic mice at the indicated ages. One step RT-PCR was carried out using an RT-PCR kit (Invitrogen) with the forward (c-myc) and the reverse (Runx2) primers. To normalize the amounts of RNA used in the experiment,  $\beta$ -actin was included as a control. The products were identified on a 2% agarose gel.

The wild-type and transgenic mice overexpressing Runx2 transcription factor under the control of the MMP-13 promoter were examined for changes in their bone phenotype. The proximal tibiae of wild-type and transgenic mice (14 days and 6 weeks) were analyzed by micro-computed tomography (microCT) measurements. There were no statistically significant differences between the wild-type and transgenic animals. However, the  $\mu$ CT analysis of the proximal tibiae of the 6 weeks old animals suggested a tendency for the transgenic animals to have slightly greater trabecular bone than the wild-type (Table IA,B). Histological sections of the midtibial metaphysis from wild-type and transgenic mice (6 weeks old) showed that there is increased size and number of trabeculae in transgenic mice, compared to wild-type mice (Fig. 5).

To further understand if the slightly increased bone in 6 weeks old transgenic mice is due to increased bone formation or reduced bone resorption, kinetic analyses of bone formation were performed. Calcein was injected twice at 12 and 2 days before sacrifice in 6 weeks old mice. Bone histomorphometric studies were carried out with proximal tibial metaphyses, a standard site for this study. As shown by histomorphometric data (Fig. 6), the percentage of mineralizing surface, the amount of secreted and mineralized matrix per osteoblast (i.e., the mineral apposition rate), and the rate of bone formation were significantly increased in transgenic mice. Moreover, there was no change in the number of osteoblasts but the number of osteoclasts was significantly decreased in the transgenic mice.

In order to evaluate the molecular events that underlie the slightly modified phenotype in transgenic mice, we analyzed the mRNA expression patterns of genes that are involved in bone formation (ALP, OC, OPN) and bone resorption (MMP-13, OPG, RANKL). Total RNA was obtained from tibiae, calvariae, teeth, skin, and liver and was subjected to real time (quantitative) RT-PCR. In the immature skeleton (14 days old animals), mRNA expression of bone formation genes such as OC and OPN were significantly increased in the tibiae of transgenic mice (Fig. 7A) and had returned to normal in 6 weeks old transgenic mice (Fig. 7B). In calvariae, there was increased mRNA expression of OC in both 14 days and 6 weeks old transgenic mice (Figs. 7A,B) and there was

**TABLE IA. Structural Parameters of Trabecular Bone in the Proximal Tibia of 6 Week Old Mice Measured by Micro-Computed Tomography**

| Parameter                              | Wild-type          | Transgenic         |
|----------------------------------------|--------------------|--------------------|
| Percent bone volume, BV/TV (%)         | 22.33 $\pm$ 0.036  | 23.14 $\pm$ 0.016  |
| Trabecular number, Tb.N (1/mm)         | 6.22 $\pm$ 0.361   | 6.48 $\pm$ 0.374   |
| Trabecular thickness, Tb.Th ( $\mu$ m) | 48.9 $\pm$ 0.003   | 48.6 $\pm$ 0.001   |
| Trabecular separation, Tb.Sp (mm)      | 155.18 $\pm$ 0.005 | 148.74 $\pm$ 0.011 |
| Connectivity density, Conn.D (1/mm)    | 221.99 $\pm$ 29.98 | 266.88 $\pm$ 25.97 |

Data was tabulated as the mean  $\pm$  SEM. The number of animals/group was five.

**TABLE IB. Structural Parameters of Cortical Bone in the Mid-Shaft Femur of 6 Week Old Mice Measured by Micro-Computed Tomography**

| Parameter                          | Wild-type         | Transgenic        |
|------------------------------------|-------------------|-------------------|
| Percent bone volume, BV/TV (%)     | 52.77 $\pm$ 0.87  | 53.10 $\pm$ 0.44  |
| Periosteal perimeter, Ps.Pm (mm)   | 8.41 $\pm$ 0.19   | 8.37 $\pm$ 0.10   |
| Endocortical perimeter, Ec.Pm (mm) | 5.33 $\pm$ 0.23   | 5.29 $\pm$ 0.33   |
| Cortical thickness, Ct.Th (mm)     | 0.194 $\pm$ 0.006 | 0.191 $\pm$ 0.001 |

Data was tabulated as the mean  $\pm$  SEM. The number of animals/group was five.

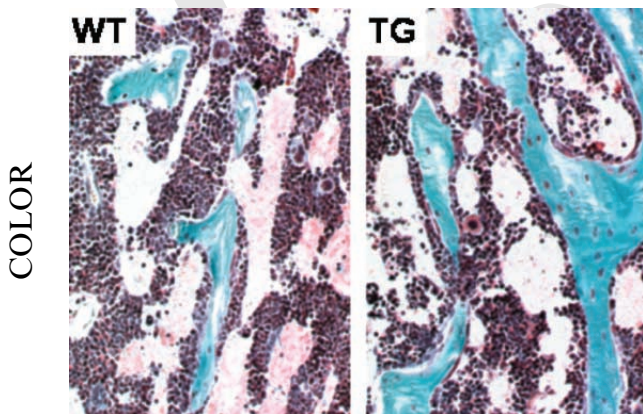
increased expression of OPG and RANKL mRNAs in 6 weeks old transgenic mice (Fig. 7B). In teeth, OPG and RANKL mRNAs were increased in 14 days old transgenic mice (Fig. 7A); whereas expression of OC and MMP-13 mRNAs were increased in this tissue in 6 weeks old transgenic mice (Fig. 7B). In skin, mRNA expression of the bone forming genes and bone resorbing genes was not significantly altered in either 14 days or 6 weeks old transgenic mice (Figs. 7A,B). Interestingly, MMP-13 mRNA expression was significantly reduced in the tibiae and calvariae of both 14 days and 6 weeks old transgenic mice (Figs. 7A,B).

When we examined the endogenous Runx2 mRNA levels, there was no significant change between the wild-type and the transgenic mice.

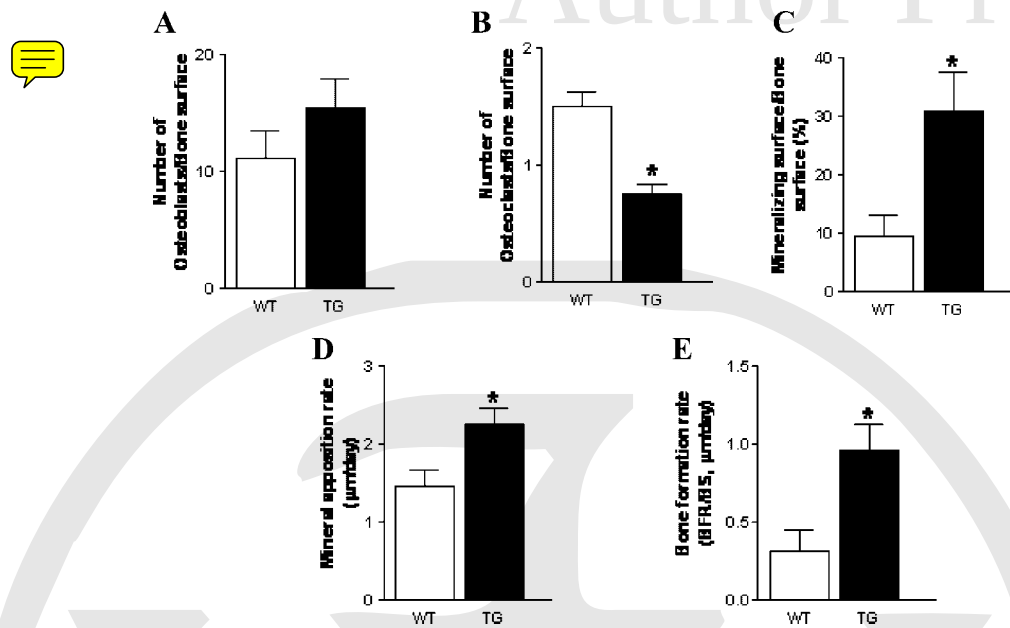
## DISCUSSION

Our previous work has determined the elements and proteins regulating the MMP-13 promoter in PTH-treated and differentiating osteoblastic cells in vitro [Selvamurugan et al., 1998; Winchester et al., 2000]. In both cases, the AP-1 and Runx/RD/Cbfa sites are involved. However, this does not mean that these elements are the functional elements directing osteoblast-preferential expression of this gene in vivo. While transient and stable transfection studies can provide insight into the biochemical and molecular interaction, it is only within the intact tissue that the true biological importance of the promoter and transcriptional environment can be appreciated. This is the rationale for the extensive and expensive effort required to carry out a promoter analysis in intact mice. Transgenic mice provide an excellent setting for studying complex regulatory systems that cannot be modeled in isolated experimental systems in vitro or in cellulo.

In this study, we show that wild-type transgenic lines (–456 and –148) express  $\beta$ -galactosidase expression in bone, teeth, and skin and none in heart, liver, or lung, compared to the mutant and non-transgenic lines (Fig. 2). Since

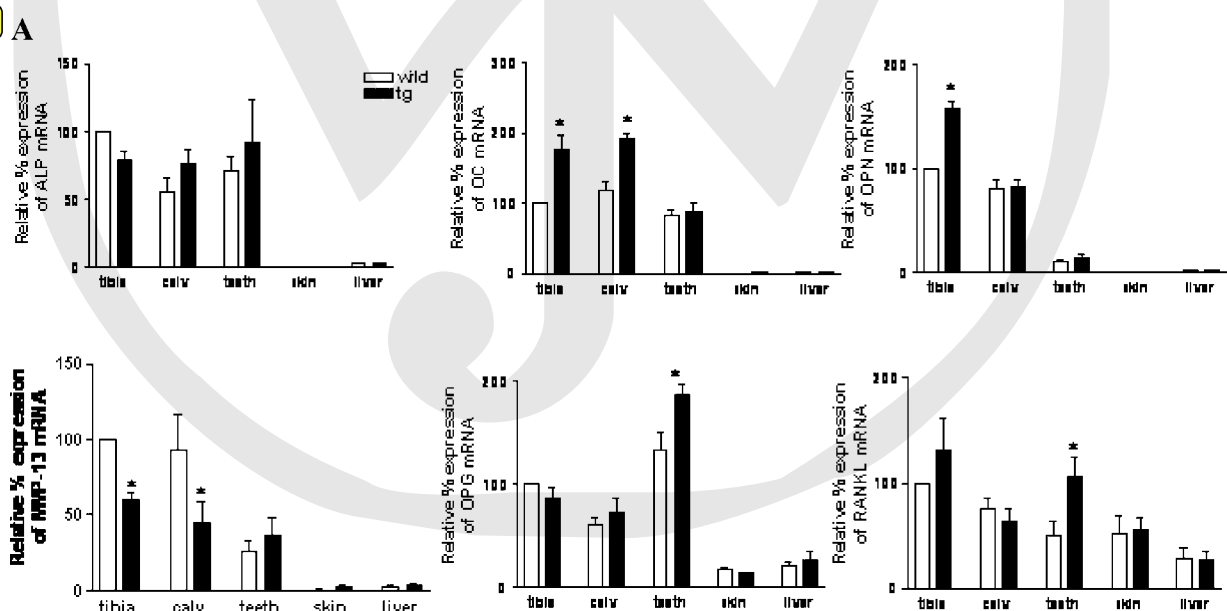


**Fig. 5.** Histological appearance of Runx2 transgenic bone. Longitudinal sections through the proximal tibiae of wild-type (WT) and transgenic (TG) mice at 6 weeks of age. The trabecular structure of both WT and TG tibiae has been magnified.



**Fig. 6.** [Histomorphometric](#)<sup>Q4</sup> bone formation and resorption parameters in Runx2-overexpressing mice. Wild-type and transgenic female mice were labeled with calcein and sacrificed at 6 weeks of age. **A:** Number of osteoblasts and **(B)** number of osteoclasts/bone surface are compared between wild-type (white bars) and transgenic mice (black bars) at 6 weeks of age.

The analyses were done using proximal parts of tibiae. **C:** mineralizing surface, **(D)** mineral apposition rate, and **(E)** bone forming rate in trabecular bone of wild-type (white bars) and transgenic (black bars) mice. Bars show means  $\pm$  SEM ( $n = 4$ ). \*Significant difference compared to the wild-type mice;  $P < 0.05$ .



**Fig. 7.** Quantitative analysis of expression of genes involved in bone formation and bone resorption. Total RNA was isolated from tibiae, calvariae, teeth, skin, and liver of 14 d **(A)**, and 6 weeks **(B)** old wild-type and Runx2 transgenic mice and subjected to real time quantitative RT-PCR using specific primers as outlined in the figure and methods section. The mRNAs were normalized to  $\beta$ -actin. The mRNA level for each gene in the tibiae

of wild-type mice has been converted to 100% and from this the relative mRNA expression was compared in other tissues of both wild and transgenic mice. The data are represented as mean  $\pm$  SEM ( $n = 3$ ). The experiment was carried out at least three times. \*Significant increase compared to the tissues of the wild-type mice;  $P < 0.05$ .

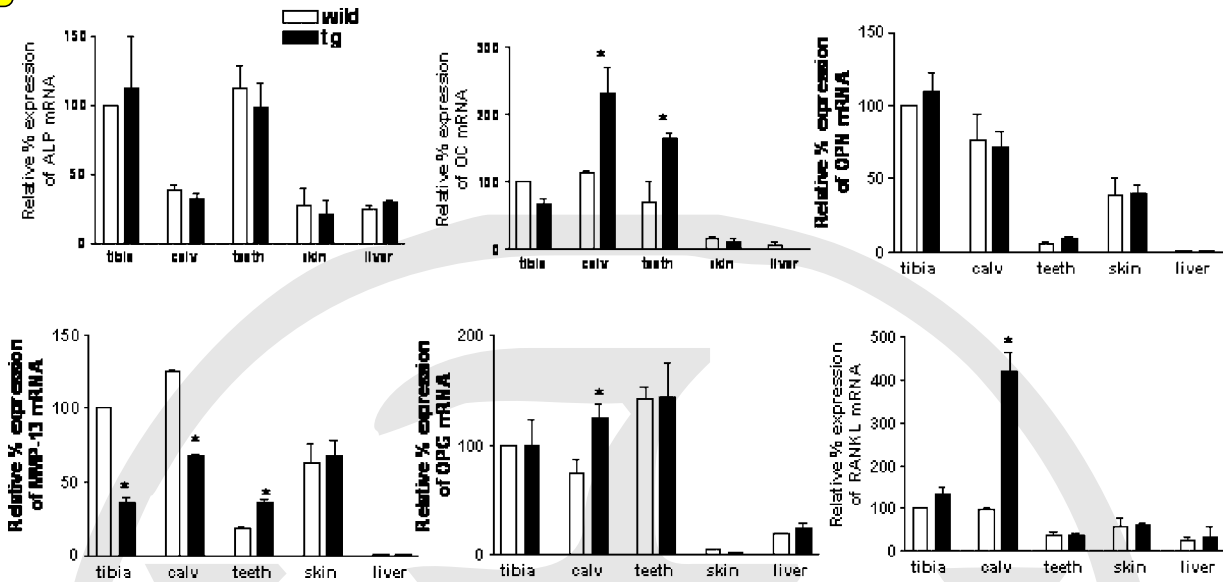


Fig. 7. (Continued)

both -456 and -148 reporter constructs exhibited a similar regulatory expression in bone, teeth, and skin, we suggest that the regulatory elements (AP-1 and Runx/RD/Cbfa) within the 148 bp upstream of the MMP-13 promoter are enough to confer this effect. In addition, it is evident that transgenic mice containing mutated AP-1 and Runx/RD/Cbfa sites (-148A<sub>3</sub>R<sub>3</sub>) in the MMP-13 promoter expressed very low  $\beta$ -galactosidase expression, compared to the non-transgenic lines (Fig. 2). Hence, it is reasonable to assume that activation of the MMP-13 promoter requires only the AP-1 and Runx/RD/Cbfa sites in both in vitro and in vivo conditions.

Runx2 is a major regulator of bone development [Karsenty, 2000; Komori, 2000]. Mouse models have enhanced our understanding of the basic functions of Runx2. Mice heterozygously mutated in the *Runx2* locus show a phenotype similar to that of cleidocranial dysplasia (CCD) in humans, in whom mutations of *Runx2* have been found [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997]. A homozygous mutation of this gene in mice induced a complete lack of bone formation with arrest of osteoblast differentiation [Komori et al., 1997; Otto et al., 1997]. The dominant negative form of Runx2 developed an osteopenic phenotype in mice and was used to indicate the indispensability of the gene for postnatal bone formation by regulating the functions of mature osteoblasts [Ducy et al., 1999]. Through deletion of the C-terminal

intranuclear targeting signal by homologous recombination, it has been shown that subnuclear targeting and the associated regulatory functions are essential for control of Runx-dependent genes [Choi et al., 2001]. A large number of in vitro studies have also implied that Runx2 is a positive regulator that can stimulate the expression of bone matrix genes, including *type I collagen*, *osteopontin*, *bone sialoprotein*, *osteocalcin*, and *fibronectin* [Banerjee et al., 1997; Ducy et al., 1997; Sato et al., 1998; Harada et al., 1999; Xiao et al., 1999; Lee et al., 2000; Kern et al., 2001; Prince et al., 2001].

A fundamental tool that is used in the transgenic experimental approach is a promoter that has tissue-restricted activity. Within the lineage of bone and cartilage cells, the type I and type II collagen promoters can be designed to have preferential expression at specific stages of differentiation. OC and BSP expression is specific to bones and thrombocytes and the OC promoter has been widely used in the transgenic mouse model system [Merk and Rowe, 2002]. When the MMP-13 promoter was used to overexpress the  $\beta$ -galactosidase reporter gene, we found its expression not only in bone but also in teeth, and skin (Fig. 2).

Liu et al. [2001] reported that transgenic mice expressing Runx2 directed by the pro- $\alpha$ -type I collagen promoter had osteopenia and fragility of bone that were caused by the inhibition of osteoblast maturation, and immature osteoblasts



accumulated in the bone of adult mice [Liu et al., 2001]. Their transgenic mice showed decreases in bone formation rate, matrix apposition rate, and mineralized surface area in trabecular bone as well as in cortical bone compared to those of the wild-type mice. Geoffroy et al. [2002] have reported that Runx2 controls not only genes that are important for osteoblast differentiation [Ducy et al., 1997] and function [Ducy et al., 1999] but also genes that are involved in osteoclast differentiation and bone formation-resorption coupling [Geoffroy et al., 2002]. Even though MMP-13 has been shown to be expressed in skin, uterus, and ovary, it is mostly expressed in bone [Balbin et al., 1996; Davis et al., 1998; Tuckermann et al., 2000; Shum et al., 2002]. We report here that overexpression of Runx2 directed by the MMP-13 promoter increases the bone mineralization surface, bone formation rate, and matrix apposition rate (Fig. 6). Since there was no change in the number of osteoblasts, this effect could be due to uncoupling and unbalancing of bone formation and bone resorption processes. Even though OPG and RANKL mRNA expression were not altered in the tibiae of transgenic mice at the ages examined, there was significantly reduced MMP-13 mRNA expression during development of transgenic mice (Fig. 7A,B) and it has been shown that MMP-13 is necessary for osteoclast-mediated bone resorption [Zhao et al., 2000]. The downregulation of MMP-13 at the ages examined by overexpression of Runx2 in transgenic mice could be due to negative feedback regulation of Runx2. This also could be due to the fact that MMP-13 is expressed at greater levels in long bones, in the fetus, and maximally at 14 days in the calvariae, while OC is mostly expressed postnatally [Davis et al., 1998; Tuckermann et al., 2000]. It is possible that the maturation of the bones is advanced and the usual peak in MMP-13 expression is at an earlier age. Perhaps the reduced expression level of MMP-13 in the transgenic mice could have led to decreased recruitment of osteoclasts (Fig. 6) to the bone surface, resulting in reduced bone-resorptive activity, reflected by increased bone formation in transgenic mice.

Overall, we provide evidence that the 148 base pairs of MMP-13 promoter is sufficient and necessary for tissue-restricted (bone, teeth, and skin) gene expression in vivo, and the AP-1 and Runx/RD/Cbfa sites are likely to regulate this. Using these regulatory elements, we

further document that overexpression of Runx2 appears to alter the balance between the bone formation-bone resorption processes in vivo and does regulate the expression of MMP-13 and other bone marker genes.

## ACKNOWLEDGMENTS

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