

AD _____

Award Number: W81XWH-04-1-0687

TITLE: MMP-8: A Breast Cancer Bone Metastasis Suppressor Gene

PRINCIPAL INVESTIGATOR: Nagarajan Selvamurugan

CONTRACTING ORGANIZATION: University of Medicine and Dentistry
of New Jersey
Piscataway, NJ 08854

REPORT DATE: August 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20060503009

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE 01-08-2005		2. REPORT TYPE Annual		3. DATES COVERED 22 Jul 2004 -21 Jul 2005	
4. TITLE AND SUBTITLE MMP-8: A Breast Cancer Bone Metastasis Suppressor Gene				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0687	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Nagarajan Selvamurugan				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Medicine and Dentistry of New Jersey Piscataway, NJ 08854				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT 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 in vitro. 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 pCMV-MMP-1-V5 was sequenced to verify cloning of the MMP-8 cDNA insert in frame. The molecular mechanisms of how TGF-beta1 mediates stimulation of invasion and formation of bone metastases have yet to be completely determined. In my laboratory, we have found that ATF-3 (activating transcription factor-3) is strongly stimulated and its level is sustained by TGF-beta1 in highly invasive and bone metastatic human breast cancer cells. A defect in repression of ATF-3 expression in breast cancer cells could lead to activation of genes that participate in multi-step breast cancer progression.					
15. SUBJECT TERMS MATRIX METALLOPROTEINASE, TRANSFORMING GROWTH FACTOR, ACTIVATING TRANSCRIPTION FACTOR-3, BONE INVASION AND METASTASIS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	9
Appendices.....	11

Introduction:

Bone metastasis of breast cancer induces severe osteolysis (bone degradation) and associated matrix metalloproteinase (MMP)-dependent matrix degradation. MMPs are classified into several subgroups, *i.e.*, collagenases (MMP-1, -8, and MMP-13), gelatinases (MMP-2 and -9), stromelysins, membrane-type MMPs, and other subfamilies. MMPs have diverse and important functions in normal physiology and can have multiple substrates, which can each have a distinct biological activity. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) are overexpressed in a variety of malignant tumors. Unlike other members of the MMP family, MMP-8 (collagenase-2) has not previously been implicated in the processes of tumorigenesis or metastasis. MMP-8 is a potent collagenolytic enzyme produced mainly by neutrophils.

TGF- β Signaling TGF- β (transforming growth factor-beta), a multipotent cytokine has a wide range of physiological and pathological effects (1, 2). TGF- β 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- β antiproliferative and apoptotic responses may compromise the turnover of the mammary epithelium, thus favoring tumor formation (5-7). TGF- β signaling involves the type I receptor T β R-I, the type II receptor T β 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- β receptors or Smads are rare in breast cancer (9, 10). Moreover, for breast cancer cells, TGF- β 1 is a crucial molecule in metastatic breast cancer stimulating invasion (11, 12) and formation of TGF- β -dependent bone metastases in model systems (13).

ATF-3 Independent observations over the years have defined a small group of immediate TGF- β target genes that contribute to the effect of TGF- β 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- β 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 the 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). ATF-3 is a common target of TGF- β 1 and stress signals and serves to inhibit cell growth in normal epithelial cells (24). ATF-3 is upregulated in sensory neurons that innervate the tumor-bearing femur (31). 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 cancer (38-42). TGF- β 1 stimulates MMP-13 (collagenase-3) 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.

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 TOPO 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 pCMV-MMP-1-V5 was sequenced to verify cloning of the MMP-8 cDNA insert in frame. This construct will be transfected into COS-7 cells using the GeneJammer (Stratagene) according to the guidelines provided by the company. Cells will be lysed and subjected to Western blot analysis. The V5-epitope tagged MMP-8 protein will be identified by the V5-epitope antibody.

In order to get bone specific expression of MMP-8, we will utilize the rat osteocalcin promoter (OC; ~1.0 kb). It was kindly provided by Dr. Gary Stein, University of Massachusetts Medical School, Worcester, MA. The pOC-V5-MMP-8 will be constructed by replacing the CMV promoter sequence from pCMV-MMP-8-V5 with the rat osteocalcin promoter sequence. The ability of osteocalcin promoter to drive V5-MMP-8 expression will be carried out by transient transfection assays and Western blot analysis. The DNA fragment containing the osteocalcin promoter and V5-MMP-8 cDNA sequence will be excised from pOC-V5-MMP-8 with appropriate enzymes. This purified transgene will be used for generation of transgenic mice.

My collaborator (Dr. Susan Rittling, Rutgers University, NJ) generated a series of metastatic murine mammary epithelial cell lines using normal mice rather than using nude mice. Mammary tumors were induced in strain 129 female mice by treatment with the carcinogen DMBA and their growth was accelerated by implanted progesterone (MPA) pellets. An epithelial cell line was isolated from one of these tumors: however, this cell line (1029) was not tumorigenic in mice even in the presence of MPA. Introduction of the oncogene polyoma middle T into these cells (1029 GP+E) was insufficient to allow hormone-independent tumor growth, although in the presence of hormone (MPA) rare tumors were observed. Thus, a second oncogene, v-Ha-ras was introduced. Cells expressing both ras and polyoma middle T were able to form tumors efficiently in the mammary fat pad, and even formed spontaneous metastases in the lungs and sometimes liver after mammary tumor growth (1029 GP+E r3). Two additional cell lines were derived from the 1029 GP+E r3 cells – one from a tumor and

one from a lung metastasis arising from injected cells: these cells were called r3T and r3L, respectively. These two cell lines have similar metastatic properties.

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. The r3T cells (5×10^5) were injected into the left ventricle of the heart, and the mice were sacrificed three weeks later. Arrows indicate positions of extensive bone loss visible in these x-ray images.

Figure 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.

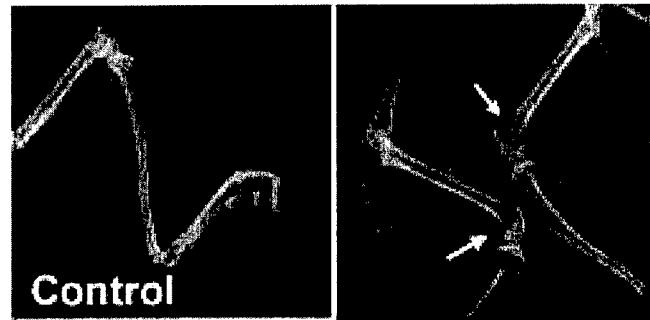
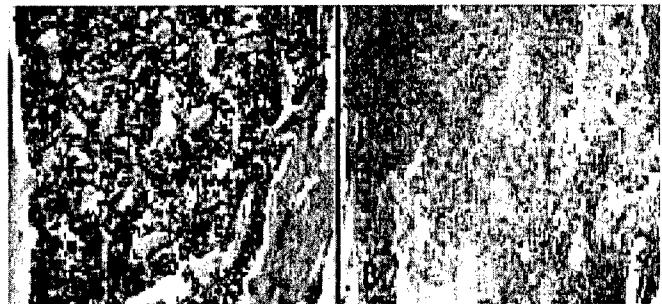


Figure 2. Histological appearance of metastatic tumor cells.

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.

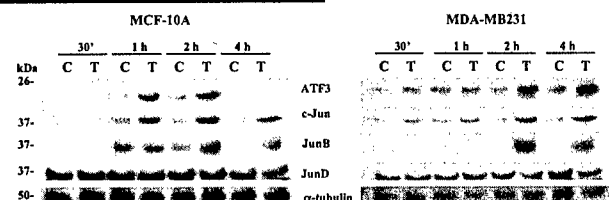


TGF- β 1 is the most potent known growth inhibitor for epithelial cells. In breast tissue, loss of TGF- β 1 anti-proliferative response favors tumor formation. Moreover, in breast cancer cells, TGF- β 1 is a crucial molecule for stimulation of invasion and formation of bone metastases. The molecular mechanisms of how TGF- β 1 mediates these effects have yet to be completely determined. In my laboratory, we have found that ATF-3 (activating transcription factor-3) is strongly stimulated and its level is sustained by TGF- β 1 in highly invasive and bone metastatic human breast cancer cells. A defect in repression of ATF-3 expression in breast cancer cells could lead to activation of genes that participate in multi-step breast cancer progression.

We first examined TGF- β 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- β 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. 3). There was no significant change in the level of JunD expression by TGF- β 1 after normalization with α -tubulin expression in MCF-10A and MDA-MB231 cells.

Figure 3. TGF- β 1 stimulates expression of ATF-3, c-Jun, and JunB.

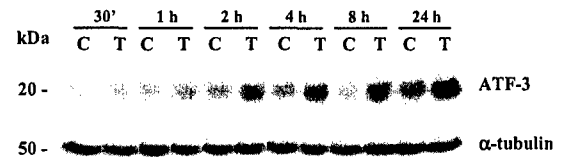
MCF-10A and MDA-MB231 cells were treated with control or TGF- β 1 (1 ng/ml)-containing media for the indicated times. Total lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure. α -tubulin represents the loading control.



My collaborator (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. TGF- β 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- β 1 and then declined (data not shown).

Figure 4. TGF- β 1 stimulates ATF-3 expression.

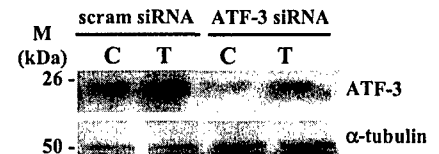
Mouse mammary pad tumor cells (r3T) were treated with control or TGF- β 1 (1 ng/ml)-containing media for the indicated times. Total lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure. α -tubulin represents the loading control.



To determine the functional role of ATF-3 in breast cancer metastasis, 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- β 1-stimulated ATF-3 expression, compared with the nonspecific target sequences (Fig. 5).

Figure 5. ATF-3 siRNA reduces both basal and TGF- β 1-stimulated ATF-3 expression.

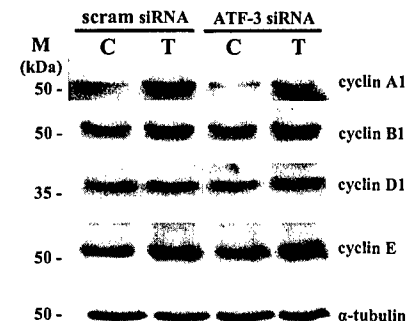
MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA vectors for 24 h and then treated with control or TGF- β 1 (1 ng/ml)-containing media for 4 h. Total lysates were prepared and subjected to Western blot analysis using the ATF-3 and α -tubulin (loading control) antibodies.



Since TGF- β 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. 5, TGF- β 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- β 1-stimulated MDA-MB231 cells. Thus, ATF-3 must be the mediator of TGF- β 1-stimulation of cyclin A1 and cyclin A1 is likely to be an ATF-3 target gene. Cyclin A1 contributes cell cycle progression from G1 to S phase.

Figure 6. ATF-3 siRNA reduces both basal and TGF- β 1-stimulated cyclin A1 expression.

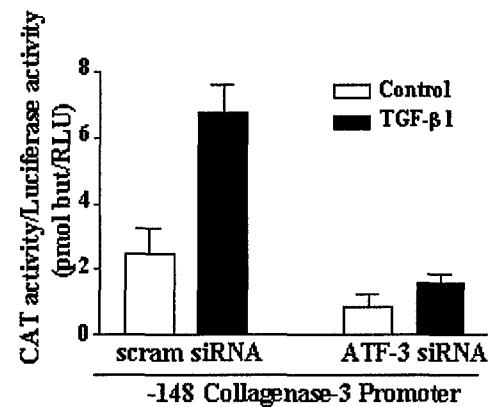
MDA-MB231 cells were transiently transfected with either scrambled siRNA or ATF-3 siRNA plasmids for 24 h and then treated with control or TGF- β 1 (1 ng/ml)-containing media for 24 h. Total lysates were prepared and subjected to Western blot analysis using the antibodies as indicated. α -tubulin was a loading control.



Since MDA-MB231 cells are highly invasive and bone metastatic in nature and TGF- β 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- β -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. 7, TGF- β 1 stimulated MMP-13 promoter activity and ATF-3 siRNA reduced both the control and TGF- β 1-stimulated MMP-13 promoter activity in these cells. Hence, the MMP-13 gene (another potential ATF-3 target gene) is regulated by TGF- β 1 via ATF-3.

Figure 7. ATF-3 siRNA reduces both the basal and TGF- β 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- β 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.



Key Research Accomplishments:

- The construct pCMV-V5-MMP-8 containing the human cytomegalovirus promoter sequence, V5-epitope tag, and a human matrix metalloproteinase-8 cDNA was made.
- Cardiac injection of tumor cells into mice and histology of bone metastasized cells were standardized.
- TGF- β 1 (transforming growth factor- β 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- β 1 stimulated expression of cyclin A1, -B1, -D1, and -E in MDA-MB231 cells while ATF-3 siRNA only decreased expression of cyclin A1 in both control and TGF- β 1-stimulated MDA-MB231 cells.
- ATF-3 must be the mediator of TGF- β 1-stimulation of cyclin A1 and cyclin A1 is likely to be an ATF-3 target gene.
- TGF- β 1 stimulated MMP-13 promoter activity and ATF-3 siRNA reduced both the control and TGF- β 1-stimulated MMP-13 promoter activity in MDA-MB231 cells.
- MMP-13 gene is another potential ATF-3 target gene and regulated by TGF- β 1 via ATF-3

Reportable Outcomes:

Manuscript:

Parathyroid hormone stimulation and PKA signaling of latent transforming growth factor- β binding protein-1 (LTBP-1) mRNA expression in osteoblastic cells
S. Kwok., L. Qin., Partridge, N. C. and N. Selvamurugan (2005)
Journal of Cellular Biochemistry 95: 1002-1011

Abstract:

TGF- β 1 Regulation of ATF-3 and its Target Genes in Bone Metastasizing Breast Cancer Cells
Presented at the 27th 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 underlying 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- β 1-regulation of ATF-3 and its target genes, cyclin A1 and MMP-13 in bone metastasizing breast cancer cells. The dysregulation of ATF-3 by TGF- β 1 in breast cancer cells may be key to the subsequent metastasis of these cells to bone.

References:

- 1 Massague, J. and Wotton, D. (2000) EMBO Journal 19, 1745-54
- 2 Piek, E., Heldin, C. H. and Ten Dijke, P. (1999) FASEB Journal 13, 2105-24
- 3 Piek, E. and Roberts, A. B. (2001) Advances in Cancer Research 83, 1-54
- 4 Engle, S. J., Hoving, J. B., Boivin, G. P., Ormsby, I., Gartside, P. S. and Doetschman, T. (1999) Cancer Research 59, 3379-86
- 5 Massague, J., Blain, S. W. and Lo, R. S. (2000) Cell 103, 295-309
- 6 Barcellos-Hoff, M. H. and Ewan, K. B. (2000) Breast Cancer Research 2, 92-9
- 7 Nguyen, A. V. and Pollard, J. W. (2000) Development 127, 3107-18
- 8 Massague, J. (2000) Nature Reviews Molecular Cell Biology 1, 169-78
- 9 Tomita, S., Deguchi, S., Miyaguni, T., Muto, Y., Tamamoto, T. and Toda, T. (1999) Breast Cancer Research & Treatment 53, 33-9
- 10 Anbazhagan, R., Bornman, D. M., Johnston, J. C., Westra, W. H. and Gabrielson, E. (1999) Cancer Research 59, 3363-4
- 11 Welch, D. R., Fabra, A. and Nakajima, M. (1990) Proceedings of the National Academy of Sciences of the United States of America 87, 7678-82

- 12 Off, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H. and Reichmann, E. (1996) *Genes & Development* 10, 2462-77
- 13 Yin, J. J., Selander, K., Chirgwin, J. M., Dallas, M., Grubbs, B. G., Wieser, R., Massague, J., Mundy, G. R. and Guise, T. A. (1999) *Journal of Clinical Investigation* 103, 197-206
- 14 Turner, B. C., Zhang, J., Gumbs, A. A., Maher, M. G., Kaplan, L., Carter, D., Glazer, P. M., Hurst, H. C., Haffty, B. G. and Williams, T. (1998) *Cancer Research* 58, 5466-72
- 15 Chen, C. R., Kang, Y. and Massague, J. (2001) *Proceedings of the National Academy of Sciences of the United States of America* 98, 992-9
- 16 Talukder, A. H., Wang, R. A., and Kumar, R. (2002) *Oncogene* 21, 4289-4300
- 17 Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R. A. (2004) *Cell* 177, 927-939
- 18 Barnes, G. L., Hebert, K. E., Kamal, M., Javed, A., Einhorn, T. A., Lian, J. B., Stein, G. S., and Gerstenfeld, L. C. (2004) *Cancer Res.* 64, 4506-4513
- 19 Dechow, T. N., Pedranzini, L., Leitch, A., Leslie, K., Gerald, W. L., Linkov, I., and Bromberg, J. F. (2004) *Proceedings of the National Academy of Sciences of the United States of America* 101, 10602-10607
- 20 Fan, W., Huang, X., Chen, C., Gray, J., and Huang, T. (2004) *Cancer Research* 64, 5132-5139
- 21 Huber, M. A., Azoitei, N., Baumann, B., Grunert, S., Sommer, A., Pehamberger, H., Kraut, N., Beug, H., and Wirth, T. (2004) *J Clin Invest.* 114, 569-581
- 22 Chakrabarti, J., Turley, H., Campo, L., Han, C., Harris, A. L., Gatter, K. C., and Fox, S. B. (2004) *Br J Cancer* 91, 954-958
- 23 Darnell, J. E. (2002) *Nature Medicine* 8, 1068-1071
- 24 Kang, Y., Chen, C. R. and Massague, J. (2003) *Molecular Cell* 11, 915-26
- 25 Chen, B. P., Liang, G., Whelan, J. and Hai, T. (1994) *Journal of Biological Chemistry* 269, 15819-26
- 26 Hsu, J. C., Bravo, R. and Taub, R. (1992) *Molecular & Cellular Biology* 12, 4654-65
- 27 Hsu, J. C., Cressman, D. E. and Taub, R. (1993) *Cancer Research* 53, 3789-94
- 28 Weir, E., Chen, Q., DeFrances, M. C., Bell, A., Taub, R. and Zarnegar, R. (1994) *Hepatology* 20, 955-60
- 29 Drysdale, B. E., Howard, D. L. and Johnson, R. J. (1996) *Molecular Immunology* 33, 989-98
- 30 Hai, T., Wolfgang, C. D., Marsee, D. K., Allen, A. E. and Sivaprasad, U. (1999) *Gene Expression* 7, 321-35
- 31 Sevcik, M. A., Luger, N. M., Mach, D. B., Sabino, M. A., Peters, C. M., Ghilardi, J. R., Schwei, M. J., Rohrich, H., De Felipe, C., Kuskowski, M. A. and Mantyh, P. W. (2004) *Pain* 111, 169-80
- 32 Wolfgang, C. D., Chen, B. P., Martindale, J. L., Holbrook, N. J. and Hai, T. (1997) *Molecular & Cellular Biology* 17, 6700-7
- 33 Wolfgang, C. D., Liang, G., Okamoto, Y., Allen, A. E. and Hai, T. (2000) *Journal of Biological Chemistry* 275, 16865-70
- 34 Allan, A. L., Albanese, C., Pestell, R. G. and LaMarre, J. (2001) *Journal of Biological Chemistry* 276, 27272-80
- 35 Yoshizumi, M., Hsieh, C. M., Zhou, F., Tsai, J. C., Patterson, C., Perrella, M. A. and Lee, M. E. (1995) *Molecular & Cellular Biology* 15(6):3266-72
- 36 Sakai, T., Ohtani, N., McGee, T. L., Robbins, P. D. and Dryja, T. P. (1991) *Nature* 353, 83-6
- 37 Linardopoulos, S., Gonos, E. S. and Spandidos, D. A. (1993) *Cancer Letters* 71, 67-74

- 38 Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A. and Engler, J. A. (1993) *Critical Reviews in Oral Biology & Medicine* 4, 197-250
- 39 Stetler-Stevenson, W. G., Liotta, L. A. and Kleiner, D. E., Jr. (1993) *FASEB Journal* 7, 1434-41
- 40 Crawford, H. C. and Matrisian, L. M. (1994) *Invasion & Metastasis* 14, 234-45
- 41 Crawford, H. C. and Matrisian, L. M. (1996) *Enzyme & Protein* 49, 20-37
- 42 Benaud, C., Dickson, R. B. and Thompson, E. W. (1998) *Breast Cancer Research & Treatment* 50, 97-116
- 43 Selvamurugan, N., Fung, Z. and Partridge, N. C. (2002) *FEBS Letters* 532, 31-5
- 44 Selvamurugan, N., Kwok, S. and Partridge, N. C. (2004) *Journal of Biological Chemistry* 279, 27764-73

Appendice:

1. Parathyroid hormone stimulation and PKA signaling of latent transforming growth factor- β binding protein-1 (LTBP-1) mRNA expression in osteoblastic cells
S. Kwok., L. Qin., Partridge, N. C. and N. Selvamurugan (2005)
Journal of Cellular Biochemistry 95: 1002-1011

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

Sukye Kwok, Ling Qin, Nicola C. Partridge, and Nagarajan Selvamurugan*

Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

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- β)-binding protein is stimulated by PTH. Latent TGF- β binding proteins (LTBPs) are required for the proper folding and secretion of TGF- β , thus modifying the activity of TGF- β , 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- β 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- β 1 together. We further examined the signaling pathways responsible for PTH-stimulated LTBP-1 and TGF- β 1 mRNA expression in UMR 106-01 cells. The PTH stimulation of LTBP-1 and TGF- β 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- β in bone remodeling. *J. Cell. Biochem.* 95: 1002–1011, 2005. © 2005 Wiley-Liss, Inc.

Key words: PTH; TGF- β ; LTBP-1; osteoblast; PKA signaling

Abbreviations used: PTH, parathyroid hormone; LTBP-1, latent transforming growth factor-beta binding protein-1; TGF- β , transforming growth factor-beta; ECM, extracellular matrix; SLC, small latent TGF- β complex; LLC, large latent TGF- β 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.

Grant sponsor: Department of Defense; Grant number: W81XWH-04-1-0687; Grant sponsor: New Jersey Commission on Cancer Research; Grant sponsor: Foundation of the University of Medicine and Dentistry of New Jersey; Grant sponsor: NIH; Grant numbers: DK48109, DK47420.

*Correspondence to: Nagarajan Selvamurugan, PhD, Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. E-mail: selvamn2@umdnj.edu

Received 24 November 2004; Accepted 28 January 2005
DOI 10.1002/jcb.20453

© 2005 Wiley-Liss, Inc.

Parathyroid hormone (PTH) is one of the major calciotropic 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- β), which is a multipotent cytokine [Seyedin et al., 1985; Hauschka et al., 1986]. TGF- β 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- β propeptide binds to TGF- β , and the proteins are secreted as a complex [Annes et al., 2003]. In this small latent complex (SLC), TGF- β 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- β from LAP is a critical regulatory event as all TGF- β is secreted in a latent form. The LAP dimer is usually disulfide bonded to a second gene product, latent TGF- β 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- β in bone matrix is via its association with the latent TGF- β 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- β , thus modifying the activity of TGF- β [Miyazono et al., 1991]. In human and rats, *LTBP-1* appears as two mRNA species, which encode for two different NH₂-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- β 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- β 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- β 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- β , LTBP-1 seems to be a mediator in controlling PTH action on osteoblasts via TGF- β . 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- β 1 mRNA expression in rat osteoblastic cells.

MATERIALS AND METHODS

Materials

Rat PTH (1–34) and human TGF- β 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 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ 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 6.4×10^3 cells/cm² and grown in minimal essential medium (MEM) supplemented with 10% FBS. After reaching confluence (day 7), the medium was switched to BGJ_b 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'-TTAGGAAGGACCTGGGTTGA, 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

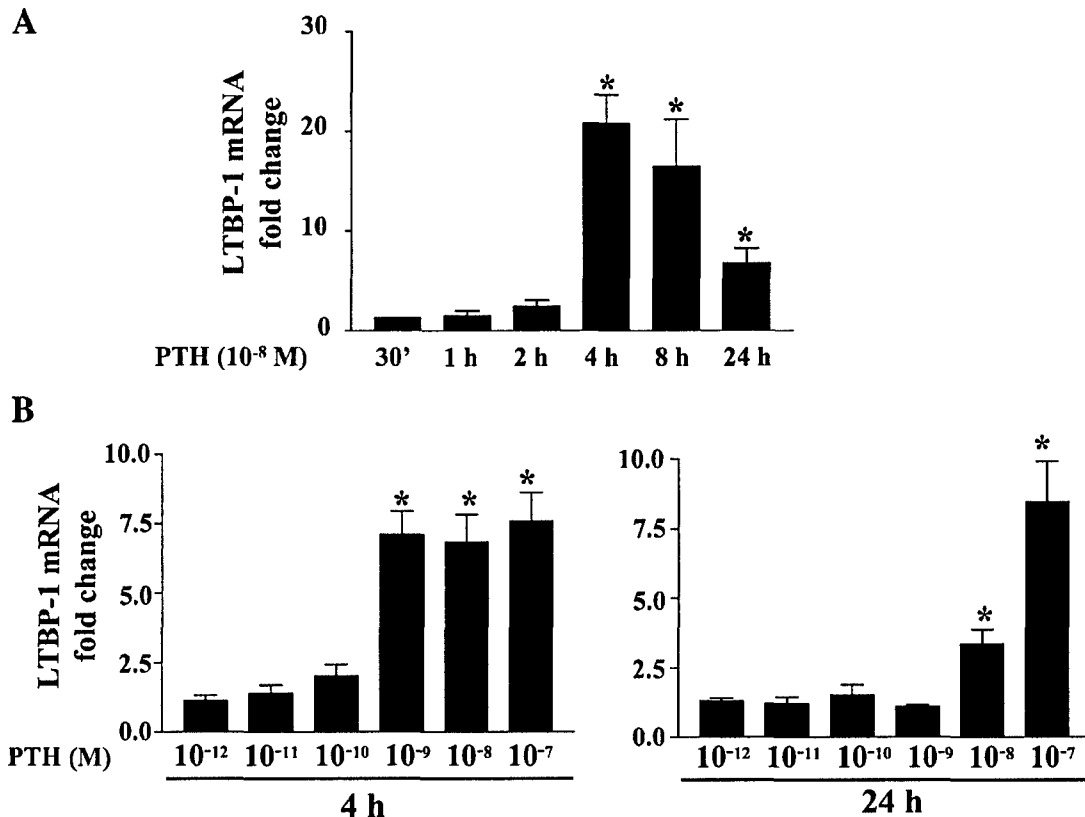


Fig. 1. Effect of PTH on expression of *LTBP-1* mRNA levels in the rat osteoblastic cell line, UMR 106-01. **A:** Time course of the PTH stimulation of *LTBP-1*. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH ($1-34$, 10^{-8} M) for different times as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -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 β -actin. The relative levels of mRNAs were normalized to β -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^{-8} M) for 4 h in the presence or absence of 30 μ 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 β -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- β 1 mRNA Expression and Both PTH and TGF- β 1 Have an Additive Effect on *LTBP-1* mRNA Expression

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

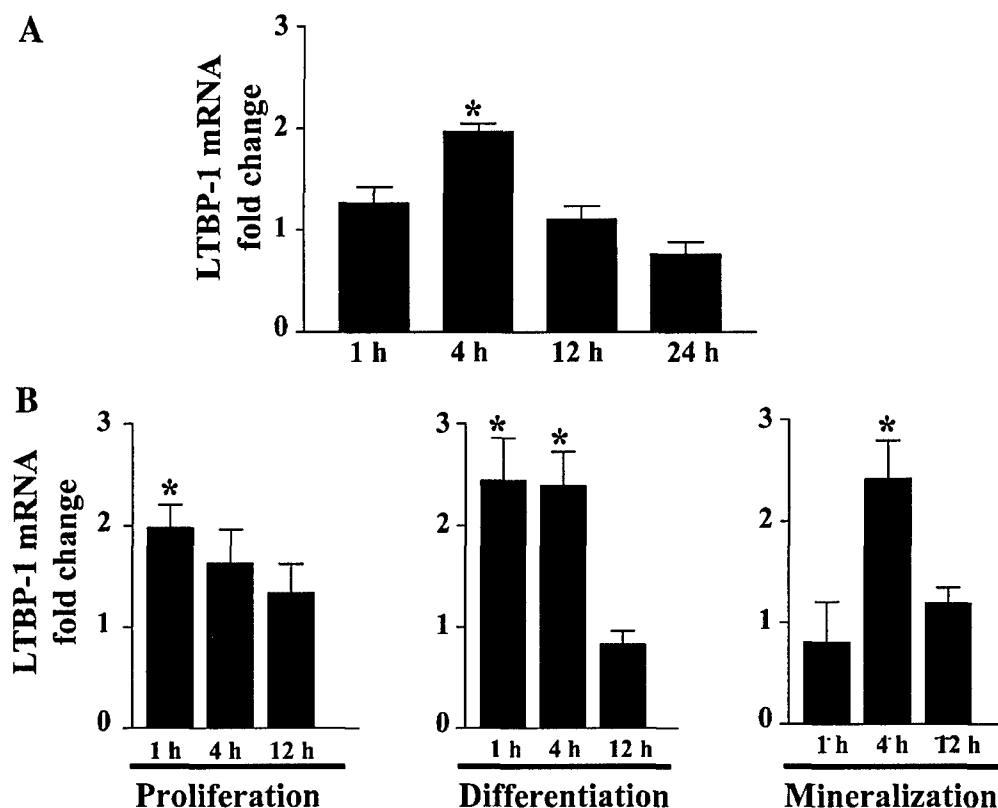


Fig. 2. Effect of PTH on expression of *LTBP-1* mRNA levels in mouse preosteoblastic cells and in rat primary osteoblastic cells. **A:** MC3T3 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for different time periods as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -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 μ g/

ml ascorbic acid, and 10 mM β -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 β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

PTH stimulated TGF- β 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- β 1 mRNA expression in differentiating and mineralizing osteoblasts at 4 h treatment indicating that osteoblasts respond to PTH and synthesize TGF- β 1 only at these stages. Since PTH stimulates mRNA expression of both *LTBP-1* and TGF- β 1 mRNAs, we wanted to determine whether there is a synergistic effect with combined treatment with PTH and TGF- β 1 on *LTBP-1* expression in rat osteoblastic cells. UMR 106-01 cells were treated with human TGF- β 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- β 1 and PTH stimulated *LTBP-1* mRNA expression to 3.5 ± 1.1 -fold and 11.7 ± 2.1 -fold, respectively at 4 h and to 1.4 ± 0.2 -fold and 7.3 ± 2.4 -fold, respectively at 24 h in UMR 106-01 cells. When cells were treated with TGF- β 1 and PTH together, an additive effect was observed at both 4 h (17.1 ± 1.6 -fold) and 24 h (9.5 ± 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

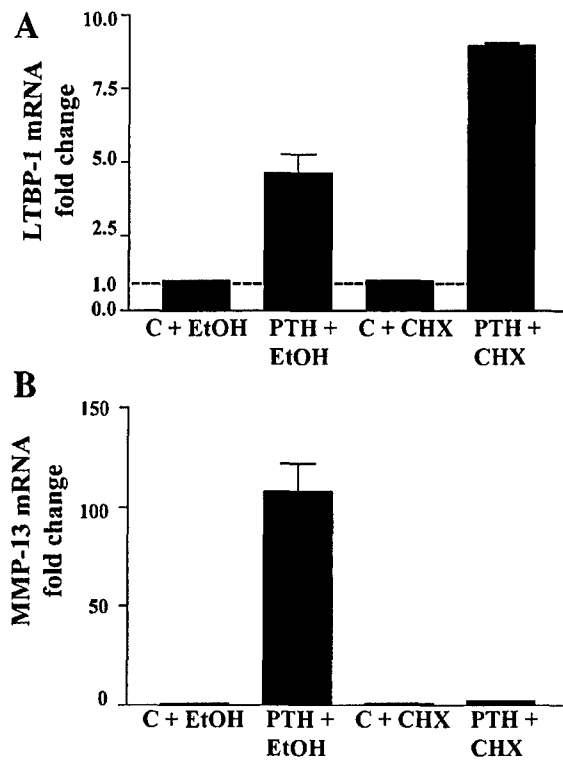


Fig. 3. PTH-stimulated *LTBP-1* mRNA expression is a primary effect. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 4 h in the presence or absence of 30 μ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -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 μ 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 β -actin. The relative levels of mRNAs were normalized to β -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- β 1 mRNA Expression Is a Secondary Effect and Is Dependent on the MAPK Signaling Pathway

Since PTH stimulated TGF- β 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 μ g/ml cycloheximide added 1 h before treatment. Total RNA was subjected to real time RT-PCR analysis using specific primers for rat TGF- β 1 and β -actin. As shown in Figure 7A, cycloheximide inhibited PTH induction of TGF- β 1 mRNA expression, indicating that PTH stimulation of TGF- β 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- β 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- β 1 mRNA expression was not significantly decreased by JNK, PKA, and PKC inhibitors; whereas MEK and p38 MAPK inhibitors inhibited PTH-stimulated TGF- β 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- β [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- β [Seyedin et al., 1985; Hauschka et al., 1986]. This ECM-bound TGF- β ,

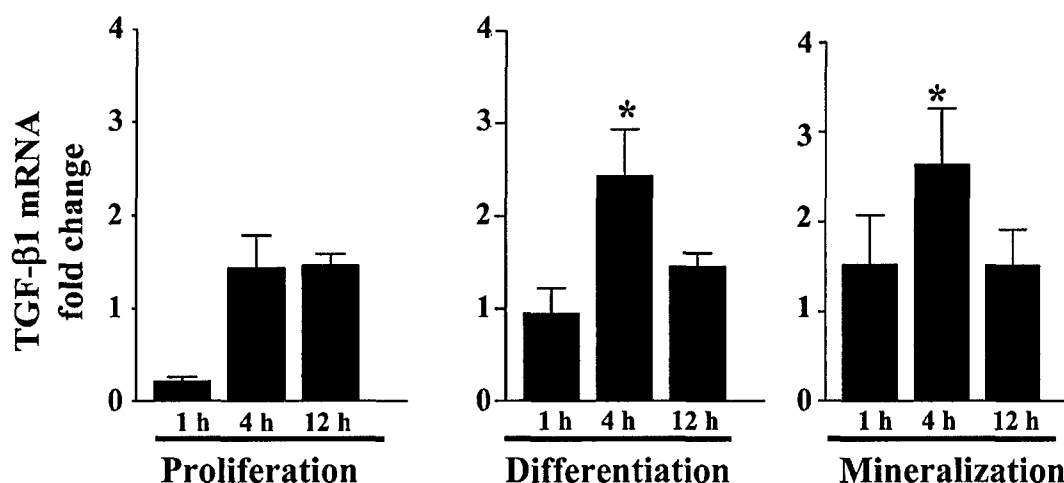


Fig. 4. Effect of PTH on expression of *TGF-β1* mRNA levels in rat primary osteoblastic cells. Proliferating, differentiating, and mineralizing rat primary osteoblasts were treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 1, 4, and 12 h at days 7, 14, and 21 of culture.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

and subjected to real time RT-PCR using specific primers for rat *TGF-β1* and β -actin. The relative levels of mRNAs were normalized to β -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

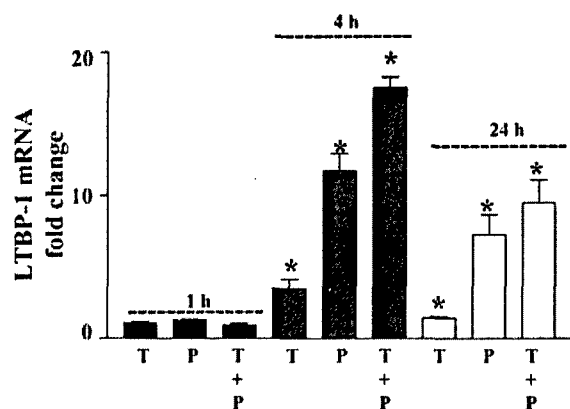


Fig. 5. Effect of PTH and *TGF-β1* on expression of *LTBP-1* mRNA levels in rat osteoblastic cells. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) (P), human *TGF-β1* (1 ng/ml) (T), or both together for 1, 4, and 24 h. Total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

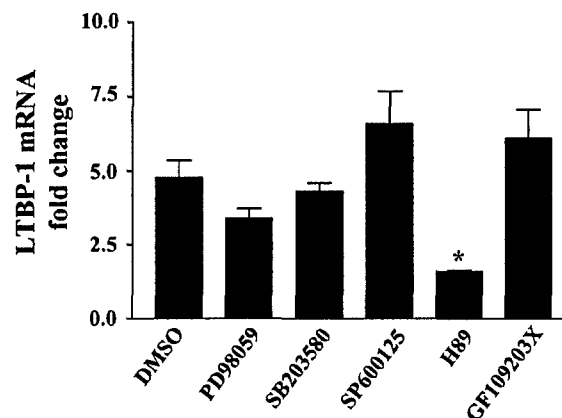


Fig. 6. PTH-stimulated *LTBP-1* mRNA expression depends on the PKA signaling pathway. UMR 106-01 cells were serum-starved for 24 h and then treated with control or rat PTH (1–34, 10^{-8} M)-containing medium for 4 h in the presence or absence of inhibitors PD98059, SB203580, SP600125, H89, and GF109203X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

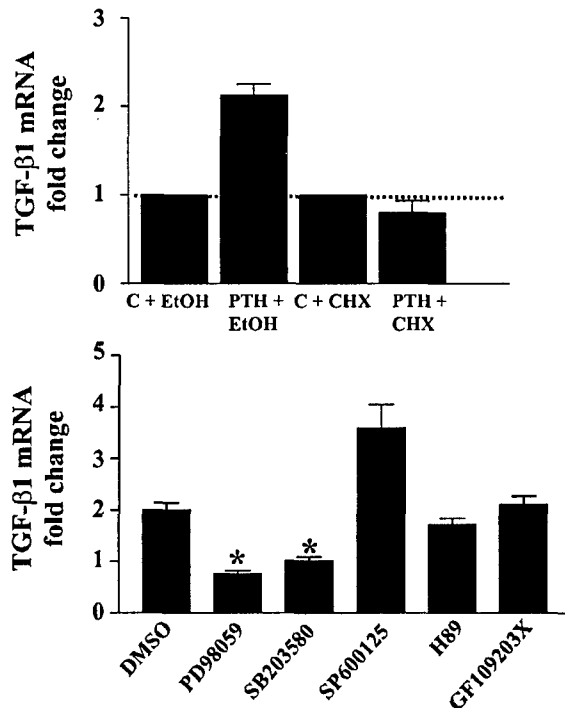


Fig. 7. PTH-stimulated TGF- β 1 mRNA expression requires de novo protein synthesis and depends on the MEK and p38 MAPK signaling pathways. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 4 h in the presence or absence of 30 μ 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- β 1 and β -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- β 1 and β -actin. The relative levels of mRNAs were normalized to β -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- β 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- β has emerged using antibodies to LTBP-1 [Miyazono et al., 1991]. TGF- β 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- β 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- β 1 in rat osteoblastic cells and this effect may be required to maintain the level of LTBP

and its bound latent TGF- β in bone matrix. It is most likely that the PTH stimulation of LTBP-1 expression in osteoblasts has an effect on osteoclasts via TGF- β . It is possible that LTBP-1 may play an important role to link signaling between the systemic (PTH) and local (TGF- β) 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- β 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- β 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- β 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- β mediates its effects by TGF- β 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- β 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- β system to mediate bone remodeling activities.

ACKNOWLEDGMENTS

This research was supported by grants from the Department of Defense (W81XWH-04-1-

0687), the New Jersey Commission on Cancer Research and the Foundation of the University of Medicine and Dentistry of New Jersey (to N.S.) and by NIH grants DK48109 and DK47420 (to N.C.P.).

REFERENCES

- Annes JP, Munger JS, Rifkin DB. 2003. Making sense of latent TGF β activation. *J Cell Sci* 116:217–224.
- Attisano L, Wrana JL. 1998. Mads and Smads in TGF β signalling. *Curr Opin Cell Biol* 10:188–194.
- Bellows CG, Ishida H, Aubin JE, Heersche JN. 1990. Parathyroid hormone reversibly suppresses the differentiation of osteoprogenitor cells into functional osteoblasts. *Endocrinology* 127:3111–3116.
- Bonewald LF. 1999. Regulation and regulatory activities of transforming growth factor β . *Crit Rev Eukaryot Gene Expr* 9:33–44.
- Bonewald LF, Dallas SL. 1994. Role of active and latent transforming growth factor β in bone formation. *J Cell Biochem* 55:350–357.
- Bonewald LF, Wakefield L, Oreffo RO, Escobedo A, Twardzik DR, Mundy GR. 1991. Latent forms of transforming growth factor- β (TGF β) derived from bone cultures: Identification of a naturally occurring 100-kDa complex with similarity to recombinant latent TGF β . *Mol Endocrinol* 5:741–751.
- Dallas SL, Park-Snyder S, Miyazono K, Twardzik D, Mundy GR, Bonewald LF. 1994. Characterization and autoregulation of latent transforming growth factor β (TGF β) complexes in osteoblast-like cell lines. Production of a latent complex lacking the latent TGF β binding protein. *J Biol Chem* 269:6815–6821.
- Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF. 1995. Dual role for the latent transforming growth factor- β binding protein in storage of latent TGF- β in the extracellular matrix and as a structural matrix protein. *J Cell Biol* 131:539–549.
- Dallas SL, Keene DR, Bruder SP, Saharinen J, Sakai LY, Mundy GR, Bonewald LF. 2000. Role of the latent transforming growth factor β binding protein 1 in fibrillin-containing microfibrils in bone cells in vitro and in vivo. *J Bone Miner Res* 15:68–81.
- Dallas SL, Zhao S, Cramer SD, Chen Z, Peehl DM, Bonewald LF. 2005. Preferential production of latent transforming growth factor β -2 by primary prostatic epithelial cells and its activation by prostate-specific antigen. *J Cell Physiol* 202:361–370.
- Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 425:577–584.
- Dobnig H, Turner RT. 1997. The effects of programmed administration of human parathyroid hormone fragment (1–34) on bone histomorphometry and serum chemistry in rats. *Endocrinology* 138:4607–4612.
- Hauschka PV, Mavrakos AE, Iafrafi MD, Doleman SE, Klagsbrun M. 1986. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. *J Biol Chem* 261:12665–12674.
- Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. 1999. Activated T lymphocytes support osteoclast formation in vitro. *Biochem Biophys Res Commun* 265:144–150.
- Hughes DE, Dai A, Tiffie JC, Li HH, Mundy GR, Boyce BF. 1996. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF- β . *Nature Med* 2:1132–1136.
- Kanzaki T, Olofsson A, Moren A, Wernstedt C, Hellman U, Miyazono K, Claesson-Welsh L, Heldin CH. 1990. TGF- β 1 binding protein: A component of the large latent complex of TGF- β 1 with multiple repeat sequences. *Cell* 61:1051–1061.
- Kanzawa M, Sugimoto T, Kanatani M, Chihara K. 2000. Involvement of osteoprotegerin/osteoclastogenesis inhibitory factor in the stimulation of osteoclast formation by parathyroid hormone in mouse bone cells. *Eur J Endocrinol* 142:661–664.
- Koli K, Saharinen J, Karkkainen M, Keski-Oja J. 2001. Novel non-TGF- β -binding splice variant of LTBP-4 in human cells and tissues provides means to decrease TGF- β deposition. *J Cell Sci* 114:2869–2878.
- Koski C, Saharinen J, Keski-Oja J. 1999. *J Biol Chem* 274:32619–32630.
- Marra F, Bonewald LF, Park-Snyder S, Park IS, Woodruff KA, Abboud HE. 1996. Characterization and regulation of the latent transforming growth factor- β complex secreted by vascular pericytes. *J Cell Physiol* 166:537–546.
- Massague J, Wotton D. 2000. Transcriptional control by the TGF- β /Smad signaling system. *EMBO J* 19:1745–1754.
- Miyazono K, Olofsson A, Colosetti P, Heldin CH. 1991. A role of the latent TGF- β 1-binding protein in the assembly and secretion of TGF- β 1. *EMBO J* 10:1091–1101.
- Munger JS, Harpel JG, Gleizes PE, Mazzieri R, Nunes I, Rifkin DB. 1997. Latent transforming growth factor- β : Structural features and mechanisms of activation. *Kidney Int* 51:1376–1382.
- Oklu R, Hesketh R. 2000. The latent transforming growth factor β binding protein (LTBP) family. *Biochem J* 352:601–610.
- Oreffo RO, Mundy GR, Seyedin SM, Bonewald LF. 1989. Activation of the bone-derived latent TGF β complex by isolated osteoclasts. *Biochem Biophys Research Commun* 158:817–823.
- Oursler MJ. 1994. Osteoclast synthesis and secretion and activation of latent transforming growth factor β . *J Bone Miner Res* 9:443–452.
- Pedrozo HA, Schwartz Z, Robinson M, Gomes R, Dean DD, Bonewald LF, Boyan BD. 1999. Potential mechanisms for the plasmin-mediated release and activation of latent transforming growth factor- β 1 from the extracellular matrix of growth plate chondrocytes. *Endocrinology* 140:5806–5816.
- Pfeilschifter J, Mundy GR. 1987. Modulation of type β transforming growth factor activity in bone cultures by osteotropic hormones. *Proc Natl Acad Sci USA* 84:2024–2028.
- Qin L, Qiu P, Wang L, Li X, Swarthout JT, Steropoulos P, Tolias P, Partridge NC. 2003. Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. *J Biol Chem* 278:19723–19731.
- Qin L, Raggatt LJ, Partridge NC. 2004. Parathyroid hormone: A double-edged sword for bone metabolism. *Trends Endocrinol Metab* 15:60–65.

- Ramirez F, Pereira L. 1999. The fibrillins. *Int J Biochem Cell Biol* 31:255–259.
- Rifkin DB. 2005. Latent TGF- β binding proteins: Orchestrators of TGF- β availability. *J Biol Chem* 280:7409–7412.
- Roberts AB. 1998. Molecular and cell biology of TGF- β . *Miner Electrolyte Metab* 24:111–119.
- Saharinen J, Hyytiäinen M, Taipale J, Keski-Oja J. 1999. Latent transforming growth factor- β binding proteins (LTBPs)—structural extracellular matrix proteins for targeting TGF- β action. *Cytokine Growth Factor Rev* 10:99–117.
- Scott DK, Brakenhoff KD, Clohisy JC, Quinn CO, Partridge NC. 1992. Parathyroid hormone induces transcription of collagenase in rat osteoblastic cells by a mechanism using cyclic adenosine 3',5'-monophosphate and requiring protein synthesis. *Mol Endocrinol* 6:2153–2159.
- Sells Galvin RJ, Gatlin CL, Horn JW, Fuson TR. 1999. TGF- β enhances osteoclast differentiation in hematopoietic cell cultures stimulated with RANKL and M-CSF. *Biochem Biophys Res Commun* 265:233–239.
- Selvamurugan N, Fung Z, Partridge NC. 2002. Transcriptional activation of collagenase-3 by transforming growth factor- β 1 is via MAPK and Smad pathways in human breast cancer cells. *FEBS Lett* 532:31–35.
- Selvamurugan N, Kwok S, Alliston T, Reiss M, Partridge NC. 2004. Transforming growth factor- β 1 regulation of collagenase-3 expression in osteoblastic cells by cross-talk between the Smad and MAPK signaling pathways and their components, Smad2 and Runx2. *J Biol Chem* 279:19327–19334.
- Seyedin SM, Thomas TC, Thompson AY, Rosen DM, Piez KA. 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc Natl Acad Sci USA* 82:2267–2271.
- Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB. 1992. Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. *J Cell Biochem* 50:425–440.
- Swarthout JT, D'Alonzo RC, Selvamurugan N, Partridge NC. 2002. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene* 282:1–17.
- Taipale J, Miyazono K, Heldin CH, Keski-Oja J. 1994. Latent transforming growth factor- β 1 associates to fibroblast extracellular matrix via latent TGF- β binding protein. *J Cell Biol* 124:171–181.
- Tam CS, Heersche JN, Murray TM, Parsons JA. 1982. Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: Differential effects of intermittent and continuous administration. *Endocrinology* 110:506–512.
- Wakefield LM, Smith DM, Flanders KC, Sporn MB. 1988. Latent transforming growth factor- β from human platelets. A high molecular weight complex containing precursor sequences. *J Biol Chem* 263:7646–7654.
- Winchester SK, Bloch SR, Fiocco GJ, Partridge NC. 1999. Regulation of expression of collagenase-3 in normal, differentiating rat osteoblasts. *J Cell Physiol* 181:479–488.