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ARTICLES

Transcriptional Activation of the Tyrosine Phosphatase Gene, OST-PTP, During Osteoblast Differentiation

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Abstract Protein tyrosine phosphatases (PTPs) are critical regulators of cellular phosphorylation functioning in processes such as cell growth, differentiation, and adhesion. Osteotesticular PTP (OST) is the only characterized member of this superfamily whose expression is regulated in osteoblasts and critical for their in vitro differentiation. Such evidence would suggest that this molecule is a key modulator of signaling events during osteogenesis, yet little is known about its genetic regulation. In an effort to examine the molecular mechanisms involved in the cellular regulation of OST, we have characterized its expression in MC3T3 osteoblasts during differentiation. Northern analysis revealed that murine OST mRNA is dramatically regulated during the preosteoblast to osteoblast progression, with predominant expression in differentiated and early mineralizing osteoblasts. This expression pattern is unique to this phosphatase since, in comparison, the structurally similar receptor PTP, LAR, and the intracellular PTP1B show little change during differentiation. Cell density contributes to this upregulated expression as confluent cultures display an increase in OST transcripts within 4 h post-plating. Transient transfection of the OST promoter in differentiating MC3T3 results in a significant increase in transcriptional activation from day 0 to day 5 of differentiation, similar in timing and intensity to the observed upregulation of the endogenous gene. This activation appears to be specific to osteoblasts, since progression to a myoblast phenotype results in no change in reporter gene activity. Culturing these preosteoblast cells in the absence of critical co-factors results in an inhibition of differentiation and leads to a delayed induction of OST transcripts as well as the attenuation of transcriptional activation. These results show that the murine OST gene is regulated at the transcriptional level in an osteoblast-specific, differentiation-dependent manner during the differentiation of MC3T3 osteoblasts. Future studies will help determine the essential regulatory elements within the OST-PTP promoter and the critical signaling pathways important in this regulation. J. Cell. Biochem. 87: 363–376, 2002.

Key words: tyrosine phosphatase; gene expression; osteoblast; differentiation; osteogenesis

Protein tyrosine phosphatases (PTPs) are enzymes which modulate signaling pathways by removing (i.e., hydrolyzing) a phosphoryl group from the tyrosine residues of specific cellular proteins [Zhang, 2002]. In concert with protein tyrosine kinases (PTKs), these molecules mediate phosphorylation and maintain a level of phosphotyrosine proteins that influences the functional state of the cell. PTPs can act as biochemical "on" or "off" switches, regulating such processes as passage through the cell cycle, proliferation, differentiation, and cell-to-cell interactions [Hunter, 1998; Hunter, 2000]. Novel drugs have been designed for specific PTPs to ameliorate symptoms of diabetes mellitus [Wagman and Nuss, 2001; Cheng et al., 2002] or inhibit oncogenesis [Pestell et al., 2000], attesting to the critical role of this enzyme superfamily in cell function.

The molecule known as osteotesticular PTP, OST-PTP, is a unique member of this superfamily which may function as an essential regulator of phosphotyrosine-dependent signaling during osteogenesis. Originally isolated from primary rat calvariae and osteosarcoma

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cell lines [Mauro et al., 1994], it is a receptor PTP possessing an extracellular region with 10 fibronectin-type-III like (FN-III) repeats, a single membrane-spanning region and an intracellular region with two tandem PTP domains. The enzymatically active membrane-proximal domain contains the signature motif, (H/V) $C(X)_5R(S/T)$, common to all classical PTP proteins [Zhang, 2002]. The extracellular FN-III protein motifs share sequence homology with matrix proteins such as fibronectin, tenascin and type VII collagen [Mauro et al., 1994] and are often found in adhesion proteins [Hutter et al., 2000]. The presence of these motifs is suggestive of interactions of OST-PTP with a ligand yet to be identified. One could speculate that such an interaction might result in modulation of this molecule's phosphatase activity and by extension, the regulation of cell–cell or cell–matrix adhesion events.

Our laboratory and others have shown that this phosphatase is the only characterized PTP whose expression is tightly regulated and critical for the differentiation of a preosteoblast to a mature, mineralizing osteoblast phenotype. During proliferation of cultured primary rat osteoblasts (rOBs), OST-PTP mRNA expression is low to nondetectable, increasing to peak levels during differentiation and matrix secretion. In late-stage mineralizing cultures, expression of this transcript declines to levels observed during proliferation [Mauro et al., 1994; Chengalvala et al., 2001]. The amount of immunoprecipitated OST-PTP protein and associated PTP activity in rOBs follows the same trend [Chengalvala et al., 2001]. Interestingly, hormonal factors important in bone remodeling can modulate the expression of this phosphatase. Parathyroid hormone (PTH), a potent regulator of osteoblast function, can enhance OST-PTP mRNA 5–6-fold in osteosarcoma cells [Mauro et al., 1996]. In addition, PTH as well as 1α, 25-dihydroxyvitamin D3 can modify OST-PTP expression in mineralizing MC3T3 osteoblasts [Mauro et al., 2001]. Evidence that this regulated OST-PTP expression is essential was found during in vitro studies of rOBs incubated with antisense oligonucleotides directed to the 5′ untranslated region of this gene. Lack of OST expression resulted in an 80% reduction in the number of bone nodules formed by day 18 of differentiation as well as a 60% decrease in osteocalcin, a matrix protein secreted by mature osteoblasts [Chengalvala et al., 2001].

The mechanisms and associated signaling pathways responsible for regulating the expression of OST-PTP are unknown. In the studies presented here, we have begun to address the molecular mechanisms involved in the regulation of this gene. Using the MC3T3-E1 cells as the osteoblast cell model, we show that OST-PTP mRNA is uniquely regulated during osteoblast differentiation in comparison to other PTPs. This regulation is dependent on cell to cell interactions and the proper progression to a mature osteoblast. The putative murine OST-PTP promoter, which we previously characterized [Morrison and Mauro, 2000], is capable of conferring osteoblast-specific, differentiation-dependent transcriptional activation during differentiation of MC3T3 osteoblasts. These results suggest that transcriptional activation is one of the mechanisms involved in the regulation of this phosphatase gene during osteoblast differentiation.

MATERIALS AND METHODS

Materials and Reagents

Tissue culture reagents were purchased from Gibco-Invitrogen (Carlsbad, CA), Hyclone (Logan, UT), and Sigma (St. Louis, MO). Restriction endonucleases were purchased from New England BioLabs (Beverly, MA) and Gibco-Invitrogen. Reagents for the polymerase chain reaction (PCR) amplification and subcloning were purchased from Perkin-Elmer Corp. (Norwalk, CT), Promega Corp. (Madison, WI), and Roche Molecular Biochemicals (Indianapolis, IN). Reagents for Northern analyses were purchased from Ambion (Austin, TX), Amersham Biosciences (Piscataway, NJ), Eppendorf 5Prime (Boulder, CO), and Promega Corp. (Madison, WI). Sources of additional reagents are indicated in the following sections.

Cell Culturing

The MC3T3-E1 osteoblast-like cell line was maintained as previously described [Morrison and Mauro, 2000]. Prior to experiments, cells were seeded at a density of 5×10^5 cells per 100 mm plate in maintenance media, also referred to as non-differentiation (ND) media. This ND media consisted of MEM, 10% fetal bovine serum, 0.1 mM non-essential amino acids (NEAA), pencillin G (50 U/ml) and streptomycin sulfate (50 μg/ml). After 14–16 h, media was replaced with ND media, as above, or

differentiation media (DIFF; α-MEM, 10% FBS, NEAA, antibiotics) which was supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate to promote differentiation and matrix mineralization. The HeLa cells were maintained in DMEM high glucose with 10% calf serum and antibiotics. The C2C12 were maintained in DMEM high glucose with 10% FBS and differentiated to myoblasts at high cell density in DMEM plus 2% horse serum.

RNA Harvesting and Northern Analysis

Total RNA was extracted with TRlzol reagent (Invitrogen) with subsequent enrichment of poly A containing mRNA from samples, conducted using the PolyATract kit (Promega Corp.) as previously described [Mauro et al., 1994; Mauro et al., 1996]. Poly A+ enriched RNA samples (2–5 μg/lane) were resolved by gel electrophoresis, transferred to Hybond N+ (Amersham) and hybridizations conducted [Mauro et al., 1996]. Templates for synthesis of random-primed cDNA probes encoding PTPs were as follows: (1) ~900 bp *Eco RI/Xho I* fragment of murine OST-PTP cDNA; (2) *BamHI fragment* of rLAR cDNA; and (3) and *EcoRI* fragment of rPTP1B cDNA. Expression of phenotypic markers including alkaline phosphatase, type I collagen, and osteocalcin was determined to verify the progression of osteoblast differentiation [Mauro et al., 1994]. In addition, partial cDNA for rat cyclophilin was used as a control for RNA integrity and concentration [Mauro et al., 1994]. Transcripts were visualized by overnight exposure in a phosphorscreen cassette followed by analysis using Phospholmager and related software (Molecular Dynamics, Sunnyvale, CA).

Cells from additional plates were also collected for alkaline phosphatase activity or von Kossa staining. Alkaline phosphatase activity was determined using a colorimetric assay [Franceschi and Iyers, 1992]. The staining of mineralized matrix on day 22 cultures was conducted as previously described [Franceschi and Iyers, 1992].

Plasmid Construction

The promoter-reporter gene plasmids, pmOST(H3)-luc sense and antisense, were constructed by ligating the 3,002 bp *HindIII* fragment of the mOST-PTP promoter (−2,833/+169) into the *HindIII* site of the pGL3 Basic luciferase reporter vector (Promega Corp.). This promoter fragment was originally isolated as previously described [Morrison and Mauro, 2000]. The plasmid, pmOC-luc, was constructed by ligating the 1.3 kb *Smal/Hind III* fragment of the mouse osteocalcin OG2 promoter (G. Karsenty) into the *Smal/Hind III* site of the pGL3 Basic luciferase reporter vector. All insert identities and orientations were confirmed by automated DNA sequencing performed by the Advanced Genomics Analysis Center at the University of Minnesota.

Transient Transfections and Luciferase Assays

Long-term transient transfections of MC3T3 cells, conducted over several days, have been used successfully in regulation studies of other osteoblast-specific genes [Xiao et al., 1997, Benson et al., 2000]. To insure that the calculated luciferase activity units were a true representation of promoter activity by day 5, the following controls were routinely included in experiments: (1) antisense mOST-PTP promoter construct as a negative control; and (2) osteocalcin promoter construct, pmOC-luc, as a positive control. Cells were transfected using lipofectAMINE (Invitrogen) according to the manufacturer's instructions. For differentiation experiments, 2×10^5 cells were plated in 6-well plates, cultured overnight, and transfected with 1 μg of the appropriate promoter construct. The Renilla luciferase vector, pRL-CMV (Promega), was co-transfected, at a concentration of 10 ng/well, as a control for transfection efficiency. Cultures were given differentiating media 24 h after transfection and harvested at specified days of differentiation. For confluency experiments, 5×10^5 cells were plated in 100 mm plates, cultured overnight, and transfected with 4 μg of the mOST-PTP reporter construct plus 40 ng pRL-CMV. Cells were grown for 24 h, trypsinized and 5×10^5 cells were plated in 6-well or 100 mm plates. Cultures were harvested at time points post-plating as indicated (Fig. 5A). To harvest samples, cells were rinsed and scraped into 1× PBS and resuspended in 250 μl of 1× Passive Lysis Buffer (Promega). Samples were stored at −80°C for later analysis.

For quantitation of luciferase activity, cell samples were initially lysed by sonication and two freeze-thaw cycles. Luciferase activity in these lysates was assayed with the Dual Luciferase Reporter Kit (Promega). Aliquots of cell lysates were mixed with 100 μl of luciferase

reagent 2 and measurements of firefly luciferase activity taken using a Lumat LB 9507 luminometer (PerkinElmer Life Sciences, Gaithersburg, MD). Subsequently, 100 μl of Stop-glo reagent was added to samples and Renilla luciferase activity measured. The protein content of cell lysates was quantitated by the Bradford method with Bovine Gamma Globulin as a standard (Bio-Rad Laboratories, Hercules, CA). Firefly luciferase activity in relative light units were normalized with Renilla luciferase activities. Final luciferase activities were expressed as normalized luciferase units per mg protein. All transfection experiments were conducted with triplicate wells, multiple lots of plasmid DNA and repeated at least three times. Unless otherwise indicated, data presented in figures is a combination of two independent experiments with an $n = 5-12$ replicate samples.

Statistical Analyses

The statistical significance of treatments on luciferase activity was tested using the scientific graphics and statistics program, GraphPad Prism (version 3.0; San Diego, CA). Data was initially tested for Gaussian or normal distribution using the Kolmogorov-Smirnov (KS) test. Then, one-way ANOVA was performed with the threshold value set at 0.05. If a P value less then 0.05 was calculated, then selected multiple pairwise comparisons were made using the Bonferroni's post test. A P value less then 0.05 for these post-tests was interpreted to indicate statistical significance.

RESULTS

Expression of OST-PTP mRNA and Other "Classical" PTPs During MC3T3 Differentiation

To insure that the MC3T3-E1 cell line is an adequate model to explore the regulation of OST-PTP, the expression pattern of mRNA encoding this phosphatase was determined. This expression was compared to the transcripts encoding two other PTPs, LAR (leucocyte antigen-related) and PTP1B. The LAR gene was chosen because this receptor PTP is structurally similar to OST, possessing FN-III repeats in its extracellular domain. The PTP1B gene is a representative member of the subfamily of intracellular PTPs and is a well-studied molecule in other systems. In addition to these genes, established markers of osteoblast differentiation were also examined including type I collagen, alkaline phosphatase, and osteocalcin.

Northern analysis showed that levels of the OST-PTP transcript increase dramatically by day 3 of differentiation showing ~11–14-fold change that is maintained to day 14 (Fig. 1A,B). At day 22 in mineralizing cultures, mRNA levels decline to 3-fold above that of day 1 cultures. In comparison, both LAR and PTP1B mRNA are expressed in MC3T3 osteoblasts but show little change during differentiation. Levels of LAR mRNA increase slightly and are maintained until a decline to basal on day 22 (Fig. 1B). The expression of PTP1B actually declines between days 1 and 6 and remains at this level through differentiation and mineralization. As expected, the markers of osteoblast differentiation are expressed and delineate the periods of differentiation and mineralization in the cultures. Alkaline phosphatase mRNA increases and is maintained from day 3 through 14, declining on day 22 (Fig. 1C). This trend is similar to that observed for OST-PTP mRNA. Alkaline phosphatase enzymatic activity increases in these cultures, also an indication of proper differentiation (Fig. 1D). In addition, type I collagen is expressed throughout differentiation and mineralization. In contrast, osteocalcin mRNA, a gene marker induced later at the initiation of mineralization, was detectable in our cultures at day 9, peaking in day 14 mineralizing cultures.

Dependence of mOST-PTP Expression on the Progression of Differentiation

To determine if the expression of OST-PTP is coupled to osteoblast differentiation, the progression to the mature mineralizing phenotype was inhibited by culturing cells without ascorbic acid (AA; vitamin C) and β-glycerol phosphate (β-GP). Ascorbic acid is a co-factor necessary for collagen processing and β-GP is needed for mineralization of the collagen matrix [Franceschi and Iyer, 1992; Franceschi et al., 1994]. MC3T3 cells were plated with (DIFF; differentiation media) or without (ND: non-differentiation media) these additives and the expression of OST and alkaline phosphatase (AP) was determined. Lack of AA and β-GP leads to a substantial attenuation in OST-PTP transcript at days 4 and 6 of culture. Transcript levels of OST at day 4 and 6 were ~11- and

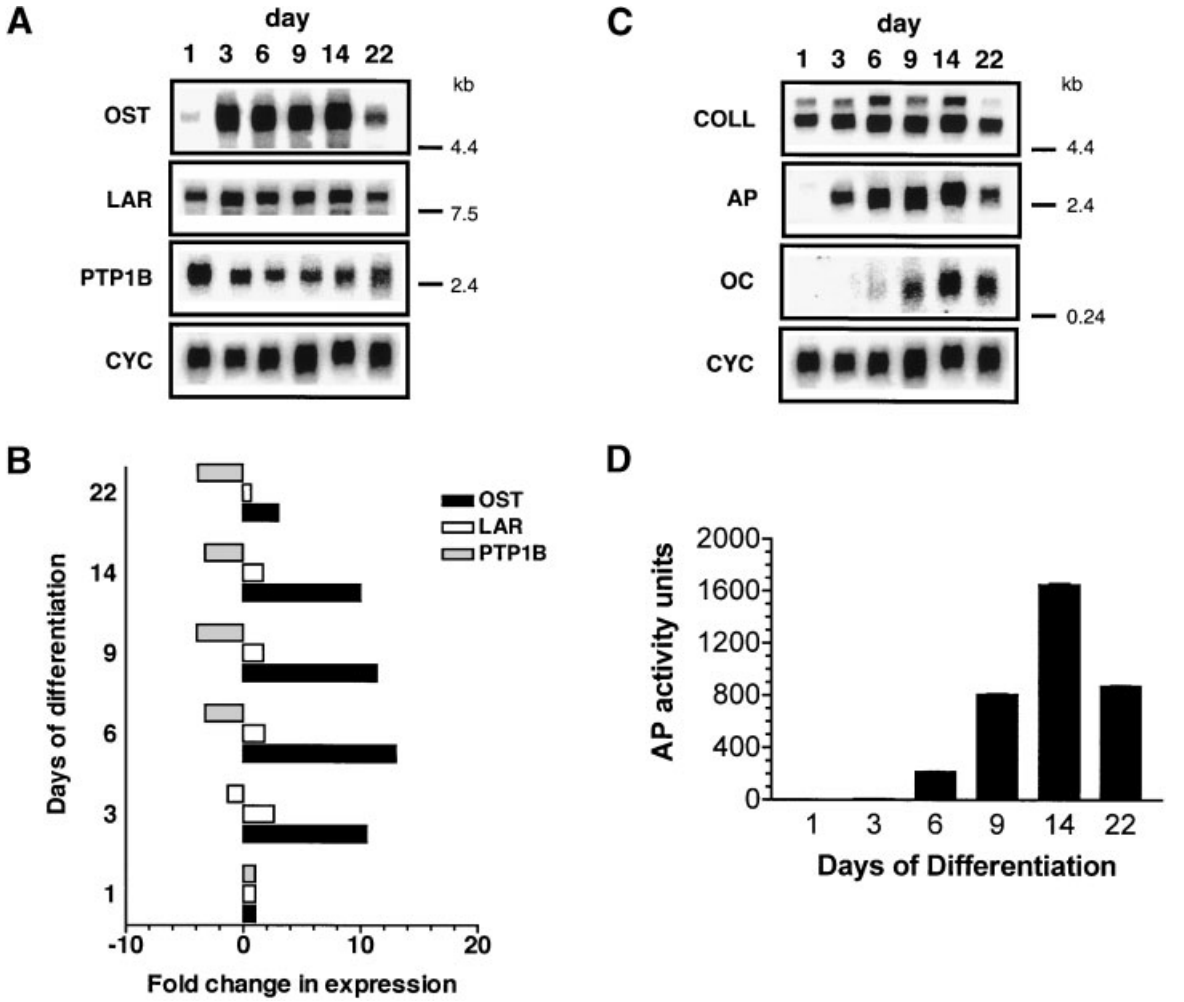

Fig. 1. Expression of mOST-PTP mRNA is uniquely regulated during differentiation of MC3T3 osteoblasts. MC3T3-E1 cells were cultured in differentiation media (DIFF) and cell lysates collected for RNA extraction on the indicated days. Resolution of transcripts by agarose-formaldehyde electrophoresis (3–5 μg poly A RNA/lane) was followed by blotting and Northern analysis. Separate plates were also harvested for determination of alkaline phosphatase activity, an indicator of osteoblast maturation. **A**: Northern blot depicting mRNA transcripts encoding the PTPs: mOST-PTP (OST), LAR, and PTP1B. Transcripts for CYC were also visualized as a control for RNA integrity and loading. Molecular size markers are indicated in kilobases (kb). **B**: Graphical depiction of the fold change in mRNA expression of the PTPs. Densitometric readings of digitized phosphoimages were determined for each transcript using NIH Image v. 1.61. These units were normalized across all lanes using the day 1 cyclophilin value. Fold change is expressed as normalized units/normalized units of day 1 cultures. **C**: Northern blot depicting expression of osteoblast markers. cDNA probes for alkaline phosphatase (AP), type I collagen (COLL), and osteocalcin (OC) were used as described. Transcripts for cyclophilin (CYC) were also visualized as a control for RNA integrity and loading. Molecular size markers are indicated in kilobases (kb). **D**: Alkaline phosphatase activity of MC3T3 osteoblasts during differentiation. Activity of cell lysates was determined using the substrate, *p*-nitrophenol phosphate (pNPP), in a colorimetric assay. Activity was expressed as nmoles of pNPP hydrolyzed/min-plate.

14-fold greater than day 2 levels in DIFF cultures whereas those of ND cultures were only 2.5- and 4-fold greater, respectively (Fig. 2A). By day 14, OST mRNA levels in ND cultures have reached those of DIFF cultures at day 6, reflecting the "escape" and progression to differentiation often observed with prolonged culturing of confluent MC3T3 cells. Both mRNA expression and enzymatic activity of the differentiation marker, AP, revealed the effectiveness of the ND culturing. AP transcripts were also attenuated and barely detectable until day 14. Alkaline phosphatase activity was very low in ND cultures as compared to DIFF cultures (day 6 ND 6.9 ± 1.4 vs. DIFF 168.9 ± 6.0 nmoles pNPP hydrolyzed/min-plate). The "escape" was also evident in increasing AP activity at day 14 with ND cultures at 114 ± 0.5 vs. ND cultures on

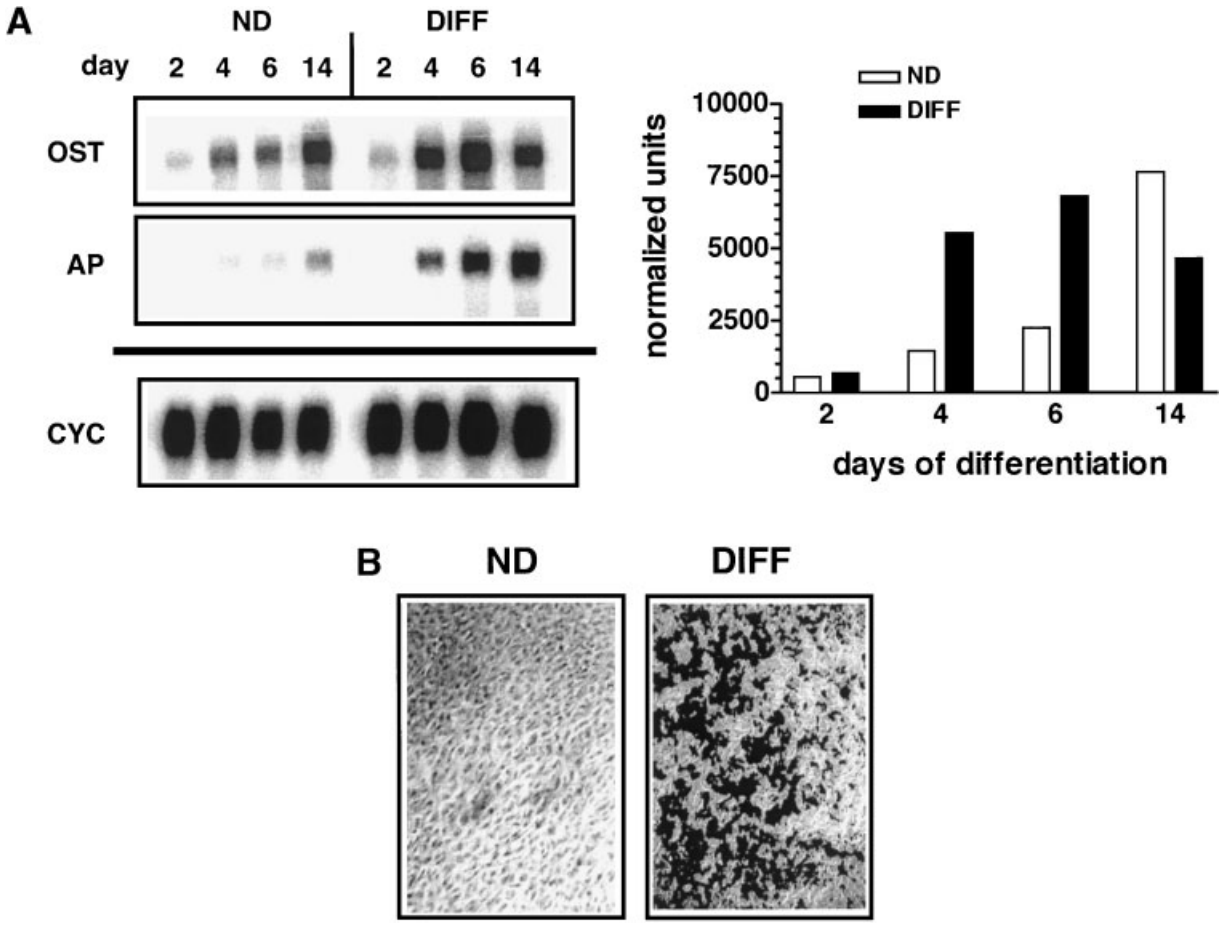

Fig. 2. Expression of mOST-PTP is dependent on the proper progression of differentiation. MC3T3-E1 osteoblasts were cultured in differentiating (DIFF) or non-differentiating (ND) media lacking βGP and AA. Cells were harvested for RNA extraction and Northern analysis performed as described. Mineralization of day 22 ND and DIFF cultures was determined using the von Kossa stain. **A**: **Left**: Northern blot depicting expression of mOST-PTP (OST) and the differentiation marker, alkaline phosphatase (AP). **Right**: Graphical depiction of transcript levels expressed as densitometic units normalized to cyclophilin. **B**: Photomicrographs showing results of von Kossa staining for day 22 ND and DIFF cultures. Darkly stained regions indicate mineralized matrix.

day 6 at 6.9 ± 1.4. As expected, ND cultures showed no detectable mineralization as compared to the extensive von Kossa staining of mineralized matrix in DIFF cultures (Fig. 2B).

The observation that OST-PTP expression still occurred early regardless of treatment suggested that factors, in addition to adequate matrix secretion and maturation, were relevant. Enhanced cell–cell interactions (i.e., experimentally manipulated by high cell density or greater percent confluency) is known to be critical for progenitor or stem cell differentiation to specific cell lineages. Therefore, we examined if confluency could be a factor in the differentiation-dependent expression of this PTP. MC3T3 cells were plated at a subconfluent (~50%) or confluent (~100%) state (Fig. 3A) and cells harvested for RNA at 4, 8, and 24 h after plating. Subconfluent cultures represent the cell density used for initiation of all experiments, corresponding to day 1. Northern analysis revealed detectable OST-PTP expression at 4 h in confluent cultures, whereas this transcript was not detectable in subconfluent cultures until 24 h post-plating (Fig. 3B). In both cultures, expression increased from 4 to 24 h. Longer exposure times to visualize transcripts and lower normalized densitometric units suggest that the peak expression levels observed during differentiation (see Fig. 1 and 2) were not attained in the confluent cultures by 24 h post-plating.

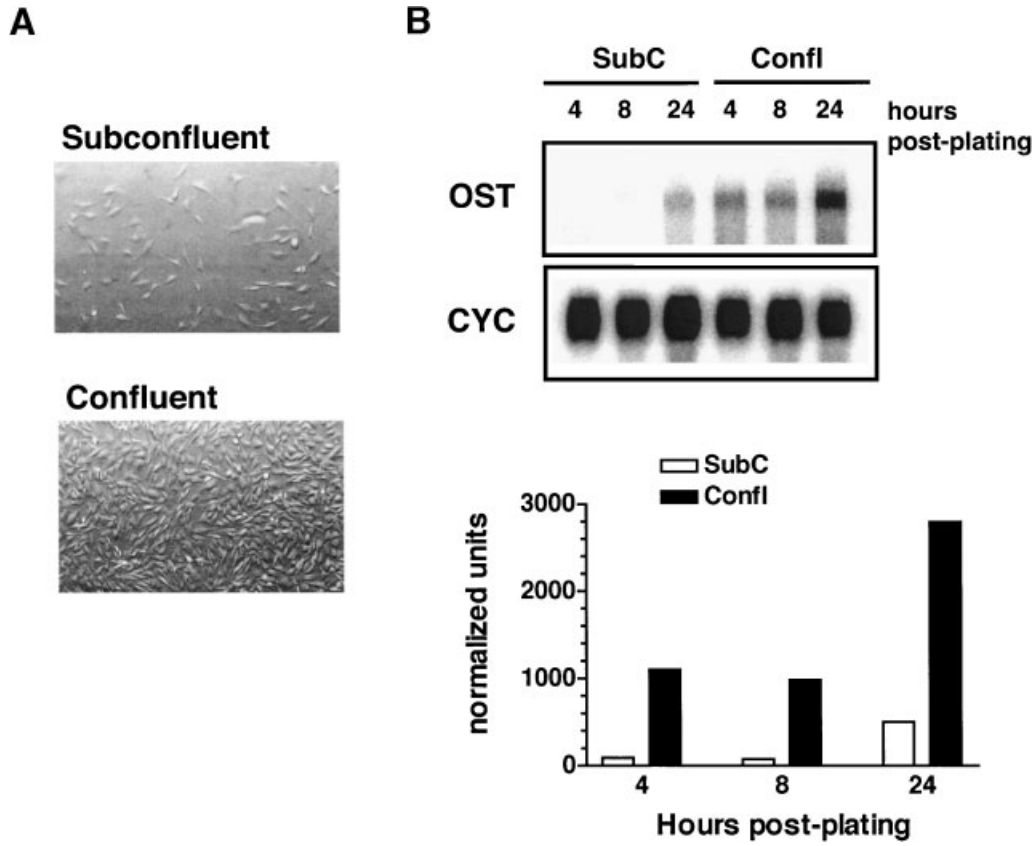

Fig. 3. Cell–cell interactions (i.e., increased cell density) contributes to the differentiation-associated expression of mOST-PTP. MC3T3 cells were plated at an initial density of 5×10^5 cells for subconfluent cultures (SubC; ~50% confluency comparable to day 1 of differentiation experiments) or 2.0×10^6 cells for confluent cultures (Confl; ~100% comparable to day 3–4). RNA extractions and Northern analysis was conducted as described in Figure 1. **A**: Representative photomicrographs showing subconfluent and confluent cultures. **B**: Northern blot depicting expression of mOST-PTP (OST). SubC and Confl cultures were harvested for RNA at 4, 8, and 24 h post-plating. Below is graphical depiction of transcript levels expressed as normalized densitometric units.

Transcriptional Activation of the mOST-PTP Gene

To explore potential regulation of mOST-PTP expression at the transcriptional level, the putative murine OST-PTP promoter was subcloned, in the sense and antisense orientation, into the luciferase reporter vector, pGL3-Basic. This created the pmOST(H3)-luc sense and antisense constructs (Fig. 4A). This OST-PTP promoter is a 3,002 bp Hind III fragment previously isolated in our laboratory from gene-positive BAC clones [Morrison and Mauro, 2000]. It includes 2,833 bp of sequence upstream of the transcriptional start site as well as 169 bp of the downstream 5′ untranslated region. Initial transient transfection experiments in undifferentiated MC3T3 cells, the pluripotent C3H10T1/2 progenitor cells, and the osteosarcoma lines, UMR and ROS, established that this fragment possessed basal transcriptional activity (data not shown). Transient transfection experiments were then conducted where undifferentiated MC3T3 cells were transfected, switched to DIFF or ND media and cells harvested at 24, 72, 96, and 144 h post-transfection. These time points approximated days 0, 2, 3, and 5 of differentiation. Analysis of DIFF cultures revealed a strong increase in luciferase activity of the OST-PTP sense promoter (Fig. 4B). This activation was significantly different on days 3 and 5 of differentiation as compared to day 0 values. Disruption of differentiation by culturing cells in ND media significantly attenuated this activation (Fig. 4B). These cultures exhibited only a ~1.5-fold change in luciferase activity on day 5 as compared to an ~9-fold change for DIFF cultures. Little activity was evident from the control antisense constructs in DIFF or ND cultures. To

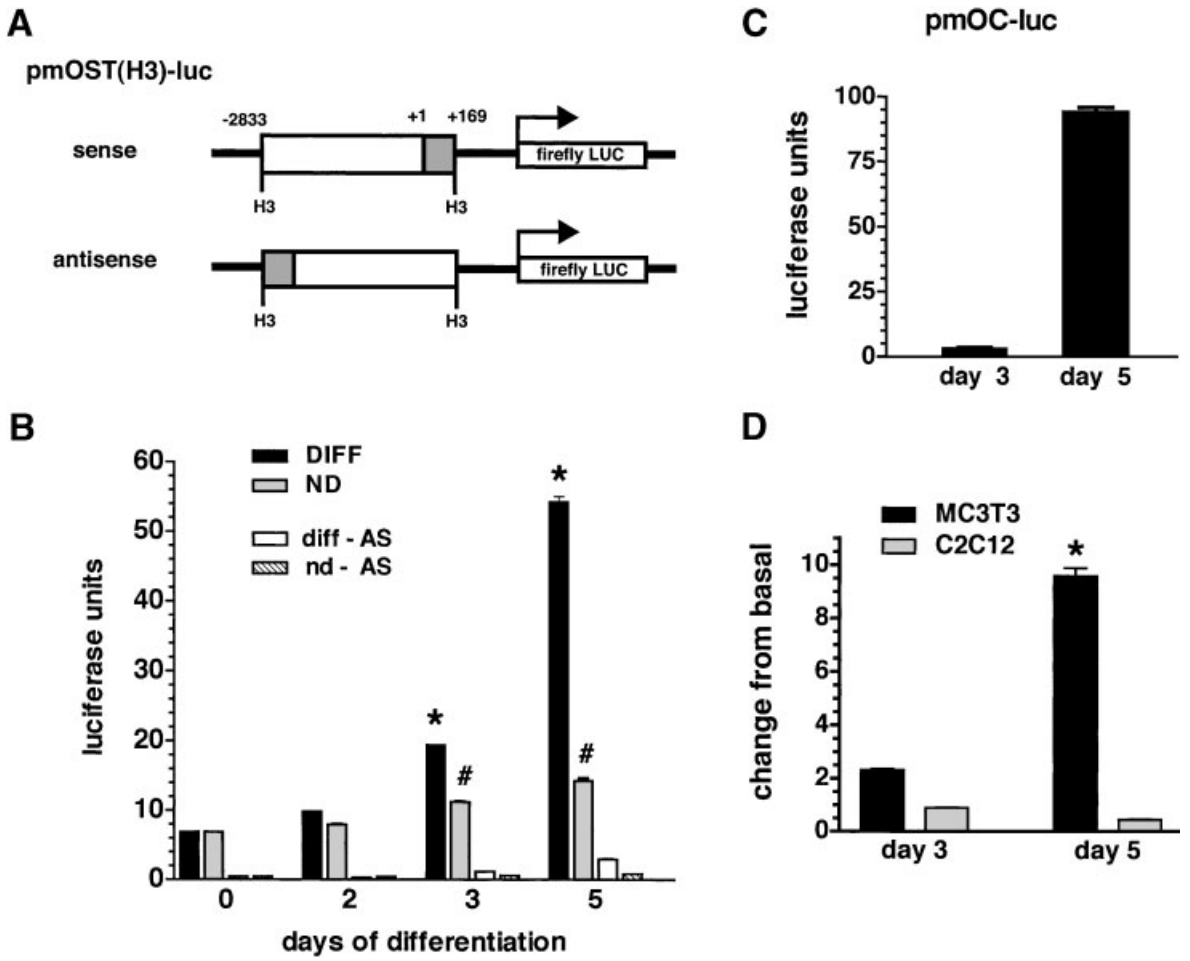

Fig. 4. Upregulation of expression is due, in part, to osteoblast-specific transcriptional activation of the mOST-PTP gene. Cells were co-transfected with 1 μg of the mOST-PTP promoter construct, pmOST(H3)-luc (sense or antisense) as well as 10 ng of the transfection control plasmid, pRL-CMV. Twenty-four hours after transfection (day 0), non-differentiating (ND) or differentiating (DIFF) media was added to the cultures. Cells were harvested at indicated times during differentiation and luciferase and protein assays conducted as described in Materials and Methods. Luciferase units are expressed as relative light units (RLU) of firefly luciferase/RLU of renilla luciferase/mg protein. **A**: Schematic depicting the mOST-PTP promoter constructs. The mOST-PTP promoter is the 3.0 kb Hind III fragment, previously described [Morrison and Mauro, 2000], which contains 2,833 bp upstream of the transcriptional start site (+1) as well as 169 bp of 5′ UTR (depicted by the shaded region). This fragment is in the sense or antisense orientation upstream of the firefly luciferase gene in the parent vector, pGL3-Basic (Promega). The resulting constructs are referred to as pmOST(H3)-luc sense or antisense. **B**: Activity of the mOST-PTP promoter during differentiation of MC3T3 osteoblasts. Cells were transfected, cultured in ND or DIFF media, and harvested on days 0, 2, 3, and 5 of differentiation. Activity of the pmOST(H3)-luc are depicted for the DIFF sense (black bar), the ND sense (gray bar), the DIFF antisense (AS; white bar), and the ND antisense (AS; hatched bar) cultures. An asterisk (*) indicates statistically significant difference of DIFF sense cultures as compared to day 0 DIFF sense cultures. A pound sign (#) indicates difference of ND sense cultures as compared to DIFF sense. **C**: Activity of promoter in osteoblasts vs. myoblasts. MC3T3 and C2C12 cells were transfected and cultured to promote differentiation to the osteoblast and myoblast phenotype, respectively. Activity of the mOST-PTP promoter (pmOST(H3) sense) is expressed as fold change from basal with basal values as day 0 luciferase units. An asterisk (*) indicates statistically significant difference in activity of day 5 osteoblast cultures as compared to day 3 cultures. No statistical significance in activity was noted for myoblast cultures. **D**: Activity of positive control, the mouse osteocalcin promoter. Parallel MC3T3 cultures were transfected with the pmOC-luc plasmid and activity of the osteocalcin promoter assayed on day 3 and 5 of differentiation.

prove that these long-term transient transfections were accurately testing activation, parallel DIFF cultures were transfected with the positive control, the mouse osteocalcin OG2 promoter (Fig. 4C). As expected, these cultures showed a strong activation of the osteocalcin promoter by day 5 of differentiation.

Additional experiments were performed to test if this differentiation-dependent transcriptional activation of the mOST promoter was

specific for the progression to an osteoblast phenotype (Fig. 4D). MC3T3 and C2C12 cells were transiently transfected with the OST-PTP promoter construct and cultured to promote differentiation to the osteoblast or myoblast phenotype, respectively. Essentially no change in luciferase activity was observed in the day 3 or 5 cultures when C2C12 cells were exhibiting elongation and fusion typical of cultured myoblasts (Fig. 4D). In contrast, differentiated MC3T3 osteoblasts exhibited a significant change in activity by day 5 as observed in previous experiments. Little activity was evident from control antisense constructs (data not shown).

Since our previous experiments suggested that confluency could modulate the differentiation-dependent expression of this gene, the ability of such enhanced cell–cell interactions to contribute to the activation of the OST-PTP promoter was determined. Twenty-four hours following transfection, MC3T3 cells were trypsinized and replated to mimic the subconfluent and confluent cell densities previously described (Fig. 5A). Initial experiments were conducted where these cultures were harvested at 1, 4, 8, and 24 h post-plating to test the effect of confluency alone. Analysis of luciferase activity revealed that high cell density leads to enhanced activity of the OST-PTP promoter, with

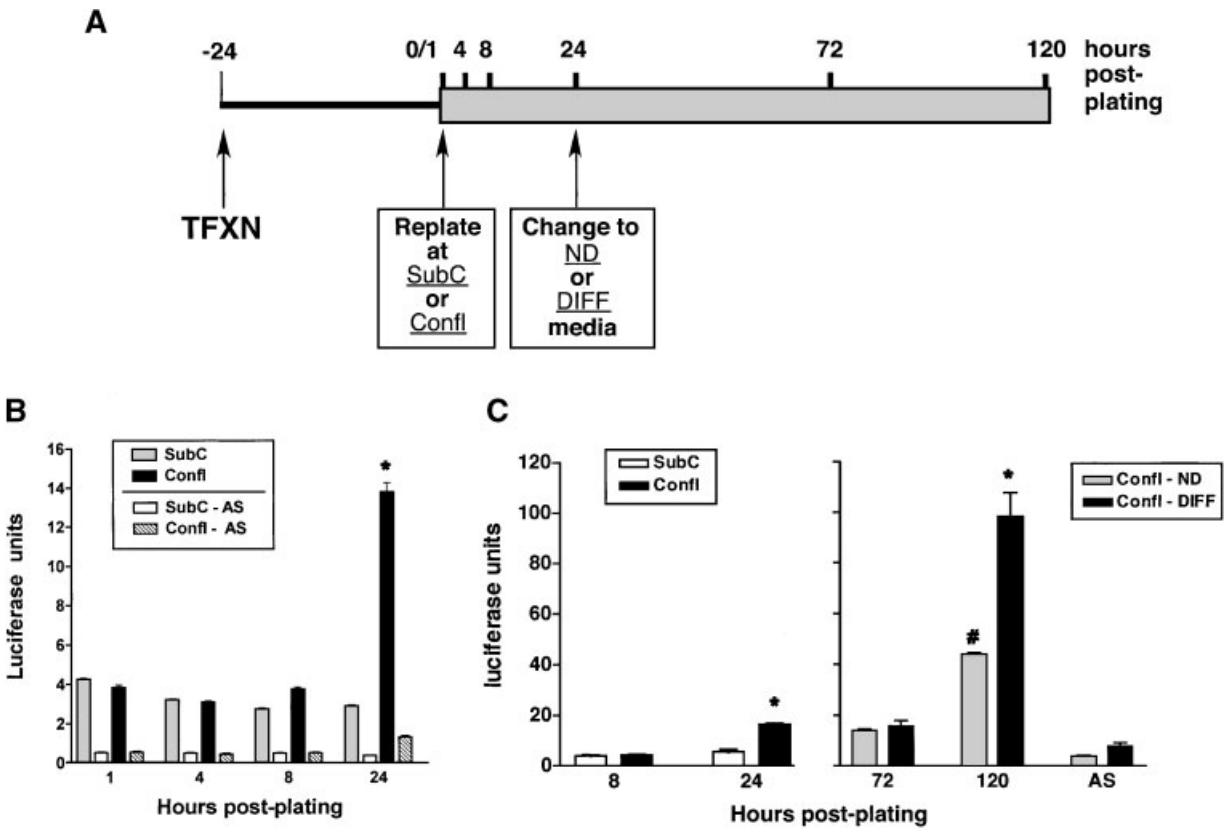

Fig. 5. The transcriptional activation observed during osteoblast differentiation is dependent on both cell–cell interactions and the proper differentiation environment. **A**: Schematic drawing illustrating design for confluency/differentiation experiments reported in parts **B** and **C**. **B**: Activity of the mOST-PTP promoter with increased cell density. MC3T3 cells were transfected with the constructs, pmOST(H3)-luc sense or AS +pRL-CMV, and identical cell numbers replated on 100 mm or 6-well plates to attain subconfluent (SubC) or confluent (Confl) cultures, respectively. Cell lysates were collected at 1, 4, 8, and 24 h post-plating and luciferase untis determined. Activities are depicted for the SubC sense (gray bar), the Confl sense (black bar), the SubC antisense (AS; white bar), and the Confl antisense (AS; hatched bar) cultures. An asterisk (*) indicates statistically significant difference of Confl sense cultures from day 1 Confl cultures. No statistical significance in activity was noted for SubC sense cultures. **C**: Activity of the mOST-PTP promoter with confluency and differentiation. As illustrated in (A), cells were transfected (pmOST(H3)-luc sense +pRL-CMV), replated at SubC and Confl and harvested at 8 and 24 h. Parallel Confl cultures were switched to ND or DIFF media and harvested at 72 and 120 h post-plating, corresponding to day 3 and 5 of differentiation. An asterisk (*) indicates statistically significant difference of Confl or DIFF Confl cultures as compared to day 8 Confl cultures. A pound sign (#) indicates difference of ND Confl cultures as compared to DIFF Confl cultures.

significant differences observed between subconfluent and confluent cultures at 24 h post-plating (Fig. 5B). Parallel experiments performed in HeLa cells, a cell line of non-osseous tissue origin, showed no effect of confluency on promoter activity (24 h SubC at 0.179 ± 0.02 ($n = 7$) vs 24 h Confl at 0.184 ± 0.02 ($n = 8$)).

The relative contribution of confluency in the activation of transcription during osteoblast differentiation was determined by a final set of experiments. Subconfluent and confluent cultures were harvested at 8 and 24 h post-plating as previously described. Parallel confluent cultures, originally plated at 0 h post-plating, were switched to DIFF or ND media at 24 h post-plating (Fig. 5A). These DIFF and ND cultures were then harvested at 72 and 120 h post-plating which would correspond approximately to days 3 and 5 of differentiation. As expected, confluent cultures exhibited enhanced promoter activity at 24 h as compared to 8 h post-plating, whereas subconfluent cultures showed no change (Fig. 5C, left). Confluent DIFF cultures carried since 0 h post-plating showed a dramatic increase in OST-PTP promoter activity at 120 h which was significantly different from the activity of 8 h subconfluent cultures. The requirement for proper matrix secretion and maturation in the progression of differentiation is again observed in the strong attenuation of promoter activation in the confluent ND cultures (Fig. 5C, right). These ND cultures did exhibit some increase in activity as seen in earlier experiments (Fig. 4B) but this may again reflect the initial "escape" to a differentiated phenotype with prolonged culturing of confluent MC3T3 cells.

DISCUSSION

The tyrosine phosphatase, OST-PTP, is the only PTP identified to date whose expression is thought to be specific to osteoblasts and critical for their in vitro differentiation. Although evidence would suggest that this molecule could be a key regulator of signaling events during osteogenesis, very little is known about the mechanisms important in regulating its expression. With the long-term goal of understanding the regulation and function of this PTP, our laboratory has begun to explore the molecular mechanisms responsible for its expression during osteoblast differentiation. In this report, we show that the mOST-PTP (OST) gene is regulated at the transcriptional level in a osteoblast-specific, differentiation-dependent manner during the differentiation of MC3T3 osteoblasts.

Unique Expression of mOST-PTP

Like cultured primary osteoblasts [Owen et al., 1990], the MC3T3 cell line recapitulates the progression from the proliferation of immature preosteoblasts through differentiation to the mature, mineralizing phenotype [Franceschi and Iyer, 1992; Quarles et al., 1992]. These phases are marked by the sequential activation of specific genes including type I collagen, alkaline phosphatase, and osteocalcin [Stein et al., 1996]. In our studies, OST-PTP mRNA is barely detectable during the proliferative phase when alkaline phosphatase mRNA also is not expressed. During the differentiation phase, when alkaline phosphatase expression serves as a transition marker, OST-PTP expression increases dramatically, peaking between days 6 and 9. Expression of osteocalcin serves as the marker of early mineralization which occurs in our cultures between day 9 and 14. During this time, OST-PTP is strongly expressed and does not decline until after day 14. These results suggest that expression of this phosphatase is dramatically regulated during this preosteoblast-osteoblast progression, being primarily expressed during differentiation and early mineralization. This pattern is very similar to that reported for in vitro differentiation of primary rat osteoblasts [Mauro et al., 1994; Chengalvala et al., 2001]. As for the other PTPs examined, LAR and PTP1B, this is the first report demonstrating that these phosphatases are expressed in MC3T3 osteoblasts, but they do not exhibit the same punctuated, differentiation-associated regulation as observed for the OST-PTP gene. Although we have not performed an exhaustive examination of all known PTP genes, we feel this finding is significant, especially in the case of LAR. LAR, a receptor PTP, is structurally similar to OST-PTP, possessing FN-III "adhesive" motifs in its extracellular domain [O'Grady et al., 1994]. It is thought to mediate cell adhesion events with putative ligands such as laminin [O'Grady et al., 1998] and it appears to be critical for cell differentiation in organs such as the mammary gland [Schaapveld et al., 1997]. We would conclude that if the highly

regulated expression of OST-PTP was the result of a non-specific response of PTPs, particularly receptor PTPs, to conditions such as confluency, cell adhesion, differentiation or culturing, a similar change in expression of other PTPs might be observed. Therefore, it appears that the murine OST-PTP gene is rather selectively regulated during differentiation of MC3T3 osteoblasts and its pattern of expression is distinct from other PTPs. It appears to be expressed primarily in differentiated and early mineralizing MC3T3 osteoblasts, in parallel with other well-characterized markers of osteoblast differentiation. These results also suggest that this cell line is a robust and valuable model for studying the regulation of this gene.

Expression Coupled to Differentiation

The in vitro progression from a preosteoblast to a mature mineralizing phenotype requires the proper culturing environment. This includes necessary co-factors and adequate cell density to support maturation of the matrix and the appropriate cell–cell and cell–matrix interactions. These requirements are thought to reflect, in part, the microenvironment necessary for in vivo osteogenesis. In MC3T3 osteoblasts and primary osteoblasts, the induction of alkaline phosphatase and osteocalcin genes during differentiation is dependent on the presence of ascorbic acid (vitamin C), a co-factor that is essential for the hydroxylation, secretion and processing of type I procollagen [Aronow et al., 1990; Franceschi, 1999]. Ascorbic acid (AA) is required early in the progression, possibly to establish a matrix-associated signal necessary for differentiation [Franceschi et al., 1994]. In addition, the presence of β-glycerol phosphate (β-GP), a source of organic phosphate, is required in late cultures to facilitate the formation of hydroxyapatite complexes and mineralization of the matrix. The observation that inhibition of MC3T3 differentiation results in the delayed induction of OST-PTP transcripts and attenuation of transcriptional activation suggests that expression of this phosphatase gene is coupled to and dependent upon the optimal progression to a mature osteoblast. It appears that AA-dependent matrix secretion and maturation may be key factors in the regulation of this expression, as it is for other osteoblast-specific genes.

Cell density or confluency is known to modulate many aspects of gene expression and hormonal responsiveness during the phases of proliferation and differentiation of the osteoblast. Cell density-induced growth inhibition results in the downregulation of both DNA synthesis [Peterson and Yamaguchi, 1996] and proliferation-specific histone H4 and H2B expression [van den Ent et al., 1993] with subsequent up-regulation of other osteoblast-specific genes [Chiba et al., 1994]. The ability of cytokines, like leukemia inhibitory factor [Malaval and Aubin, 2001], interleukin-1 or fibroblast growth factor [Laulederkind et al., 2000], to modulate osteoblast functions such as prostaglandin synthesis, proliferation or bone nodule formation can be highly cell density-dependent. We observed a profound effect of cell density on the induction of OST-PTP expression, with detectable transcripts as early as 4 h post-plating in confluent cultures. Transcriptional activation of the OST-PTP promoter also occurred in the confluent culture as compared to the subconfluent cultures by 24 h post-plating. This difference in timing is most probably due to the disruption of cultures due to necessary transfections, trypsinization, and re-plating. Nonetheless, in both cases, confluency, or higher cell density resulted in enhanced gene expression in non-differentiated MC3T3 osteoblasts in the absence of the co-factors, ascorbic acid or β-GP. In addition, this appears to be specific to an osteoblast-like cell, since confluent cultures of HeLa cells showed no change in luciferase activity. The potency and specificity of this density-dependent modulation is very interesting considering the observation that confluent UMR 106.06 osteoblast-like cells show a 9-fold higher level of membrane-associated tyrosine phosphatase activity compared to cells of low and medium density [Southey et al., 1995]. The identities of the PTPs in this membrane fraction were not determined, but one could speculate that an increase in OST-PTP gene transcription could conceivably result in increased protein and therefore PTP activity. Finally, this is the first report of a specific PTP being modulated by cell density in osteoblasts, although this means of regulating PTPs has been observed in other tissues. For example, the mRNA and protein expression as well as the activity of the receptor PTP, DEP-1, are dramatically increased in dense lung fibroblasts [Ostman et al., 1994]. Enhanced cell–cell interactions through higher cell density can also change the subcellular localization of PTPs such

as SHP-2 [Xu et al., 2001] or Pez/PTPD2/PTP36 [Wadham et al., 2000] and thereby modulate cell function.

Transcriptional Activation of the mOST-PTP Gene

Throughout the progression from proliferating preosteoblasts to differentiated, mineralizing osteoblasts, key transitions must occur in the transcriptional regulation of numerous ubiquitous and osteoblast-specific genes. Our results indicate that the murine OST-PTP gene is a unique member in this cohort of critical genes, which is transcriptionally regulated early during this progression. Using the previously isolated OST-PTP promoter, we have shown that this fragment can confer differentiation-dependent transcriptional activation similar to that of the endogenous gene. Transfection experiments have shown increased luciferase activity of 9–11-fold by day 5 of differentiation, very similar to the 11–14-fold increase in endogenous transcript levels usually observed by day 6. Lack of the co-factors, AA and β-GP, was shown to inhibit differentiation as evidenced by alkaline phosphatase expression and ultimately results in the suppression of the activation of the OST-PTP promoter. Slight activation is observed in the non-differentiating cultures and is most likely due to the stimulatory effects of confluency as well as "escape" of long-term MC3T3 cultures. Not only is this transcriptional activation dependent on the proper progression of differentiation, but it also appears to be osteoblast-specific since progression from multipotent cell to fused myoblast results in no activation of the OST-PTP promoter. Finally, the activation of this PTP gene appears to be dependent on the effects of cell density as well as several other factors, including AA and β-GP, which provide the optimal environment for differentiation. Our combined confluency/differentiation experiment showed that confluency results in a ~3–4-fold increase in activation followed by an additional ~9-fold increase during differentiation. This indicates that confluency has an effect very early in the progression of cells to a differentiated phenotype.

In the context of the PTP superfamily, these results should be very relevant to our understanding of the biological importance of the receptor PTPs. Of all the RPTP genes known, this gene is one of only several where the promoter has been successfully isolated and shown to possess transcriptional activity. Promoter analysis of the RPTPs has been complicated by their genomic size which often spans 75–750 kb of sequence [Hall et al., 1988; O'Grady et al., 1994]. In addition, some of these genes like *CD45* [Timon and Beverley, 2001] and *RPTPε* [Tanuma et al., 1999] utilize multiple promoters within intronic sequences which can also hamper identification. Therefore, the success of these and future studies on the molecular mechanisms important for the murine OST-PTP gene expression will help in developing a model for the regulation and potential function of this subclass of PTPs in cell differentiation.

The transcriptional control of osteogenesis involves key transcription factors critical for the commitment and differentiation of the osteoblast lineage [Stein et al., 1996; Karsenty and Wagner, 2002]. During embryonic bone development or bone remodeling in the adult vertebrate, this progression from osteoprogenitor to mature, mineralizing osteoblast requires factors like Cbfa1/Runx2 [Merriman et al., 1995; Ducy et al., 1997; Komori et al., 1997] and the recently discovered Osterix [Nakashima et al., 2002] for the sequential activation of osteoblast-specific genes. In this report, we have shown that the murine OST-PTP gene is selectively expressed early during this progression in an osteoblast-specific, differentiation-dependent manner that is, in part, due to transcriptional activation. Analysis of the promoter has revealed numerous putative binding sites for Cbfa1/Runx2 [Morrison and Mauro, 2000] and could contain elements for unidentified factors important for its osteoblast-specific regulation. Considering this evidence and the proposed critical function of this molecule, it is feasible this PTP could serve as a new marker of osteoblast commitment and differentiation. Future studies will establish if this is a viable role for this gene and will help determine essential regulatory elements and the critical signaling pathways important for its expression.

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