Endothelin signaling in osteoblasts: global genome view and implication of the calcineurin/NFAT pathway

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Abstract
Patients with prostate cancer develop osteoblastic metastases when tumor cells arrive in the bone and stimulate osteoblasts by secreting growth-promoting factors. Endothelin 1 (ET-1) is believed to be a key factor in promoting osteoblastic metastasis. Selective blockade of the ETA receptor is an established strategy in the development of cancer therapeutics. However, the molecular mechanisms whereby prostate cancer promotes abnormal bone growth are not fully understood. In this study, we have applied genomic approaches to elucidate the molecular mechanism of stimulation of osteoblasts by ET-1. To examine the ET-1 axis, we generated genomic signatures for osteoblasts treated with ET-1, in the presence and absence of a selective ETA antagonist (ABT-627). The ET-1 signature was comprised of several motifs, such as osteoblastic differentiation, invasion, and suppression of apoptosis. The signature also pointed at possible activation of the calcineurin/NFAT pathway. We showed that ET-1 activate calcineurin and causes nuclear translocation of NFATc1, implicating the pathway in the ET-1-mediated stimulation of osteoblasts. We also showed that ET-1 inhibits apoptosis in osteoblasts, implying that the suppression of apoptosis may be an important factor in the promotion of osteoblastic growth by ET-1. [Mol Cancer Ther 2007;6(1):253–61]

Introduction
Osteoblastic metastases frequently develop in advanced cases of prostate cancer and in several other common malignancies, such as breast cancer (1). The development of metastases at distant sites is driven by interactions between disseminated tumor cells and the host tissue environment. In the case of osteoblastic metastases, it is believed that the excessive bone growth at the metastatic site is caused by stimulation of the osteoblasts by factors secreted by tumor cells (1). Several factors have been implicated in this process, including fibroblast growth factors 1 and 2, insulin-like growth factors 1 and 2, urokinase-type plasminogen activator, bone morphogenetic proteins, and endothelin 1 (ET-1; ref. 2). The role of ET-1 deserves particular attention because it is secreted by prostate cancer cells and is elevated in plasma from patients with advanced prostate cancer (3).

ET-1 is a vasoconstrictor that belongs to a family of three 21-amino acid peptides (endothelins 1, 2, and 3; ref. 4). It exerts its effects by binding to two cell surface receptors, ETA and ETB, the latter functioning primarily in ligand clearance (4). A selective ETA receptor antagonist, atrasentan (ABT-627), is currently undergoing clinical trials in prostate cancer. The compound extended time to disease progression in patients with metastatic hormone-refractory prostate cancer (2).

A significant amount of evidence has been accumulated to support the role of ET-1 in the formation of osteoblastic metastases. Injection of several ET-1–secreting breast cancer cell lines into mice caused the formation of osteoblastic metastases, whereas administration of the ETA receptor antagonist, ABT-627, suppressed metastatic growth (5, 6). However, the precise molecular mechanism whereby ET-1 stimulates osteoblasts remains to be elucidated. Additionally, there is a clear need for biomarkers that would facilitate monitoring the metastatic load in patients and thus enable monitoring the efficacy of antimitastatic therapies. Here, we report a genomic study of the effects of ET-1 on osteoblasts and present evidence in favor of an involvement of the calcineurin/NFAT pathway in ET1-induced stimulation of osteoblasts.

Materials and Methods
Cell Culture and Reagents
Mouse MC3T3 preosteoblastic cells (subclone 4) were purchased from American Type Culture Collection (Manassas, VA) and propagated in α-MEM medium without ascorbic acid (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). MC3T3-E1 cell line is a well-characterized osteoblast line derived from mouse calvariae. This line exhibits a high degree of differentiation potential and forms a well-mineralized extracellular matrix during this process (7). Human mesenchymal stem cells (MSC) and NHOst cells were purchased from Cambrex (Walkersville, MD) and propagated in MSCGM and OGM media (Cambrex), respectively. To initiate differentiation of
the MSC into human osteoblasts, the growth medium was replaced by osteogenic differentiation medium (Cambrex). ET-1 and cycloheximide were from Sigma (St. Louis, MO). Thapsigargin and cyclosporin A were from EMD Biosciences (La Jolla, CA). Tumor necrosis factor α (TNF-α) was from R&D Systems (Minneapolis, MN). Anti rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase staining was done using an AP detection kit (procedure no. 85; Sigma).

**Microarray Analysis of Gene Expression**

Total RNA was isolated by using the Trizol reagent (Invitrogen) and purified on RNeasy columns (Qiagen, Valencia, CA). Labeled cRNA was prepared according to the microarray manufacturer’s protocol and hybridized to either mouse 430A 2.0 or human U133A 2.0 arrays (Affymetrix, Santa Clara, CA). The 430A 2.0 and U133A 2.0 chips contain 14,000 and 14,500 well-characterized genes, respectively, as well as several thousand expressed sequence tags. The microarray data files were loaded into the Rosetta Resolver software for analysis. Gene expression fold changes for each treatment were calculated by combining three biological replicates for each treatment using the Rosetta Resolver’s Affymetrix error model and building a ratio from the resulting values. All genes regulated ≥1.5-fold at \( P \leq 0.01 \) were retained for further analysis. Two-dimensional clustering was done by using the agglomerative hierarchical clustering algorithm. For gene clustering, pairwise similarity metrics were calculated based on the expression ratios for all treatments. For experiment clustering, pairwise similarity metrics were calculated based on the expression ratios across all genes with \( P \leq 0.01 \). The Euclidean distance was used as the similarity metric.

**Analysis of Calcineurin Activity**

Cells were grown on 100 mm tissue culture plates (Corning, Corning, NY) and treated with 10 nmol/L of ET-1 or 1 μmol/L of thapsigargin for 2 or 10 min, rinsed with TBS, and scraped into 350 μL of lysis buffer. To remove inorganic phosphate, the lysate was purified by gel filtration. The lysate was diluted 1:5 and tested for calcineurin activity using a PP2B activity kit (EMD Biosciences) according to the manufacturer’s protocol. The specific calcineurin activity was determined as the difference between the total phosphatase activity and the phosphatase activity in the presence of EGTA and measured in nanomoles of released phosphate.

**Detection of NFATc1 Nuclear Translocation by Immunofluorescence**

Cells were seeded on eight-well slides at \( 2 \times 10^4 \) cells/well, allowed to grow to 80% confluency, starved for 20 h, subjected to treatments, and immersed into ice-cold methanol for 2 h. The slides were dried, rehydrated with PBS, and blocked with 20% fetal bovine serum in PBS for 1 h at room temperature. The slides were then incubated overnight at 4°C with a rabbit anti-NFATc1 antibody (Santa Cruz Biotechnology). The cell layer was washed with PBS and incubated with a goat anti-rabbit FITC secondary antibody for 2 h and with 4′,6-diamidino-2-phenylindole for 10 min at room temperature. The slides were washed, air-dried, and mounted in mounting media (Vector Laboratories, Burlingame, CA). Images were obtained on an AxioScope (Zeiss, Jena, Germany) and recorded using AxioVision software. The FITC channel was collected for 1.5 s.

**Quantitation of NFATc1 Nuclear Translocation**

Cells were seeded at 5 × 10^5 cells per well in a 96-well viewplate (Packard, Boston, MA). The cells were treated as above, followed by immersion in 3.7% formaldehyde. After PBS washing, cells were blocked in 1% bovine serum albumin for 2 h, followed by permeabilization with 0.5% Triton X-100 for 15 min. Nuclear translocation of NFATc1 was quantitated on a Cellomics ArrayScan VTI system. The NFATc1 translocation HitKit (Cellomics, Pittsburgh, PA) quantitates the colocalization of the NFATc1 signal with Hoechst nuclear staining. Approximately 200 cells or 16 fields were analyzed in triplicate. The cytoplasm-to-nucleus translocation algorithm divides the cell into two regions based on the masks created, the nucleus and the cytoplasm. The response variable for the assay is the difference between the average intensity of the nuclear and the cytoplasmic regions.

**Detection of Apoptosis by Annexin V Quantitation**

Cells were seeded in six-well dishes at 3 × 10^5 cells/well, allowed to grow to 80% confluency, starved for 20 h, and treated at the indicated times with either 20 ng/mL TNF-α + 100 nm of cycloheximide (MC3T3) or 30 ng/mL TNF-α + 150 nmol/L of cycloheximide (MSC) with or without pretreatment with 10 nmol/L of ET-1. The cells were trypsinized and pelleted at 250 × g for 10 min at 4°C. The cell pellets were carefully resuspended in a 1 μg/mL of Annexin V–phycocerythrin solution in PBS and incubated for 15 min, followed by fixation in 3.7% formaldehyde in PBS. Flow cytometry analysis was conducted on a LSRII flow cytometer (BD Biosciences, San Jose, CA). For each treatment, 30,000 events were measured in duplicate wells.

**Detection of Apoptosis by Caspase 3 and Poly(ADP-Ribose) Polymerase Cleavage Assays**

Cells were seeded in six-well dishes at 3 × 10^5 cells/well, allowed to grow to 80% confluency, starved for 20 h, and treated at the indicated times with apoptosis inducers (20 ng/mL TNF-α/100 nmol/L cycloheximide for MC3T3 cells and 30 ng/mL TNF-α/150 nmol/L cycloheximide for MSCs) with or without pretreatment with 10 nmol/L ET-1 for 1 h. ABT-627 at a concentration of 5 μmol/L was added 1 h prior to the ET-1 treatment. The cells were scraped into PBS and pelleted at 1,000 × g for 10 min. The pellets were resuspended in lysis buffer containing 6 mol/L urea, 2% β-mercaptoethanol, 2% SDS, 1% glycerol, and 0.006% Bromophenol Blue in 50 mmol/L Tris-HCl (pH 6.8) and heated to 65°C for 15 min, followed by freeze-thawing and sonication for 15 s. The lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk (Bio-Rad, Hercules, CA) and incubated overnight at 4°C with either rabbit anti–caspase 3 or rabbit anti–poly(ADP-ribose) polymerase (PARP) antibodies (Cell Signaling, Beverly, MA). The nitrocellulose sheet was
washed with T-TBS and incubated for 2 h with a goat anti-rabbit AP antibody (Bio-Rad) or a donkey anti-rabbit horseradish peroxidase secondary antibody (Santa Cruz Biotechnology).

Results

Global Genome View of ET-1 Signaling in Osteoblasts

We began to study the ET-1 axis in osteoblasts by generating the genome-wide view of ET-1 signaling using gene expression microarrays. Mouse preosteoblastic MC3T3 cells as well as primary human osteoblasts (NHOsts) were treated with 10 nmol/L of ET-1 for 2, 4, and 6 h in the absence or presence of an ETA receptor antagonist (ABT-627, 5 μmol/L). The drug was added 1 h prior to the addition of ET-1. The raw microarray data for the mouse and human cells are presented in Supplementary Tables S1 and S2, respectively. To minimize the false discovery rate, only genes regulated 1.5-fold or higher with a \( P \leq 0.01 \) were considered differentially expressed.

We did two-dimensional hierarchical clustering of the gene expression signatures for ET-1 (Fig. 1A). Clearly, ET-1 induced a significant number of genes at all three time points, whereas pretreatment with the ABT-627 abrogated almost all of these gene induction events. The antagonist alone caused very few gene expression changes. The ET-1 treatments clustered together because of the similarity of the signatures, whereas the rest of the treatments showed a random clustering pattern because very few genes were regulated. Table 1 summarizes the microarray data for the mouse osteoblasts. ET-1 induced 608 genes at 2 h; the number of up-regulated genes decreased with time. The overwhelming majority of the gene induction events were abrogated by ABT-627, implying that ET-1 signals exclusively through the ETA receptor. The microarray experiment for NHOsts revealed very similar statistics (see Supplementary Table S2 for raw data).

Pathway analysis of the ET-1 signature in osteoblastic cells revealed several dominant motifs (Fig. 1B). First, an osteoblastic maturation motif was represented in the ET-1 signature by such genes as osteoprotegerin, COX-2, Dmp1, Tgfbi, connective tissue growth factor, Kruppel-like factor 10 (Klf10), and others (8–11). Because of the early time points chosen, the genes induced were implicated primarily into the differentiation process, rather than into the maintenance of the mature osteoblastic phenotype. The induction of all these genes by ET-1 was blocked by pretreatment with ABT-627. Second, an invasion signature included such genes as uroplasminogen activator, uroplasminogen activator receptor, plasminogen activator inhibitor 1, transforming growth factor \( \beta \), interleukin 6, interleukin 8, and connective tissue growth factor (12–15). The products of these genes have been previously implicated in metastasis and were shown to be elevated in patients with metastatic cancer (16–18). Finally, the third theme in the ET-1 signature was the suppression of apoptosis. This group was comprised of Nur77, Fhl1, and NFATc1 (19–21). Again, these genes were induced by ET-1, and the induction was blocked by ABT-627.

Table 1. Regulation of gene expression by ET-1

<table>
<thead>
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<th>Time point (h)</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
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<tr>
<td>Induced by ET-1</td>
<td>608</td>
<td>472</td>
<td>194</td>
</tr>
<tr>
<td>Blocked by ABT-627</td>
<td>581 (96%)</td>
<td>390 (83%)</td>
<td>189 (97%)</td>
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<td>Down-regulated by ET-1</td>
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<td>295</td>
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<tr>
<td>Blocked by ABT-627</td>
<td>403</td>
<td>262</td>
<td>93</td>
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</tbody>
</table>

Figure 1. A, a global genome view of ET-1 signaling in mouse MC3T3 osteoblastic cells. The heatmap contains all 548 genes regulated at least 2-fold in at least one of the experiments with a \( P \leq 0.01 \). Each row represents a treatment (combined biological triplicates) and each column represents a gene. The dendrogram (left) reflects the degree of similarity between the expression signatures. B, ET-1 induces genes involved in osteoblastic differentiation, invasion and metastasis, and suppression of apoptosis.

1 Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
The ET-1 signatures in MC3T3 cells and primary human osteoblasts revealed a coordinated induction of several members of the calcineurin/NFAT pathway, i.e., NFATc1, calcineurin, several transcriptional targets of NFAT (COX-2, tissue factor, and interleukin 8), and a regulator of the pathway, DSCR1. This coordinated induction led us to hypothesize that the calcineurin/NFAT pathway was activated by ET-1 in osteoblasts. Therefore, we investigated the activity of this pathway at several levels, starting with the calcineurin phosphatase activity.

**ET-1 Activates Calcineurin in Mouse and Human Osteoblasts**

To find out whether ET-1 affects the phosphatase activity of calcineurin in osteoblasts, we treated MC3T3 cells and human MSCs with 10 nmol/L of ET-1 for 2 and 10 min in the presence or absence of 5 μmol/L ABT-627. As a positive control, we used 1 μmol/L of thapsigargin, a known activator of the calcineurin/NFAT pathway (22). All treatments were done in quadruplicate (two biological replicates run in duplicate). The results at 2 min (Fig. 2) show that ET-1 markedly increases the calcineurin activity in MC3T3 cells (A, Student’s test, \( P = 0.001 \)) and MSCs (B, \( P = 0.024 \)) similarly to thapsigargin. This induction of calcineurin phosphatase activity is completely blocked by ABT-627 in both MC3T3 cells (\( P = 0.001 \)) and MSCs (\( P = 0.025 \)). Calcineurin activation leads to rapid translocation of NFAT family members from the cytoplasm to the nucleus and activation of transcription of NFAT-controlled genes (23). Therefore, the observed activation of calcineurin prompted us to investigate the activation of NFAT by ET-1.

**ET-1 Causes Nuclear Translocation of NFATc1 in Osteoblasts**

We used immunofluorescence to determine whether ET-1 induces the translocation of NFATc1 from the cytoplasm into the nucleus. Images in Fig. 3A are representative of three independent experiments. The cells treated with the vehicle have an even distribution of NFATc1 throughout the cell; 1 μmol/L of thapsigargin and 10 nmol/L of ET-1 cause NFATc1 translocation to the nucleus. Pretreatment with 5 μmol/L of ABT-627 completely blocks ET1-induced nuclear translocation of NFATc1. Interestingly, ABT-627 pretreatment abolishes trace nuclear accumulation seen in the vehicle-treated cells, leaving the nucleus vacant. This may be due to blocking of the effect of the endogenous ET-1 present in the media. Importantly, the aforementioned effects of ET-1 and ABT-627 were observed in all cells on the respective slides, rather than a subset of cells. These data indicate that ABT-627 is a potent inhibitor of ET-1–induced NFAT activation.

To confirm and quantify these results, we analyzed NFATc1 translocation using an independent anti-NFATc1 monoclonal antibody in a high-content screening analysis. Cyclosporin A was used as a negative control. The effects of thapsigargin peaked at 20 and 40 min after the induction (Fig. 3B) and were abrogated by 200 nmol/L of cyclosporin A. The nuclear translocation of NFATc1 caused by ET-1 was comparable to that induced by thapsigargin, implying that ET-1 is a potent activator of NFAT. Translocation of NFAT induced by ET-1 was blocked by pretreatment with ABT-627, indicating that the effect is exerted through the ET\(_A\) receptor. At 40 min, the \( P \) value for ET-1–treated cells versus vehicle-treated cells was 0.031 and the \( P \) value for ET-1/ABT-627–treated cells versus ET-1–treated cells was 0.011 (Student’s \( t \) test). The quantitation presented in Fig. 3B and D suggests residual nuclear NFAT in the presence of ABT-627, which is inconsistent with the vacant nucleus observed by NFATc1 immunofluorescence (Fig. 3A and C). Quantitation of NFATc1 translocation relies on a computer-generated mask drawn just outside the nucleus, as defined by Hoechst staining. In ABT-627–treated cells, the intense NFATc1 staining around the nuclear membrane (Fig. 3A and C) coincides with established nuclear masks, hindering accurate quantitation of the resulting empty nucleus.

To determine whether cells committed to the osteoblastic lineage also respond to ET-1 by NFATc1 activation, we induced MSCs to differentiate for 4 and 8 days into osteoblasts and treated them with ET-1. We confirmed osteoblastic differentiation by bone alkaline phosphatase staining in triplicate (Supplemental Fig. S1). Additionally, it has previously been reported that a 4-day incubation in the osteogenic medium causes osteoblastic differentiation in MSCs (24, 25). The images in Fig. 3C are representative of two separate experiments and show that ET-1 causes NFATc1 to translocate into the nucleus in MSCs after 4 or 8 days of differentiation. Pretreatment with ABT-627 completely abrogated nuclear translocation of NFATc1. To determine whether the stimulation of the pathway by ET-1 is abrogated by cyclosporine A, we also evaluated the effect of ET-1 on cyclosporine A–pretreated MSCs. NFAT localization, in this case, did not change and was similar to vehicle-treated or cyclosporine A–treated cells (Supplementary Fig. S2). To quantify NFATc1 translocation in differentiated MSCs, we propagated the cells in the differentiation medium for 4 or 8 days, treated them with...
ET-1, and quantified the colocalization of the NFATc1 signal with Hoechst nuclear staining. For the cells differentiated for 8 days, the quantitation proved impossible because of overlapping cell layers and mineralization. The results for the cells differentiated for 4 days are presented in Fig. 3D. Clearly, the trend observed for undifferentiated MSCs remained unchanged: the induction of NFATc1 nuclear translocation by ET-1 was comparable to that by the positive control (thapsigargin). In summary, our results indicate that ET-1 activates the NFAT pathway in osteoblastic cells regardless of their differentiation status.

**ET-1 Is a Survival Factor in Osteoblasts**

Pathway analysis of the ET-1 signature in osteoblastic cells revealed an antiapoptotic motif. This finding prompted us to examine the effects of ET-1 on apoptosis in osteoblasts. A combination of TNF-α and cycloheximide (TNF-α/cycloheximide) has previously been used in osteoblastic cells to induce apoptosis (26, 27). In order to ascertain whether ET-1 protects osteoblastic cells from apoptosis, undifferentiated and differentiated MSCs were treated with TNF-α/cycloheximide in the presence of 10 nmol/L of ET-1. The cells were then analyzed for Annexin V positivity, an early apoptotic event (28). The analysis was conducted for biological triplicates tested in duplicate; gating was used to select for intact cells that were Annexin V–positive. The data shown in Fig. 4A includes Annexin V positivity in undifferentiated MSCs undergoing the apoptotic insult. The ET-1–pretreated group showed a significant attenuation in apoptotic induction compared with the cells that received no ET-1. Although apoptotic rates were low in MSCs, ET-1 protected 43.2% of intact undifferentiated MSC cells. P values of $6.0 \times 10^{-4}$ and $2.8 \times 10^{-4}$ were generated when contrasting the ET-1–treated undifferentiated MSC with TNF-α/cycloheximide and TNF-α/cycloheximide/ET-1/ABT-627 MSCs, respectively. In differentiated MSCs, ET-1 was also a survival factor protecting 18.1% of cells (Fig. 4A). P values of $2.9 \times 10^{-6}$ and $6.0 \times 10^{-3}$ were generated when contrasting the ET-1–treated differentiated MSCs with the cells treated with TNF-α/cycloheximide only and TNF-α/cycloheximide/ET-1/ABT-627, respectively. Thus, ABT-627 abrogates the survival advantage that ET-1 provides to human preosteoblasts.

Figure 4A also shows Annexin V positivity data for mouse MC3T3 cells induced to apoptose with TNF-α/cycloheximide in the presence or absence of ET-1. Even with lower concentrations of inducers (100 nmol/L cycloheximide/20 ng/mL TNF-α), the majority of cells during the analysis were positive for Annexin V. However, as was the case for human cells, ET-1 reduced the apoptotic rate by 15.8% (P values of $1.1 \times 10^{-3}$ and $8.9 \times 10^{-4}$ for the comparison of ET-1–pretreated MC3T3 with cycloheximide/TNF-α alone and with the TNF-α/cycloheximide/ET-1/ABT-627 group, respectively). Representative flow
cytometry histograms are shown in Supplemental Fig. S3.1. In summary, our data show that ET-1 attenuates early events in the apoptosis of osteoblastic cells regardless of their differentiation status. Noteworthy, the ETa receptor antagonist ABT-627 abrogated the protective effect of ET-1 in osteoblasts, thus validating the observations for ET-1 and providing clues into the mechanism of action of the drug in suppressing osteoblastic metastases.

Apoptotic pathways rely on protease activation to effect downstream, irreversible cellular destruction. The activity of the proteases involved (caspases) could be monitored by analyzing the cleavage of their targets. In order to validate our Annexin V findings, we examined caspase 3 and PARP cleavage in MC3T3 cells. Immunoblotting was conducted on MC3T3 cell lysates for caspase 3 and PARP cleavage products. As shown in Fig. 4B, nonapoptotic, uninduced cells contain only the full-size caspase 3 across all treatment groups. The observed low background level of PARP cleavage products in uninduced osteoblasts is consistent with the observations of others (29, 30) and is considered a baseline for this evaluation. Upon apoptotic induction, all of the full-length caspase 3 and PARP proteins were cleaved in all groups, except the ET-1 group. Pretreatment with ET-1 provided protection from apoptosis, as evidenced by the presence of uncleaved caspase 3 and PARP. The addition of ABT-627 before ET-1 treatment abrogated the protective effect of ET-1, as indicated by the complete cleavage of caspase 3 and PARP.

The Annexin V analysis of early apoptotic events, in combination with later caspase activation data, imply that ET-1 suppresses apoptosis in osteoblastic cells regardless of their differentiation status. The protective effect of ET-1 is exerted through the ETa receptor because blockade of this receptor with a selective antagonist restores apoptosis.

Discussion

The bone is the most common site of metastasis in prostate cancer, with osseous metastases detected in 90% of patients dying from the disease (31). Although the majority of cancers produce lesions characterized by bone lysis, prostate cancer most often results in increased bone production (osteoblastic metastases). The mechanisms whereby prostate cancer promotes increased bone growth are not fully understood. It is believed that osteoblastic metastases arise when tumor cells arrive in the bone and stimulate osteoblasts by secreting growth-promoting factors (1, 2). Several factors have been shown to affect osteoblasts, including fibroblast growth factors 1 and 2, insulin-like growth factors 1 and 2, insulin-like growth factors 1 and 2, urokinase-type plasminogen activator, bone morphogenetic proteins, and ET-1 (2).

In this study, we have applied genomic approaches to elucidate the molecular mechanism of stimulation of osteoblasts by ET-1. This factor is believed to be particularly important in prostate cancer cell interactions with osteoblasts because it is secreted by prostate cancer cells and is elevated in the plasma of patients with advanced prostate cancer (3). The role of ET-1 in the pathogenesis of osteoblastic metastases has been previously addressed using in vivo models. Injection of several ET-1–secreting breast cancer cell lines into the heart of a mouse caused the formation of osteoblastic metastases, whereas administration of an ETa antagonist, ABT-627, suppressed metastatic growth (5, 6). Selective blockade of the ETa receptor is an accepted strategy in the development of cancer therapeutics (2). Atrasentan (ABT-627) is the lead ETa receptor antagonist for the treatment of hormone-refractory metastatic prostate cancer (32). Although a substantial amount of supportive evidence has been accumulated for the tumor cell/osteoblast interaction model, the exact molecular mechanism of the ET-1 effects on osteoblasts remains to be ascertained.

The main goals of this study were to elucidate the molecular mechanism of osteoblast stimulation by ET-1.

![Figure 4](image-url)
and identify mechanism-based biomarkers for ET\textsubscript{A} receptor antagonists in the treatment of metastatic prostate cancer. Here, we used established osteoblastic models, i.e., mouse osteoblastic MC3T3 cells, human primary osteoblasts, and MSCs differentiated to osteoblasts (33, 34). To determine the signaling pathways involved in the ET-1 axis, we generated genomic signatures for both cell types following an ET-1 treatment in the presence and absence of a selective ET\textsubscript{A} antagonist (ABT-627).

The drug almost completely blocked all gene induction events caused by ET-1, implying that all of the ET-1 effects on osteoblasts are mediated by the ET\textsubscript{A} receptor. The role of the ET\textsubscript{B} receptor in ET-1 signaling has not been unambiguously defined. It has been suggested that its main function is in ligand clearance (2, 4). Indeed, ET\textsubscript{A}-selective antagonists strongly inhibited the proliferation of osteoblasts \textit{in vitro} and the growth of osteoblastic metastases \textit{in vivo} (5, 35), suggesting that ET\textsubscript{A} is the main transducer of ET-1 signals. However, other studies have shown that the ET\textsubscript{B} receptor plays a critical role in the progression of different cancers and mediates the proliferative effects of ET-1 (36–39). Antagonists of the ET\textsubscript{B} receptor blocked the proliferation of melanoma cells and induced their death (36, 37). Here, we used microarray analysis, an extremely sensitive method for dissecting intracellular pathways. Based on the fact that ABT-627 abrogates almost all of the effects of ET-1 on osteoblasts, we conclude that ET\textsubscript{A} receptor is the primary mediator of ET-1 signaling in these cells.

Next, we used the ET-1 signatures in MC3T3 preosteoblasts and primary human osteoblasts to delineate the pathways activated by ET-1 in these cells. There were several prominent motifs in these signatures. First, we observed the induction of genes associated with osteoblastic differentiation, including osteoprotegerin, Cox-2, Dmp-1, Tgfbi, connective tissue growth factor, Klf10, and others. This is not surprising, given the previously established prodifferentiation effects of ET-1 on osteoblasts (40). The second motif in the ET-1 signature was invasion and metastasis. It was comprised of such genes as uropasminogen activator, uroplasminogen activator receptor, plasminogen activator inhibitor-1, transforming growth factor \(\beta\), interleukin 6, interleukin 8, and connective tissue growth factor. Again, this finding was not surprising in the context of osteoblastic metastases. These genes are known to be overexpressed in metastatic tumor cells (12–15, 41–46). In our case, however, these genes were activated in normal osteoblasts under the influence of a factor produced by the tumor cells (ET-1), thereby providing a redundant mechanism for the accumulation of factors required for the maintenance of the metastatic site. A similar mechanism was recently established for indirect stimulation of bone lysis by tumor cells, whereby tumor cells stimulate MSCs in the bone to produce osteolytic factors (47). Finally, the third theme in the ET-1 profiling experiment was the suppression of apoptosis. This signature of genes included Nur77, FlhI, and NFATc1 (21, 48). This observation is consistent with the previously established antiapoptotic effects of ET-1 in different cell types (49–52).

The most important finding in our microarray-based pathway analysis was the induction of several components of the calcineurin/NFAT pathway (calcineurin, NFATc1, and several transcriptional targets of NFAT) as well as a regulator of the pathway, DSCR1. This prompted us to further examine the activation of the pathway by ET-1 in osteoblasts. The calcineurin/NFAT pathway is one of the central controllers of signaling in eukaryotes (23). The pathway is activated by an increase in the intracellular Ca\(^{2+}\) concentration, followed by activation of calcineurin, a Ca\(^{2+}\)-dependent phosphatase. Calcineurin dephosphorylates NFATs, which represent cytoplasmic components of NFAT transcription complexes. The dephosphorylation of NFATs causes their activation and nuclear translocation followed by the assembly of the respective transcription complexes, which initiate the transcription of genes with broad functions in cell-cell interactions (23).

We examined the activation of the pathway at two levels. First, we showed the induction of the calcineurin activity by ET-1. Pretreatment of the cells with ABT-627 blocked calcineurin activation, suggesting that ET-1 affects the intracellular Ca\(^{2+}\) levels through the ET\textsubscript{A} receptor. Next, we determined the NFATc1 activation status. To visualize nuclear translocation of NFATc1, we used immunofluorescence analysis following treatment of human osteoblasts with ET-1. Significant nuclear accumulation of the transcription factor was observed after ET-1 treatment. However, when the cells were pretreated with ABT-627, no nuclear translocation occurred. Moreover, we observed clearing of the nucleus relative to the untreated control, most likely due to the abrogation by the antagonist of the effects of endogenous ET-1 present in the medium. Thus, we clearly showed the effects of the drug on the NFAT pathway. Most likely, ET-1 activates the calcineurin/NFAT pathway in osteoblasts by inducing the influx of Ca\(^{2+}\) into the cells through activation of the ET\textsubscript{A} receptor. It has previously been shown that ET-1 causes a dose-dependent increase in intracellular Ca\(^{2+}\) in HeLa cells (53) and vascular smooth muscle cells (54).

Several studies have addressed the function of the calcineurin/NFAT pathway in osteoblasts. It has recently been shown that calcineurin is expressed in osteoblasts (55). It has also been found that calcineurin overexpression enhances osteoblastogenesis and that calcineurin deletion or inhibition diminishes bone formation and reduces osteoblast differentiation (55). Koga et al. (56) have shown that NFAT binding is important for the transcriptional activity of osterix, an essential factor in osteoblastogenesis. Our data suggest that the effects of ET-1 on osteoblasts are mediated by the calcineurin/NFAT pathway, and thus, may link the existing evidence for the role of ET-1 in bone metastasis with the function of NFAT in bone formation.

The NFATs have previously been implicated as survival factors in different cell types (48, 57), suggesting that the calcineurin/NFAT pathway is antiapoptotic. It has been shown that ET-1 is a survival factor in prostate (49) and ovarian (50) cancer cell lines, as well as in endothelial cells (53), fibroblasts (51), smooth muscle cells (52), and other...
cell types. This prompted us to study the effects of ET-1 on apoptosis in preosteoblasts. We induced apoptosis in undifferentiated and differentiated MSCs in the presence or absence of ET-1. Taken together, our data suggest that ET-1 is an antipapoptotic factor in osteoblasts, providing a possible mechanism for its function in promoting bone metastasis.

In conclusion, we have used genomic analysis to analyze the effects of ET-1 on osteoblasts and discovered the involvement of a calcineurin/NFAT pathway in ET-1-induced osteoblastic stimulation. Our data contribute to the understanding of the molecular mechanism of ET-1-mediated stimulation of osteoblasts. Our findings may have implications on the future development of endothelin receptor antagonists by providing a path for biomarker identification.

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References


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