

## Prostate-Specific Antigen Modulates Genes Involved in Bone Remodeling and Induces Osteoblast Differentiation of Human Osteosarcoma Cell Line SaOS-2

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**Abstract Purpose:** The high prevalence of osteoblastic bone metastases in prostate cancer involves the production of osteoblast-stimulating factors by prostate cancer cells. Prostate-specific antigen (PSA) is a serine protease uniquely produced by prostate cancer cells and is an important serologic marker for prostate cancer. In this study, we examined the role of PSA in the induction of osteoblast differentiation.

**Experimental Design:** Human cDNA containing a coding region for PSA was transfected into human osteosarcoma SaOS-2 cells. SaOS-2 cells were also treated with exogenously added PSA. We evaluated changes in global gene expression using cDNA arrays and Northern blot analysis resulting from expression of PSA in human osteosarcoma SaOS-2 cells.

**Results:** SaOS-2 cells expressing PSA had markedly up-regulated expression of genes associated with osteoblast differentiation including runx-2 and osteocalcin compared with the controls. Consistent with these results, the stable clones expressing PSA showed increased mineralization and increased activity of alkaline phosphatase *in vitro* compared with controls, suggesting that these cells undergo osteoblast differentiation. We also found that osteoprotegerin expression was down-regulated and that the receptor activator of NF- $\kappa$ B ligand expression was up-regulated in cells expressing PSA compared with controls.

**Conclusions:** Modulation of the expression of osteogenic genes and alteration of the balance between osteoprotegerin – receptor activator of NF- $\kappa$ B ligand by PSA suggests that PSA produced by metastatic prostate cancer cells may participate in bone remodeling in favor of the development of osteoblastic metastases in the heterogeneous mixture of osteolytic and osteoblastic lesions. These findings provide a molecular basis for understanding the high prevalence of osteoblastic bone metastases in prostate cancer.

Bone is the frequent site of many types of cancer metastasis including prostate cancer. Advanced prostate cancer is frequently accompanied by the development of unique bone metastases characterized as osteoblastic (bone forming), which results in significant complications including bone pain, fractures, and spinal cord compression, even hemiparesis, leading to morbidity with no curable treatment (1–3). Although osteoblastic lesions

are the most dominant bone metastases associated with prostate cancer, osteoclastic lesions (bone resorption) also infrequently occur in prostate bone metastases (4, 5). In contrast, cancers from other tissues that metastasize to bone are frequently associated with osteoclast formation. Osteoblastic characterization associated with prostate bone metastases suggests that factors derived from prostate cancer cells which influence bone remodeling may be unique from other types of cancer cells.

Several osteogenic factors produced by prostate cancer cells have been identified including bone morphogenetic proteins (BMP; refs. 6, 7), endothelin-1 (8), insulin-like growth factors (IGF; ref. 9), parathyroid hormone-related peptide (10), transforming growth factor- $\beta$  (TGF- $\beta$ ; ref. 11), and prostate-specific antigen (PSA; refs. 11, 12). Among them, only PSA is uniquely produced and secreted abundantly by prostate cancer cells, although recently, trace amounts of PSA were found to be produced by breast cancer cells. PSA is a widely used serologic marker for prostate cancer. The physiologic functions of PSA are largely unknown. PSA is a serine protease which could cleave the parathyroid hormone-related peptide, resulting in abolishment of the ability of parathyroid hormone-related peptide to stimulate cyclic AMP production leading to a decrease in bone resorption (13). PSA could enhance IGF function by cleaving IGF-binding protein and

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activating latent TGF- $\beta$ , leading to an increase in bone formation (14). These studies suggest that PSA may be involved in prostate cancer bone metastases associated with the osteoblastic phenotype.

In the present study, we examined the role of PSA in the differentiation of human osteosarcoma SaOS-2 cells by ectopically expressing a cDNA encoding human PSA. Using cDNA arrays and Northern blot analysis, we evaluated the changes in global gene expression resulting from the expression of PSA in human osteosarcoma SaOS-2 cells. We found a number of osteogenic genes induced by PSA to be implicated in bone remodeling, and unveiled a molecular basis for prostate cancer bone metastases associated with osteoblastic phenotype.

## Materials and Methods

**Cell culture.** Human SaOS-2 osteosarcoma cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were grown at 37°C in 5% CO<sub>2</sub> and 95% air.

**Cloning of human PSA cDNA and construction of PSA expression vectors.** Human PSA cDNA was cloned by RT-PCR from the LNCaP human prostate cancer cells. Total RNAs were isolated by TRIZOL kit (Life Technologies, Inc., Gaithersburg, MD) and cDNAs were reverse-transcribed by using first-strand cDNA synthesis reagents (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. A full open reading frame of the human PSA cDNA was isolated by PCR using primers forward (5'-GATGACTCCAGCCACGACCT) and reverse (5'-CACAGACACCCCATCTATC) based on the human PSA cDNA sequence. Briefly, 5  $\mu$ g of total RNA was reverse-transcribed and 2  $\mu$ L of the reverse transcription reaction mix was amplified. The PCR was done for 30 seconds at 94°C, 1 minute at 55°C, and 2 minutes at 72°C for 35 cycles. The PCR products were cloned into pCR II vector (Invitrogen, San Diego, CA) and subcloned into pCDNA3.1<sup>+</sup> (Invitrogen). The sense and antisense PSA constructs were selected and confirmed by DNA sequencing using DNasequenseII in accordance with the manufacturer's instructions (Amersham, Arlington Heights, IL).

**Northern blot.** Total RNA was extracted from cells with TRIZOL reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.2-kb BamHI fragment of the PSA cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out for 3 hours at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 minutes at 65°C in 2 $\times$  SSC, 0.1% SDS (twice), 0.5 $\times$  SSC, 0.1% SDS, and 0.1 $\times$  SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad, Hercules, CA).

**PSA protein analysis.** PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA). An equal number of cells were plated in phenol red-free RPMI containing 10% fetal bovine serum. Cells were allowed to grow for 24 hours and 50  $\mu$ L of supernatant was assayed for PSA.

**Production of cDNA microarrays.** The 6K Cancer-Specific arrays used in this experiment were produced at the RPCI Microarray and Genomics Core Facility. A total of 6,116 cDNA clones (Research Genetics, Huntsville, AL) were selected based on their association with oncogenesis. Each clone was amplified from 100 ng plasmid DNA by performing PCR amplification of the insert using M13 universal primers for the plasmids represented in the clone set (5'-TGAGCGGATAACAATTTCACACAG-3', 5'-GTTTCCAGTCACGACGTTG-3'). Each PCR product (75  $\mu$ L) was purified by ethanol precipitation, resuspended in 25% DMSO and adjusted to 200 ng/ $\mu$ L. The PCR amplicons were

spotted in duplicate on type A glass slides (Schott North American, Inc., Elmsfor, NY) using a MicroGrid II TAS arrayer and MicroSpot 2500 split pins (Genomic Solutions, Inc., Ann Arbor, MI).

**Preparation and hybridization of fluorescent-labeled cDNA.** To screen the samples for gene expression, cDNA was synthesized and indirectly labeled using the Atlas Powerscript Fluorescent Labeling Kit (BD Biosciences, San Jose, CA). Total RNA isolated from the transfected cells was labeled with Cy5 or Cy3. For each reverse transcription reaction, 2.5  $\mu$ g total RNA was mixed with 2  $\mu$ L random hexamer primers (Invitrogen) in a total volume of 10  $\mu$ L, heated to 70°C for 5 minutes and cooled to 42°C. An equal volume of reaction mix was added to this sample (4  $\mu$ L 5 $\times$  first-strand buffer, 2  $\mu$ L 10 $\times$  deoxynucleotide triphosphate mix, 2  $\mu$ L DTT, 1  $\mu$ L of deionized H<sub>2</sub>O, and 1  $\mu$ L Powerscript reverse transcriptase) according to the manufacturer's instructions. After 1 hour of incubation at 42°C, the reverse transcriptase was inactivated by incubating at 70°C for 5 minutes. The mixture was cooled to 37°C and incubated for 15 minutes with 0.2  $\mu$ L RNase H (10 units/ $\mu$ L). The resultant amino-modified cDNA was purified, precipitated, and fluorescently labeled as described by the manufacturer's instructions. Uncoupled dye is removed by washing twice using a Qiaquick PCR Purification Kit (Qiagen, Chatsworth, CA). The probe is eluted in 60  $\mu$ L elution buffer and dried down to completion in a SpeedVac.

Prior to hybridization, the two separate probes were resuspended in 10  $\mu$ L dH<sub>2</sub>O, combined and mixed with 2  $\mu$ L of human Cot-1 DNA (20  $\mu$ g/ $\mu$ L, Invitrogen) and 2  $\mu$ L of polyadenylic acid (20  $\mu$ g/ $\mu$ L, Sigma, St. Louis, MO). The probe mixture was denatured at 95°C for 5 minutes, placed on ice for 1 minute and prepared for hybridization with the addition of 110  $\mu$ L of preheated (65°C) SlideHyb no. 3 buffer (Ambion). After a 5-minute incubation at 65°C, the probe solution was placed on the array in an assembled GeneTAC hybridization station module (Genomic Solutions, Inc.). The slides were incubated overnight at 55°C for 16 to 18 hours with occasional pulsation of the hybridization solution. After hybridization, the slides were automatically washed in the GeneTAC station with reducing concentrations of SSC and SDS. The final wash was 30 seconds in 0.1 $\times$  SSC, followed by a 5-second 100% ethanol dip. Two hybridizations for each RNA sample were done, switching the dyes in the second hybridization to account for possible dye bias.

**Microarray image and data analysis.** The hybridized slides were scanned using a GenePix 4200A scanner (Molecular Devices Corp., Sunnyvale, CA) to generate high-resolution (10  $\mu$ m) images for both the Cy3 and Cy5 channels. Image analysis was done using the ImaGene program (version 6.0.1, BioDiscovery, Inc., El Segundo, CA). Each cDNA spot is defined by a circular region. For each spot, the size of which is programmed to adjust to match the size of the spot. The local background for a spot is determined by ignoring a two- to three-pixel buffer region around the spot and then measuring the signal intensity in a two- to three-pixel-wide area outside the buffer region. Raw signal intensity values for each spot and its background region are segmented using a proprietary segmentation algorithm, which excludes pixels that are not representative of the majority of pixels in that region. The background-corrected signal for each cDNA spot was obtained by subtracting the mean local background from the mean signal of all the pixels in the region. The output of the image analysis is two raw fluorescence data files, one for each channel, and is further processed by the RPCI-developed Perl program. Spots that are not significantly above background or have a poor coefficient of variance are excluded. For each spot, a ratio is calculated from the background subtracted mean signal of the two channels. The ratios are then normalized on the log scale across the entire slide using a linear normalization algorithm. For each slide, the expression ratios are displayed as the log<sub>2</sub> mean of all replicate spots. The results from the two slides that make up the dye flip are then averaged on the log scale and becomes the final expression ratio of that clone.

**Gene annotation and classification.** The accession numbers of the cDNAs on the microarray were first mapped to the UniGene database (build 184) at the National Center for Biotechnology Information

to extract full information of the genes represented by these cDNA sequences. The UniGene cluster IDs of these genes were then used to query the SOURCE database at Stanford University (<http://source.stanford.edu/cgi-bin/source/sourceSearch>) to extract the Gene Ontology terms associated with these genes. The genes were classified into 11 categories based on their Gene Ontology annotations. These analyses were carried out using custom Perl scripts. Due to space limitations, we could only list the 10 most modulated genes in each category. These genes are ranked by the absolute values of their folds of changes. The full list can be found at our web site (<http://falcon.roswellpark.org/publication/Gao/>).

**von Kossa staining.** SaOS-2 cells, vector controls, and PSA-expressing clones were seeded at  $10^5$  cells/plate in 60 mm plates in medium supplemented with 1.4 mmol/L  $\text{CaCl}_2$ , 10 nmol/L dexamethasone, and 50  $\mu\text{g}/\text{mL}$  ascorbic acid.  $\beta$ -Glycerophosphate (10 mmol/L) was added to the plates on day 8 and incubation continued for 3 more days. At the end of 11 days, cells were fixed in neutral buffered formalin and stained *in situ* using the standard von Kossa technique. Briefly, cells were fixed overnight and subjected to dehydration using ethanol gradients of 70%, 90%, and 100%. The plates were rinsed in distilled water and stained with 5% silver nitrate solution by placing in front of a UV light source until calcium deposits turned black. Plates were then rinsed in distilled water with three changes and treated with 5% sodium thiosulfate for 5 minutes, rinsed in distilled water and allowed to dry. Plates were stored in the dark for analysis.

**Treatment of SaOS-2 cells with PSA.** Enzymatically active PSA was obtained from Calbiochem (La Jolla, CA). SaOS-2 cells were grown in RPMI supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin. Cells were plated in 24-well plates at a density of  $5 \times 10^4$  cells/well and treated after 24 hours with 0 to 1,000 ng/mL PSA in differentiation medium containing 1.4 mmol/L  $\text{CaCl}_2$ , 10 nmol/L dexamethasone, 50  $\mu\text{g}/\text{mL}$  ascorbic acid, and 10 mmol/L  $\beta$ -glycerophosphate. The cells were incubated at 37°C for 4 days with PSA being replenished every 36 hours.

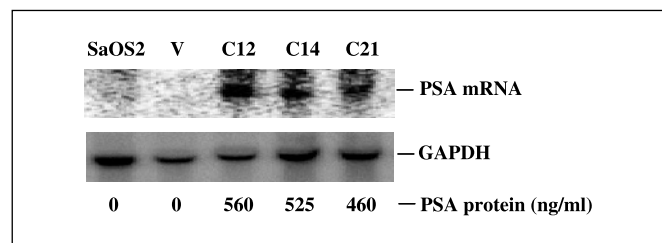
**Alkaline phosphatase activity assay.** After PSA treatment, alkaline phosphatase activity was assayed in cell lysates by determining the release of *p*-nitrophenol from *p*-nitrophenyl phosphate using Sigma Fast *p*-nitrophenyl phosphate tablet sets according to the manufacturer's instructions. Cells were lysed in 20 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L  $\text{ZnCl}_2$ , 1 mmol/L  $\text{MgCl}_2$ , and 0.5% Triton X-100 and the release of *p*-nitrophenol was determined by measuring the absorbance at 405 nm over a period of 4 minutes. Total protein content was determined using Coomassie blue protein assay reagent (Pierce, Rockford, IL) and alkaline phosphatase activity was normalized to milligrams of protein. The experiments were repeated at least thrice.

## Results

### Generation of PSA expressing osteosarcoma SaOS-2 cells.

Human osteosarcoma SaOS-2 cells were transfected with cDNA encoding a full-length PSA protein under a cytomegalovirus promoter. Clones were selected in G418 and PSA mRNA expression was assessed by Northern blots and PSA protein secreted in the medium was assessed by ELISA. Several PSA-producing clones and vector controls were selected and PSA expression was shown in Fig. 1. Northern blot analysis revealed that PSA-producing clones express PSA mRNA; parental SaOS-2 and vector control lack PSA expression (Fig. 1). These clones also express ~500 ng/mL of secreted PSA in the medium (Fig. 1).

**Identification of PSA-regulated genes in osteosarcoma SaOS-2 cells using cDNA microarrays.** To investigate whether PSA regulates genes involved in bone remodeling, we investigated genome-wide changes in gene expression in PSA-producing and



**Fig. 1.** PSA expression in SaOS-2, vector (V), and PSA cDNA-transfected clones (C12, C14, and C21). Top, Northern blot analysis using PSA cDNA labeled probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a RNA loading control. The levels of PSA protein secretion were measured by ELISA and expressed as ng/mL of medium.

PSA-nonproducing vector control osteosarcoma SaOS-2 cells. To control for biological and experimental variation, the same cell numbers of PSA-producing and vector control cells were seeded. Total RNA was extracted when the cells reached ~90% confluence and were used for microarray analysis using the 6K Cancer-Specific arrays. Differentially expressed genes between PSA-producing and vector control SaOS-2 cells were identified. A treatment to control signal ratio of  $\geq 2$  or  $\leq 0.5$  was chosen as the criteria for induction or repression, respectively. We identified total of 1,000 genes that are differentially regulated by PSA expression in SaOS-2 cells. Among them, the expression of ~454 genes are increased by PSA that were considered significant and ~400 genes are repressed by PSA. The top 50 genes in each of these two groups are shown in Tables 1 and 2, respectively. Complete lists of the significant up-regulated and down-regulated genes are included in the Supplementary Data. The PSA regulated genes were then grouped into distinct known biological functions including signal transduction, cell death, cell motility, metabolism, cell growth and/or maintenance, and transporter functions. Functional annotation of transcripts was done by using the Gene Ontology database and literature review (Table 3).

We analyzed the array data based on the known osteogenic function of the genes by manual examination of published literature using PubMed. Genes involved in bone remodeling that show at least 3-fold changes are listed in Table 4. It is interesting to note that among the most significant changes induced by overexpression of PSA in SaOS-2 cells are the factors involved in bone remodeling (Table 4). These include an increase in some of the important genes involved in bone remodeling such as *runx-2* (25-fold), *cadherin 11* (10-fold), *osteopontin* (7-fold), *TGF- $\beta$*  (5-fold), *BMP4* (4-fold), and *BMP8* (3-fold), and most significantly, decrease in the expression of *osteoprotegerin* (>95-fold decrease).

### Validation of microarray results using Northern blot analysis.

To validate the oligonucleotide array data, we did Northern blot analysis on a select number of osteogenic genes identified as being differentially regulated by PSA expression in osteosarcoma SaOS-2 cells. The Northern blot results for the selective genes relative to bone remodeling are shown in Fig. 2. Similar to the array data from *runx-2*, *osteopontin* expression is considerably elevated in PSA-producing clones, and *osteoprotegerin* is significantly reduced (Fig. 2). In general, there is a good agreement between array data and Northern blotting with regard to genes identified as being differentially expressed in SaOS-2 cells producing PSA.

**Table 1.** Top 50 up-regulated genes

Accession no.	Unigene ID	Symbol	Description	Fold of change
R55809	Hs.284122	<i>WIF1</i>	WNT inhibitory factor 1	31.12
R53942	Hs.246506	<i>SLC25A4</i>	Solute carrier family 25, member 4	28.43
AA858175	Hs.535845	<i>RUNX2</i>	Runt-related transcription factor 2	25.80
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
AA425628	Hs.77578	<i>USP9X</i>	Ubiquitin specific protease 9, X-linked (fat facets-like, <i>Drosophila</i> )	17.23
H06516	Hs.212838	<i>A2M</i>	$\alpha$ -2-macroglobulin	16.64
T51538	Hs.368592	<i>SORL1</i>	Sortilin-related receptor, L (DLR class) A repeats-containing	14.85
AA677306	Hs.158560	<i>TAF1</i>	TAF1 RNA polymerase II, TATA box binding protein (TBP) -associated factor, 250 kDa	13.32
AI016456	Hs.333497	<i>CYP2D7P1</i>	Cytochrome P450, family 2, subfamily D, polypeptide 7 pseudogene 1	13.28
AA442092	Hs.476018	<i>CTNNB1</i>	Catenin (cadherin-associated protein), $\beta$ 1, 88 kDa	13.15
AA055979	Hs.524484	<i>ITGA7</i>	Integrin, $\alpha$ 7	13.06
AA490459	Hs.417948	<i>TCN2</i>	Transcobalamin II; macrocytic anemia	12.68
AA464532	Hs.164226	<i>THBS1</i>	Thrombospondin 1	12.53
AA705237	Hs.370480	<i>ABCB7</i>	ATP-binding cassette, subfamily B (MDR/TAP), member 7	12.20
AA676805	Hs.336994	<i>MTSS1</i>	Metastasis suppressor 1	11.36
AA404486	Hs.522767	<i>SLC25A5</i>	Solute carrier family 25, member 5	10.28
AA487231	Hs.148641	<i>CTSH</i>	Cathepsin H	10.14
H96738	Hs.116471	<i>CDH11</i>	Cadherin 11, type 2, OB-cadherin (osteoblast)	9.82
AA446600	Hs.136905	<i>UREB1</i>	HECT, UBA and WWE domain containing 1	9.70
AA227885	Hs.80395	<i>MAL</i>	Mal, T cell differentiation protein	8.97
N29914	Hs.82002	<i>EDNRB</i>	Endothelin receptor type B	8.93
AA810225	Hs.552573	<i>GPR30</i>	G protein – coupled receptor 30	8.39
AA452149	Hs.173135	<i>DYRK2</i>	Dual-specificity tyrosine- (Y) -phosphorylation regulated kinase 2	8.34
AA443093	Hs.533683	<i>FGFR2</i>	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crozon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	8.29
AA682819	Hs.470171	<i>ACVR2</i>	Activin A receptor, type II	8.26
AA970865	Hs.21145	<i>ODAG</i>	GATA zinc finger domain containing 1	7.48
AA775616		<i>SPP1</i>	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein 1)	7.45
N69689	Hs.310645	<i>RAB1A</i>	RAB1A, member RAS oncogene family	7.40
R85213	Hs.22543	<i>UBE3A</i>	Ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)	7.28
AA706987	Hs.514806	<i>GALNT1</i>	UDP-N-acetyl- $\alpha$ -D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	7.28
R41839	Hs.154073	<i>SLC35B1</i>	Solute carrier family 35, member B1	7.27
N71628	Hs.437905	<i>SPIB</i>	Spi-B transcription factor (Spi-1/PU.1 related)	7.26
AI129421	Hs.83077	<i>IL18</i>	Interleukin 18 (IFN- $\gamma$ -inducing factor)	7.25
AA101617	Hs.220971	<i>FOSL2</i>	FOS-like antigen 2	7.18
AA180742	Hs.75318	<i>TUBA1</i>	Tubulin, $\alpha$ 1 (testis specific)	7.15
AA446108	Hs.76753	<i>ENG</i>	Endoglin (Osler-Rendu-Weber syndrome 1)	7.11
T60048	Hs.516105	<i>ACTG2</i>	Actin, $\gamma$ 2, smooth muscle, enteric	7.06
AA454585	Hs.73965	<i>SFRS2</i>	Splicing factor, arginine/serine-rich 2	6.96
AA418813	Hs.309090	<i>SFRS7</i>	Splicing factor, arginine/serine-rich 7, 35 kDa	6.93
AA626845	Hs.380774	<i>DDX3X</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	6.82
T66839	Hs.325846	<i>FLJ11806</i>	Nuclear protein UKp68	6.78
T50498	Hs.433795	<i>SHC1</i>	SHC (Src homology 2 domain containing) transforming protein 1	6.73
AA496691	Hs.76111	<i>DAG1</i>	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	6.72
AA598670	Hs.533273	<i>UBE1</i>	Ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature sensitivity complementing)	6.63
AA281784	Hs.518451	<i>PIK3CD</i>	Phosphoinositide-3-kinase, catalytic, $\delta$ polypeptide	6.37
H20138	Hs.503222	<i>RAB6A</i>	RAB6A, member RAS oncogene family	6.35
AA912448	Hs.46523	<i>ELK3</i>	ELK3, ETS-domain protein (SRF accessory protein 2)	6.33
N68166	Hs.185172	<i>GNB2</i>	Guanine nucleotide binding protein (G protein), $\beta$ polypeptide 2	6.22
AI392759	Hs.515840	<i>DNMT3A</i>	DNA (cytosine-5-) -methyltransferase-3 $\alpha$	6.21
AA281635	Hs.411311	<i>IL24</i>	Interleukin 24	6.16

Table 2. Top 50 down-regulated genes

Accession no.	Unigene ID	Symbol	Description	Fold of change
AA194983	Hs.81791	<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-95.28
AA486444	Hs.505687	<i>PYM</i>	Within bgcn homologue ( <i>Drosophila</i> )	-30.49
AA598794	Hs.410037	<i>CTGF</i>	Connective tissue growth factor	-27.45
N35316	Hs.153952	<i>NT5E</i>	5'-nucleotidase, ecto (CD73)	-27.19
AA629591	Hs.35052	<i>TEGT</i>	Testis enhanced gene transcript (BAX inhibitor 1)	-26.86
AA917374	Hs.104839	<i>TIMP2</i>	Tissue inhibitor of metalloproteinase 2	-25.98
AI262978	Hs.435974	<i>MTHFD1</i>	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	-20.70
H13623	Hs.26139	<i>EPS8</i>	Epidermal growth factor receptor pathway substrate 8	-20.60
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ -polypeptide	-20.51
H88540	Hs.187199	<i>MALAT1</i>	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	-16.50
AA136710	Hs.268849	<i>GLO1</i>	Glyoxalase I	-16.36
AA495936	Hs.389700	<i>MGST1</i>	Microsomal glutathione S-transferase 1	-15.68
AA487429	Hs.352018	<i>TAP1</i>	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	-14.88
R09561	Hs.527653	<i>DAF</i>	Decay accelerating factor for complement (CD55, Cromer blood group system)	-13.88
AA598817	Hs.30743	<i>PRAME</i>	Preferentially expressed antigen in melanoma	-13.75
T62048	Hs.458355	<i>C1S</i>	Complement component 1, s subcomponent	-13.35
AA670438	Hs.518731	<i>UCHL1</i>	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	-13.12
AA130042	Hs.438970	<i>TBL1XR1</i>	Transducin ( $\beta$ )-like 1X-linked receptor 1	-12.99
N66644	Hs.132513	<i>HSD17B12</i>	Hydroxysteroid (17- $\beta$ ) dehydrogenase 12	-12.60
AA464246	Hs.534125	<i>HLA-C</i>	MHC, class I, C	-12.07
H11003	Hs.511899	<i>EDN1</i>	Endothelin 1	-12.02
AA937895	Hs.495605	<i>CD99</i>	CD99 antigen	-11.79
AA043133	Hs.75231	<i>SLC16A1</i>	Solute carrier family 16 (monocarboxylic acid transporters), member 1	-11.70
AA181300	Hs.180062	<i>PSMB8</i>	Proteasome (prosome, macropain) subunit, $\beta$ type, 8 (large multifunctional protease 7)	-11.59
N21573	Hs.514685	<i>TWSG1</i>	Twisted gastrulation homologue 1 ( <i>Drosophila</i> )	-11.10
H99676	Hs.474053	<i>COL6A1</i>	Collagen, type VI, $\alpha$ 1	-10.72
H15574	Hs.127826	<i>EPOR</i>	Erythropoietin receptor	-10.53
AA447561	Hs.90691	<i>NPM3</i>	Nucleophosmin/nucleoplasmin, 3	-10.53
AA630771	Hs.413812	<i>RAC1</i>	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	-10.48
W73874	Hs.418123	<i>CTSL</i>	Cathepsin L	-10.42
AA878257	Hs.173894	<i>CSF1</i>	Colony-stimulating factor 1 (macrophage)	-10.21
R76394	Hs.25338	<i>PRSS23</i>	Protease, serine, 23	-10.13
AA865464	Hs.521903	<i>LY6E</i>	Lymphocyte antigen 6 complex, locus E	-10.11
AA504482	Hs.185597	<i>SPG7</i>	Spastic paraplegia 7, paraplegin (pure and complicated autosomal recessive)	-9.82
AA056232	Hs.75652	<i>GSTM5</i>	Glutathione S-transferase M5	-9.80
AA486570	Hs.348387	<i>GSTM4</i>	Glutathione S-transferase M4	-9.62
N52911	Hs.436687	<i>SET</i>	SET translocation (myeloid leukemia-associated)	-9.46
AI356709	Hs.332197	<i>D2S448</i>	Melanoma associated gene	-9.45
AI084613	Hs.56186	<i>EGFL3</i>	EGF-like-domain, multiple 3	-9.36
AA424956	Hs.151777	<i>EIF2S1</i>	Eukaryotic translation initiation factor 2, subunit 1 $\alpha$ , 35 kDa	-9.34
R25377	Hs.484813	<i>DEK</i>	DEK oncogene (DNA binding)	-9.22
AA453749	Hs.506748	<i>HDGF</i>	Hepatoma-derived growth factor (high-mobility group protein 1-like)	-8.98
AA487486	Hs.523852	<i>CCND1</i>	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	-8.93
AA485353	Hs.514535	<i>LGALS3BP</i>	Lectin, galactoside-binding, soluble, 3 binding protein	-8.83
AA504348	Hs.156346	<i>TOP2A</i>	Topoisomerase (DNA) II $\alpha$ 170 kDa	-8.80
AA479090	Hs.295137	<i>AMFR</i>	Autocrine motility factor receptor	-8.69
AA464849	Hs.434367	<i>TXNRD1</i>	Thioredoxin reductase 1	-8.57
AA676466	Hs.160786	<i>ASS</i>	Argininosuccinate synthetase	-8.54
AA410636	Hs.445403	<i>IARS</i>	Isoleucine-tRNA synthetase	-8.51
R38933	Hs.491582	<i>PLAT</i>	Plasminogen activator, tissue	-8.32

**Table 3.** PSA-regulated genes grouped into function classes

Accession no.	Unigene ID	Symbol	Description	Fold of change
Metabolism (566 genes)				
R53942	Hs.246506	<i>SLC25A4</i>	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	28.43
AA598794	Hs.410037	<i>CTGF</i>	Connective tissue growth factor	-27.45
N35316	Hs.153952	<i>NT5E</i>	5'-nucleotidase, ecto (CD73)	-27.19
AA858175	Hs.535845	<i>RUNX2</i>	Runt-related transcription factor 2	25.80
AI262978	Hs.435974	<i>MTHFD1</i>	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	-20.70
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ polypeptide	-20.51
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
AA425628	Hs.77578	<i>USP9X</i>	Ubiquitin specific protease 9, X-linked (fat facets-like, <i>Drosophila</i> )	17.23
AA136710	Hs.268849	<i>GLO1</i>	Glyoxalase I	-16.36
T51538	Hs.368592	<i>SORL1</i>	Sortilin-related receptor, L (DLR class) A repeats-containing	14.85
Catalytic activity (399 genes)				
R55809	Hs.284122	<i>WIF1</i>	WNT inhibitory factor 1	31.12
N35316	Hs.153952	<i>NT5E</i>	5'-nucleotidase, ecto (CD73)	-27.19
AI262978	Hs.435974	<i>MTHFD1</i>	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	-20.70
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ polypeptide	-20.51
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
AA425628	Hs.77578	<i>USP9X</i>	Ubiquitin specific protease 9, X-linked (fat facets-like, <i>Drosophila</i> )	17.23
AA136710	Hs.268849	<i>GLO1</i>	Glyoxalase I	-16.36
AA495936	Hs.389700	<i>MGST1</i>	Microsomal glutathione S-transferase 1	-15.68
AA487429	Hs.352018	<i>TAP1</i>	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	-14.88
T62048	Hs.458355	<i>C1S</i>	Complement component 1, s subcomponent	-13.35
Cell growth and/or maintenance (307 genes)				
R53942	Hs.246506	<i>SLC25A4</i>	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	28.43
AA598794	Hs.410037	<i>CTGF</i>	Connective tissue growth factor	-27.45
H13623	Hs.26139	<i>EPS8</i>	Epidermal growth factor receptor pathway substrate 8	-20.60
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ polypeptide	-20.51
H06516	Hs.212838	<i>A2M</i>	$\alpha$ -2-macroglobulin	16.64
AA487429	Hs.352018	<i>TAP1</i>	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	-14.88
T51538	Hs.368592	<i>SORL1</i>	Sortilin-related receptor, L (DLR class) A repeats-containing	14.85
AA677306	Hs.158560	<i>TAF1</i>	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250 kDa	13.32
AA055979	Hs.524484	<i>ITGA7</i>	Integrin, $\alpha$ 7	13.06
AA490459	Hs.417948	<i>TCN2</i>	Transcobalamin II; macrocytic anemia	12.68
Cell communication (277 genes)				
AA194983	Hs.81791	<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-95.28
R55809	Hs.284122	<i>WIF1</i>	WNT inhibitory factor 1	31.12
AA598794	Hs.410037	<i>CTGF</i>	Connective tissue growth factor	-27.45
H13623	Hs.26139	<i>EPS8</i>	Epidermal growth factor receptor pathway substrate 8	-20.60
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ polypeptide	-20.51
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
AA442092	Hs.476018	<i>CTNNB1</i>	Catenin (cadherin-associated protein), $\beta$ 1, 88 kDa	13.15
AA055979	Hs.524484	<i>ITGA7</i>	Integrin, $\alpha$ 7	13.06
AA464532	Hs.164226	<i>THBS1</i>	Thrombospondin 1	12.53
H11003	Hs.511899	<i>EDN1</i>	Endothelin 1	-12.02

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**Table 3.** PSA-regulated genes grouped into function classes (Cont'd)

Accession no.	Unigene ID	Symbol	Description	Fold of change
Signal transduction (141 genes)				
AA194983	Hs.81791	<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-95.28
H13623	Hs.26139	<i>EPS8</i>	Epidermal growth factor receptor pathway substrate 8	-20.60
H23235	Hs.74615	<i>PDGFRA</i>	Platelet-derived growth factor receptor, $\alpha$ polypeptide	-20.51
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
T51538	Hs.368592	<i>SORL1</i>	Sortilin-related receptor, L (DLR class) A repeats-containing	14.85
AA055979	Hs.524484	<i>ITGA7</i>	Integrin, $\alpha$ 7	13.06
AA464246	Hs.534125	<i>HLA-C</i>	MHC, class I, C	-1 2.07
H11003	Hs.511899	<i>EDN1</i>	Endothelin 1	-12.02
AA676805	Hs.336994	<i>MTSS1</i>	Metastasis suppressor 1	11.37
H15574	Hs.127826	<i>EPOR</i>	Erythropoietin receptor	-10.53
Transcriptional regulation (117 genes)				
AA858175	Hs.535845	<i>RUNX2</i>	Runt-related transcription factor 2	25.80
R25377	Hs.484813	<i>DEK</i>	DEK oncogene (DNA binding)	-9.22
AA970865	Hs.21145	<i>ODAG</i>	GATA zinc finger domain containing 1	7.48
N71628	Hs.437905	<i>SPIB</i>	Spi-B transcription factor (Spi-1/PU.1 related)	7.26
AA101617	Hs.220971	<i>FOSL2</i>	FOS-like antigen 2	7.18
AA664389	Hs.507916	<i>TGFB114</i>	TSC22 domain family 1	-6.68
AI364369	Hs.493096	<i>PBX1</i>	Pre - B cell leukemia transcription factor 1	-6.58
R42479	Hs.517296	<i>ETS2</i>	V-ets erythroblastosis virus E26 oncogene homologue 2 (avian)	-6.34
AA912448	Hs.46523	<i>ELK3</i>	ELK3, ETS-domain protein (SRF accessory protein 2)	6.33
AA495962	Hs.412293	<i>NCOA1</i>	Nuclear receptor coactivator 1	6.08
Cell death (70 genes)				
AA194983	Hs.81791	<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-95.28
AA629591	Hs.35052	<i>TEGT</i>	Testis enhanced gene transcript (BAX inhibitor 1)	-26.86
AA227885	Hs.80395	<i>MAL</i>	Mal, T cell differentiation protein	8.97
AI129421	Hs.83077	<i>IL18</i>	Interleukin 18 (IFN $\gamma$ -inducing factor)	7.25
AA281635	Hs.411311	<i>IL24</i>	Interleukin 24	6.16
AA598601	Hs.450230	<i>IGFBP3</i>	Insulin-like growth factor binding protein 3	-5.96
N62514	Hs.283454	<i>BNIP2</i>	BCL2/adenovirus E1B 19 kDa interacting protein 2	-5.83
AA418744	Hs.55220	<i>BAG2</i>	BCL2-associated athanogene 2	-5.77
N69521	Hs.397465	<i>HIPK2</i>	Homeodomain interacting protein kinase 2	-5.62
N70463	Hs.255935	<i>BTG1</i>	B cell translocation gene 1, antiproliferative	5.32
Transporter (54 genes)				
R53942	Hs.246506	<i>SLC25A4</i>	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	28.43
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
H06516	Hs.212838	<i>A2M</i>	$\alpha$ -2-macroglobulin	16.64
AA487429	Hs.352018	<i>TAP1</i>	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	-14.88
T51538	Hs.368592	<i>SORL1</i>	Sortilin-related receptor, L (DLR class) A repeats-containing	14.85
AA490459	Hs.417948	<i>TCN2</i>	Transcobalamin II; macrocytic anemia	12.68
AA705237	Hs.370480	<i>ABCB7</i>	ATP-binding cassette, subfamily B (MDR/TAP), member 7	12.20
AA043133	Hs.75231	<i>SLC16A1</i>	Solute carrier family 16 (monocarboxylic acid transporters), member 1	-11.70
AA404486	Hs.522767	<i>SLC25A5</i>	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	10.28
AA227885	Hs.80395	<i>MAL</i>	Mal, T cell differentiation protein	8.97
Cell motility (18 genes)				
AA156964	Hs.471200	<i>NRP2</i>	Neuropilin 2	19.22
AA496691	Hs.76111	<i>DAG1</i>	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	6.72
AA056693	Hs.405156	<i>PPAP2B</i>	Phosphatidic acid phosphatase type 2B	-6.70
H20759	Hs.224008	<i>FEZ1</i>	Fasciculation and elongation protein $\zeta$ 1 (zygin I)	-6.17
N94616	Hs.213861	<i>LAMA4</i>	Laminin, $\alpha$ 4	-5.6

(Continued on the following page)

**Table 3.** PSA-regulated genes grouped into function classes (Cont'd)

Accession no.	Unigene ID	Symbol	Description	Fold of change
N70463	Hs.255935	<i>BTG1</i>	B cell translocation gene 1, antiproliferative	5.32
R59598	Hs.371720	<i>SYK</i>	Spleen tyrosine kinase	3.23
AA398400	Hs.465929	<i>CNN1</i>	Calponin 1, basic, smooth muscle	-3.21
AA496565	Hs.476209	<i>PLXNB1</i>	Plexin B1	3.12
AA676840	Hs.133135	<i>UTRN</i>	Utrophin (homologous to dystrophin)	-2.98
Translational regulation (15 genes)				
AA424956	Hs.151777	<i>EIF2S1</i>	Eukaryotic translation initiation factor 2, subunit 1 $\alpha$ , 35 kDa	-9.34
AA878570	Hs.534314	<i>EIF5A</i>	Eukaryotic translation initiation factor 5A	4.84
AA669443	Hs.433702	<i>EIF5</i>	Eukaryotic translation initiation factor 5	4.49
AA460838	Hs.355348	<i>GTF2H3</i>	General transcription factor IIH, polypeptide 3, 34 kDa	-3.85
AA459999	Hs.534582	<i>MTIF3</i>	Mitochondrial translational initiation factor 3	-3.84
AA053129	Hs.396644	<i>PAIP2</i>	Poly(A) binding protein interacting protein 2	3.60
R43766	Hs.515070	<i>EEF2</i>	Eukaryotic translation elongation factor 2	3.34
AA486233	Hs.528780	<i>GSPT1</i>	G1 to S phase transition 1	3.30
R15111	Hs.530727	<i>CUGBP1</i>	CUG triplet repeat, RNA binding protein 1	2.92
R51607	Hs.150580	<i>SUI1</i>	Putative translation initiation factor	-2.64
Other/unknown (146 genes)				
AA486444	Hs.505687	<i>PYM</i>	Within bgcn homologue ( <i>Drosophila</i> )	-30.49
AA917374	Hs.104839	<i>TIMP2</i>	Tissue inhibitor of metalloproteinase 2	-25.98
H88540	Hs.187199	<i>MALAT1</i>	Metastasis associated lung adenocarcinoma transcript 1 (noncoding RNA)	-16.50
R09561	Hs.527653	<i>DAF</i>	Decay accelerating factor for complement (CD55, Cromer blood group system)	-13.88
AA598817	Hs.30743	<i>PRAME</i>	Preferentially expressed antigen in melanoma	-13.75
N21573	Hs.514685	<i>TWSG1</i>	Twisted gastrulation homologue 1 ( <i>Drosophila</i> )	-11.10
AI084613	Hs.56186	<i>EGFL3</i>	EGF-like domain, multiple 3	-9.36
AA400234	Hs.523789	<i>TncRNA</i>	Trophoblast-derived noncoding RNA	-7.81
AA464417	Hs.374650	<i>IFITM3</i>	IFN induced transmembrane protein 3 (1-8U)	-7.65
AA424824	Hs.304192	<i>DSTN</i>	Destrin (actin depolymerizing factor)	-7.40

**Overexpression of PSA enhances SaOS-2 cell calcium deposition and elevates alkaline phosphatase activity in vitro.** The PSA-expressing SaOS-2 cells express elevated runx-2, osteocalcin, and bone sialoprotein compared with PSA-negative SaOS-2 cells. Because the increase in the expression of these genes is closely associated with osteoblastic differentiation, PSA-expressing SaOS-2 cells may result in the stimulation of differentiation and bone mineralization in culture. One of the characteristics of osteoblastic differentiation is bone formation. The von Kossa staining was used to detect the presence of mineral deposits of calcium in bone nodules formed during culture of osteoblasts. The mineralized bone nodule formation in SaOS-2 cells and SaOS-2 cells expressing PSA cultured for 11 days in medium supplemented with dexamethasone, ascorbic acid, and  $\beta$ -glycerophosphate was analyzed by von Kossa staining and is illustrated in Fig. 3A. The PSA-expressing SaOS-2 cells show a considerable increase in mineralized bone nodule formation compared with PSA-negative SaOS-neo cells. The activity of alkaline phosphatase was elevated in the PSA-expressing SaOS-2 cells compared with PSA-negative SaOS-neo control (Fig. 3B). These results suggest that overexpression of PSA induces SaOS-2 cells undergoing osteoblastic differentiation *in vitro*.

**Exogenously added PSA increases runx-2 expression and elevates alkaline phosphatase activity in SaOS-2 cells.** To determine whether exogenously added PSA has similar effects as transfecting expressed PSA, SaOS-2 cells were treated with increasing doses of PSA. Cells were harvested and alkaline phosphatase activity was determined. PSA increased alkaline phosphatase activity in a dose-dependent manner (Fig. 4A). PSA also increased runx-2 and Wif-1 mRNA expression in SaOS-2 cells (Fig. 4B). These results suggested that exogenously added PSA has similar effects as transfecting expressed PSA on the induction of osteoblasts in SaOS-2 cells.

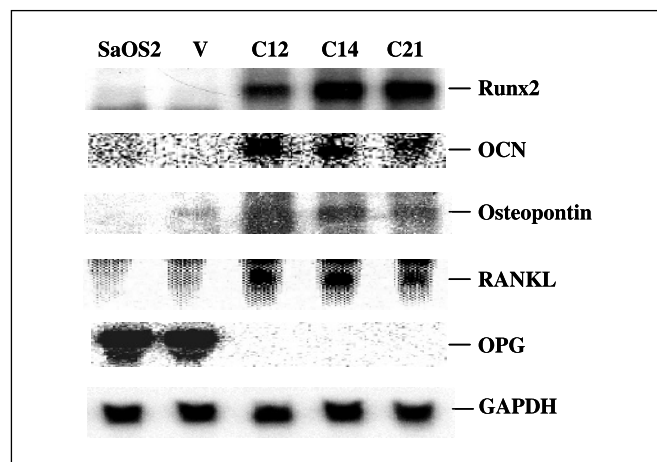
## Discussion

Prostate cancer preferably metastasizes to bone and produces a primarily osteoblastic phenotype. Despite recent studies on the cellular and molecular interactions between prostate cancer and bone cells, which revealed several factors produced by prostate cancer cells that are involved in osteoblastic differentiation, the underlying mechanisms of prostate cancer-induced osteoblasts are poorly understood.

**Table 4.** Changes on the expression of osteogenic associated genes

Accession no.	Unigene ID	Symbol	Description	Fold of change
Up-regulated (nine genes)				
R55809	Hs.284122	<i>WIF1</i>	WNT inhibitory factor 1	31.12
AA858175	Hs.535845	<i>RUNX2</i>	Runt-related transcription factor 2	25.80
H96738	Hs.116471	<i>CDH11</i>	Cadherin 11, type 2, OB-cadherin (osteoblast)	9.82
AA775616		<i>SPP1</i>	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	7.45
N20203	Hs.471119	<i>BMPR2</i>	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	5.35
TGF- $\beta$		<i>TGFB</i>	Transforming growth factor- $\beta$	5.05
AA449300	Hs.481022	<i>SFRP2</i>	Secreted frizzled-related protein 2	4.71
AA463225	Hs.68879	<i>BMP4</i>	Bone morphogenetic protein 4	3.87
AA779480	Hs.494158	<i>BMP8A</i>	Bone morphogenetic protein 8a	3.37
Down-regulated (six genes)				
AA194983	Hs.81791	<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-95.28
R38933	Hs.491582	<i>PLAT</i>	Plasminogen activator, tissue	-8.32
AA490172	Hs.489142	<i>COL1A2</i>	Collagen, type I, $\alpha$ 2	-6.47
AA598601	Hs.450230	<i>IGFBP3</i>	Insulin-like growth factor binding protein 3	-5.96
AA143331	Hs.83169	<i>MMP1</i>	Matrix metalloproteinase 1 (interstitial collagenase)	-5.66
AA936799	Hs.513617	<i>MMP2</i>	Matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	-5.46

PSA is the most commonly used biomarker for prostate cancer, but its role in bone metastasis is still unclear. In the present study, we show that PSA modulates the expression of genes involved in bone remodeling, including up-regulation of some of the most important osteoblastic genes such as runx-2, osteopontin, TGF- $\beta$ , receptor activator of NF- $\kappa$ B ligand (RANKL), and down-regulation of osteoprotegerin. Furthermore, PSA overexpression increased mineralization *in vitro* using von Kossa staining. These results suggest that factors such as PSA produced by prostate cancer cells modulate bone remodeling, resulting in the induction of bone cells' osteoblastic differentiation.



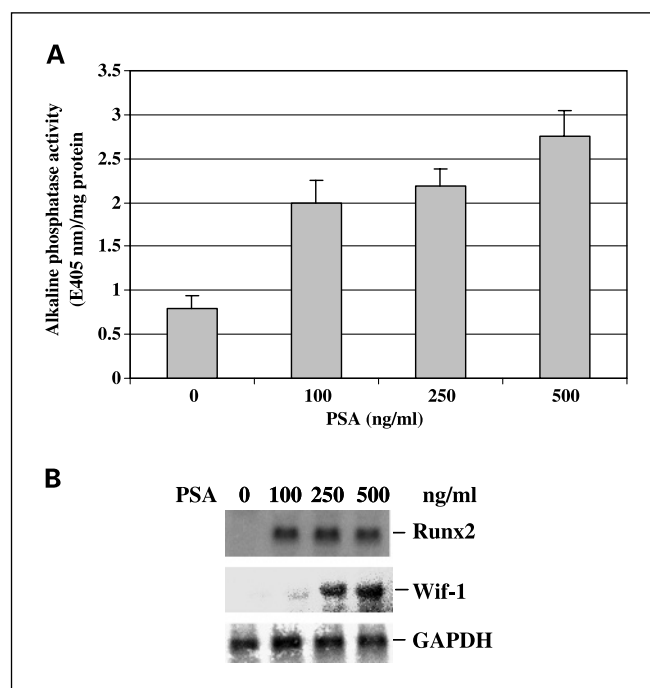
**Fig. 2.** Northern blot analyses of selective osteogenic genes in SaOS-2, vector (V), and PSA cDNA-transfected clones (C12, C14, and C21). OCN, osteocalcin. Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) was used as a RNA loading control.

We have identified 15 osteogenic associated genes which are dysregulated by the overexpression of PSA in SaOS-2 cells using 6K Cancer-Specific arrays. Many of those which are up-regulated by PSA are associated with osteoblast differentiation including runx-2, osteopontin, cadherin 11, and TGF- $\beta$ . Overexpression of PSA enhances runx-2 mRNA expression by 25-fold. Runx-2 is a transcription factor essential for osteoblast differentiation (15, 16). Runx-2-deficient mice showed a complete lack of bone formation due to the absence of osteoblasts (17). Runx-2 has been identified as a key factor which mediates osteoblast differentiation induced by soluble factors produced by prostate cancer cells (18). Runx-2 is a transcription factor that regulates the expression of many bone matrix genes including osteopontin and osteocalcin (18, 19). Of those two genes, the expression of osteopontin was increased to  $\sim$ 7-fold by PSA (Table 4). To examine whether overexpression of PSA also enhances osteocalcin expression in SaOS-2 cells, Northern blot analysis was done. Osteocalcin mRNA expression was considerably elevated in SaOS-2 cells expressing PSA, but not in the parental SaOS-2 or vector control cells (Fig. 2). Osteocalcin is expressed solely in osteoblasts (20), further suggesting that PSA induces osteoblast differentiation, possibly through up-regulation of runx-2 expression.

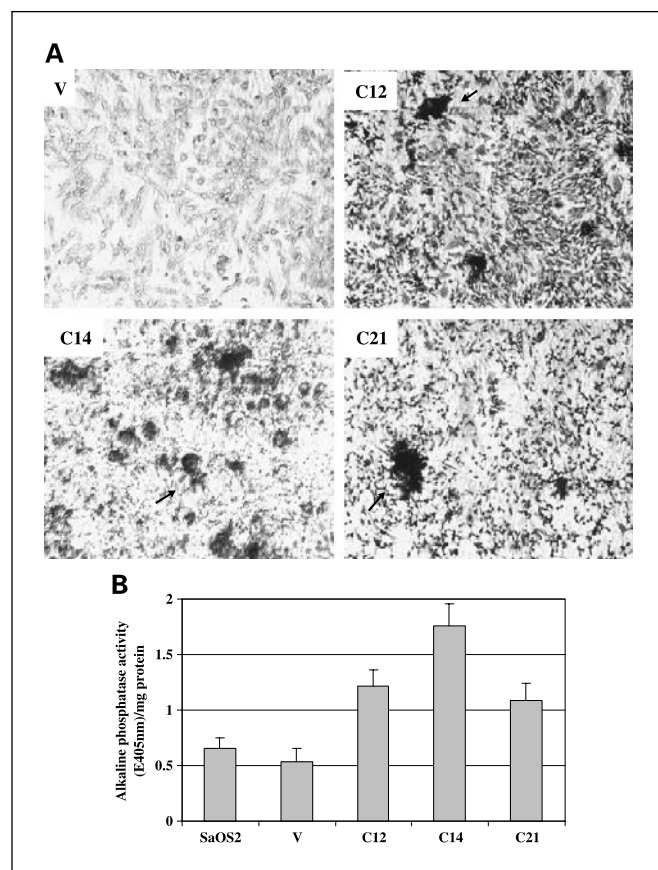
Besides runx-2, one of the well known factors which induce osteoblast differentiation is TGF- $\beta$ . TGF- $\beta$  is one of the most abundantly deposited growth factors in bone matrix that modulates proliferation and differentiation of osteoblastic cells, and enhances the accumulation of extracellular matrix components (21, 22). Direct injection of TGF- $\beta$  in the periosteum increases bone volume in rodents (23). Prostate cancer cells produce TGF- $\beta$  which can be further activated by PSA (11). In addition, TGF- $\beta$  secretion in SaOS-2 cells can be elevated by PSA (12). Growth stimulation of SaOS-2 cells by PSA can be inhibited by TGF- $\beta$  antibody, suggesting that

PSA-induced growth of osteoblasts is mediated, at least in part, by TGF- $\beta$  (12). These results are in agreement with the findings of our study that PSA enhances TGF- $\beta$  mRNA expression in SaOS-2 cells. Collectively, these studies further show the involvement of TGF- $\beta$  in osteoblast differentiation induced by prostate cancer cells.

Some of the lesser known genes involved in osteoblast differentiation regulated by PSA are cadherin 11, Wnt inhibitory factor-1 (Wif1), and secreted frizzled-related protein 2 (Sfrp2; Table 4). Cadherin 11 is a member of the cadherin family and can directly regulate the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage, resulting in the formation of bone and cartilage tissues (24, 25). Wif1 and Sfrp2 are Wnt antagonists and their expressions are elevated during osteoblast differentiation (26). Wif1 is a secreted protein that binds Wnts and antagonizes their activity (27). Wif1 has been identified as a novel marker for osteoblast differentiation (28). It was hypothesized that Wnt maintains a proliferative signal for osteoblasts, which should be turned off by Wnt antagonists such as Wif1 and Sfrp2, allowing osteoblast differentiation to proceed (26). In this study, we showed that PSA enhances Wif1 and Sfrp2 mRNA expression in SaOS-2 cells by ~31-fold and 5-fold, respectively. These data are in concordance with the role of Wif1 and Sfrp2 in osteoblast differentiation (26), suggesting that dysregulation of Wnt signaling pathways by



**Fig. 4.** Exogenously added PSA affects bone remodeling in SaOS-2 cells. *A*, PSA increased the activity of alkaline phosphatase in SaOS-2 cells. *B*, PSA increased runx-2 and Wif-1 mRNA expression.



**Fig. 3.** *A*, von Kossa staining of vector control (V) and PSA cDNA-transfected clones (C12, C14, and C21). Arrows, mineralized bone nodules. *B*, overexpression of PSA increased alkaline phosphatase activity in SaOS-2 cells.

PSA may contribute to osteoblast differentiation induced by prostate cancer cells.

It is interesting to note that osteoprotegerin is the most down-regulated gene by PSA (Table 2). Osteoprotegerin is a soluble member of the tumor necrosis factor receptor superfamily that prevents the association of RANKL with RANK by acting as a decoy receptor (29). The RANKL-osteoprotegerin system plays a very important role in bone remodeling. The binding of RANKL to the osteoclast surface receptor RANK induces osteoclastogenesis (29, 30). Osteoprotegerin is a soluble decoy receptor of RANKL, which neutralizes its interaction with RANK, resulting in reduction of osteoclast formation (30). Several studies show a role for RANKL and osteoprotegerin in regulating the bone remodeling in prostate cancer bone metastases (31–33). In contrast to the reduction of osteoprotegerin, PSA enhances RANKL mRNA expression in SaOS-2 cells (Fig. 2). These data suggest that in addition to the induction of osteoblast differentiation, PSA also induces osteoclast formation. This is in concordance with clinical observations that prostate cancer metastases forms a heterogeneous mixture of osteolytic and osteoblastic lesions, although osteoblastic lesions are predominant (4, 34).

In summary, our results show that overexpression of PSA in SaOS-2 cells modulates genes involved in bone remodeling and induces osteoblast differentiation, suggesting that in addition to serving as a marker, PSA plays a functional role in bone metastases of prostate cancer. Further investigation of the biological effects of PSA and genes regulated by PSA in osteogenesis should enhance the current understanding of prostate cancer metastases in bone.

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