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Prostate Cancer Cells Regulate Growth and Differentiation of Bone Marrow Endothelial Cells Through TGFβ and Its Receptor, TGFβRII

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BACKGROUND. The underlying mechanisms permitting prostate cancer bone metastasis are poorly understood. We previously showed that the highly metastatic prostate cancer cell line, PC-3, inhibits bone marrow endothelial (HBME-1) cell growth in collagen gels and induces them to differentiate into cords, resembling angiogenesis in vivo.

METHODS. cDNA microarray analysis was performed to identify cytokines responsible for the effects of PC-3 cells on HBME-1 cells. Cytokine and neutralizing antibody studies were done to further investigate specific angiogenic factors, such as transforming growth factor β (TGFβ). TGFβ RNA and protein were detected by real-time RT-PCR and enzyme-linked immunosorbent assay (ELISA) analysis to measure their production by prostate cancer cell lines. Conditioned media experiments using TGFβ neutralizing antibodies were used to analyze TGFβ activation by prostate cancer cells.

RESULTS. PC-3 conditioned media altered the expression of several TGFβ-regulated or -associated genes in HBME-1 cells. Low concentrations of TGFβ cytokines inhibited HBME-1 cell growth to a similar level as PC-3 conditioned media and partially induced differentiation. Inhibitors and neutralizing antibodies directed against TGFβ isoforms and TGFβ receptor type 2 (TGFβRII) reversed the growth inhibition of HBME-1 cells conferred by PC-3 conditioned media. Yet, only TGFβRII neutralizing antibodies significantly inhibited HBME-1 differentiation. Also, prostate cancer cell lines produced low levels of TGFβ RNA and protein, and were shown to activate serum-derived TGFβ.

CONCLUSIONS. These results suggest that prostate cancer cells mediate growth inhibition and differentiation of bone marrow endothelial cells both through production and activation of

Abbreviations: FBS, fetal bovine serum; ANOVA, analysis of variance between groups; TGFβ, transforming growth factor β; TGFβRII, TGFβ receptor type 2; VEGF, vascular endothelial growth factor; FGFb, fibroblast growth factor basic; PDGF, platelet-derived growth factor; LAP, latency-associated peptide; Fc:sTGFβRII, soluble TGFβ receptor 2 fusion construct; ELISA, enzyme-linked immunosorbent assay; β_2-M, β_2-microglobulin; BMP, bone morphogenetic protein.

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TGFβ as well as alteration of TGFβRII-mediated signal transduction. This could contribute to the establishment and growth of bone metastases. *Prostate 66: 632–650, 2006.*

KEY WORDS: **prostate cancer; endothelium; TGFβ; angiogenesis; bone metastasis**

INTRODUCTION

Prostate cancer preferentially metastasizes to the bone, causing high morbidity and often resulting in death [1–5]. About 80% of patients with advanced disease have skeletal metastasis [6]. For these patients treatment options are few and none are curative [7–9]. It is critical that we identify and understand the factors that promote bone metastasis in order to develop more effective diagnostic and therapeutic tools.

It has been postulated that bone marrow endothelial cells are the initial contact site for metastatic prostate cancer cells [10–12]. Lehr and Pienta [10] demonstrated that prostate cancer cells preferentially adhere to endothelial cells derived from the bone marrow compared to those from other sites. Both normal and malignant prostate epithelial cells have been shown to bind well to bone marrow endothelial cells *in vitro*, but only tumor cells are capable of invasion through basement membrane in response to bone marrow endothelial cells [13] or invasion through endothelial cell monolayers [11,14]. In order for metastatic prostate cancer cells to form sizable lesions in the bone, stimulation of angiogenesis is necessary to provide a blood supply to the growing tumor [15,16].

Using a co-culture collagen gel system, we recently showed a strong bi-directional interaction between highly metastatic prostate cancer cells (PC-3) and bone marrow endothelial cells (HBME-1) [17]. HBME-1 cells stimulated proliferation and migration of PC-3 cells; and concurrently the prostate cancer cells inhibited HBME-1 cell growth and induced them to differentiate into highly organized cords, resembling capillary networks. PC-3 conditioned media had a similar effect on HBME-1 cell growth and morphology in collagen gels. In this study, we analyzed factors potentially responsible for the effects of PC-3 cells on HBME-1 cells grown in collagen gels. The results presented here may yield further insights as to how prostate cancer cells metastasize to and grow in the bone.

MATERIALS AND METHODS

Cell Culture

PC-3, DU-145, and LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD). HBME-1 cells were a generous gift of Dr. Kenneth J. Pienta (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI). All cell lines were maintained and conditioned media was collected as described previously [17].

cDNA Microarray Analysis

HBME-1 cells growing in collagen gels [17] were exposed to conditioned media from PC-3 and LNCaP cells or normal growth media. After 48 hr, cells were harvested and total RNA was extracted using Trizol® Reagent (Invitrogen Corp., Carlsbad, CA). DNA was removed using DNA-Free™ (Ambion, Inc., Austin, TX) and RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quantity was determined using a spectrophotometer and RNA integrity was determined by agarose gel electrophoresis. Generation of labeled cDNA probes and hybridization to a 4.1K Cancer Array were done at the University of Michigan cDNA Microarray Core Facility according to their standard protocols. Briefly, Cy3 and Cy5 fluorescently-labeled cDNA probes were generated using the CyScribe™ First-Strand cDNA Labeling Kit (Amersham Biosciences, Inc., Piscataway, NJ) followed by hybridization to target cDNAs on glass slides. Hybridized microarrays were scanned with the Axon GenePix 4000a Scanner (Molecular Devices Corp., Union City, CA). Raw data was exported as Excel files and then imported into the R statistical language. Each microarray was normalized using a rank-invariant normalization [18]. After normalization, missing data was imputed using a nearest neighbor's algorithm. The resulting data was log-transformed and then fit using the analysis of variance between groups (ANOVA) model. Differential expression was determined by calculating 99.9% confidence intervals using a semi-parametric bootstrap method. All genes listed in the ''Results'' and in the ''Appendix'' are considered statistically significant using this data analysis method.

Cytokine and Neutralizing Antibody Experiments

All cytokines (transforming growth factor β (TGFβ)1, 2, and 3) and all neutralizing antibodies [anti-TGFβ1, 2, and 3; anti-TGFβ receptor type 2 (TGFβRII); monoclonal anti-TGFβ1, 2, 3; anti-pan-specific TGFβ; anti-vascular endothelial growth factor (VEGF); anti-fibroblast growth factor basic (FGFb); and anti-platelet-derived growth factor (PDGF)] and latency-associated peptide (LAP) were purchased from R&D Systems, Inc. (Minneapolis, MN). The soluble TGFβ receptor 2 fusion construct (Fc:sTGFβRII) [19–21]

was generously provided by Dr. Philip Gotwals (Biogen Idec, Inc., Cambridge, MA).

For cytokine studies, HBME-1 cells growing in collagen gels [17] were exposed to active TGFβ1, 2, or 3 added directly to normal growth media. For neutralizing antibody studies, conditioned media experiments were performed as described previously [17]. HBME-1 cells (1×10^4) were plated in 24-well plates in a single layer of collagen gel and fed either normal media or conditioned media plus or minus neutralizing antibodies (affinity-purified, if available). On day 6, HBME-1 cells were harvested and counted using a Z2™ Coulter Counter® (Beckman Coulter, Inc., Fullerton, CA).

Fluorescent Staining and Digital Microscopy

Morphological changes induced in HBME-1 cells by prostate cancer cells were assessed using fluorescence staining and digital imaging microscopy. Collagen gel layers were fixed with 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 45 min, followed by washes in phosphate-buffered saline. Gel layers were then stained with 1× SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) or a 1:5,000 dilution of a 1 mg/ml stock of Hoechst 33258 trihydrochloride (Sigma Chemical Co., St. Louis, MO). Stained cells were imaged by digital fluorescent microscopy as described previously [17]. The number of nuclei per aggregate were counted.

Analysis of TGFβ Expression by Prostate Cancer Cell Lines

TGFβ expression was determined by real-time RT-PCR and enzyme-linked immunosorbent assay (ELISA) analysis. The β_2-microglobulin (β_2-M) and TGFβ2 Lightcycler™ RT-PCR assays (Roche Diagnostics Corp., Indianapolis, IN) were developed in our laboratory and the TGFβ1 assay was based on the work of Quan et al. [22]. Hybridization probes (fluorescence energy transfer probes for β_2-M and TGFβ2 and TaqMan® probes for TGFβ1) were used to determine mRNA expression. Values obtained for TGFβ1 and 2 mRNA expression were normalized to expression of β_2-M. Cycle parameters were as follows: β_2-M = 55°C for 10 min; 95°C for 30 sec; 35 cycles of 95°C for 10 sec, 62°C for 5 sec, and 72°C for 15 sec; 95°C for 2 min; 60°C for 2 min; 95°C for 0 sec; and 35°C for 0 sec. TGFβ1 = 50°C for 10 min; 95°C for 3 min; 50 cycles of 95°C for 10 sec, 66°C for 20 sec; 72°C for 15 sec; and 40°C for 0 sec. TGFβ2 = 55°C for 10 min; 95°C for 30 sec; 50 cycles of 95°C for 10 sec, 53°C for 5 sec, and 72°C for 20 sec; 95°C for 2 min; 60°C for 2 min; 95°C for 0 sec; and 40°C for 0 sec. Primer and probe sequences are listed in Table I.

Production of TGFβ protein was measured by Quantikine® ELISA analysis (R&D Systems) according to the manufacturer's instructions. Serum-containing conditioned media previously frozen at −20°C was thawed and activated prior to assay in order to measure total (active + acid-activatable) TGFβ protein. Serum-containing media was tested since HBME-1 cells did not grow well in serum-free conditions. TGFβ production was measured in normal growth media as a background control, since 10% serum can contain up to 1,600 pg/ml TGFβ protein. To obtain protein concentrations, the normal media control value was subtracted from the values for each cell line, and then resulting values were normalized to the total number of cells at the time of conditioned media collection.

TGFβ Activation by Prostate Cancer Cells

TGFβ activation was assessed by comparing the effect on HBME-1 cell growth of serum-containing media conditioned by PC-3 cells to that of serum-free conditioned media to which serum was added after exposure to prostate cancer cells. Experiments were performed as described previously [17] with the addition of TGFβ neutralizing antibodies; and cells were harvested and counted.

Statistical Analysis

All numerical data is expressed as mean ± standard deviation (when possible). Statistical analysis was conducted using ANOVA (Tukey's Multiple Comparison Post-Test), unless otherwise noted. A P-value < 0.05 was considered statistically significant.

RESULTS

Our previous studies showed that prostate cancer cells (PC-3, DU-145, and LNCaP) and their conditioned media inhibited growth of HBME-1 cells in collagen gels [17]. For the more aggressive PC-3 cell line (and to a lesser extent, DU-145), the growth inhibition was accompanied by a strong induction of aggregation of cells into cord-like structures, resembling capillaries [17].

Prostate Cancer Cell Conditioned Media Induces Significant Gene Expression Changes of Several TGFβ-Regulated and -Associated Genes

As a screening tool to begin to identify the factors involved in the effects of the prostate cancer cells on HBME-1 cell growth and differentiation, cDNA microarray analysis was performed. Exposure of HBME-1 cells grown in collagen to PC-3 conditioned media for 48 hr induced statistically significant changes in expression of hundreds of genes compared to growth

TABLE I. Primer and Probe Sequences for Lightcycler™ RT-PCR Assays

Gene	Primer sequences	Probe sequences
β_2-Microglobulin (β_2-M)	Fwd: 5′-CTTGTCTTTCAGCAAGGACTG-3′	5′-ACATGGTTCACACGGCAGGCA/6-FAM/-3′
	Rev: 5′-CCTCCATGATGCTGCTTACAT-3′	5′-5Cy5.5/ACTCATCTTTTT-CAGTGGGGGTGA/3Phos/-3′
Transforming growth factor β (TGFβ)1 [22]	Fwd: 5′-TGACAAGTTCAAGCAGAGTACA-CACA-3′	5′/6-FAM/TCAACACATCAGAGCTCC-GAGAAGCG/TAMRA/-3′
	Rev: 5′-AGAGCAACACGGGTTCAGGTA-3′	
TGFβ2	Fwd: 5′-GAAGAAGCGTGCTTT-3′	5′-TGAGTGTCTGAACTCCAT/6-FAM/-3′
	Rev: 5′-TTTGCCAATGTAGTAGAG-3′	5′-/5Cy5.5/AATACGGGCATGCTCCAG/SpC3/-3′

in normal media, while LNCaP conditioned media affected expression of only a few genes. A complete list of affected genes showing statistically significant up- or down-regulation by PC-3 conditioned media is shown in the ''Appendix.''

The cDNA microarray analysis revealed several of the genes with altered expression are regulated by or associated with TGFβ signaling (Table II). Although none of the expression changes were greater than two-fold, the interwoven loop design used for microarray analysis as well as the careful normalization across arrays and data analysis eliminated false signals. Many of the altered TGFβ-regulated and -associated genes include factors involved in cell proliferation (activated p21/cdc42 Hs kinase, cell division cycle 25C and cyclin-dependent kinase 6), cell adhesion (integrins, laminins, and fibronectin), invasion (tissue inhibitor of metalloproteinase 2), and angiogenesis (Rho GTPase activating protein 5 and TGFβ3). Based on these results, we decided to analyze the role of TGFβ, in addition to other cytokines, in the regulation of HBME-1 cell growth and differentiation conferred by PC-3 conditioned media.

TGFβ Cytokines at Low Concentrations Inhibit Growth of HBME-1 to a Similar Level as Prostate Cancer Conditioned Media and Induce Aggregate Formation

Active TGFβ1, 2, and 3 cytokines at various concentrations from 1 pg/ml to 10 ng/ml (0.04–400 pM) were added to HBME-1 cells to determine their effects on cell proliferation and morphology. All three TGFβ isoforms inhibited growth of HBME-1 cells in a dose-dependent manner with TGFβ1 being most potent. At high concentrations [1–10 ng/ml (40–400 pM)] each TGFβ isoform inhibited cell proliferation by more than 80% (Fig. 1). At more physiologically relevant concentrations [10–100 pg/ml (0.4–4 pM)], the TGFβ isoforms inhibited HBME-1 cell growth similarly to PC-3 conditioned media (by ~30%). Also, low concentrations of active TGFβ1 induced HBME-1 cells to form cords, resembling angiogenesis (Table III). Maximal aggregate size, as measured by the number of nuclei per aggregate, was achieved at a concentration of 5 pg/ml (0.2 pM) TGFβ1, yet these aggregates were smaller than the aggregates formed in the presence of PC-3 conditioned media. The reduced effect may be a result of suboptimal TGFβ concentration or the need for a cooperative effect with other cytokines. Low concentrations (pg/ml range) of active TGFβ2 and 3 cytokines also induced aggregate formation (data not shown), suggesting redundancy of action for TGFβ isoforms *in vitro*.

TGFβ Neutralizing Antibodies Reverse the Growth Inhibition of HBME-1 Cells Induced by Prostate Cancer Cell Conditioned Media

To further determine the mediator(s) of the effects of PC-3 cells on HBME-1 cell growth and differentiation, neutralizing antibodies were used to target various angiogenesis inducers, including TGFβ, VEGF, FGFb, and PDGF. HBME-1 cells were grown in serum-containing media, since they did not grow well in serum-free conditions. Thus, any inhibitor tested could also inhibit factors present in the serum. Only antibodies/inhibitors directed against TGFβ signaling reversed HBME-1 growth inhibition conferred by PC-3 conditioned media (Table IV). Blockage of multiple TGFβ isoforms resulted in even higher proliferation than the normal media control, suggesting serum-derived TGFβ was being inhibited. Neutralizing antibodies to VEGF and PDGF had little or no effect on cell growth and anti-FGFb caused further growth

TABLE II. Significant Gene Expression Changes in HBME-1 Cells Induced by PC-3 Conditioned Media (TGFβ-Regulated and -Associated Genes)

Gene	Accession number	Fold change
Increased		
Laminin, alpha 3	AA001432	1.24
Fibronectin 1	R62612	1.20
Tissue inhibitor of metalloproteinase 2	AA486280	1.16
Plasminogen activator inhibitor 1 mRNA-binding protein	AA487070	1.16
Activated p21/cell division cycle 42 Hs kinase	AA427891	1.15
Janus kinase 2	AA284634	1.15
Cyclin-dependent kinase 6	H73724	1.14
Platelet-derived growth factor (PDGF) beta polypeptide	T49539	1.10
Decreased		
Rho GTPase activating protein 5	W86145	1.48
Latent transforming growth factor β binding protein 2	AA424629	1.42
Integrin, alpha 9	AA865557	1.24
Transforming growth factor β 3	N76883	1.22
Cell division cycle 25C	W95001	1.22
Jun D proto-oncogene	N66278	1.19
Bone morphogenetic protein 7	W73473	1.19
Activin A receptor, type II	AA682819	1.18
Laminin, gamma 2	AA677534	1.17
Forkheadbox J2	AA902249	1.15
Integrin, beta 8	R74357	1.15
p21/cell division cycle 42/Rac1-activated kinase 1	AA173411	1.15

cDNA microarray data was normalized across arrays, log-transformed, and then fit using the analysis of variance between groups (ANOVA) model. A semi-parametric bootstrap method using 99.9% confidence intervals was used to determine differential expression. Expression changes for all genes listed were deemed significant (see ''Materials and Methods'').

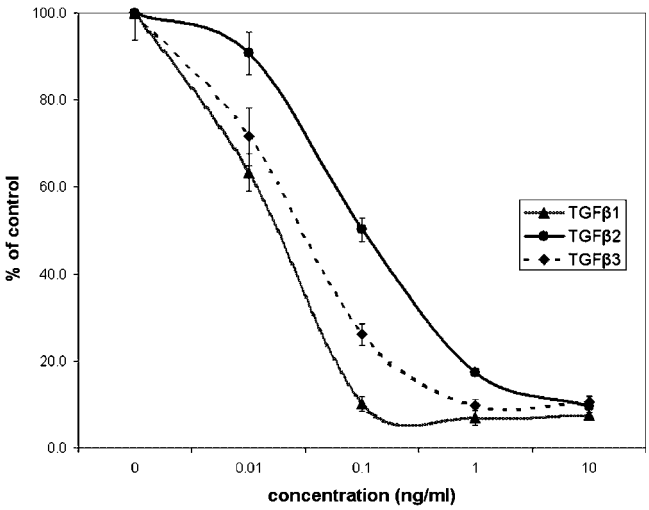

Fig. 1. Effect of transforming growth factor β (TGFβ) cytokines on HBME-1 cell growth. HBME-1 cells growing in collagen gels were subjected to activeTGFβ1, 2, or 3 cytokines at concentrations of 0.01, 0.1, 1, and 10 ng/ml. After 6 days, cells were harvested and counted using a Z2™ Coulter Counter®. EachTGFβ isoform inhibited growth in a dose-dependent manner. Low concentrations of TGFβ cytokines best mimicked the effects of prostate cancer cells on HBME-1 cells. Individual data points are means of total number of cells and standard deviations are represented by error bars; and results are presented as the average of two experiments for each cytokine tested.

inhibition. This strongly suggests primarily TGFβ mediates the growth inhibition of HBME-1 cells by prostate cancer cells.

Anti-TGFβRII Neutralizing Antibodies Inhibit HBME-1 Cord Formation Induced by Prostate Cancer Cell Conditioned Media

In addition to their effect on cell proliferation, the neutralizing antibodies and inhibitors to various angiogenic factors were tested for their ability to block HBME-1 cell differentiation induced by PC-3 conditioned media. Only anti-TGFβRII neutralizing antibodies (2 μg/ml) inhibited cord formation (Fig. 2), reducing it by about 50%. None of the other neutralizing antibodies inhibited HBME-1 cord formation (data not shown), including inhibitors of TGFβ (Table V). This suggests that signaling through TGFβRII plays an important role in angiogenesis.

In contrast to the inhibition of cords by anti-TGFβRII antibodies, inhibitors targeting multiple TGFβ isoforms actually induced formation of larger and more multicellular aggregates than PC-3 conditioned media alone (Table V). These TGFβ inhibitors include anti-pan-specific TGFβ, monoclonal anti-TGFβ1, 2, 3, Fc:sTGFβRII, and LAP. Each of these inhibitors blocks

TABLE III. Size of HBME-1 Aggregates Induced by TGFβ1 Cytokine

Condition	Mean no. of nuclei per aggregate (n = 5)[a]	*P*-value[b]
Normal media	37.2 ± 7.4	NA
PC-3 conditioned media	320.0 ± 65.3	<0.01
Normal media + vehicle	27.2 ± 7.5	N.S.
Normal media + X ng/ml TGFβ1		
+0.001	41.4 ± 3.1	N.S.
+0.005	95.6 ± 54.6	<0.01
+0.01	58.5 ± 25.2	N.S.
+0.02	38.2 ± 28.6	N.S.
+0.1	16.5 ± 6.6	N.S.
+1.0	16.2 ± 5.7	N.S.
+10	17.6 ± 7.0	N.S.

N.S., not statistically significant.
[a]Means of total number of nuclei per aggregate ± standard deviation.
[b]Versus normal media control.

more than one TGFβ isoform, including TGFβ1. For instance, LAP is associated with multiple TGFβ isoforms *in vivo* and is partly responsible for maintaining TGFβ in a latent state. Thus, LAP is a potent inhibitor of TGFβ, and so the low concentrations used were sufficient to cause a similar effect as the other TGFβ inhibitors. The increased cord formation due to inhibition of multiple TGFβ isoforms was also significant compared to an additional control in which twice the number of HBME-1 cells was seeded initially and then grown in the presence of PC-3 conditioned media. This suggests that the presence of more cells is not solely responsible for increased cord formation. However, this does not rule out the possibility that a higher proliferation rate may play a role in the increased cord formation due to inhibition of multiple TGFβ isoforms. The difference in effect for anti-TGFβRII antibodies and inhibitors of TGFβ isoforms may be attributable to the way in which TGFβ signaling is disrupted in each case and also due to TGFβ superfamily receptor/ligand promiscuity.

TGFβ mRNA and Protein Expression by Prostate Cancer Cells

We measured TGFβ1 and 2 mRNA and protein expression in PC-3, DU-145, and LNCaP cells by real-time RT-PCR and Quantikine® ELISA analysis. As shown in Table VI, PC-3 and DU-145 cells expressed TGFβ1 mRNA, while LNCaP cells did not. All three cell lines produced TGFβ2 mRNA. ELISA analysis of serum-containing conditioned media revealed TGFβ1 protein in PC-3 and DU-145 conditioned media (36 pg and 17 pg per 1×10^5 cells, respectively), but none above that normally present in serum-containing media was found in LNCaP conditioned media (Table VI). However, only PC-3 conditioned media contained TGFβ2 protein (48 pg per 1×10^5 cells). In

TABLE IV. Effect of Various Neutralizing Antibodies and Specific TGFβ Inhibitors on HBME-1 Cell Growth in Collagen in the Presence of PC-3 Conditioned Media

Condition	% of control[a]	% change versus normal media (*P*-value)	% change versus PC-3 conditioned media (*P*-value)
Normal media	100.0 ± 8.4	NA	40.3 ↑ (<0.001)
PC-3 conditioned media	71.3 ± 6.6	28.7 ↓ (<0.001)	NA
+10 μg/ml anti-TGFβ1	94.4 ± 10.2	5.6 ↓ (N.S.)	32.4 ↑ (<0.001)
+10 μg/ml anti-TGFβ2	103.8 ± 6.1	3.8 ↑ (N.S.)	45.6 ↑ (<0.001)
+10 μg/ml anti-TGFβ3	102.9 ± 8.0	2.9 ↑ (N.S.)	44.3 ↑ (0.001)
+20 μg/ml anti-TGFβR2	88.6 ± 2.9	11.4 ↓ (<0.001)	24.3 ↑ (<0.005)
+50 μg/ml anti-pan-specific TGFβ	117.9 ± 3.2	17.9 ↑ (<0.001)	65.4 ↑ (<0.001)
+10 μg/ml monoclonal anti-TGFβ1,2,3	135.0 ± 10.0	35.0 ↑ (<0.001)	89.4 ↑ (<0.001)
+1 μg/ml LAP	128.0 ± 19.3	28.0 ↑ (<0.05)	79.6 ↑ (<0.001)
+10 μg/ml Fc:sTGFβR2	120.6 ± 11.3	20.6 ↑ (<0.01)	69.1 ↑ (<0.001)
+10 μg/ml anti-VEGF	72.4 ± 7.4	27.7 ↓ (<0.001)	1.5 ↑ (N.S.)
+10 μg/ml anti-FGFb	53.8 ± 11.6	46.2 ↓ (<0.001)	24.5 ↓ (<0.001)
+50 μg/ml anti-PDGF	62.0 ± 3.0	38.0 ↓ (<0.001)	13.0 ↓ (<0.05)
2 × no. of HBME-1 cells	105.6 ± 5.2	5.6 ↑ (<0.5)	48.2 ↑ (<0.001)

Data normalized to control antibodies for each condition and to normal media control.
N.S., not statistically significant.
[a]Represented as mean percentage compared to normal media control ± standard deviation.

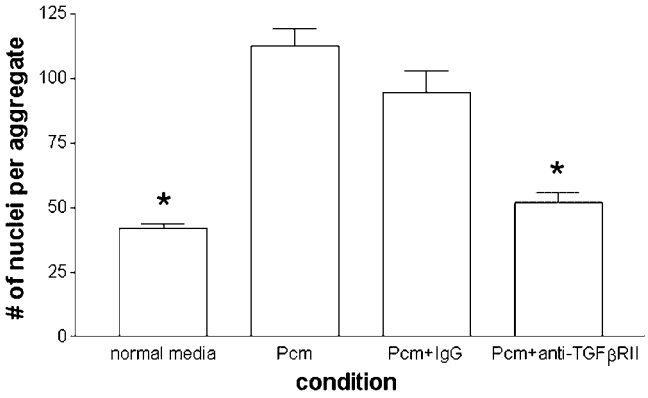

Fig. 2. Anti-TGFβ receptor type 2 (TGFβRII) antibodies block HBME-1 cord formation induced by PC-3 conditioned media (Pcm). Anti-TGFβRII neutralizing antibodies (2 μg/ml) were tested for their ability to prevent formation of cords by HBME-1 cells grown in collagen in the presence of PC-3 conditioned media. After 6 days, cells were fixed with 4% formaldehyde, stained with SYBR® Green I, and nuclei were counted in cord-like aggregates for each condition. The values are means of total number of nuclei per aggregate (largest five aggregates per condition) and error bars represent standard deviations. Results are presented as the average of two experiments. Significant values compared to PC-3 conditioned media are represented by a * symbol.

comparison, HBME-1 conditioned media contained both TGFβ isoforms (28 pg TGFβ1 and 49 pg TGFβ2 per 1×10^5 cells). Overall, the amount of TGFβ detected in the prostate cancer conditioned medias was low, suggesting that prostate cancer cells mediate some of their effects by activating TGFβ in the serum.

Activation of Serum-Derived TGFβ by Prostate Cancer Cells Contribute to Their Growth-Inhibitory Effect on HBME-1 Cells

Since TGFβ protein levels were low in the prostate cancer cell conditioned medias, we compared growth of HBME-1 cells in prostate cancer cell conditioned media collected in two different ways to determine if activation of TGFβ in the serum was the reason for growth inhibition of HBME-1 cells. As shown in Figure 3, when prostate cancer cells conditioned serum-free media to which serum was added just prior to use on HBME-1 cells, a reduced effect on HBME-1 cell growth was observed (in comparison to conditioned media prepared containing serum that was exposed to the prostate cancer cells). The addition of anti-pan-specific TGFβ antibodies to serum-containing PC-3 conditioned media reversed the growth inhibition normally observed, suggesting that TGFβ was responsible for the growth inhibition of HBME-1 cells by serum-containing PC-3 conditioned media (Fig. 3). Thus, exposure of serum-derived TGFβ to prostate cancer cells is necessary for the full effect of their conditioned media on HBME-1 cells.

DISCUSSION

Our previous results indicate that prostate cancer cells secrete factors that affect growth and differentiation of bone marrow endothelial cells grown in collagen gels [17]. In this study, we demonstrated that TGFβ production and activation by prostate cancer cells and signaling through TGFβRII in HBME-1 cells mediate

TABLE V. Effect of TGFβ Antibodies and Inhibitors on Size and Number of HBME-1 Aggregates Formed in the Presence of PC-3 Conditioned Media

	Size of aggregates		Number of aggregates	
Condition	Mean	Fold change versus PC-3 conditioned media (*P*-value)	Mean	Fold change versus PC-3 conditioned media (*P*-value)
Normal media	58 ± 22	0.39 (<0.0001)	4.5 ± 1.9	0.31 (<0.0005)
PC-3 conditioned media	147 ± 53	NA	14.8 ± 5.0	NA
+50 μg/ml rabbit IgG	119 ± 25	0.81 (N.S.)	12.0 ± 2.2	0.81 (N.S.)
+50 μg/ml anti-pan-specific TGFβ	232 ± 70	1.58 (<0.005)	90.8 ± 57	6.15 (N.S.[a])
+10 μg/ml mouse IgG1	111 ± 38	0.75 (<0.05)	14.5 ± 7.0	0.98 (N.S.)
+10 μg/ml monoclonal anti-TGFβ1, 2, 3	179 ± 55	1.22 (N.S.)	158.3 ± 149	10.73 (N.S.[a])
+10 μg/ml mouse IgG2a	126 ± 36	0.86 (N.S.)	24.0 ± 6.2	1.63 (<0.05)
+10 μg/ml Fc:sTGFβRII	226 ± 70	1.54 (<0.0005)	100.8 ± 30	6.83 (<0.01)
+1 μg/ml LAP	244 ± 80	1.67 (<0.005)	169.0 ± 56	11.46 (<0.01)
2 × no. of HBME-1 cells initially	115 ± 23	0.79 (N.S.)	24.5 ± 5.3	1.66 (N.S.)

The Student's *T*-test was used to determine statistical significance (*P*-value < 0.05).
N.S., not significant.
[a]*P*-value is <0.001 using ANOVA to determine statistical significance.

TABLE VI. TGFβ mRNA and Protein Production by Prostate Cancer Cells and HBME-1 Cells

	mRNA expression (normalized[a] C_T value)		Protein levels in conditioned media pg per 10^5 cells/**pM** (*P*-value)[b]	
Cell line	TGFβ1	TGFβ2	TGFβ1	TGFβ2
PC-3	22.06	27.04	35.85/**6.05** (<0.0005)	48.10/**8.1** (<0.005)
DU-145	20.82	30.19	17.05/**4.5** (<0.05)	0[c]
LNCaP	Negligible	33.22	0[c]	0[c]
HBME-1	N.D.	N.D.	28.47/**9.0** (<0.01)	49.05/**15.5** (<0.0001)

N.D., not determined.
For comparison of RNA expression, lower normalized C_T values represent higher relative expression levels compared with other cell lines. To obtain the protein concentrations for each cell line, the normal media control values were first subtracted out and then resulting values were normalized to the total number of cells at collection of conditioned media.
[a]Normalized to β_2-M C_T value for each cell line.
[b]Statistically significant compared to normal media control (*P*-value < 0.05).
[c]Any TGFβ1 or 2 protein measured was below baseline levels in the normal media control.

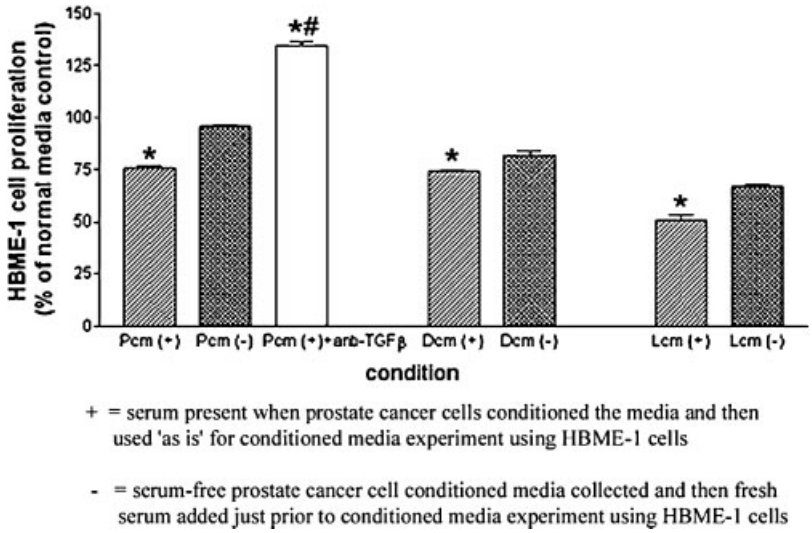

Fig. 3. Activation of serum-derived TGFβ by prostate cancer cell lines. A comparison of growth was made for HBME-1 cells exposed to serum-containing prostate cancer cell conditioned media versus serum-free conditioned media to which serum was later added. Conditioned media experiments were performed as described in the "Materials and Methods." For PC-3 conditioned media, a comparison was also made to HBME-1 cells exposed to serum-containing conditioned media plus the addition of anti-pan-specificTGFβ antibody. Data is normalized to normal media controls (normal media with serum and serum-free normal media plus serum later). Normalized mean values are expressed as percentage of control and error bars represent standard deviations. For each prostate cancer cell line, there is a reduced effect on HBME-1 cell growth when serum-free conditioned media is used. Values with statistically significant differences compared with the serum-free condition for each cell line are denoted with a * symbol. Anti-pan-specificTGFβ antibody completely reversed the growth inhibition conferred by serum-containing PC-3 conditioned media and significance (*P*-value < 0.05) is denoted with a # symbol.

growth and differentiation of HBME-1 cells. TGFβ isoforms are involved in several cellular responses, including growth inhibition and regulation of angiogenesis [23]. In addition, TGFβ is thought to have a dual role in prostate cancer progression, involving inhibition of tumor growth early, but stimulation of progression in later stages [23–26]. This study provides further insight into the role TGFβ signaling plays in prostate cancer bone metastasis.

Microarray analysis revealed that PC-3 conditioned media altered expression of several TGFβ-regulated and -associated genes in HBME-1 cells. Consequently, we did functional studies to determine the role of TGFβ, and other angiogenic factors, in the effects of PC-3 conditioned media on HBME-1 cell growth and differentiation in collagen gels. TGFβ cytokine studies and neutralizing antibody experiments demonstrated that TGFβ is primarily responsible for the growth

inhibition of HBME-1 cells by PC-3 conditioned media. TGFβ cytokine experiments clearly showed that HBME-1 cells are strongly inhibited by active recombinant TGFβ. Additionally, of all the neutralizing antibodies tested, only antibodies targeting TGFβ signaling reversed HBME-1 growth inhibition caused by PC-3 conditioned media. An interesting finding was that blocking multiple TGFβ isoforms yielded even higher cell proliferation than the normal media control, suggesting that serum-derived TGFβ was being inhibited. Another factor that may be important is FGFb, since neutralizing antibodies also caused growth inhibition of HBME-1 cells.

TGFβ and its type II receptor are key regulators of HBME-1 cell differentiation. Low concentrations of TGFβ cytokines induced HBME-1 to differentiate to form cords in collagen gel cultures, resembling angiogenesis. Although the effect was lower than with PC-3 conditioned media, it does indicate that TGFβ plays a key role in HBME-1 cord formation (differentiation). The growth inhibition conferred by low concentrations of TGFβ may prime the bone marrow endothelial cells to undergo differentiation, being an initiating step of angiogenesis [27].

Of all the inhibitors tested, only anti-TGFβRII neutralizing antibodies inhibited differentiation of HBME-1 cells in the presence of PC-3 conditioned media. No other antibodies significantly inhibited cord formation, including several direct inhibitors of TGFβ isoforms. It is possible that a higher concentration of inhibitors is required to block HBME-1 differentiation. Also, our list of inhibitors was not comprehensive, so other angiogenic factors may be involved. Regardless, inhibition of TGFβRII appears to be a potent means of blocking HBME-1 differentiation induced by PC-3 conditioned media.

It is unclear why there were such different results for blockage with anti-TGFβRII antibodies compared to use of TGFβ antibodies and inhibitors. One possible cause may be anti-TGFβRII antibodies completely block all signaling through the TGFβRII receptor in HBME-1 cells, but inhibitors of TGFβ isoforms may not completely block receptor signaling.

Another possibility for the difference in blocking with anti-TGFβRII antibodies versus direct inhibition of TGFβ isoforms is the potential ability of TGFβ to signal through other receptors and/or the ability of other ligands to bind and signal through TGFβRII. TGFβs are members of a large superfamily of cell growth and differentiation mediators, which includes activins, inhibins, bone morphogenetic proteins (BMPs), and the Müllerian inhibitory substance [24,26]; and there is evidence of promiscuity among the various ligands and receptors of this superfamily [28,29]. For instance, activin receptor-like kinase 2, a type I receptor, has been shown *in vitro* to bind activin [30] as well as BMP2 and 4 [31,32]; and its mouse homolog has been shown to bind TGFβ when overexpressed [33]. A better understanding is needed as to how TGFβ and its related family members mediate cell signaling, including their overlapping receptor usage and downstream activities.

It was surprising that inhibition of multiple TGFβ isoforms using anti-pan-specific TGFβ, monoclonal anti-TGFβ1, 2, 3, Fc:sTGFβRII, and LAP resulted in a dramatic increase in the size and number of cords. TGFβ is generally considered an angiogenesis inducer [16,34,35], yet there is also evidence that blocking TGFβ signaling can induce angiogenesis and/or a more aggressive phenotype [36–38]. The concentrations of TGFβ inhibitors used may not have completely blocked TGFβ activity. Thus, low concentrations of TGFβ may have induced further cell differentiation. In addition, factors other than TGFβ may be present in the PC-3 conditioned media that also induce HBME-1 cells to differentiate. Further analysis is needed to understand the role of TGFβ signaling in prostate cancer-induced bone marrow endothelial cell differentiation.

Our findings also suggest that activation, not simply production, of TGFβ by prostate cancer cells is important for their effects on HBME-1 cells grown in collagen gels. Activation of TGFβ in serum *in vitro* may correlate to an extent the activation of latent TGFβ in the bone. Large quantities of latent TGFβ are stored within the bone and activation of just a small portion of TGFβ is enough to mediate cell growth and differentiation [39,40]. Prostate cancer cells may activate latent TGFβ in the bone either directly as suggested by our studies or through its secreted proteases [40–42], leading to stimulation of angiogenesis to promote metastatic growth [34].

Further studies are needed to analyze the mechanisms by which TGFβ and other growth factors work together to regulate bone marrow endothelial cell growth and angiogenesis. To begin to identify other angiogenic factors besides TGFβ that can also induce angiogenesis in bone marrow endothelial cells, we are utilizing cytokine arrays to identify other proteins secreted by prostate cancer cells that are capable of inducing angiogenesis in HBME-1 cells. Revealing these factors and understanding their role, along with TGFβ and TGFβRII, in prostate cancer cell-induced angiogenesis should lead to more effective anti-angiogenic treatments for metastatic prostate cancer.

ACKNOWLEDGMENTS

We thank Tracy Roberts for her assistance with imaging and Jing Lu for her help with statistical analysis.

APPENDIX: EFFECT OF PROSTATE CANCER CELL CONDITIONED MEDIA ON HBME-1 GENE EXPRESSION IN COLLAGEN GELS

See 'Materials and Methods'

PC-3 Conditioned Media on HBME-1 Cells		
Gene	Accession number	Fold change
Decreased expression		
Potassium inwardly-rectifying channel, subfamily J, member 13	H97186	1.78
Glutamate receptor, metabotropic 3	N62328	1.75
Rho GTPase activating protein 5	W86145	1.48
Ankylosis, progressive (mouse) homolog	AA007428	1.46
Kruppel-like factor 6	AA055585	1.46
Xeroderma pigmentosum, complementation group A	AA453300	1.44
Hypothetical protein MGC15523	AI016677	1.44
Hypothetical protein FLJ13941	AA005329	1.44
Latent transforming growth factor beta binding protein 2	AA424629	1.42
Ferrochelatase (protoporphyria)	AA025142	1.40
Tissue factor pathway inhibitor 2	AA399473	1.38
Serine/threonine kinase 4	T94961	1.37
Glutathione S-transferase M1	AA290737	1.36
MT-protocadherin	AA707714	1.35
Hypothetical protein FLJ10890	AA489394	1.35
Hypothetical protein DKFZp566I133	N78263	1.32
FAT tumor suppressor (*Drosophila*) homolog 2	H10939	1.32
Apolipoprotein C-IV	T71886	1.31
Hypothetical protein FLJ10648	AA007402	1.31
Hypothetical protein MGC5347	AA451905	1.31
ATP-binding cassette, sub-family G (WHITE), member 2	W84773	1.30
CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	AA111969	1.30
Spectrin SH3 domain binding protein 1	R16667	1.28
HIR (histone cell cycle regulation defective homolog A (*S. cerevisiae*)	W94880	1.28
Valosin-containing protein	AA489681	1.28
Annexin A8	AA252968	1.28
Stromal cell-derived factor 1	AA447115	1.28
FGFR1 oncogene partner	AA884755	1.27
Metallothionein 1E (functional)	R95882	1.27
Alcohol dehydrogenase 1B (class I), beta	AA682469	1.27
Kininogen	R89067	1.27
Galactosidase, alpha	AA251784	1.27
CDP-diacylglycerol synthase	R31562	1.27
Dead ringer (*Drosophila*)-like 1	AA705382	1.26
Bromodomain adjacent to zinc finger domain, 2B	T41078	1.26
KIAA1488 protein	T52311	1.26
Hypothetical protein MGC35048	H98742	1.25
Aldehydede hydrogenase 1 family, member A1	AA664101	1.25
Hypothetical protein MGC3133	R78514	1.25
Integrin, alpha 9	AA865557	1.24
Alcohol dehydrogenase 5 (class III), chi polypeptide	AA453859	1.24
YME1 (*S. cerevisiae*)-like 1	N53511	1.24
Up-regulated by BCG-CWS	AA521384	1.24
KIAA0530 protein	T60850	1.24
Cntrl-7	Cntrl-7	1.24
Transforming growth factor, beta 3	AA040617	1.24
Inhibitor of growth family, member 3	AA996230	1.24
KIAA1389 protein	AA464598	1.24
Calpastatin	T47601	1.24
Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	AA193405	1.24

(*Continued*)

Gene	Accession number	Fold change
N-acetylgalactosaminidase, alpha-	R25825	1.23
KIAA0096 protein	W68396	1.23
Sarcomeric muscle protein	AA418414	1.23
CD38 antigen (p45)	R00276	1.23
Angiogenin, ribonuclease, RNase A family, 5	AA682399	1.23
Joined to JAZF1	AI023724	1.23
Tumor differentially expressed 2	R10545	1.22
c-Myc target JPO1	N45440	1.22
RecQ protein-like (DNA helicase Q1-like)	AA456585	1.22
Unactive progesterone receptor, 23kD	N48708	1.22
Hydroxyacyl-Coenzyme Adehydrogenase/3-k	T69767	1.22
KIAA1856 protein	H40023	1.22
Highly charged protein	H95716	1.22
Hypothetical protein FLJ20080	AA402965	1.22
CCR4-NOT transcription complex, subunit 4	W88693	1.22
Peter Pan (*Drosophila*) homolog	AI000807	1.22
Cell division cycle 25C	W95001	1.22
Transforming growth factor, beta 3	N76883	1.22
VAMP (vesicle-associated membrane protein)-associated protein B and C	AA148402	1.22
Clathrin, heavy polypeptide (Hc)	H54366	1.22
Apolipoprotein B (including Ag (x) antigen)	H57779	1.22
Advillin	AA427733	1.22
EPH receptor B6	AA609284	1.21
Protocadherin 9	N63057	1.21
Hypothetical protein EDAG-1	H57052	1.21
GTP-binding protein Rho 7	AA700934	1.21
Uteroglobin	T63761	1.21
Putative ankyrin-repeat containing protein	AA621188	1.21
Kh domain containing, RNA binding, signal transduction associated 3	AA456299	1.21
cDNA clone IMAGE:4079668, partial cds	AA456629	1.20
Major histocompatibility complex, class II, DP alpha 1	AA278767	1.20
KIAA1105 protein	AA045176	1.20
UDP-Gal:beta GlcNAc beta 1 ,4-galactosyltransferase, polypeptide 1	AA284292	1.20
Homo sapiens clone IMAGE:5303499, mRNA	AA281793	1.20
Semadomain, immunoglobulin domain (Ig), short basic domain, secreted, 3C	AA042990	1.20
Amiloride binding protein 1 (amine oxidase (copper-containing))	T46924	1.20
Proteasome (prosome, macropain) inhibitor subunit1 (PI31)	AA873845	1.20
B-factor, properdin	AA401441	1.20
KIAA0746 protein	AA456569	1.20
Glutamate-cysteine ligase, catalytic subunit	H56069	1.20
Pyruvate dehydrogenase kinase, isoenzyme 4	AA169469	1.20
Cytochrome P450, subfamily IVB, polypeptide 1	AA291484	1.19
Full length insert cDNA clone YI46G04	R70541	1.19
Myosin, light polypeptide 3	AA192166	1.19
RAD51 (*S. cerevisiae*)-like 1	N70362	1.19
Chromosome 6 open reading frame 5	R78513	1.19
JunD proto-oncogene	N66278	1.19
Bladder cancer overexpressed protein	N94362	1.19
Interferon, gamma-inducible protein 30	AA630800	1.19
KIAA1479 protein	AA452824	1.19
Zinc finger protein 37a (KOX21)	AA069549	1.19
Microtubule-associated protein 7	R77251	1.19
Progestin induced protein	H27554	1.19
Bone morphogenetic protein 7	W73473	1.19
Cathepsin B	AA130998	1.19

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Gene	Accession number	Fold change
8D6 antigen	AA434403	1.18
Chromosome 20 open reading frame 3	AA190882	1.18
Dual specificity phosphatase 10	AA056608	1.18
N-acylsphingosine amidohydrolase (acid ceramidase)-like	W47576	1.18
Metallothionein 1E (functional)	T99140	1.18
Ribosomal protein S15a	AA411682	1.18
Hypothetical protein FLJ20730	AA983410	1.18
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	AA457158	1.18
Hypothetical protein FLJ20396	AA486092	1.18
Activin A receptor, type II	AA682819	1.18
Cell death regulator aven	AA055221	1.18
ATP-binding cassette, sub-family B (MDR/TAP), member 10	AA434409	1.18
ADP-ribosylation factor-like 7	N35301	1.17
CD2-associated protein	AA173981	1.17
Monoamine oxidase B	AA682423	1.17
Stromal interaction molecule 2	R28541	1.17
Signal transducer and activator of transcription 1, 91kDa	AA486367	1.17
Hypothetical protein FLJ10875	N25240	1.17
CHK1 (checkpoint, *S. pombe*) homolog	N73242	1.17
Kynureninase (L-kynureninehydrolase)	H87471	1.17
Myeloid leukemia factor 1	AA707847	1.17
c-Myc target JPO1	AA406348	1.17
Selectin L (lymphocyte adhesion molecule 1)	H00756	1.17
Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	T95930	1.17
EGF-TM7-latrophilin-relatedprotein	W72803	1.17
Laminin, gamma 2	AA677534	1.17
Methyl-CpG binding domain protein 1	AA459922	1.16
Hypothetical protein MGC2747	R91577	1.16
Paired box gene 8	AA115328	1.16
Lymphocyte-specific protein tyrosine kinase	AA469965	1.16
Hypothetical protein	AA447746	1.16
Hypothetical protein	AA455284	1.16
Chemokine (C-C motif) receptor 1	AA036881	1.16
Chromosome 5 open reading frame 7	H26156	1.16
Hypothetical protein FLJ21069	AA284277	1.16
Transforming, acidic coiled-coil containing protein	AA456316	1.16
Arginase, type II	H17612	1.16
Chromosome 19 open reading frame 3	AA434159	1.16
Adrenergic, beta-2-, receptor, surface	H90431	1.16
Major histocompatibility complex, classII, DR beta 4	W88967	1.16
p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	AA173411	1.15
Methionine adenosyltransferase II, beta	AA521303	1.15
Gamma-aminobutyric acid (GABA) B receptor, 1	N70841	1.15
S100 calcium-binding protein A13	AA070489	1.15
FOXJ2 forkhead factor	AA902249	1.15
Telomeric repeat binding factor (NIMA-interacting) 1	AA151295	1.15
Nebulette	AA461473	1.15
UDP glycosyltransferase 2 family, polypeptide B4	N53031	1.15
Hypothetical protein FLJ20079	R99573	1.15
Mal, T-cell differentiation protein 2	AA453783	1.15
NY-REN-58 antigen	W56770	1.15
Mannosidase, alpha, class 1A, member 1	AA489636	1.15
Integrin, beta 8	R74357	1.15
Glutathione S-transferase A2	T73468	1.15
Chaperonin containing TCP1, subunit 8 (theta)	AA630016	1.15

(*Continued*)

Gene	Accession number	Fold change
Tumor necrosis factor (ligand) superfamily, member 10	H54629	1.15
WD40 repeat domain 11 protein	AA134743	1.15
Spastin	AA171421	1.15
Neural precursor cell expressed, developmentally down-regulated 9	T61428	1.14
Ath.-3	Ath.-3	1.14
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	R83837	1.14
Serum response factor	AA487973	1.14
Ankylosis, progressive (mouse) homolog	H81907	1.14
Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	AA173453	1.14
Mitochondrial ribosomal protein S25	N71078	1.14
Cyclin M4	AA599138	1.14
Non-metastatic cells 5	AA129736	1.14
Hypothetical protein MGC17791	AA055992	1.14
Growth arrest-specific 2	R15728	1.14
FYVE and coiled-coil domain containing 1	AA489626	1.14
Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	W44685	1.14
NAG18 protein	N67878	1.14
Hypothetical protein LOC283507	AA035452	1.14
Prominin (mouse)-like 1	R40057	1.14
Keratin 13	W60057	1.14
RPB5-mediating protein	R63137	1.14
Transmembrane 4 superfamily member 6	H87106	1.14
Syndecan 2 (heparin sulfate proteoglycan 1, cell surface-associated, fibroglycan)	H64346	1.14
Hyaluronan-mediated motility receptor	R10284	1.14
Antigen identified by monoclonal antibody Ki-67	AA425973	1.14
Syntaxin 11	R33851	1.14
TNF receptor-associated factor 3	H48096	1.13
G protein-binding protein CRFG	H94929	1.13
Protein kinase C, zeta	AA458993	1.13
M5-14 protein	AA496948	1.13
v-raf-1 murine leukemia viral oncogene homolog 1	N25425	1.13
Secreted frizzled-related protein 4	AA486838	1.13
Asparagine synthetase	AA894927	1.13
EGL nine (*C. elegans*) homolog 3	AA150198	1.13
Sorting nexin 5	AA459393	1.13
KIAA0475 gene product	N73927	1.13
Hypothetical protein DKFZp761K1423	AA460826	1.13
Glycogenin	AA411678	1.13
Hypothetical protein	AA446864	1.13
Pleckstrin homology, Sec7 and coiled/coil domains, binding protein	AA490903	1.13
Ataxin 3	N51479	1.13
Ubiquitin-like 1 (sentrin)	AA488626	1.12
Replication protein A1 (70kD)	AA629900	1.12
Eukaryotic translation initiation factor 5A	AA878570	1.12
Phospholipase A2, group IIA	T61323	1.12
Palmitoyl-protein thioesterase 1(ceroid-lipofuscinosis, neuronal 1, infantile)	AA063637	1.12
ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	AA598652	1.12
Secreted protein, acidic, cysteine-rich	H95960	1.12
Glutamate receptor, ionotropic, kainite 1	R44776	1.12
Zinc finger protein 200	R01941	1.12
p53-inducible p53 DINP1	AA883711	1.12
Thrombopoietin	AA479058	1.12
Hypothetical protein	N53445	1.12

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Gene	Accession number	Fold change
Increased expression		
DR1-associated protein 1 (negative cofactor 2 alpha)	AA421977	1.10
Platelet-derived growth factor beta polypeptide	T49539	1.10
Clusterin	AA130017	1.11
A disintegrin and metalloproteinase domain 12 (meltrin alpha)	AA190993	1.11
Cullin 4B	AA780712	1.11
Eukaryotic translation elongation factor	AA644657	1.11
Arachidonate 5-lipoxygenase-activating protein	H02307	1.12
SEC63 protein	N66750	1.12
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	H27564	1.12
Non-metastatic cells 1, protein (NM23A)	AA644092	1.12
Glutathione peroxidase 4 (phospholipids hydroperoxidase)	AA455197	1.12
Cyclin E1	T54121	1.12
Polymerase (RNA) II (DNA directed) polypeptide I, 14.5 kDA)	AA777192	1.12
Carboxypeptidase D	T56021	1.13
Soares pregnant uterus NbHPU *Homo sapiens* cDNA clone	AA029997	1.13
Ataxin 3	AA702422	1.13
Host cell factor homolog	AA488367	1.13
Mitochondrial ribosomal protein L2	N94366	1.13
Hypothetical protein FLJ12619	AA418914	1.13
3′-phosphoadenosine 5′-phosphosulfatesynthase 1	AA169832	1.13
Dual specific ityphosphatase 5	W65461	1.13
Angiopoietin-like 4	W30988	1.13
DKFZP586F1524 protein	AA428604	1.13
5′ nucleotidase (CD73)	R60343	1.13
Hypothetical protein	AA176785	1.13
Alpha-actinin-2-associated LIM protein	AA972352	1.13
Ribosomal protein S6 kinase, 90kDa, polypeptide 3	H55921	1.13
Signal transducer and activator of transcription 3	AA399410	1.13
Dyskeratosis congenita1, dyskerin	AA052960	1.13
API5-like 1	AA451935	1.13
Protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform	H99661	1.13
Coiled-coil domain containing 6	AA699864	1.13
Eukaryotic translation initiation factor	AA676471	1.13
Cyclin-dependent kinase 6	H73724	1.14
Non-metastatic cells 4	H54417	1.14
HYA22 protein	R01638	1.14
Telomeric repeat binding factor 2	AA676590	1.14
Bruno-like4, RNA binding protein (*Drosophila*)	H92642	1.14
Ornithine decarboxylase 1	AA460115	1.14
Beta-2-microglobulin	AA670408	1.14
Eukaryotic translation initiation factor	AA936783	1.14
Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (*Drosophila*)	AA188416	1.14
Fibrillarin	AA663986	1.14
KIAA1813 protein	AA456143	1.14
Golgi phosphoprotein 3 (coat-protein)	R63022	1.14
Protein phosphatase 1, regulatory (inhibitor) subunit 12A	W32763	1.14
Hypothetical protein PP591	R38171	1.14
Zinc finger protein 33a (KOX 31)	AA779415	1.14
Pim-1 oncogene	N63635	1.14
Desmoglein 2	T97257	1.14
Thioredoxin-like2	H99205	1.14
ARP1 (actin-related protein 1, yeast) homolog A, centractin alpha	AI014416	1.15
KIAA1275 protein	N70837	1.15

(*Continued*)

Gene	Accession number	Fold change
IGF-II mRNA-binding protein 3	AA187143	1.15
Janus kinase 1	AA284634	1.15
DKFZP564A122 protein	N59690	1.15
Karyopherin alpha 3 (importin alpha 4)	AA668178	1.15
Minichromosome maintenance deficient 5, cell division cycle 46 (*S. cerevisiae*)	AA285155	1.15
E2F transcription factor 3	N92519	1.15
NS1-associated protein 1	AA186327	1.15
Centromere protein F (350/400 kDa, mitosin)	AA701455	1.15
Chromogranin B (secretogranin 1)	W37769	1.15
Actin related protein 2/3 complex, subunit 5, 16 kDa	W55964	1.15
Synovial sarcoma translocation	N59206	1.15
Transmembrane protease, serine 4	AA999953	1.15
KIAA0205 gene product	R91264	1.15
Carcinoembryonic antigen-related cell adhesion molecule	AA054073	1.15
Inositol polyphosphate phosphatase-like 1	AA279072	1.15
Hypothetical protein	T70922	1.15
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	AA630082	1.15
Transmembrane trafficking protein	W94609	1.15
Hypothetical protein from EUROIMAGE 1669387	R32354	1.15
CHK1 (checkpoint, *S. pombe*) homolog	AA463256	1.15
v-Ki-ras 2 Kirsten rat sarcoma 2 viralon	N95249	1.15
Activated p21 cdc42 Hs kinase	AA427891	1.15
Ras homolog gene family, member B	N41062	1.16
Amyloid beta precursor protein (cytoplasmic tail) binding protein 2	AA046411	1.16
Myosin IXB	N51705	1.16
Helicase, lymphoid-specific	W25169	1.16
Eukaryotic translation initiation factor	H54752	1.16
Soares fetal liver spleen 1 NFLS *Homo sapiens* cDNA clone	H80215	1.16
Lectin, galactoside-binding, soluble, 3 binding protein	AA485353	1.16
PAI-1 mRNA-binding protein	AA487070	1.16
Retinoblastoma-binding protein 7	AA130591	1.16
Transcription elongation factor B (SIII)	AA630017	1.16
Ras-related C3 botulinum toxin substrate	N54221	1.16
Growth factor receptor-bound protein 10	AA136336	1.16
Mitochondrial ribosomal protein S17	AA733080	1.16
Misshapen/NIK-related kinase	R10659	1.16
Secreted frizzled-related protein 1	T68892	1.16
Chromosome 5 open reading frame 3	T74768	1.16
Frizzled (*Drosophila*) homolog 4	R26355	1.16
Signal peptidase complex subunit 3 homolog (*S. cerevisiae*)	AA411686	1.16
Aldehyde dehydrogenase 7 family, member A1	AA101299	1.16
Hypothetical protein MGC2383	H95253	1.16
FEM-1 (*C. elegans*) homolog b	H82273	1.16
Tissue inhibitor of metalloproteinase 2	AA486280	1.16
Serum-inducible kinase	AA460152	1.16
tRNA isopentenylpyrophosphate transferase 1	AA207083	1.16
E1A binding protein p300	N94428	1.17
Corticotropin releasing hormone receptor	H07088	1.17
PTD008 protein	AA427691	1.17
Sarcoma amplified sequence	AA664211	1.17
RAN binding protein 1	R14822	1.17
Hypothetical protein FLJ10853	T68430	1.17
Hypothetical protein MGC5297	R28412	1.17
Farnesyl-diphosphate farnesyltransferase 1	AA679352	1.17

(*Continued*)

(*Continued*)

Gene	Accession number	Fold change
RAB32, member RAS oncogene family	AA057378	1.17
Actinin, alpha1	AA669042	1.17
Protein tyrosine phosphatase	AA679180	1.17
Heterogeneous nuclear ribonucleoprotein U	T97593	1.18
Presenilins associated rhomboid-like protein	AA131464	1.18
Cntrl-1	Cntrl-1	1.18
Ribophorin I	AA127100	1.18
Ribonucleotide reductase M2 polypeptide	AA187351	1.18
Protein disulfide isomerase-related protein	R02609	1.18
Bleomycin hydrolase	AA417881	1.18
Eukaryotic translation elongation factor 2	R20379	1.18
Abl-interactor 2	N21334	1.19
ElaC (E.coli) homolog 2	AA455121	1.19
Protocadherin 1 (cadherin-like 1)	R77512	1.19
Rho GDP dissociation inhibitor (GDI) alpha	AA459400	1.19
Immediate early response 3	AA480815	1.19
Aldehyde dehydrogenase 18 family, member A1	R20638	1.19
Retinoic acid receptor, alpha	AA705069	1.19
Peroxiredoxin 6	AA598874	1.19
Frizzled (*Drosophila*) homolog 6	T68333	1.19
Dual specificity phosphatase 12	AA485951	1.19
Peroxiredoxin 2	H68845	1.19
CDC14 (cell division cycle 14, *S. cerevisiae*)	AA417319	1.20
Caspase recruitment domain protein 12	AA443290	1.20
Calnexin	AA126356	1.20
Chaperonin containing TCP1, subunit 4 (delta)	AA598637	1.20
Villin 1	AA876039	1.20
Keratin 8	AA598517	1.20
Fibronectin 1	R62612	1.20
Arrestin, beta 1	H20859	1.20
BENE protein	AA778392	1.21
Chaperonin containing TCP1, subunit 7 (eta)	AA676588	1.21
Potassium intermediate/small conductance calcium-activated channel, N4	AA443903	1.21
Death-associated protein kinase 1	AA025275	1.21
Amino acid transporter system A1	R66556	1.21
ATPase, Na^+/K^+ transporting, beta 1 polypeptide	AA598814	1.22
Suppressor of G2 allele of SKP1 (*S. cerevisiae*)	AA416876	1.22
KIAA0226 gene product	N51014	1.22
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	AA485052	1.22
Hepatitis B virus x associated protein	R16760	1.22
cAMP responsive element binding protein 1	H12320	1.22
Ras homolog gene family, member E	W86282	1.22
DKFZP434F091 protein	H15274	1.23
Drebrin-like	R68360	1.23
Ribosomal protein L23	AA463200	1.23
Olfactory receptor, family 2, subfamily A, member 9 pseudogene	AA001222	1.23
Microtubule-associated protein, RP/EB family, member 1	AA922700	1.23
CD27-binding (Siva) protein	AA167823	1.23
B-Actin	B-Actin	1.23
ADP-ribosylation factor 4-like	H15085	1.23
Adrenergic, alpha-2A-, receptor	T48692	1.24
Stromal cell derived factor receptor 1	AA130671	1.24
Protein tyrosine phosphatase, non-receptor type 2	AA428195	1.24
Laminin, alpha 3	AA001432	1.24
Protein kinase, cAMP-dependent, catalytic, beta	AA018980	1.24

(*Continued*)

Gene	Accession number	Fold change
Phosphofructokinase, muscle	AA099169	1.24
Protein-L-isoaspartate (D-aspartate) O-m	T68518	1.24
Zinc finger RNA binding protein	T50389	1.24
Hsp70-interacting protein	AA401391	1.25
Protein kinase, cAMP-dependent, catalytic, beta	AA459980	1.25
Transmembrane 4 superfamily member 1	AA487893	1.26
Cell growth regulatory with ring finger domain 1	AA676705	1.26
Cot-2	Cot-1	1.26
Peptidylprolyl isomerase B (cyclophilin B)	AA481464	1.26
Ribosomal protein S16	AA668301	1.26
Hypothetical protein FLJ22060	H56147	1.26
Keratin 18	AA664179	1.26
Transcribed locus	H30547	1.26
Lymphoidblast crisis oncogene	AA113166	1.26
Succinate dehydrogenase complex, subunit D, integral membrane protein	AA035384	1.27
Gamma-aminobutyric acid (GABA) A receptor, pi	AA102670	1.27
Death-associated protein 6	N73287	1.28
Amplified in osteosarcoma	AA013336	1.29
Placental growth factor, vascular endothelial growth factor-related protein	AA130714	1.30
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	AA018676	1.30
Splicing factor, arginine/serine-rich 8	AA702973	1.31
FK506-binding protein 1A (12kD)	AA427899	1.32
Retinoic acid induced 14	AA490456	1.35
Phosphoribosylaminoimidazole-carboxylase, -succinocarboxamidesynthetase	N33274	1.36
Hypothetical protein FLJ10468	N63744	1.37
Suppression of tumorigenicity 13 (coloncarcinoma) (Hsp70 interacting protein)	H65676	1.49

LNCaP Conditioned Media on HBME-1 Cells

Gene	Accession number	Fold change
Decreased expression		
Bromodomain adjacent to zinc finger domain, 2B	T41078	2.26
Sciellin	AA455012	2.08
Dead ringer (*Drosophila*)-like 1	AA705382	2.01
Chaperonin containing TCP1, subunit 6A (zeta 1)	AA872690	1.98
Potassium inwardly-rectifying channel, subfamily J, member 13	H97186	1.92
Hypothetical protein MGC15523	AI016677	1.80
Serine/threonine kinase 4	T94961	1.70
Hypothetical protein FLJ10890	AA489394	1.59
KIAA1488 protein	T52311	1.59
Tissue factor pathway inhibitor 2	AA399473	1.58
myosin, light polypeptide 3	AA192166	1.54
Galactosidase, alpha	AA251784	1.51
Kininogen	R89067	1.41
Increased expression		
Minichromosome maintenance deficient 5, cell division cycle 46 (*S. cerevisiae*)	AA285155	1.58

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