Temporal Expression Profiling of the Effects of Secreted Factors From Prostate Stromal Cells on Embryonal Carcinoma Stem Cells

Laura E. Pascal,[1,2]* Ricardo Z.N. Vêncio,[2] Young Ah Goo,[2,3] Laura S. Page,[1,2] Christina P. Shadle,[2] and Alvin Y. Liu[1,2]

[1]*Department of Urology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, Washington*
[2]*Institute for Systems Biology, Seattle, Washington*
[3]*Department of Medicinal Chemistry, University of Washington, Seattle, Washington*

BACKGROUND. There is a growing body of evidence indicating that epigenetic influences originating from stromal cells in the immediate microenvironment may play a role in carcinogenesis. Determining the molecular mechanisms involved in stromal–stem cell interaction could provide critical insight into prostate development and disease progression, particularly with regard to their relationship to and influence on the putative cancer stem cell.
METHODS. Prostate and bladder stromal cells prepared from tissue specimens were co-cultured with the pluripotent embryonal carcinoma cell line NCCIT. Transcriptome analysis was used to characterize NCCIT cell response to prostate or bladder signaling.
RESULTS. A systems approach demonstrated that prostate stromal cells were capable of inducing gene expression changes in NCCIT through secreted factors. Induction led to a loss of embryonic stem cell markers, with concurrent up-regulation of many genes characteristic of stromal mesenchyme cells as well as some of epithelial and cancer stem cells. Bladder stromal signaling produced gene expression changes different from those of prostate signaling.
CONCLUSIONS. This study indicates that paracrine stromal cell signaling can affect cancer stem cell response in an organ-specific manner and may provide insight for future development of treatment strategies such as differentiation therapy.

KEY WORDS: **prostate; stromal; cancer stem cell**

INTRODUCTION

Cancer stem cells, or tumor-initiating cells, are thought to be a rare subset of tumor cells capable of self-renewal and driving tumor formation and maintenance in much the same manner as normal adult stem cells direct homeostasis. Stem cells differentiate according to signaling by surrounding cells through intercellular interaction and secreted factors. The stem cell niche or microenvironment regulates the balance between self-renewal and differentiation, and disruption of prostate stem cell homeostasis is thought to be a major factor in disease progression. In the adult

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIDDK; Grant number: DK63630; Grant sponsor: NCI; Grant number: CA111244; Grant sponsor: Center for Systems Biology; Grant number: PM50 GMO76547.

Ricardo Z.N. Vêncio's present address is Department of Genetics, University of Sao Paulo's Medical School at Ribeirão Preto, Ribeirão Preto, Sao Paulo, Brazil.

*Correspondence to: Dr. Laura E. Pascal, Department of Urology, University of Pittsburgh Medical Center, Pittsburgh, PA 15232.
E-mail: pascalle@upmc.edu
Received 11 February 2009; Accepted 16 April 2009
DOI 10.1002/pros.20982
Published online in Wiley InterScience (www.interscience.wiley.com).

prostate, each acinus is lined with secretory luminal cells and an underlying layer of basal cells, and is embedded in a fibromuscular stroma. A stem cell model for the prostate postulates that multipotent cells reside within the basal epithelium and are the progenitor of luminal epithelial cells and a minor population of epithelial cells with neuroendocrine differentiation [1,2]. In normal prostate, one primary function of stromal cells is to provide a regulatory extracellular matrix and to direct epithelial differentiation and development through growth factors and androgen stimulation [3]. The critical role of stromal cells in prostate development was demonstrated by co-implantation in animals of putative stem cells and stromal cells to achieve functional prostate glandular development [4–7]. Although the majority of prostate cancers are epithelial in origin, there is a growing body of evidence suggesting that the stromal microenvironment plays a significant role in tumor progression [8–13].

The goal of this study was to utilize an in vitro co-culture model to characterize the potential influence of secreted prostate stromal cell factors on cancer stem cells. Tissue recombination studies using human embryonic stem (hES) cells or preparations containing prostate progenitor cells have previously demonstrated that the stromal mesenchyme was a key determinant in prostate differentiation [14–22]. Recently, groups have isolated and characterized several putative prostate stem cell and cancer stem cell populations based on differential expression of ABCG2 [23], cell surface markers, ITGA2/ITGB1 ($\alpha2\beta1$) [24], and most recently, PROM1 (CD133) [4,25–27] and CD117 [28]. These previous studies provide an important framework for examining the potential influence of stromal cells on cancer stem cell differentiation in this study. Here, we used the human embryonal carcinoma (hEC) cell line NCCIT to examine prostate stromal influence on a cancer stem cell. NCCIT is a nonseminomatous germ cell-derived keratin-negative cell line that has features intermediate between seminoma and embryonal carcinoma. It is developmentally pluripotent and can differentiate into derivatives of the three embryonic germ layers of ectoderm, mesoderm, and endoderm. In culture, NCCIT cells grow in aggregates and respond to retinoic acid by growth arrest and change in morphology [29]. NCCIT expresses markers of hES cells including the protein antigens CD9, Thy1 (CD90), tissue-nonspecific alkaline phosphatase (ALP), and major histocompatibility complex, class I (HLA), as well as the strongly developmentally regulated genes SRY (sex determining region Y)-box 2 (SOX2) [30], NANOG, POU5F1, teratocarcinoma-derived growth factor 1 (TDGF1, or Cripto), DNA cytosine-5-methyltransferase 3βDNMT3B), GABA A receptor β3 (GABRB3), and growth differentiation factor 3 (GDF3) [29,31,32]. They also express CD133, which recent evidence suggests is also a marker for prostate stem cells [4] and the putative cancer stem cells [33].

Previously, we have determined the differential gene expression between cultured prostate and bladder stromal cells [34,35]. Prostatic stromal cells in culture can be readily obtained, and the resultant cells appear to be myofibroblasts containing smooth muscle actin and vimentin (VIM) [36]. Although stromal cells of the prostate and bladder are indistinguishable histomorphologically, they are phenotypically and genotypically different [34,35]. The prostate is characterized by the expression of genes PENK, STC1, GALNT7, RIS1, ChGN, TNC, and EDNRB; and secreted proteins CTSL, FSTL1, SPARC, and TIMP1. Bladder-specific genes include STC2, BF, GFRA1, and OSF2; and bladder-specific secreted proteins APOH, SERPING1, DSG2, and CADM1 [34,35]. Organ-specific candidate signaling molecules such as these might mediate stromal influence. Here, we sought to examine the role secreted factors might play in prostate stromal induction of cancer stem cells in vitro and to characterize the process by gene array analysis. We showed that NCCIT responded to prostate stromal cell secreted factors with extensive gene expression changes and phenotypic alteration. Furthermore, plasticity in response to stromal cell secreted factors was also shown by comparison to bladder stromal cell induction.

MATERIALS AND METHODS

NCCIT Cell Line and Tissue Specimens

NCCIT cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 (Cambrex BioScience, Walkersville, MD) media supplemented with 10% heat-inactivated fetal bovine serum (FBS) [29]. The tissue samples used in this study consisted of cancer-free prostate tissue specimens obtained from 10 patients undergoing radical prostatectomy and cancer-free bladder tissue specimens obtained from five patients undergoing cystoprostatectomy. All tissue samples were obtained under approval by the University of Washington Institutional Review Board. Samples of cancer-free prostate or bladder parenchyma were collected following a standard protocol. Upon receipt of a radical specimen, 3-mm thick transverse sections were made of the prostate after inking the exterior surface. Between 1 and 10 g of tissue from the anterior aspect of the prostate (transition zone) were excised. A corresponding frozen section of the tissue block was histologically assessed to confirm the specimen was free of cancer. Regions

of bladder mucosae and wall that appeared grossly normal both visually and by palpation were identified as normal urinary bladder. An approximately $2\,cm \times 2\,cm$ portion of bladder wall with minimal perivesicle fat was excised and cut into pieces. Representative pieces were fixed in buffered formaldehyde and processed for paraffin embedding and histological characterization to verify the tissue that was macroscopically determined as normal urinary bladder.

Samples of prostate or bladder were minced and digested by overnight incubation at room temperature in 0.2% collagenase type I (Invitrogen, Carlsbad, CA) in RPMI-1640 media supplemented with 5% FBS and 10^{-8} M dihydrotestosterone on a magnetic stirrer. The resultant cell suspension was filtered with a 70-μm Falcon cell strainer to remove any nondigested tissue, diluted with an equal volume of Hanks balanced salt solution (HBSS), and aspirated with an 18-gauge needle. The resultant single cell preparation was partitioned into stromal and epithelial fractions on a discontinuous Percoll density gradient (Amersham Pharmacia, Piscataway, NJ) as described previously [37,38]. Cells banding at a density of $\rho = 1.035$ were collected as the stromal cell fraction for cell culture.

Cell Culture

The stromal cell fractions isolated from Percoll density gradients were cultured for 3–5 passages in RPMI-1640 media supplemented with 10% FBS, and their identity checked as described [34]. Preliminary experiments using stromal conditioned media resulted in similar effects as found previously [39]. Co-cultures were used in order to provide for examining the reciprocal effects of NCCIT on stromal cells. For co-culture experiments, 0.4 μm polycarbonate membrane trans-well inserts (Corning, Corning, NY) to preclude cell contact were employed. NCCIT cells were seeded at 1×10^4 cells/ml in RPMI-1640, 10% FBS on six-well plates or chamber slides (for immunocytochemistry), and prostate stromal cells were seeded at 1×10^4 cells/ml on insert. Controls of NCCIT, prostate, and bladder stromal cells alone were included. Cultures were maintained for several hours to 7 days. Time points were chosen based on previous time-course study of retinoic acid-induced differentiation of NCCIT [29] and were limited to 7 days because of the higher proliferation rate of uninduced NCCIT than those of prostate and bladder stromal cells. For each time point, cells were trypsinized and lysed in RLT buffer (Qiagen, Valencia, CA) and total RNA was extracted for gene expression analysis using the RNeasy Minikit according to the manufacturer's instructions.

Alkaline Phosphatase Immunostaining

Cultures were incubated for 5 days prior to staining according to the manufacturer's protocol. On day 5, media were aspirated; cells were fixed with 90% CH_3OH/10% formalin for 1–2 min, rinsed with phosphate-buffered saline, 0.2% Tween-20 (PBST) and stained using an ALP detection kit (Chemicon, Temecula, CA). Immunostained cells were imaged with an Olympus BX41 microscope (Olympus, Melville, NY) equipped with a MicroFire digital camera (Optronics, Goleta, CA). Composite images were constructed with Photoshop CS (Adobe Systems, San Jose, CA).

Western Blot

Cell cultures were washed twice with HBSS and lysed for protein purification using M-PER (Pierce, Rockford, IL) containing protease inhibitor cocktail with a cell scraper and incubated on ice for 15 min. Protein concentrations were measured using Bradford Assay (BioRad, Hercules, CA). Sample buffer and 0.1 M DTT were added to 60 μg of protein extract. Before electrophoresis, samples were placed at 70°C for 10 min. Protein samples were resolved on a 4–20% gradient SDS–polyacrylamide gel, and electrotransferred to a PVDF membrane (Hybond-P, Amersham Pharmacia). The membrane was immersed in 5% nonfat dry milk in PBST for 30 min, and probed with ALP antibody (1:500, Santa Cruz Biotech, Santa Cruz, CA) for 60 min, followed by horseradish peroxidase-conjugated anti-mouse IgG. After washing, the membrane was incubated with Luminol Reagent (Santa Cruz) and immunoreactive bands were exposed using Biomax MR light film (Kodak, Rochester, NY).

TIMP1 Immunocytochemistry

Chamber slides were washed twice in PBS, fixed in cold acetone, and processed for immunocytochemistry. Immunostaining was performed as described previously, using a three-step indirect avidin–biotin–peroxidase procedure [40]. The primary antibody used was mouse monoclonal anti-TIMP1 (7-6C1, Chemicon) diluted 1:100 in PBS. Antigen was localized using biotinylated anti-mouse IgG (BA-2000, Vector Labs, Burlingame, CA) as the secondary antibody, and diaminobenzidine tetrahydrochloride as the chromogen. The sections were counterstained in hematoxylin. Immunostained sections were imaged as described above.

Whole Transcriptome Analysis

RNA was isolated from cultures of NCCIT (0 hr), prostate stromal cells, and co-cultures at the following time points: 3 hr, 6 hr, 24 hr, 3 days, 5 days, and 7 days.

RNA was isolated from cultures of bladder stromal cells and bladder/NCCIT co-cultures at 7 days. Quality and concentration of RNA were determined using an Agilent 2100 Bioanalyzer and RNA Nano Labchip (Agilent Technologies, Santa Clara, CA). Only RNA samples that were of sufficient concentration and showed no degradation were used for microarray experiments. Between 2 and 7 biological replicates of each prostate experimental condition or control were assayed with the Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA). The U133 Plus 2.0 array contains probesets representing 54,675 genes, splice variants, and ESTs. The GeneChips were prepared, hybridized, and scanned according to the protocols provided by Affymetrix (P/N 702232 Rev. 2). Briefly, 200 ng of RNA was reverse transcribed with poly(dT) primer containing a T7 promoter, and the cDNA was made double-stranded. In vitro transcription was performed to produce unlabeled cRNA. Next, first-strand cDNA was produced with a random primed reaction, and the cDNA was made double-stranded in a reaction with poly(dT) primer/T7 promoter. Finally, in vitro transcription was performed with biotinylated ribonucleotides. The biotin-labeled cRNA was hybridized with the GeneChips. The chips were washed and stained with streptavidin–phycoerythrin (PE) using an Affymetrix FS-450 fluidics station. Data were collected with an Affymetrix GeneChip Scanner 3000.

Bioinformatics Data Analysis

A probabilistic comparative analysis between transcriptomes of treated NCCIT was used to highlight the differentially expressed genes with respect to that of untreated NCCIT. Moreover, the principal component analysis (PCA) method was used to visualize the global patterns of NCCIT expression with respect to prostate cell-type transcriptomes previously published by our group [23,41]. The gene expression level was defined as the normalized and summarized intensities of each GeneChip probeset and was presented as its logarithmic value: $X = \log_2(\text{normalized intensity})$. This step was carried out using the standard RMA method [42], implemented in the in-house analysis pipeline SBEAMS [43].

The strength of differential expression between any pair of experiments was estimated by $M_i = \log_2(\text{ratio}) = X_i - X_{0\ hr}$, where 0 hr represented the untreated NCCIT and i represented each given group of experiments in the set: 3 hr, 6 hr, 24 hr, 3 days, 5 days, or 7 days. The reliability of the differential expression was estimated by calculating the probability $\mathbf{P}(X_i > X_{0\ hr})$, or $P = \mathbf{P}(X_i < X_{0\ hr})$, according to a statistical model that assumed a normal distribution $X_j \sim N(m_j, s_j)$ where m_j and s_j are the mean and the maximum difference, respectively, among group j's replicates. Consistently, $P = \mathbf{P}(X_i > X_{0\ hr})$ or $P = \mathbf{P}(X_i < X_{0\ hr})$ was reported if $m_j > m_{0\ hr}$ or $m_j < m_{0\ hr}$, respectively. The PCA analysis was used to obtain a gene expression subspace that could highlight the principal sources of variability among the transcriptomes of the four prostate cell types previously studied by our group: stromal (S), luminal (L), basal (B), and endothelial (E) [41]. The rotation matrix was obtained using m_S, m_L, m_B, and m_E. The NCCIT transcriptome was then projected onto this PCA-derived subspace using the rotation matrix. In addition, m_{S_LCM} and m_{S_Pr}, which referred to the transcriptomes of stromal cells obtained by laser-capture microdissection (S_LCM) and cultured prostate stromal cells (S_Pr), respectively, were used [34]. Functional and ontology enrichment analysis was performed using the DAVID web-based tool [44].

Gene Expression Validation

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to validate expression scored by DNA arrays. RNA was isolated from co-cultures at the following time points: 3 and 5 days. Controls of untreated NCCIT (0 hr) and prostate or bladder stromal cells (str) alone were cultured at the same time. For each cell sample, 1 μg RNA was reverse transcribed with Superscript II RT (Invitrogen) at 50°C for 50 min followed by 10 min at 70°C. Gene-specific primers for PCR (Additional file 1) were designed to produce amplicons of 100–650 bp in size. PCR was carried out at 95°C 30 sec, 55°C 30 sec, 72°C 1 min for 35 cycles. PCR products were resolved on 2% agarose gels. The housekeeping gene GAPDH served as the internal reference for each sample. Results are representative of three biological replicates different from those used for whole transcriptome analysis.

RESULTS

Alkaline Phosphatase Activity and Morphology of Treated NCCIT Cells

ALP expression has been used to characterize the undifferentiated state of stem cells [31,45]. To assess the differentiation of NCCIT induced by treatment with prostate stromal cell secreted factors, ALP activity was assayed by immunostaining. Treated NCCIT showed decrease in the number of ALP-positive cells (Fig. 1). Untreated NCCIT showed strong staining, while prostate stromal cells were negative. In addition to decreased ALP expression, apparent changes in cell morphology and decreased proliferation became evident at 3–5 days. NCCIT cells in culture formed

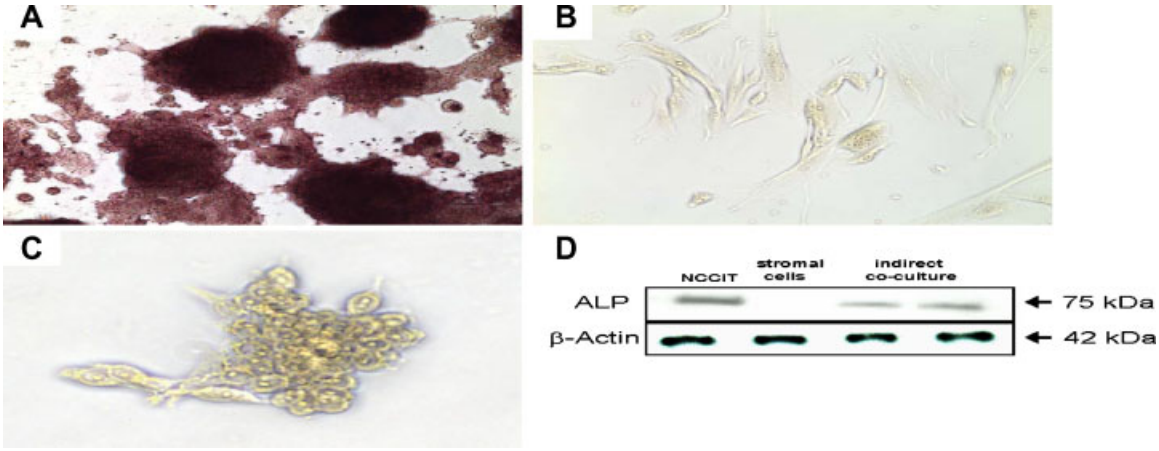

Fig. 1. Alkaline phosphatase expression. Untreated and treated NCCIT cells were analyzed for alkaline phosphatase immunostaining after 5 days in culture. **A**: Untreated NCCIT, like embryonic stem cells, were well stained for alkaline phosphatase. **B**: Prostate stromal cells were unstained. **C**: NCCIT cells treated with prostate stromal cell secreted factors are unstained and have a flattened morphology. Magnification is 100×, magnification for C is 200×. **D**: Western blot verification of alkaline phosphatase (ALP) expression in untreated and treated NCCIT cells with β actin used as a sampling control.

aggregates of small cells with larger flat cells piling up at the periphery as described in the literature [29]. Treated NCCIT cells appeared flattened in a monolayer and some radiated branching and elongated cytoplasmic processes. At 5 days of treatment, ALP expression in NCCIT was greatly reduced as verified by Western blot analysis (Fig. 1D). ALP detected by Western could be residual undegraded protein or indicative of the presence of uninduced NCCIT in that particular co-culture. The specific activity of ALP increases with increasing cell density [46]. Although efforts were made to harvest the cells at the same densities, variations between expression assayed by immunostaining and Western blot may in part be explained by these effects.

Gene Expression Changes Induced by Stromal Secreted Factors

Gene expression changes induced by trans-well co-culture of NCCIT cells and prostate stromal cells were determined using Affymetrix GeneChip arrays. The raw data were made publicly available at our UESC database [47]. A key to assessing the differentiation of NCCIT cells in context of the prostate was the availability of cell-type specific transcriptomes previously generated in our lab [23,34,41]. Genes that were differentially expressed in prostate luminal, basal, and stromal cells were compared to those genes that were differentially expressed in NCCIT cells after 3 hr, 6 hr, 24 hr, 5 days, or 7 days of trans-well co-culture with prostate stromal cells and untreated NCCIT 0 hr.

Gene expression changes induced by prostate stromal cell secreted factors became prominent after 24 hr and included decreased expression of stem cell markers NANOG, POU5F1, TDGF1, PROM1 (Fig. 2A); and, increased expression of prostate progenitor markers ITGA2/CD49b, ITGB1/CD29, and ABCG2 (Fig. 2B). Semi-quantitative RT-PCR analysis on selected genes was carried out to verify expression changes scored by arrays at 3 and 5 days. As shown in Figure 2C, down-regulation of POU5F1, CD9, NANOG, and TDGF1 was confirmed. THY1, which in addition to being a marker for hES cells [30], is also a marker for prostate stromal cells [48], and was not differentially expressed. Putative prostate stem cell gene PROM1 (CD133) was down-regulated after 3 days, while LAMP1/CD107a, ABCG2, LAMP3/CD63, and the epithelial-specific gene EGP (epithelial glycoprotein) [49] were also up-regulated, confirmed by RT-PCR (Fig. 2D). Note that expression of CD133, ABCG2, CD63, and EGP was undetectable by RT-PCR in stromal cells. Genes associated with fully differentiated prostate cells, including androgen receptor (AR) and serine proteases KLK3 (PSA), KLK4 (prostase), and KLK2 (hK2), were not significantly differentially expressed although AR expression in treated NCCIT was slightly increased at 3 days (data not shown). In silico comparison of the recently determined molecular signature of $CD133^{+}$ prostate cancer stem cells [26] in Figure 3A,B [27] to untreated NCCIT revealed similarities in gene expression as well (Fig. 3A). Similarities of note were expression of genes CD133, ANXA1, SERPINB1, TncRNA, GALNT1, ITGAV, IL6, and NFKB1.

Increased expression of prostate stromal genes such as MMP3, STC1, TNC, BMP2, PENK, EDNRB, and CNTN1 was also detected (Fig. 4A), and confirmed by

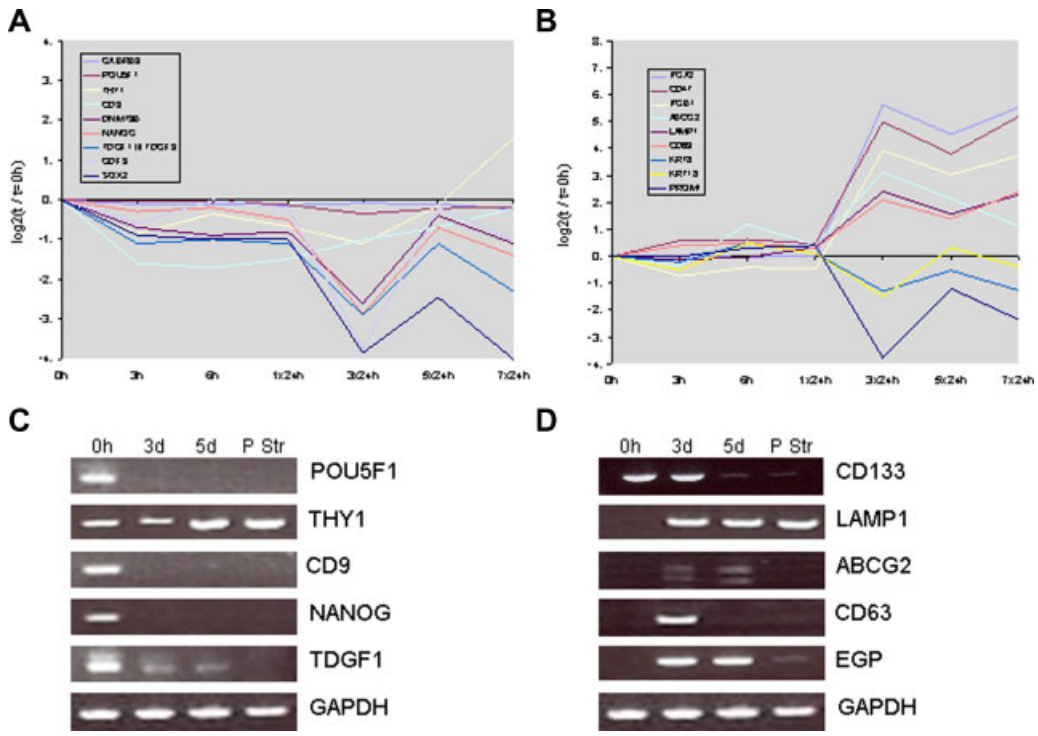

Fig. 2. Expression profile of embryonic stem cell and prostate progenitor cell genes in NCCIT. **A**: Expression of embryonic stem cell markers GABRB3, POU5FI, THYI, CD9, DNMT3B, NANOG, TDGFI, GDF3, and SOX2 by treated NCCITrelative to untreated NCCIT. **B**: Expression of putative prostate progenitor cell genes ITGA2, ITGBI, ABCG2 increases following 3 days of treatment then decreases by 5 days. Stem cell marker CDI33 decreases at 3 days. **C**,**D**: RT-PCR verification of differential expression for untreated (0 hr), 3 days, 5 days, and cultured prostate stromal cells as a control (str).

RT-PCR analysis (Fig. 4B). In addition, PCA was able to clearly show that, with respect to the principal differences in gene expression among the major prostate cell types stromal (S), luminal (L), basal (B), and endothelial (E), the NCCIT transcriptome became most similar to stromal (S) in co-culture. Figure 5 shows two different views of the PCA-derived subspace describing the gene expression resemblance of induced NCCIT to stromal cells, obtained by sorting (S), cell culture (S_Pr) or by LCM (S_LCM) methods. The 3 days time point showed the greatest similarity to the stromal data sets, whether this similarity was maintained at 5 and 7 days (i.e., without variation) was difficult to say because the analyzed cultures were not continuous.

TIMP1 is a gene involved in the generation and remodeling of extracellular matrix, and increased expression of TIMP1 in prostate stromal cells was associated with fibroblast to myofibroblast transdifferentiation [50] or with cell culturing [34]. TIMP1 up-regulation is associated with anti-metastatic potential [51] and is reported to be down-regulated in primary tumors [36]. TIMP1 is potentially a signaling molecule involved in stromal–epithelial interaction [8,15,35]. In tissue, TIMP1 expression is localized to luminal cells [36]. Treated NCCIT showed increased expression of TIMP1 as measured by differential gene expression (Fig. 4A) and verified by immunocytochemistry (Fig. 4C–F).

To assess organ-specific response of NCCIT cells, co-cultures were performed with bladder stromal cells. As shown in Figure 6A, RT-PCR detected no expression of the prostate-specific gene PENK (cf. Fig. 4B; note the absence of PENK expression in bladder str), whereas the bladder genes including GFRA1 [34] were up-regulated. We have previously shown that STC1 and STC2 are differentially expressed in prostate versus bladder stromal cells with STC1 at a higher level in prostate stromal and STC2 at a higher level in bladder stromal [34,35]. This was reflected in the array signal intensity levels in the treated NCCIT cells (Fig. 6B).

Functional Clustering Analysis of Differentially Expressed Transcripts

The set of genes that exhibited either increased or decreased expression levels at $\geq$4-fold and a *P*-value <0.05 was analyzed for significant enrichment with respect to various functional categories using the DAVID annotation tool [44]. The top 10 categories enriched in the differentially expressed genes with

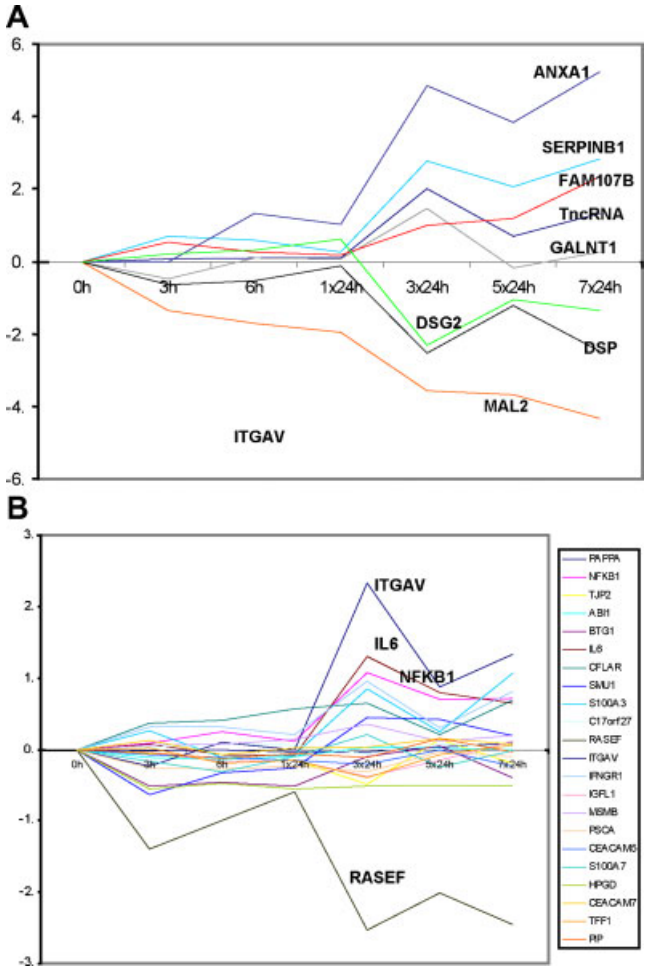

Fig. 3. Expression profile of CDI33+ prostate cancer stem cell genes in treated NCCIT. **A**: Temporal expression of selected genes up-regulated in CDI33+ cells reported by Shepherd et al., in treated NCCIT cells relative to untreated NCCIT. **B**: Temporal expression of selected genes up-regulated in CDI33+ cells reported by Birnie et al., in treated NCCIT cells relative to untreated NCCIT.

an EASE [44] score <0.05 at 3 and 7 days of prostate stromal cell induction are shown in Table I. The top 10 categories enriched by prostate compared to bladder induction at 7 days are shown in Table II. The enrichment of functional categories of response to external stimulus, cell adhesion, organ development, response to wounding, negative regulation of cellular process, negative regulation of biological process, and morphogenesis was prominent in both the 3 and 7 days data sets. In comparing prostate and bladder functional clustering, notable differences found included enrichment of functional categories such as organ development and cell proliferation in prostate versus enrichment of functional categories such as nervous system development and system development in bladder.

Expression of Stromal Cell Markers in Co-Cultured Stromal Cells

Cultured stromal cells have been previously shown to have increased expression in CD13, CD26, CD44, and CD10, as well as prolyl 4-hydroxylase β (P4Hβ) and hepatocyte growth factor (HGF) [52]. Cultured and uncultured cells were shown to be positive for VIM and smooth muscle actin (SMACT). Here, we examined the effect of co-culturing on the expression of selected stromal cell genes by comparing treated and untreated cultured stromal cells to stromal cells obtained by cell sorting and LCM. The expression of selected genes was monitored by PCR in cultured and co-cultured stromal cells (Fig. 7). The data showed that expression of these genes was predominantly unchanged by co-culture with NCCIT cells. Array analysis of co-cultured stromal cells did however show down-regulation of PENK and CNN1 (data not shown). Both PENK and CNN1 have been previously reported to be down-regulated in tumor-associated stromal cells [9,34].

DISCUSSION

Formation of prostatic glandular epithelium is governed by stromal signaling, which involves diffusible molecules and cell contact. Defects in this process could give rise to hypoplasia, hyperplasia, metaplasia, and neoplasia. Prostatic diseases constitute a major medical problem affecting a large number of men. In order to understand the potential influence of stromal cell secreted factors on cancer stem cells, we utilized an in vitro model with the hEC cell line, NCCIT. CD133, which is expressed by NCCIT, has recently been proposed as a marker of prostate cancer stem cells [26,27] and prostate cancer stem cells expressing CD133 have been found to be capable of self-renewal and producing more differentiated progeny [25].

The existence of cell type-specific [23,41] as well as organ-specific (i.e., found in prostate and not bladder) stromal factors [34] was shown by our previous analysis of differential gene expression between the prostatic cell types and between prostate and bladder stromal cells. To a large extent, expression of organ-specific stromal genes (e.g., PENK, CNTN1) appears to be maintained when stromal cells are cultured. In our experimental condition, NCCIT cells responded to stromal induction with loss of stem cell gene expression, change in morphology, and reduction in cell proliferation. This response was detectable after 24 hr. NCCIT cells were induced by prostate stromal cell secreted factors to differentiate into cells with a predominantly prostate stromal gene expression signature. This is reflected by the near but not exact match of the transcriptomes in the PCA plot. However, genes specific to epithelial cells (EGP, CD47, LAMP1/

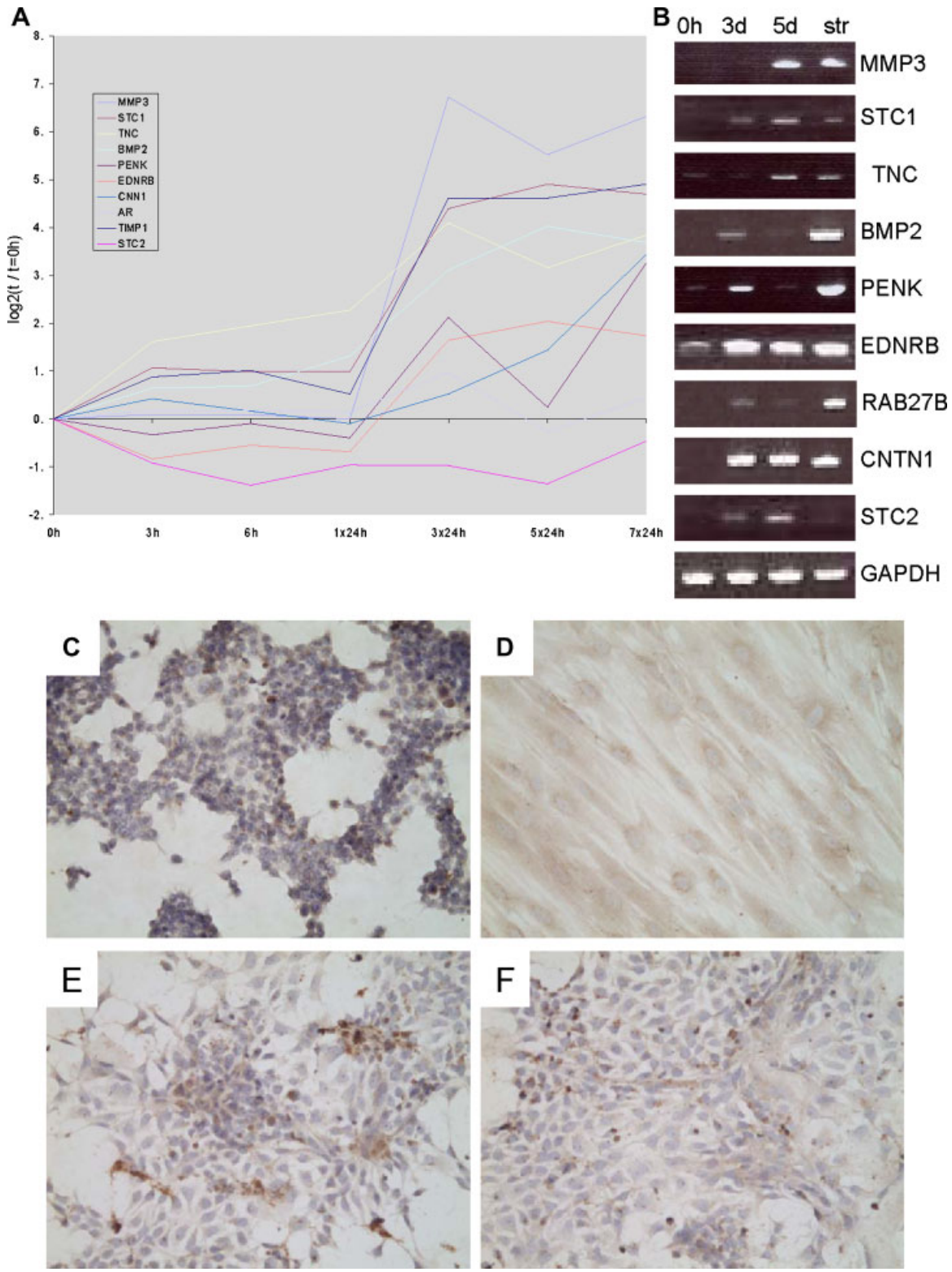

Fig. 4. Expression profile of prostate stromal cell genes in treated NCCIT. **A**: Increased expression of prostate stromal cell-specific genes relative to untreated NCCIT. Note increases in MMP3, STCI, TNC, BMP2, PENK, EDNRB, CNNI whereas genes characteristic of more fully functional prostate cell types AR (stromal and CDI33 cancer stem cell), KLK3 (luminal) remain undetected. **B**: RT-PCR verification of differential expression for untreated (0 hr), 3 days, 5 days, and prostate stromal cells as a control (str). **C**: Immunostaining for TIMPI expression in untreated NCCIT shows little expression. **D**: TIMPI immunostaining in cultured prostate stromal cells shows extensive expression. **E**,**F**: Treated NCCIT shows areas of TIMPI staining in two different fields. Original magnification for C–F at 200×.

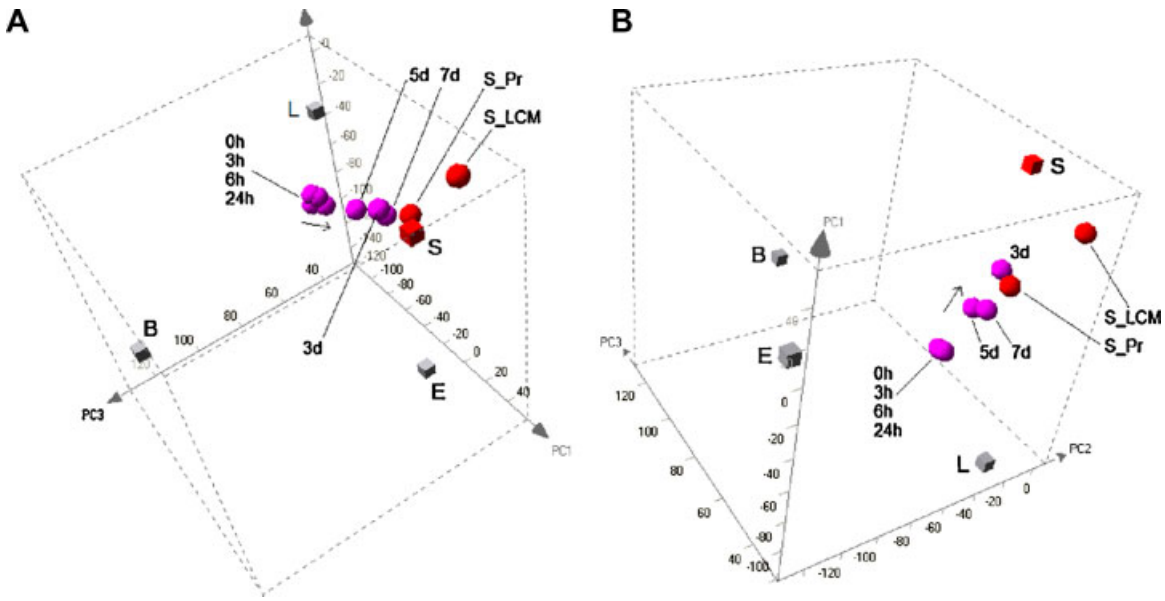

Fig. 5. PCA projections of treated NCCIT transcriptomes with respect to those of prostate cell transcriptomes. **A**: Three-dimensional projection of 0 hr, 3 hr, 6 hr, 24 hr, 3 days, 5 days, and 7 days NCCIT transcriptomes, and prostate cell-type specific transcriptomes for stromal cells obtained by cell sorting (S), cell culture (S.Pr), LCM (S.LCM), and sorted luminal cells (L), endothelial cells (E), and basal cells (B) in a PCA-derived subspace. **B**: PCA projection from a different point of perspective. The 3D coordinate system was obtained by performing the usual PCA analysis to L, E, B, and S, defining the rotation matrix related to the top three principal components and applying it to all data sets to create a subspace which highlights the particularities of each prostate cell type.

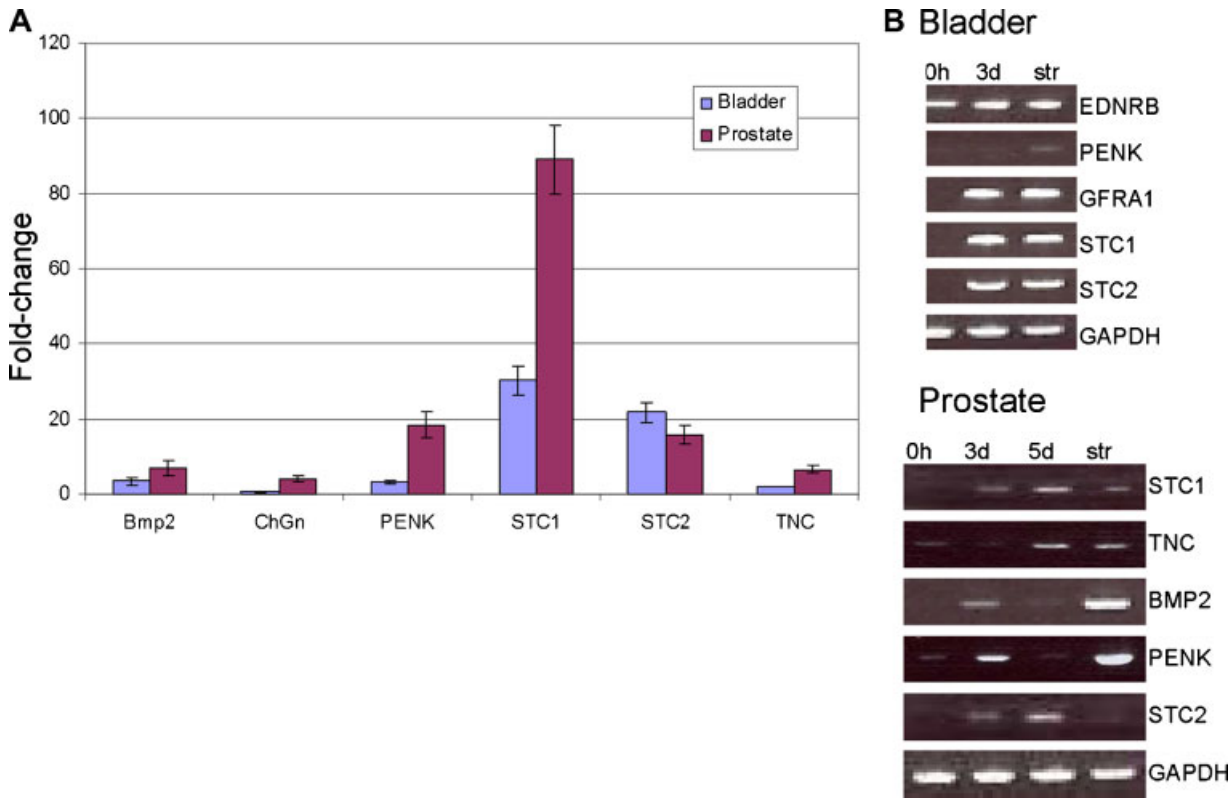

Fig. 6. Differential expression of organ-specific stromal cell genes in prostate- versus bladder-treated NCCIT. **A**: To verify organ-specific differentiation induced by secreted factors, NCCIT was treated with bladder stromal cells. **A**: Array analysis comparison of differentially expressed prostate and bladder-specific genes at 7 days of co-culture with prostate or bladder cells compared to untreated NCCIT. **B**: RT-PCR examination of differentially expressed genes by prostate and bladder at 3 and 5 days induction.

TABLE I. Induced Functions

Term	Count	*P*-value
3 days		
Response to external stimulus	51	9.92E – 14
Cell adhesion	59	1.73E – 13
Organ development	51	3.13E – 13
Response to wounding	43	5.26E – 13
Development	107	3.56E – 12
Skeletal development	22	4.34E – 10
Organismal physiological process	124	5.75E – 10
Negative regulation of cellular process	53	1.12E – 09
Negative regulation of biological process	55	1.90E – 09
Morphogenesis	47	2.34E – 09
7 days		
Development	180	4.23E – 17
Cell adhesion	91	3.06E – 16
Organ development	74	4.98E – 14
Response to wounding	58	4.71E – 12
Negative regulation of biological process	88	7.57E – 12
Negative regulation of cellular process	83	1.53E – 11
Response to external stimulus	67	2.20E – 11
Morphogenesis	71	2.79E – 10
Response to stress	107	8.14E – 10
Wound healing	24	2.02E – 09

Functional cluster analysis of differentially expressed genes involved in the top 10 biological processes which have differential expression levels in treated versus untreated NCCIT at 3 and 7 days as shown. Count refers to the number of genes in each category.

TABLE II. Prostate Versus Bladder-Induced Functions

Term	Count	*P*-value
Increased in prostate-treated NCCIT		
Cell adhesion	41	4.90E – 12
Development	68	7.73E – 10
Phosphate transport	14	2.28E – 08
Organ development	30	3.38E – 08
Cell proliferation	29	1.55E – 07
Response to external stimulus	28	2.39E – 07
Anion transport	16	1.70E – 06
Organismal physiological process	72	2.85E – 06
Inorganic anion transport	14	5.40E – 06
Response to wounding	21	1.54E – 05
Increased in bladder-treated NCCIT		
Homophilic cell adhesion	18	1.05E – 13
Nervous system development	27	7.77E – 12
System development	27	9.36E – 12
Cell–cell adhesion	19	3.77E – 11
Development	45	2.19E – 07
Cell adhesion	24	1.06E – 06
Regulation of biological process	60	8.51E – 04
Regulation of cellular process	55	2.72E – 03
Regulation of cellular physiological process	53	2.76E – 03
Regulation of physiological process	54	3.11E – 03

Functional cluster analysis of genes involved in the top 10 biological processes which have differential expression levels treated NCCIT in prostate versus bladder at 7 days.

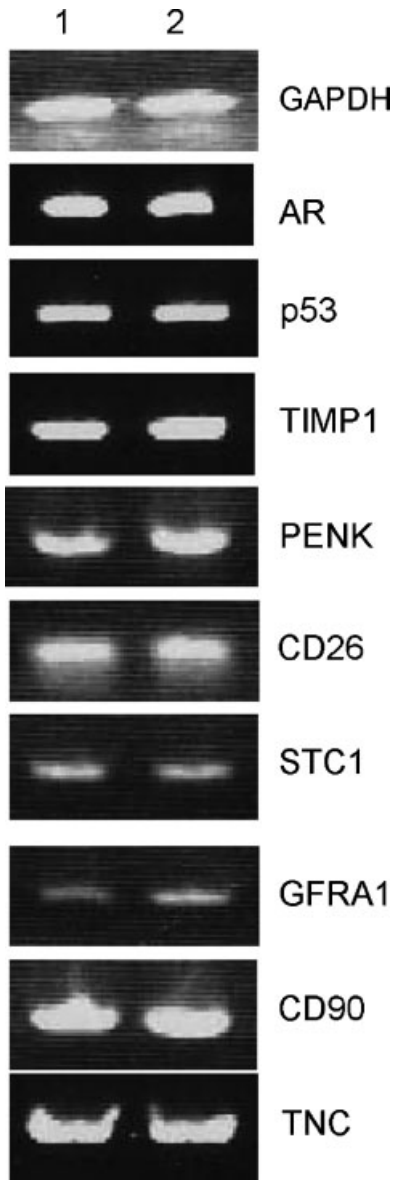

Fig. 7. Expression of stromal cell markers in co-cultured stromal cells. RT-PCR verification of expression for cultured (**1**) and co-cultured in the presence of NCCIT (**2**) prostate stromal cells at 3 days (both conditions were for stromal cells at the third passage).

CD107a, ITGA2/CD49b, ITGB1/CD29) and $CD133^{+}$ prostate cancer stem cells (ANXA1, SERPINB1, TncRNA, GALNT1, ITGAV, IL6, and NFKB1) were also induced. Whether or not this seemingly incomplete differentiation of treated NCCIT into a prostate stromal cell phenotype rather than that of a malignant epithelial cell is due to cellular mimicry or heterogeneity in the treated NCCIT cell population remains to be determined.

Future examination of genes, such as CD133, ANXA1, and NFKB1, which are both highly expressed by the putative prostate cancer stem cell and differentially expressed over time in treated NCCIT, might provide important insight into their roles in controlling and directing tumor differentiation. Determining stromal effects on isolated prostate cancer stem cell populations, which unlike NCCIT and the prostate stem cell, expresses AR [25] will be critical for fully understanding the role of stromal cells in prostate disease progression. Previous studies characterizing gene expression profiles of tumor-associated stromal cells have identified several candidate genes that could potentially have important influences on the cancer stem cell as well as the more differentiated tumor cell types [53–55]. Current thought is that tumor-associated stroma always co-exists with prostate cancer [56] and that it may contribute to the metastatic potential of the tumor by facilitating processes such as angiogenesis and progression towards androgen-independence [57]. In this study, we examined the effect of co-culturing on the stromal cells by comparing treated and untreated cultured stromal cells to stromal cells from normal tissue. Future comprehensive monitoring of cancer stem cell influence on stromal cell gene expression profiles with respect to those of tumor-associated stromal cells could provide further valuable insight into prostate tumor biology and the relationship between the cancer stem cell and its surrounding microenvironment.

ACKNOWLEDGMENTS

We are grateful to Drs. Lawrence True, Paul Lange, William Ellis, and Thomas Takayama for providing prostate and bladder tissue for this study. We also thank Adam Van Mason for collecting and preparing the prostate and bladder tissue. This work was supported by Grant Number DK63630 from NIDDK and Grant Number CA111244 from NCI. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIDDK or the NCI. Additional funding came from grant PM50 GMO76547/Center for Systems Biology.

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