

# Differential Inductive Signaling of CD90<sup>+</sup> Prostate Cancer-Associated Fibroblasts Compared to Normal Tissue Stromal Mesenchyme Cells

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**Abstract** Prostate carcinomas are surrounded by a layer of stromal fibroblastic cells that are characterized by increased expression of CD90. These CD90<sup>+</sup> cancer-associated stromal fibroblastic cells differ in gene expression from their normal counterpart, CD49a<sup>+</sup>CD90<sup>lo</sup> stromal smooth muscle cells; and were postulated to represent a less differentiated cell type with altered inductive properties. CD90<sup>+</sup> stromal cells were isolated from tumor tissue

specimens and co-cultured with the pluripotent embryonal carcinoma cell line NCCIT in order to elucidate the impact of tumor-associated stroma on stem cells, and the ‘cancer stem cell.’ Transcriptome analysis identified a notable decreased induction of smooth muscle and prostate stromal genes such as PENK, BMP2 and ChGn compared to previously determined NCCIT response to normal prostate stromal cell induction. CD90<sup>+</sup> stromal cell secreted factors

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induced an increased expression of CD90 and differential induction of genes involved in extracellular matrix remodeling and the RECK pathway in NCCIT. These results suggest that, compared to normal tissue stromal cells, signaling from cancer-associated stromal cells has a markedly different effect on stem cells as represented by NCCIT. Given that stromal cells are important in directing organ-specific differentiation, stromal cells in tumors appear to be defective in this function, which may contribute to abnormal differentiation found in diseases such as cancer.

**Keywords** Prostate · Prostate cancer · Cancer stem cell · Tumor-associated stroma · Reversion-inducing cysteine-rich protein with Kazal motifs (RECK)

## Introduction

The prostate is a glandular organ composed of secretory luminal cell-lined acini and an underlying layer of basal cells supported within a fibromuscular stroma. In urologic organ development, the stroma provides the inductive signaling to stem/progenitor cells to produce the resultant tissue [1]. Increasingly, it is thought that the stromal environment might play a significant role in disease development and progression as well. An altered stroma has been identified in association with both prostate cancer [2–4] as well as premalignant prostate diseases including prostatic intraepithelial neoplasia (PIN) and benign prostatic hyperplasia (BPH) [3, 5]. In prostate cancer, the stroma is also affected in that gene expression in cancer-associated fibroblastic (CAF) stromal cells differs significantly from that of normal tissue stromal cells [6–10]. Prostate CAFs are capable of inducing proliferation and malignant transformation [1] and have been postulated to drive tumor progression [11]. These studies strongly suggest that the prostate stroma plays a significant role in facilitating disease progression.

CAFs immediately surrounding prostate cancer can be identified by strong CD90 immunostaining [12] and CD90<sup>hi</sup> cancer-associated fibroblasts (CAFs) were postulated to have greater tumor-promoting effects than CD90<sup>lo</sup> CAFs [10]. Comparative transcriptome analysis of isolated CD90<sup>+</sup> CAFs and CD49a<sup>+</sup> normal tissue stromal cells revealed a decrease in the expression of genes involved in smooth muscle cell differentiation and those specific or restricted to the prostate [5, 7]. Genes that encode secreted proteins or hormones are likely candidates responsible for organ-specific stromal induction, and dysregulation of these genes might contribute to disease progression through stromal-stem cell signaling.

To examine the molecular mechanisms involved in prostate stromal-stem cell interaction, we previously developed an *in vitro* co-culture system with stromal cells isolated from normal prostate (referred to as NP) and a cancer stem cell type, the embryonal carcinoma (EC) cell line, NCCIT [13]. NCCIT is a pluripotent cell line that can be readily maintained in cell culture in an undifferentiated state [14]. When co-cultured without direct cell contact, NCCIT cells were induced by NP stromal cells to differentiate into a cell population that expressed predominantly prostate stromal cell genes (e.g., PENK). Induced NCCIT cells also lost expression of stem cell genes and underwent a change in morphology with reduced proliferation [13]. Bladder stromal cells isolated from human specimens induced a bladder stromal-like expression (e.g., absent PENK) [13], thus demonstrating plasticity—a property of stem cells—in NCCIT response. This cell-to-cell signaling was presumably mediated by diffusible factors. Interestingly, NP prostate stromal cells were also significantly altered by co-culture with NCCIT, whereas NCCIT had no significant effect on the gene expression of CP stromal cells [15]. In this study, NCCIT induced an increase in expression for CD90, MIRN21, HGF, SFRP1, BGN, and decreased expression of IGFBP5, HSD11B1a in NP stromal cells. These findings suggest that alterations in prostate stroma could be induced by stem or cancer stem cell influence. In this study, we examined the inductive functioning of cultured CD90<sup>+</sup> CAFs (referred to here as CP) stromal cells for comparison to that of NP stromal cells.

## Materials and Methods

### Cell Lines and Tissue Specimens

NCCIT and prostate cancer cell line PC3 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). In this culture condition, NCCIT cells maintain expression of stem cell genes and microRNA, and are alkaline phosphatase positive [13, 15]. The tissue samples used in this study consisted of cancer-enriched CP tissue specimens, characterized as CD90<sup>hi</sup>TIMP1<sup>lo</sup> by Western blot analysis of tissue digestion media [7], obtained from 2 radical prostatectomy surgery patients. Note ‘pure’ CP specimens had increased CD90 expression and minimal reactivity to TIMP1, which is secreted by luminal cells and not by cancer cells [16], whereas NP specimens were CD90<sup>lo</sup>TIMP1<sup>hi</sup>. All tissue samples were obtained under approval by the University of Washington IRB and were collected following a standard protocol. Upon receipt of a

resected specimen, 3-mm thick transverse sections were made of the gland after inking the exterior surface. Frozen sections of tissue blocks were histologically prepared to locate the tumor foci for dissection. Pathology characteristics of the 2 tumors were 08-021CP: Gleason 5+4, T3a, tumor volume 4.5 cc; 08-028CP: Gleason 3+4, T2c, tumor volume 2.5 cc. NP specimens were obtained and analyzed as described previously [13]. Briefly, between 1 and 10 g of tissue from the anterior aspect of the prostate (transition zone) were excised; corresponding frozen sections of the tissue blocks were histologically assessed to confirm specimens were free of cancer.

Tissue samples 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- $\mu$ m Falcon cell strainer to remove any non-digested tissue, diluted with an equal volume of Hanks balanced salt solution (HBSS), and aspirated with an 18-gauge needle. The single cell preparation was partitioned into stromal and epithelial fractions on a discontinuous Percoll density gradient (Amersham Pharmacia, Piscataway, NJ) as described [17, 18]. Cells banding at a density of  $\rho=1.035$  were collected as the stromal fraction for magnetic cell sorting (MACS). The cell-free tissue digestion media supernatant was analyzed by Western blotting to verify that the specimens were of cancer.

CP stromal cells were sorted using anti-CD90. Briefly, the gradient-purified stromal cell fraction was resuspended in 100  $\mu$ l 0.1% bovine serum albumin (BSA)-HBSS, and CD90-Phycoerythrin (PE) mouse monoclonal antibody (clone 5E10, BD-PharMingen, San Diego, CA) at 1:20 was added for 15 min at room temperature in the dark. The reaction was stopped by 1 ml 0.1% BSA-HBSS and centrifugation. The labeled cells were resuspended in BSA-HBSS, and 15  $\mu$ l paramagnetic microbead conjugated anti-PE (Miltenyi Biotec, Auburn, CA) was added for 15 min. After incubation, the positive and negative cells were separated by AutoMACS (Miltenyi Biotec) using the double positive sort program. Aliquots of positive and negative cell fractions were analyzed by fluorescence-activated cell sorting (FACS, Becton Dickinson, Mountain View, CA) to gauge the sort efficiency; only >85% CD90<sup>+</sup> fractions were used for experiments.

### Cell Culture

The sorted CD90<sup>+</sup> stromal cells were adapted to culture for 3–5 passages in RPMI-1640 media supplemented with 10% FBS, and their identity verified by RT-PCR analysis of gene expression as described [5]. For co-culture experiments, 0.4  $\mu$ m polycarbonate membrane trans-well

inserts (Corning, Corning, NY) to preclude cell contact were employed. NCCIT cells were seeded at  $10^4$ /ml in RPMI-1640, 10% FBS on 6-well plates, and CP stromal cells were seeded at  $10^4$ /ml on the insert. Controls were NCCIT and CP stromal cells alone. Cultures were maintained for 3 d. The 3 d-time point was chosen based on our previous time course study of NP stromal cell-induced differentiation of NCCIT [13]. In that experiment, gene expression changes in NCCIT were detected as early as 6 h in co-culture, and by the third day, nearly the entire stromal gene repertoire was induced as shown by a principal components analysis of time-point transcriptomes. Cells were trypsinized and lysed in RLT Buffer (Qiagen, Valencia, CA).

### Transcriptome Analysis

RNA was isolated from cultures of CP stromal, NCCIT, and NCCIT + CP stromal cells at 3 d. Transcriptomes of untreated NCCIT, NP stromal cells and NCCIT at 3 d of co-culture with NP stromal cells were determined previously [13], as were sorted CD90<sup>+</sup> CP stromal cells, i.e., not cultured [7]. These datasets were made available online (<http://scgap.systemsbio.net/data/>). Quality and concentration of RNA were determined using an Agilent 2100 Bioanalyzer and RNA Nano Labchip (Agilent Technologies, Santa Clara, CA). Between 2 and 7 replicates of each 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 random primers, and the cDNA was made double-stranded with poly (dT) primer/T7 promoter. A final in vitro transcription was done with biotinylated ribonucleotides. The biotin-labeled cRNA was hybridized to the GeneChips. The chips were washed and stained with streptavidin-PE using an Affymetrix FS-450 fluidics station. Data was collected with an Affymetrix GeneChip Scanner 3000.

### Bioinformatic Analysis

A probabilistic comparative analysis between transcriptomes of treated NCCIT was used to highlight differentially expressed genes with respect to that of untreated NCCIT [7]. 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 robust multi-array average (RMA) method [19], implemented in the in-house analysis pipeline SBEAMS [20]. Data were presented on a grayscale indicating RMA-normalized Affymetrix signal intensity [21]. Signals of 10 or less were represented as white and signals greater than or equal to 10,000 as black. Higher Affymetrix signal (more black) indicated higher levels of gene expression.

The strength of differential expression between any pair of experiments was estimated by  $M_i = \log_2(\text{ratio}) = X_{3d} - X_{0h}$ , where 0 h represented the untreated NCCIT and 3d represented treated NCCIT at 3 d. Reliability of the differential expression was estimated by calculating the probability  $P = \mathbf{P}(X_{3d} > X_{0h})$  or  $P = \mathbf{P}(X_{3d} < X_{0h})$  according to a statistical model that assumed a normal distribution  $X_j \sim N(m_j, s_j)$ , where  $m_j$  and  $s_j$  were the mean and maximum difference, respectively, among the replicates of group  $j$ . Consistently,  $P = \mathbf{P}(X_{3d} > X_{0h})$  or  $P = \mathbf{P}(X_{3d} < X_{0h})$  was reported if  $m_{3d} > m_{0h}$  or  $m_{3d} < m_{0h}$ . Functional and ontology enrichment analysis was performed using the DAVID web-based tool [22]. Freely available prediction software for determination of signal peptides and likely cell membrane-spanning sequences was also used. Signal peptides were predicted using SignalP 3.0 [23], and transmembrane (TM) regions were predicted using TMHMM 2.0 [24] for protein topology and the number of TM helices. Information from both SignalP and TMHMM were combined to identify proteins that contained predicted cleavable signal peptides and no predicted TM segments as reported previously [25].

## Results

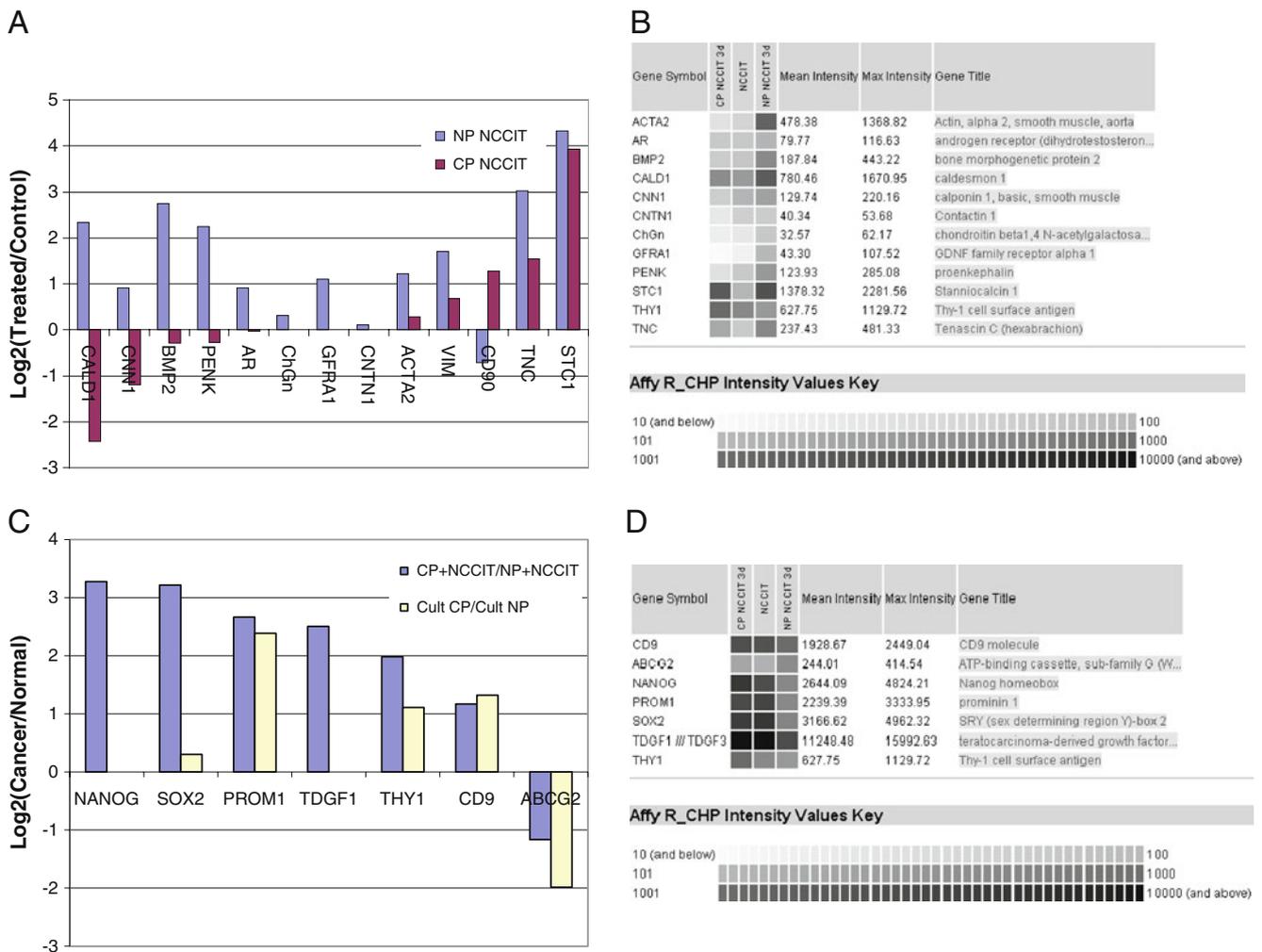
Gene expression changes induced in NCCIT by secreted factors from CP stromal cells were determined by Affymetrix DNA microarray analysis. Following 3-days co-culture with CP stromal cells, the induced expression in NCCIT cells (CP-NCCIT) of smooth muscle genes ACTA2, CALD1, CNN1, prostate stromal genes PENK, CNTN1, ChGn, BMP2 [5, 7], androgen receptor (AR) and a stromal gene GFRA1 was significantly less than that previously shown by NP stromal cells (NP-NCCIT) [13]. The CP-NCCIT transcriptome dataset contained minimal signal levels for CALD1, and CNN1 compared to that of NP-NCCIT (Fig. 1a and b). Other queried prostate genes such as tenascin C (TNC) [5] were also lacking. The similar induction of stromal gene stanniocalcin (STC1) and increased expression of CD90/THY1 in CP-NCCIT showed that gene expression changes did occur in NCCIT co-cultured with CP stromal cells. Induction of CD90 was notably higher in CP-NCCIT than in NP-NCCIT (NCCIT

cells are also positive for CD90, a stem cell marker). This reflected the increased CD90 expression in CP stromal cells.

A comparison of stem cell gene expression in treated NCCIT showed higher signal levels of NANOG, POU5F1, TDGF1, and SOX2 in CP-NCCIT than in NP-NCCIT (Fig. 1c and d). Induction of NCCIT by NP stromal cells was found to lead to almost complete down-regulation of these stem cell genes [13], whereas CP stromal cells had apparently little effect. Of note was the detection of ABCG2 (a prostate progenitor cell marker) expression in NP-NCCIT but not CP-NCCIT (Fig. 1d). NCCIT is negative for ABCG2 expression.

To identify genes encoding secreted proteins that might function in cell-cell signaling, the most differentially expressed genes in NP-NCCIT vs. CP-NCCIT (or NP stromal vs. CP stromal) were analyzed using the DAVID annotation tool. CP stromal induction led to up-regulation of several such genes including LEFTY2 and TAC1, while NP stromal induction led to up-regulation of ADAMTS1, IGFBP5, WNT5A and others (Table S1). Overall, there were many more such genes induced in NP-NCCIT than CP-NCCIT. This showed a smaller pool of candidate signaling molecules produced by CP stromal cells.

Differentially expressed genes were also analyzed for significant enrichment with respect to functional categories using DAVID. The top KEGG pathways identified were cytokine-cytokine receptor interaction, chemokine signaling pathway, extracellular matrix (ECM)-receptor interaction, cell adhesion and focal adhesion. Enrichment of these functional categories was prominent in the CP-NCCIT vs. NP-NCCIT datasets. Of particular interest were the genes that contribute to the functioning and maintenance of ECM. Matrix metalloproteinases (MMPs) are involved in the degradation of ECM proteins and have been associated with tumor cell invasion. The membrane-anchored reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a potent inhibitor of MMP activity. RECK down-regulation has been identified in many cancers, and it has been reported that high RECK expression levels were associated with favorable prognosis in prostate cancer [26, 27]. In comparing the CP-NCCIT and NP-NCCIT expression profiles, several genes associated with RECK were differentially expressed (Fig. 2a). For example, induction of MMP9, a potential prostate cancer urine biomarker [28], was greater in CP-NCCIT than in NP-NCCIT (Fig. 2b). MMP9 expression was also higher in sorted CP vs. NP stromal cell transcriptomes. In contrast, RECK was more up-regulated in NP-NCCIT than CP-NCCIT, as was tissue inhibitor of metalloproteinase TIMP1, an antagonist of MMPs. With regard to the possible genesis of CP stromal



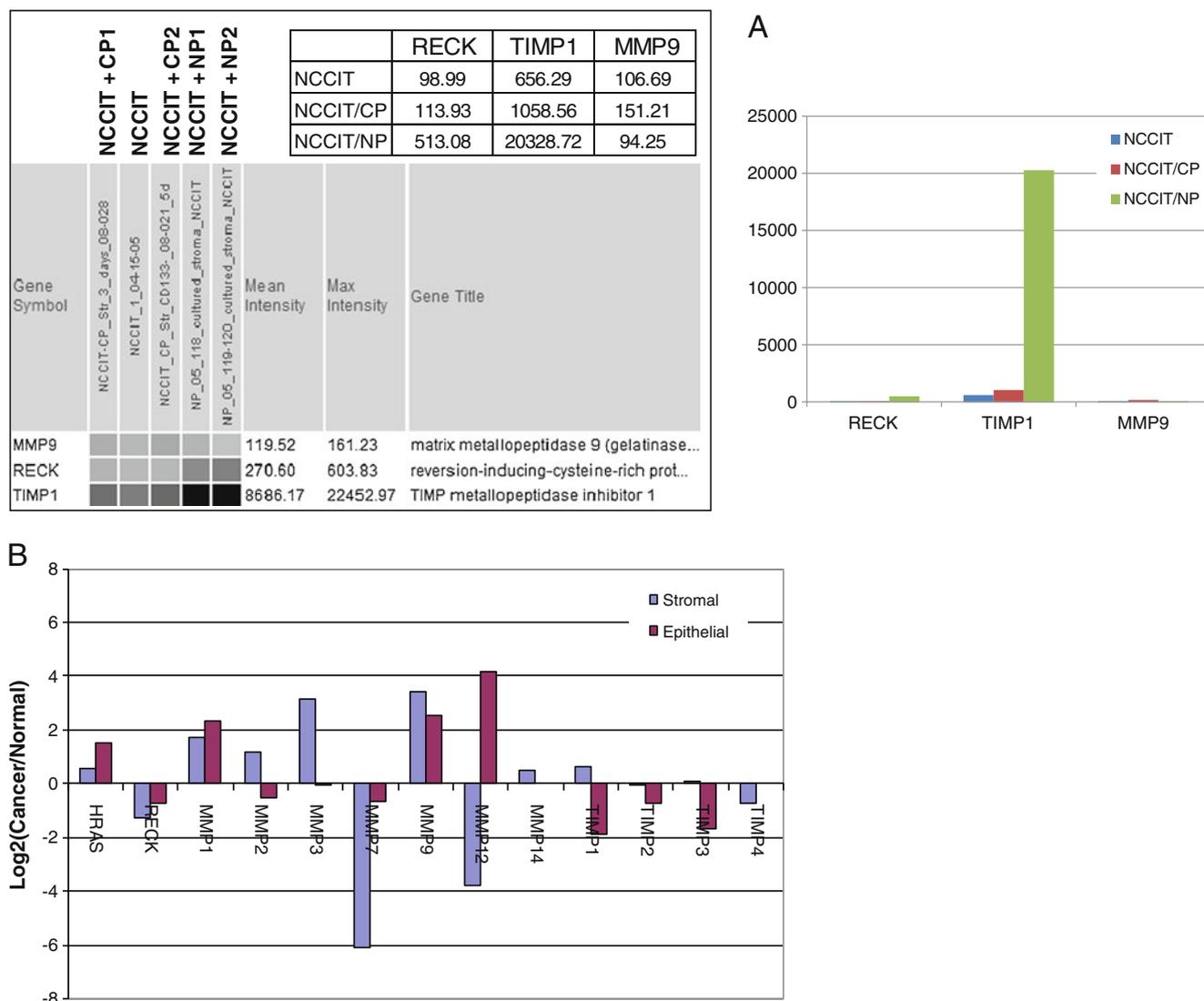
**Fig. 1** Expression profiles of stromal and stem cell genes in treated NCCIT. **a** Increased expression of prostate stromal cell-specific genes relative to untreated NCCIT was detected in co-cultures of NP stromal + NCCIT cells (labeled NP-NCCIT). Expression of these stromal genes was less pronounced in co-culture of CP stromal + NCCIT cells (CP-NCCIT). For example, PENK was not induced. Note the increase in tumor-associated stromal marker CD90/THY1. **b** The CP-NCCIT transcriptome dataset (first column) contains minimal signal for smooth muscle differentiation genes (CALD1, CNN1) present in the NP-

NCCIT transcriptome (third column) compared to untreated NCCIT transcriptome (second column) in virtual Northern blot format (darker shades of boxes indicate higher mRNA levels with background  $\leq 50$  RMA units). **c** Higher expression of several stem cell genes was detected in CP-NCCIT relative to NP-NCCIT as well as in cultured CP stromal cells relative to NP stromal cells. **d** Virtual Northern blot format shows that for stem cell genes NANOG, SOX2, CD9 and THY1, expression was increased in CP-NCCIT compared to untreated NCCIT, whereas expression was decreased in NP-NCCIT

cells, we also examined the effect of NCCIT factors on NP stromal cells [15]. NCCIT extracts, when injected into differentiated cells, can activate expression of stem cell genes [29]. Instead of extract injection, NCCIT factors were examined in the co-culture format with NP stromal cells. We found that at day 3, the co-cultured NP stromal cells showed a gene expression profile for both mRNA and microRNA resembling that of CP stromal cells. Thus, CP stromal cells appear to represent a more primitive cell type in the stromal lineage. This is certainly in line with their lower expression of smooth muscle cell genes, and CP stromal cells are characterized by a loss of smooth muscle

differentiation [3]. The basal epithelium also contains the progenitor cell population, which could affect stromal cell differentiation. To isolate enough CD90<sup>+</sup> NP stromal cells for study presents a technical challenge because of their low number, which necessitates the need to obtain large tissue specimens for sorting.

Figure 3 illustrates the RECK pathway network in which MMPs synthesized by CP stromal cells could lead to ECM degradation, which would in turn promote tumor cell escape. The MMP effect is amplified by the decrease in TIMP expression in cancer cells. Increase in MMPs is due to down-regulation of RECK.



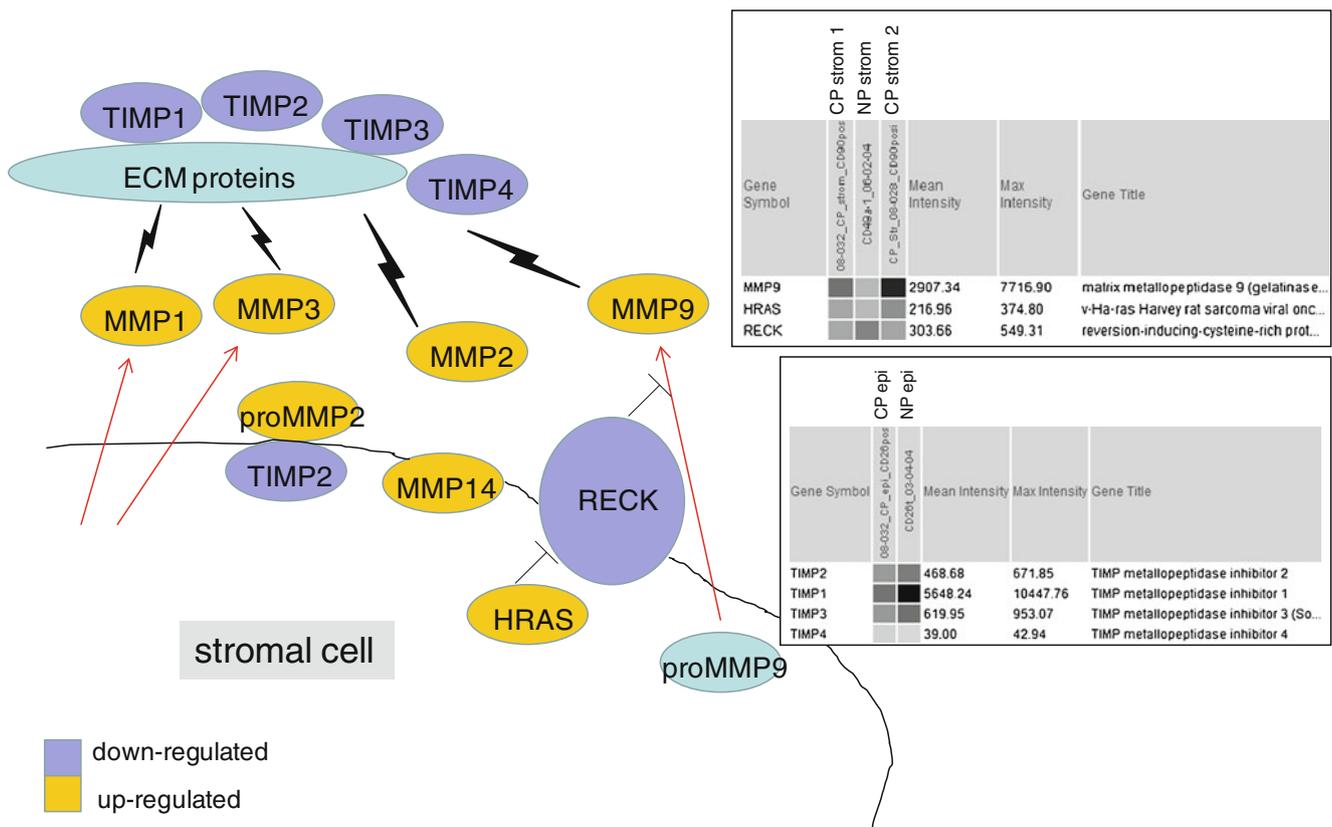
**Fig. 2** Expression of RECK genes in prostate cancer. **a** Relative expression of RECK, TIMP1 and MMP9 induced by CP vs. NP stromal cells in NCCIT. The average array signal values for 2 experiments (NP1, NP2, CP1, CP2) are tabulated, which were used to

generate the histogram. **b** Relative expression profile of genes involved in ECM remodeling in sorted stromal and epithelial cells from tumor vs. normal tissue. Positive values indicate up-regulation in cancer and negative values indicate down-regulation in cancer

## Discussion

NCCIT response to CD90<sup>+</sup> CAFs tumor-associated was significantly altered from the gene expression changes induced by normal stromal cell factors. Previously, NP stromal cells induced a loss of embryonic stem cell markers and an up-regulation of genes characteristic of stromal mesenchyme, some epithelial genes and cancer stem cell genes. NCCIT response to CP stromal cells was characterized by an absence or decreased induction of genes involved in smooth muscle cell differentiation and those expressed by the prostate but not the bladder, i.e., organ restricted. At the same time, the decrease in stem cell gene expression was not as pronounced. This altered differenti-

ation response could be due to differences in signaling factors secreted from tumor-associated stromal cells. These differences could be the result of either a reduction or loss of certain proteins such as the hormone PENK. Stromal cells are important in tissue repair and renewal as suggested by their demonstrated role in prostate and bladder formation. Organ specificity in this process could be due to the differentially expressed genes between the stromal cell types. Indeed, we previously showed that bladder stromal cells induced a different response from NCCIT than prostate stromal cells. Thus, if tumor-associated stromal cells were unable to provide the appropriate signaling, then normal histodevelopment would not occur, instead cancer development takes place.



**Fig. 3** Schematic of RECK pathway in stromal–epithelial interaction in prostate cancer. Decreased RECK expression leads to activation of MMPs and degradation of ECM proteins allowing dissemination of tumor cells. Virtual Northern blot format shows array signals for

MMP9, HRAS and RECK in NP stromal vs. CP stromal cells (1 and 2 from two different specimens) and for TIMPs in NP epithelial vs. CP cancer cells

Although only two cases of CP stromal cells were tested, they did provide a demonstration that stem cell induction was markedly different from that by NP stromal cells. These differences, including reduced induction of smooth muscle cell genes and increased induction of genes involved in ECM remodeling, are consistent with alterations to the prostate tumor microenvironment. When we can model epithelial cytodifferentiation, i.e., PSA secretion, with cell contact and ECM, then a study using multiple samples of CP stromal cells can be carried out. For example, one could contrast the effect of CP stromal cells isolated from Gleason 3 +3 vs. Gleason 4+4 tumors.

The differentially induced expression pattern of genes involved in the ECM RECK pathway in CP-NCCIT vs. NP-NCCIT appears to mimic that in primary tumors as inferred from the transcriptomes of sorted stromal and epithelial cell types. NP stromal induction produced up-regulation of TIMP1 and RECK whereas CP stromal induction produced up-regulation of MMP9 and comparatively less of TIMP1 and RECK. In the sorted cells, expression of MMP9 (and HRAS, which inhibits RECK) is higher in CP stromal than NP stromal, whereas that of RECK is higher in NP stromal. TIMP1 protein in cancer is

absent [16]. Thus, our in vitro model of CP stromal induction of NCCIT recapitulated to some degree a major pathway important in cancer development. In response to NP stromal influence, NCCIT cells were induced to express a transcriptome with a predominant but incomplete stromal mesenchyme profile. However, the response to CP stromal cell influence with regard to induction of stromal mesenchyme genes and loss of stem cell genes was significantly less. This difference could simply reflect a reduction in secreted factors from CP stromal cells compared to NP stromal cells and therefore a lesser degree of influence on NCCIT cells, or it could represent a shift in the heterogeneity of the treated NCCIT cell population.

With regard to the possible genesis of CP stromal cells, we also examined the effect of NCCIT factors on NP stromal cells [15]. NCCIT extracts, when injected into differentiated cells, can activate expression of stem cell genes [29]. Instead of extract injection, NCCIT factors were examined in the co-culture format with NP stromal cells. We found that at day 3, the co-cultured NP stromal cells showed a gene expression profile for both mRNA and microRNA resembling that of CP stromal cells. Thus, CP stromal cells appear to represent a more primitive cell type

in the stromal lineage. This is certainly in line with their lower expression of smooth muscle cell genes, and CP stromal cells are characterized by a loss of smooth muscle differentiation [3]. The basal epithelium also contains the progenitor cell population, which could affect stromal cell differentiation. To isolate enough CD90<sup>+</sup> NP stromal cells for study presents a technical challenge because of their low number, which necessitates the need to obtain large tissue specimens for sorting.

In summary, these experimental results showed that in induction of stem cells CP stromal cells were very different from NP stromal cells. The abnormal gene expression of CP stromal cells may well be the cause. Whether this would lead to cancer cell differentiation is still unknown since heterotypic cell contact and ECM were not provided for in the co-culture. Also unknown is whether other cell types beside stromal (e.g., epithelial) were induced in this system. For example, some ABCG2 expression was detected in NP-NCCIT, and this may suggest a small subpopulation with this marker. ABCG2 expression was identified in a putative prostate progenitor cell population localized to the basal epithelium [30]. It is therefore possible that more than one cell lineage, stromal and epithelial, could result from stromal induction of stem cells.

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**Conflicts of interest** The authors declare no conflict of interest.

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