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Stromal-epithelial interactions in early neoplasia

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Abstract. In prostate tumors, both the epithelial and stromal mesenchyme compartments show gene expression changes from their respective normal counterpart. In fact, there are more such changes in the stroma than the epithelium. These include down-regulated expression of genes involved in smooth muscle cell differentiation and those differentially expressed between prostate and bladder, i.e., organ-restricted. In development, the stromal cell type mediates tissue formation from differentiation of stem or progenitor cells. Diseases like cancer may arise as a result of defective stromal signaling. Stromal signaling can be demonstrated by co-culture of stromal cells and embryonal carcinoma NCCIT cells used as a stem cell substitute. In co-culture, stromal cells induce NCCIT cells through diffusible molecules to lose stem cell gene expression, gain expression of prostate genes, alter cytomorphology, and lower proliferation. This NCCIT response is varied as co-cultured bladder stromal cells induce a different gene expression. At the same time, NCCIT factors also affect gene expression of co-cultured stromal cells. NCCIT induces normal prostate tissue (NP) stromal cells to become more like cancer-associated (CP) stromal cells in both mRNA and microRNA expression. In contrast, NCCIT shows minimal effect on CP stromal cells. CP stromal cells may represent a less differentiated state in the prostate stromal cell lineage.

Keywords: Prostate cancer, cell type transcriptomes, CD26^{+} cancer cells, CD90^{+} cancer-associated stromal cells, Gleason patterns, stromal induction of stem cells, NCCIT embryonal carcinoma cells, in vitro co-culture, cell-cell interaction, intercellular signaling molecules, mRNA and miRNA expression changes

1. Introduction

The prostate is a multicellular organ and intercellular communication is essential to maintaining tissue homeostasis. Loss of this communication through, for example, aberrant expression of signaling molecules or surface receptors could be the basis for the development of diseases including cancer. The relatively small number of major cell types of the prostate can be identified by their unique complement of cell surface molecules, the so-called cluster designation (CD) antigens [1]. CD antibodies can be used to target cells for isolation: CD26/DPP4 for luminal secretory epithelial cells, CD104/ITGB4 for basal epithelial cells, CD49a/PELO for stromal fibromuscular cells, CD338/ABCG2 for putative progenitor cells, CD31/PECAM1 for endothelial cells, CD45/PTPRC for white blood cells. In tumor, the tissue composition is altered and the component cell types have a unique complement of CD molecules that differs from that of the respective normal counterpart. The cancer cells, like luminal cells, are positive for CD26 but negative for CD10/MME and CD13/ANPEP; the tumor-associated stromal cells differ from normal tissue stromal cells by increased expression of CD90/THY1. Basal cells are missing in cancer. The gene expression or transcriptomes of the respective epithelial and stromal cell types have been

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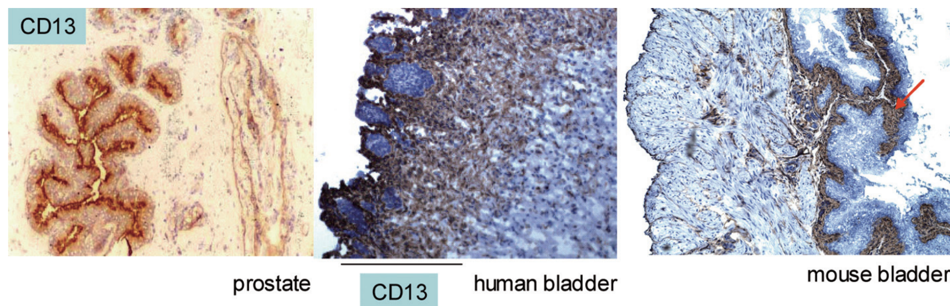

Fig. 1. CD13 immunohistostaining. CD13 expression is localized to the prostate luminal cells (left), superficial lamina propria for both human (middle), and mouse (right, red arrow) bladder.

determined [2–4]. Differential gene expression found suggests that stromal-epithelial interaction in normal prostate would differ from that in cancer prostate. What is the molecular mechanism of cell-cell interaction? We are far from answering this question. In this chapter, we will describe some of our recent experiments in our attempt to understand this process.

The title of this chapter would suggest that we are studying the interaction between cancer epithelial and stromal cell types within primary tumors. The difficulty of this type of study is manifold. First, primary tumor specimens we get from surgeries are frequently too small to yield enough quantities of the cell types for experimentation. Second, the epithelial cancer cells appear "unculturable", i.e., do not propagate in vitro, while the available cancer cell lines have very different gene expression and CD phenotype from those of primary tumor cells [5,6]. Nevertheless, we have developed cell sorting techniques to target specific cell populations for isolation. With development of microfluidics technology and nanomachines interaction between small numbers of cells could likely be studied in the future. Here, we will describe the cell signaling biology of one key cell type – stromal mesenchyme fibromuscular. In the adult prostate, these stromal cells display smooth muscle differentiation on the basis of certain characteristic marker expression.

1.1. Stromal cells as the determinant of prostatic epithelial differentiation

Gerald Cunha did the ground breaking work on stromal-epithelial interaction in prostate development [7]. The prostate develops from the urogenital sinus, which contains stromal mesenchymal and epithelial elements. In a reciprocal fashion, the mesenchyme induces epithelial development and the epithelium induces smooth muscle cell differentiation as shown by in vivo tissue recombinant experiments. This bidirectional communication appears to be evolutionarily conserved between human and rodents. The effect of androgen hormone is mediated via androgen receptor (AR)-positive mesenchyme because an epithelial component lacking functional AR can still be induced by AR-positive mesenchyme. AR-negative mesenchyme, in contrast, gives rise to vagina-like structure regardless of AR status of the epithelium. More strikingly, adult bladder epithelium can be induced by embryonic mesenchyme into prostate-like structures [8]. Presumably, if bladder-specific mesenchyme were used a prostate epithelium could be converted to a bladder urothelium. Besides the interaction between epithelium and stromal mesenchyme, morphogenesis and functional cytodifferentiation are dependent on those with the basement membrane and extracellular matrix (ECM).

1.2. Organ specific stromal genes

What determines specificity in stromal induction since prostate stromal cells can induce prostatic epithelial development from progenitor/stem cells resident in either prostate or bladder? One possibility is that induction specificity is linked to specific gene expression of stromal cells in different organs. To test this, we compared the gene and protein expression of prostate and bladder stromal cells to determine if they could be distinguished from each other. CD immunohistochemistry showed differential staining reactivity between the stromal compartments of these two organs. In particular, a layer of 10–20 cells adjacent to the bladder urothelium is positive for CD13. This is an evolutionarily conserved feature between the human and mouse bladder (Fig. 1). In the prostate, CD13 is localized to the epithelium. We designated this CD13-positive bladder region the superficial lamina propria to contrast it with the CD13-negative remainder. Because of their

Table 1
Tissue distribution of CD molecules. "+/-" indicates weak staining. CD56 is NCAM1 and CD184 is CXCR4

	bladder superficial lamina propria	bladder remainder	bladder muscle bundles	prostate stroma	blood vessels
CD13	+	−	+	−	+
CD49a	scattered	scattered	+	+	+
CD56	−	−	+	+	−
CD184	+/−	+/−	+	+	+

proximity to the urothelial cells the CD13$^+$ cell type is postulated to be the one that functions in bladder stromal/epithelial interaction, and is the functional counterpart of the prostatic stromal cell type. Table 1 shows the reactivity of other informative CD markers. Since the stromal cells are fibromuscular, it is not unexpected that these CD molecules are also found in the bladder muscularis and blood vessel wall. Our first gene profiling study was done with cultured stromal cells. Stromal cells, because of their good plating efficiency, are readily grown in vitro to produce sufficient material for analysis and experimentation. Cell culturing, however, induces changes that include expression of non-stromal CD molecules such as CD10, CD13, CD26, CD44, CD49b, CD49f, CD107a/LAMP1 as well as that of those associated with cell proliferation such as CD81, CD95, CD147 [9]. Cultured stromal cells nevertheless retain expression of CD markers detected in stromal cells in situ. Cell culturing appears not to affect their inductive property (see below).

Using initially a 40K human cDNA-gene chip 22 genes were found to be differentially expressed in prostate (ranging from 3 to 26-fold higher relative to bladder) and 29 genes in bladder (ranging from 4 to 44-fold higher relative to prostate) [10]. Expression was verified by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The prostate genes include proenkephalin (PENK, 26-fold higher than in bladder), stanniocalcin I (STC1, 21-fold), trophinin (TRO, 13-fold), while the bladder genes include properdin (CFD/BF, 44-fold), claudin 11 (CLDN11, 18-fold), stanniocalcin II (STC2, 4-fold). Transcriptome analysis showed that gene expression of individual stromal cultures established from different patient specimens was not significantly different from each other. In addition, we used mass spectrometry proteomics to profile stromal secreted N-linked glycoproteins [11], since secreted proteins are likely candidates to mediate stromal signaling. We identified 116 proteins in the prostate culture media (serum-free) and 84 in bladder culture media. The actual numbers are probably higher as some proteins are "invisible" to the analytic method used. Undersampling is another problem. These results showed that stromal cells had secretory function, and $> 75\%$ of the identified proteins were annotated as either secreted or membrane-bound in Gene Ontology (GO) [12]. The GO functions ascribed to them include cell communication, physiological process, cell adhesion, development, and regulation of biological processes. Differentially expressed proteins between prostate and bladder were verified by Western blot analysis where antibodies were available to show, for example, prostate higher expression of STC1.

Later, we used Affymetrix GeneChip arrays (Human Genome U133 Plus 2.0 containing probesets representing ∼55,000 genes) to analyze sorted CD13$^+$ (and CD13$^-$) bladder stromal cells for dataset comparison with CD49a$^+$ prostate stromal cells to identify as many organ-restricted genes encoding secreted/extracellular proteins as possible, and to remove changes induced by cell culturing. This would allow a more directed approach to target specific proteins for detection by proteomics in expression validation. Comparative dataset analysis between CD13$^+$ bladder stromal and CD49a$^+$ prostate stromal identified 91 bladder and 288 prostate differentially expressed genes including SPOCK3 (sparc/osteonectin proteoglycan/testican), CXCL13 (chemokine ligand), PAGE4 (P antigen family member), CNTN1 (contactin), MAOB (monoamine oxidase) for prostate, and TRPA1 (transient receptor potential cation channel), HSD17B2 (hydroxysteroid 17-β dehydrogenase), IL24, SALL1 (Drosophila sal-like) for bladder [3].

1.3. Down-regulation of organ-restricted stromal genes in cancer

The prostate-specific expression of PENK protein was confirmed by immunohistochemistry using a rabbit antibody raised against a synthetic PENK peptide, (C)TGDNRERSHHQDGSDNE. Immunostaining was localized to stromal fibromuscular cells of the prostate, and not to stromal cells of the bladder. Bladder muscularis and blood vessels were also positive [10]. PENK expression likely associates with smooth muscle dif-

ferentiation. PENK is normally processed into opioid pentapeptides, Met- and Leu-enkephalin. Although antibodies to these processed products could detect expression at nerve endings in the prostate none was detected in the stromal cells [13]. Therefore, stromal-derived PENK in the prostate is either not processed or processed differently. Stromal production of PENK is notable because prostate luminal epithelial cells express CD10 (also known as neutral endopeptidase) [1], and CD10 possesses enkephalinase activity. PENK and CD10 could therefore constitute a signaling pathway in stromal/epithelial interaction. PENK expression was documented in embryonic mesenchymal tissues during differentiation [14]. We postulate that cancer could be due to defects in stromal/epithelial interaction as a result of missing key signaling molecules. PENK expression was examined in cancer by RT-PCR analysis of matched cancer and non-cancer microdissected tissue specimens. PENK expression was decreased or undetectable in the cancer samples [10]. Previously, we showed that cancer epithelial cells were CD10 negative [6], which would further suggest a link between PENK and CD10. Loss of enkephalinase activity and that of expression of the substrate PENK may be linked in the cancer process.

1.4. Prostate cancer-associated stromal cells with increased CD90 expression

We previously identified CD90 as up-regulated in stromal cells surrounding the tumor epithelium [6]. We found an increase in the average intensity of CD90 staining of these cancer-associated (CP) stromal cells. The CD90$^+$ CP stroma did not extend more than 10 stromal cell layers beyond the cancer cells (Fig. 2). In non-cancer (NP), a single layer of CD90$^+$ stromal cells surrounded benign glands. Increased CD90 expression was confirmed by quantitative RT-PCR analysis of matched CP vs. NP stromal cells obtained via laser-capture microdissection (LCM). Elevated expression of CD90 protein in cancer can also be detected in tissue digestion media (Fig. 2). CD90 is a GPI-anchored cell surface antigen, and is apparently released or shed into the media. In our prostate cell sorting procedure [15], tissue specimens (CP or NP) are minced and digested by collagenase in culture media overnight. The liberated cells are centrifuged and the cell-free media contain proteins made by the different cell types of the tissue, and these proteins are detectable with specific antibodies or by mass spectrometry proteomics [16]. In Fig. 2, stromal-derived CD90 and epithelial-derived TIMP1 in digestion media were analyzed by Western blots. Note the increase in CD90 for cancer specimens. Our use of proteomics methodology targeting N-linked glycoproteins identified a CD90 proteotypic glycopeptide, LDCRHE$\underline{\text{NTS}}$SSPIQYEFSLTR ($m = 2541.15$, N-glycosylation sequon underlined) with an increased amount in media from digested cancer samples. Additional CD90 peptides detected in the media include HENTSSSPIQYEFSLTR ($m = 1994.92$, minus the N-terminal LDCR of the above) and DEGTYTCALHHSGHSPPISSQNVTVLR ($m = 2905.37$). There appears to be minimal protein degradation in these media preparations, and proteins expressed by prostatic cells such as PSA, ACPP and zinc-α2-glycoprotein (AZGP1) are readily detected for use as positive controls.

CD90 was originally identified as a T-cell marker and is expressed in primitive hematopoietic progenitor cells, thymocytes, and fibroblasts. Studies have reported differential expression of CD90 in fibroblasts from many tissues including lung, myometrium, and orbit [17]. CD90 expression distinguishes fibroblasts by their differentiative potential: myofibroblastic CD90$^+$ vs. lipofibroblastic CD90$^-$ [18], or responsiveness to growth factor signaling: CD90$^-$, but not CD90$^+$, fibroblasts respond to PDGF, IL-1β, IL-4 with increased TGFβ activity, Smad3 phosphorylation, expression of smooth muscle actin and fibronectin [19]. Although the function of CD90 in prostate is unknown, it is likely that the CD90$^+$ CP stromal cells could function differently from their NP counterpart.

Differential expression of CD90 provided us a means to isolate CP stromal cells for transcriptome analysis [3] and cell culture. Thus, CD90$^+$ cells were sorted from tumor specimens [characterized by increased CD90 and absent TIMP1 (see Fig. 2)] by MACS and analyzed by Affymetrix GeneChips. The stromal datasets, as expected, contained no epithelial genes such as CD26, CD10, AMACR and HPN. LCM could also be used but without CD90 immunostaining there would be the possibility of capturing neighboring NP stromal cells. Dataset comparison between CD90$^+$ CP stromal and CD49a$^+$ NP stromal showed the previously reported decrease in calponin (CNN1) [20], decrease in PENK, and increase in the Wnt pathway member secreted frizzled-related SFRP4 [21]. Stromal smooth muscle cells are characterized by desmin/DES, caldesmon/CALD1, α-smooth muscle actin/ACTA2 expression in contrast to myofibroblasts by ACTA2, vimentin/VIM expression and fibroblasts by VIM expression. Dataset query for expression levels of these genes as represented by array signal intensities between CP

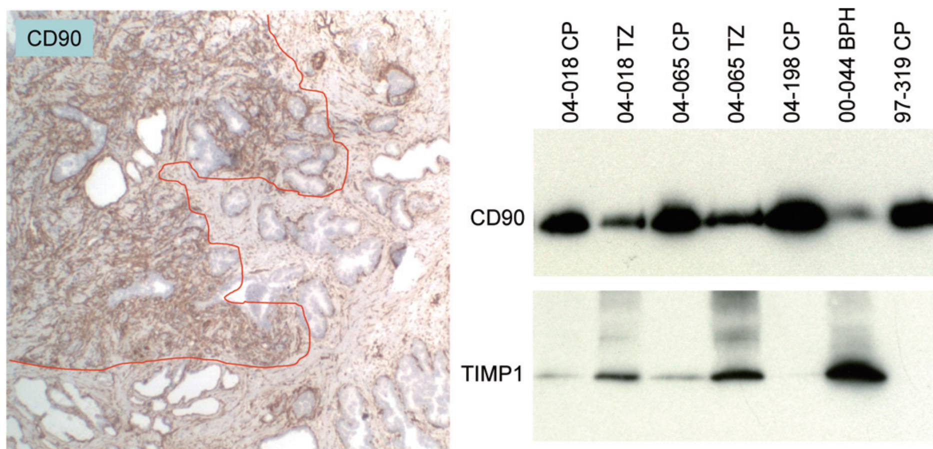

Fig. 2. Cancer-associated stromal cells. Left: CD90 immunostains the stromal cells within a tumor focus (outlined in red). Weaker staining is seen in the non-cancer area. Right: Western blot analysis shows that CD90 protein can be detected in tissue digestion media. TZ is transition zone of specimens 04-018 and 04-065 with matched TZ cancer (CP). The other 2 CP (04-198 and 97-319) are peripheral zone cancer. BPH is non-cancer. TIMP1 is not made by cancer. Presence in CP is due to non-cancer "contamination".

and NP stromal cells showed at least 4-fold decrease for ACTA2, DES, CNN1, and 2-fold for CALD1 in CP stromal cells [3]. No significant difference was seen for AR and VIM. This could indicate a decrease in smooth muscle expression in the CP stroma in contrast to NP stroma.

In addition to PENK, gene expression analysis showed decreased cancer expression of the identified prostate stromal genes CNTN1, CXCL13, MAOB, PAGE4, SPOCK3. Similarly, bladder tumor-associated stromal cells were isolated (by CD13), analyzed by Affymetrix, and the resultant transcriptome compared to that of $CD13^+$ bladder stromal cells showed decreased expression in the bladder stromal genes HSD17B2, SALL1, TRPA1 in bladder cancer [3]. Functionally, PENK is a hormone with a role in development, CNTN1 appears to mediate cell surface interactions in nervous system development through signaling between axons and myelinating glial cells, SPOCK3 encodes a secreted protein involved in diverse steps of neurogenesis, MAOB catalyzes oxidative deamination of biogenic and xenobiotic amines with important roles in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues, TRPA1 functions in signal transduction and growth control, HSD17B2 may have a role in bone development, and SALL1 a role in kidney development. References to these study results can be found in *NCBI Entrez Gene*. Interestingly, the bladder stromal SALL1 and IL24 were found expressed in prostate tumor-associated stromal cells. We postulate that these changes in CP stromal cells would be the basis for abnormal stromal signaling in cancer.

1.5. Tissue organization field theory and somatic mutation theory in carcinogenesis

Possible defects in stromal/epithelial interaction in cancer would lend support to the tissue organization theory of carcinogenesis [22]. It equates cancer to inborn errors of development where cancer arises as a result in disrupted reciprocal intercellular signaling that maintains tissue organization, repair and homeostasis. Cancer cells are cells free of their negative control to proliferate and migrate. For example, irradiation of breast stroma caused tumor formation from implanted non-irradiated mammary epithelial cells while non-irradiated stroma did not, implying an active stromal influence [23]. No loss of heterozygosity and copy number changes were detected in cancer-associated stroma of breast and ovarian tumors [24]. In contrast, the somatic mutation theory suggests that altered gene expression, due to DNA mutations, leads to uncontrolled cell proliferation in cancer. It does not require that the stroma be altered or involved. Currently, only familial cancers can be ascribed to inherited mutations, whereas for the majority of sporadic cancers the responsible genetic lesions have not been clearly defined [25].

1.6. Cancer-associated stromal cells in carcinogenesis

The involvement of stromal cells in cancer is suggested by the altered stroma in tumors. Stromal alterations could be due to DNA methylation [26], loss of heterozygosity [27], tumor promoting secretory prod-

ucts [28], or loss of p53 function [29]. A series of experiments were done with CAF (carcinoma-associated fibroblasts) that were prepared from prostate tumors. In culture, CAF produce more colonies in soft agar and expresses more TGF-β1 than stromal cells from benign tissue [30]. Normal prostate stromal cells start to resemble CAF when co-cultured with tumor cells. For the biological effect of CAF, SV40TAg-immortalized, nontumorigenic BPH-1 was used as the epithelial target. CAF was able to convert BPH-1 into a transplantable tumorigenic line after the recombined cell types were encased in the mouse kidney capsule [31]. Non-cancer stromal cells and BPH-1 did not produce any tumorous outgrowth. With testosterone and 17β-estradiol, BPH-1 (negative for androgen and estrogen receptors) in the presence of stromal cells was made to produce massive tumors [32]. The tumors that developed resembled squamous cells in appearance, they exhibited high-grade malignant features and were positive for both luminal and basal cytokeratins. In addition, the tumors responded to castration by undergoing involution. This model showed that hormone imbalance may alter the stromal/epithelial interaction, an event that can trigger cancer development. Despite these results, this model of prostate carcinogenesis, like others, displays only certain but not all features of the human disease. The transformed BPH-1 progeny are unlike the luminal-like cancer cells found in primary tumors. What is the gene expression of this luminal-like cancer cell type?

1.7. Prostate cancer cells and luminal cell counterpart gene expression difference

Like the transcriptomes of the major cell types of the prostate, the transcriptome of prostate cancer cells and that of CP stromal cells discussed above are important for our dataset analysis of cell-cell interaction experiments. Since all of our cell type-specific transcriptomes are established from cell sorting that of cancer cells was obtained by that route as well. LCM of cancer cells is the alternative route, and a large number of genomics datasets of LCM cancer samples are available. Due to the heterogeneity in prostate cancer, multiple cancer cell transcriptomes may likely be the case. Many studies have indicated that the normal counterpart of cancer cells is the luminal cell type, and certainly cells in Gleason pattern 3 tumor foci have similar features with luminal cells but not basal cells, including expression of CD26. Thus, cancer cells were sorted by using the luminal marker CD26 from Gleason 3 + 3 tumors and analyzed by Affymetrix arrays. Absent expression of CD10 and CD13 distinguished these cancer cells from $CD10^+$/$CD13^+$ luminal cells. This cancer CD phenotype, characterized by $CD10^-$ and $CD13^-$, is found in a majority ($\sim$70%) of tumors; others are $CD10^+$/$CD13^-$, $CD10^-$/$CD13^+$, or $CD10^+$/$CD13^+$ [6]. As expected, basal cell CD markers are rarely found in these tumors. Comparison between one such $CD26^+$ cancer cell transcriptome and $CD26^+$ luminal cell transcriptome identified 121 genes with increased expression and 86 genes with decreased expression by at least 8-fold relative to that of $CD26^+$ luminal cells [4]. Gene expression of CD10, CD13, CD24, CD26, and CD38 was concordant with immunohistochemistry: CD10 negative, CD13 negative, CD26 positive; increased CD24 and decreased CD38 compared to luminal cells. The elevated signal levels for known prostate cancer genes AMACR, HPN, CRISP3 and PCA3 indicated that the transcriptome was indeed of cancer.

The luminal-like cancer cells, like their luminal counterpart, are difficult to culture in vitro due in large measure to the fact that they are well-differentiated morphologically and post-mitotic. Thus, it is difficult to obtain large quantities of these cells in order to study their cell-cell interaction, for example, with stromal cells. Most tumor specimens could not provide enough cells for various experimental conditions. Although a number of prostate cancer cell lines are available. These were established mainly from metastatic lesions: lymph node-LNCaP, bone-PC3, and brain-DU145, for example. Line C4-2 was derived from LNCaP through in vivo interaction with bone stromal cells and line CL1 was derived from LNCaP via in vitro selection for androgen-independent growth [33]. The transcriptomes and CD immunoreactivity profiles of these cell lines have been determined by us. Overall, their gene expression and CD phenotype were found to differ substantially from the corresponding ones of the $CD26^+$ cancer cells. Therefore, it is highly unlikely that any of these cell lines could have derived from the $CD26^+$/$CD10^-$/$CD13^-$ cancer cell type found in primary tumors. In many reported studies, for example ref. 34, stromal influence on these cell lines appears to increase their growth potential and tumor formation in mice. However, stromal influence on $CD26^+$ cancer cells remains to be studied. For example, what is the effect of NP stromal cells on cancer cell gene expression?

1.8. Stromal induction of stem cells

Our lab has previously isolated and characterized a putative prostate stem cell population from tissue specimens based on differential expression of the membrane transporter ABCG2 [35]. This approach unfortunately cannot routinely provide sufficient quantity of cells for in vitro experimentations. Embryonic stem (ES) cells, derived from the inner cell mass of blastocyst, are capable of developing into every functional cell type [36]. In combination with either mouse urogenital sinus or rat seminal vesicle mesenchyme, human ES could be induced to form prostate-like structures with PSA synthesis in the renal capsule of immune deficient mice about 50% of the time. There were p63-labeled basal cells and periglandular smooth muscle cells indicating a full tissue development. Androgen removal led to regression of the glandular structures [37]. Without stromal signaling, the ES cells gave rise to teratomas. This stromal signaling is conserved in evolution. Similarly, mouse ES cells could be induced by fetal rat bladder mesenchyme to form bladder-like structures with expression of bladder-specific uroplakin and containing p63-positive basal cells [38]. It seems that more than one cell type (e.g., luminal and basal epithelial) could result from this stromal induction. How do stromal cells and stem cells interact? In order to gain a molecular picture, an in vitro system that can model cell-cell interaction needs to be developed. In this way, signaling molecules may be identified.

For an in vitro model, we used embryonal carcinoma (EC) cells as an ES cell surrogate. EC cells, derived from teratocarcinomas, resemble ES cells in morphology, differentiative capacity, surface antigen expression, and stem cell maintenance by the transcription factor POU5F1/OCT4 [39,40]. NCCIT is an established EC cell line, hyperdiploid (with 54-64 chromosomes), developmentally pluripotent that can differentiate into derivatives of all embryonic germ layers. It expresses ES cell markers including CD9, CD90, tissue-nonspecific alkaline phosphatase [41], histocompatibility complex antigens, SOX2 (sex determining region Y-box 2), NANOG, POU5F1, TDGF1/Cripto (teratocarcinoma-derived growth factor), DNMT3B (DNA cytosine-5-methyltransferase 3β), GABRB3 (GABA A receptor β3) and GDF3 (growth differentiation factor) [42]. Like ES cells, NCCIT cells express stem-cell antigens such as SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 [43]. In global gene expression, EC and ES cells appear to be virtually identical [42, 44] although genes from chromosome 12 are overrepresented in EC lines [45], which is possibly related to tumor biology [46]. Several laboratories have shown that when ES cells were cultured for extended periods genetic and epigenetic changes were found [47, 48]. In mice, EC xenografts showed foci of immature somatic tissue, trophoblastic giant cells in addition to embryonal carcinoma and yolk sac tumor [43]. Likewise, ES cells when implanted in mice can also develop into teratomas and undifferentiated tumors [49, 50]. In culture, EC cells grow in spherical aggregates and respond to retinoic acid with growth arrest and change in morphology. The retinoic acid-treated cells were shown to produce keratin, fibronectin, laminin, uvomorulin, desmoplakin, glial fibrillary acid protein, and neurofilaments [51]. Expression silencing of core transcription factors OCT4, SOX2, NANOG could also lead to differentiation with the resultant cells displaying a flattened morphology and showing reduced proliferation [44]. Despite their origin, EC cells and ES cells appear indistinguishable in their biology. However, ES cells in culture require embryonic fibroblasts and supplementary factors. Because EC cell lines are simpler to maintain, they provide an attractive alternative to ES cells, and are used to develop experimental techniques and approaches before use of ES cells for validation [40]. We have determined the NCCIT transcriptome, and confirmed their expression of ES genes such as NES (nestin), NANOG and BMI1.

Stromal induction of NCCIT served as our initial functional test of stromal cell biology. In the culture format, the two cell types are separated by a membrane barrier that allows diffusion of secreted stromal cell or NCCIT protein factors [52]. Multiple cultures were set up for various incubation time points. Physical separation allowed the cell types to be harvested and analyzed individually. Our experiments showed that NCCIT responded to stromal signaling with loss of stem-cell marker expression, reduction in proliferation, change in morphology and gain of prostatic marker expression. These alterations became evident in 3 to 5 days. Treated NCCIT cells were flattened in a monolayer (Fig. 3) with a decrease in the number of alkaline phosphatase (ALP)-positive cells, which was confirmed by Western blot analysis (Fig. 3). This stromal induction of NCCIT cells was effected by diffusible factors as use of stromal cell conditioned media produced the same result. This cell-free conditioned media was prepared by centrifugation and filtration of stromal cell cultures. Western blot and mass spectrometry proteomic analyses showed that stromal cell-derived proteins were present in these media preparations [10,11].

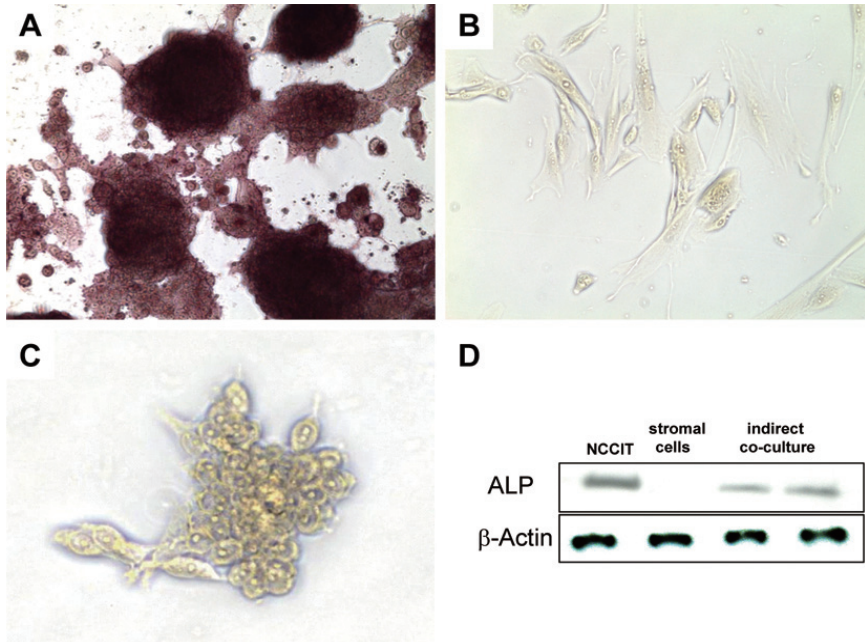

Fig. 3. Stromal induction of EC cells. Untreated and treated NCCIT cells were analyzed for alkaline phosphatase (ALP) immunostaining after 5 d in culture. (A) Untreated NCCIT cells are strongly stained. (B) Prostate stromal cells are unstained. (C) Treated NCCIT cells with stromal cell media are unstained and have a flattened morphology. (D) Western blot verified the ALP (75 kDa) expression patterns. β-actin (42 kDa) was used as sample loading control.

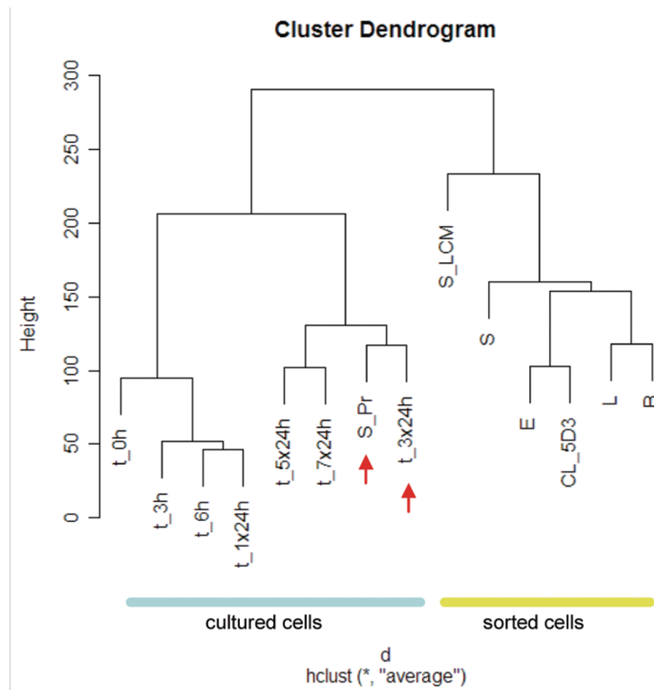

Fig. 4. Stromal expression of treated NCCIT. Clustering analysis of transcriptome datasets. S, stromal cells; E, endothelial cells; CL_5D3, progenitor cells; L, luminal; B, basal; S_LCM, laser-captured stromal cells. In clustering, whole transcriptomes were used to measure Euclidean distances. Arrows point to S_Pr, cultured stromal cells; and treated NCCIT at 3d.

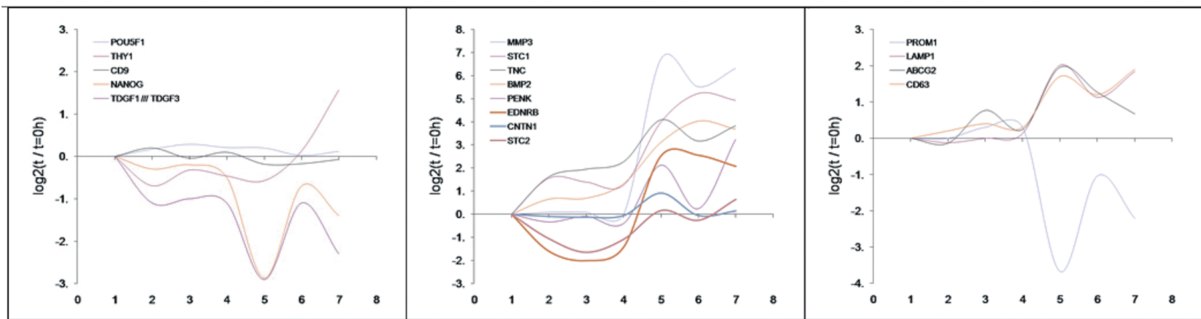

Fig. 5. Gene expression of treated NCCIT. Display of array data to show down-regulation of stem cell markers (left panel); up-regulation of prostatic stromal cell markers including PENK (middle panel), and markers not associated with stromal cells (right panel). Numbers on the x-axis indicate days of co-culture.

Gene expression of stromal induced NCCIT cells was analyzed by Affymetrix arrays. A key to probing response of NCCIT cells in the context of the prostate was the availability of previously determined prostate cell-type specific transcriptomes [2,10,35]. Data quality of these transcriptomes is described in our report [53]. The culture time points analyzed were 3h, 6h, 24h, 3d, 5d and 7d. Dataset clustering showed that treated NCCIT cells had a gene expression most similar to that of cultured prostate stromal cells (Fig. 4). Treated NCCIT showed decreased expression of stem cell markers POU5F1, NANOG, TDGF1 and increased expression of prostatic cell markers ITGA2/CD49b, CD47, ITGB1/CD29, CD63, LAMP1/CD107a and EGP (epithelial glycoprotein) (Fig. 5). RT-PCR was used to verify expression detected by DNA microarrays of these genes [52]. Increased expression of stromal cell markers MMP3, STC1, TNC, BMP2, PENK, EDNRB, CNN1, CNTN1 was similarly verified (Fig. 5). Because of the presence of non-stromal markers like EGP, the "differentiated" NCCIT cells would appear to be a hybrid cell type. Determining whether these markers are all expressed by one cell or they indicate the presence of multiple cell types (stromal and epithelial) will involve cell sorting by the use of appropriate cell surface markers for gene expression analysis and immunocytochemistry.

Despite the presence of EGP expression, genes of prostate luminal epithelial cells like AR, KLK2/hK2, KLK3/PSA, KLK4 were not detected. This is not unexpected because direct stromal cell contact and ECM are required for luminal cells to express markers of fully functional differentiation, for example, PSA [15]. Luminal cells when isolated free from tissue lose PSA expression (RNA scored by RT-PCR and protein by ELISA) within a short time. Expression is restored when stromal cells and ECM material are added to the luminal cells. Indeed, in half of the co-cultures of NCCIT and prostate stromal cells with cell contact and Matrigel (an ECM substitute), PSA was detected in the media by ELISA. Admixture of the cell types, however, makes array analysis of the experiments more difficult. There is the need to free the cells from Matrigel, separate the stromal cells (which would affect PSA expression) in reasonable time to analyze the treated NCCIT cells. Alternatively, immunocytochemistry could be developed as an analysis tool.

Viewed in another light, the above experiments demonstrated that stromal factors could be employed as cancer therapeutics for they can render a highly proliferative stem cell with tumorigenic property into a differentiated, less proliferative derivative.

1.9. Induction with bladder stromal cells

As a test of the specificity in stromal induction and plasticity in NCCIT response, we performed co-cultures using bladder stromal cells prepared from surgical tissue specimens [52]. The induced gene expression changes were dissimilar to those induced by prostate stromal cells (Fig. 6). For example, there was no up-regulation of prostate PENK; instead, up-regulation of bladder GFRA1 was detected. STC1 and STC2 are notable - expression of STC1 is higher in prostate than bladder, whereas that of STC2 is higher in bladder than prostate [10]. This was reflected by different gene expression levels of STC1 and STC2 in prostate vs. bladder induction. Thus, NCCIT in vitro, like ES cells and rodent mesenchyme in vivo, can respond to divergent signaling and produce particular cell lineages determined by that signaling.

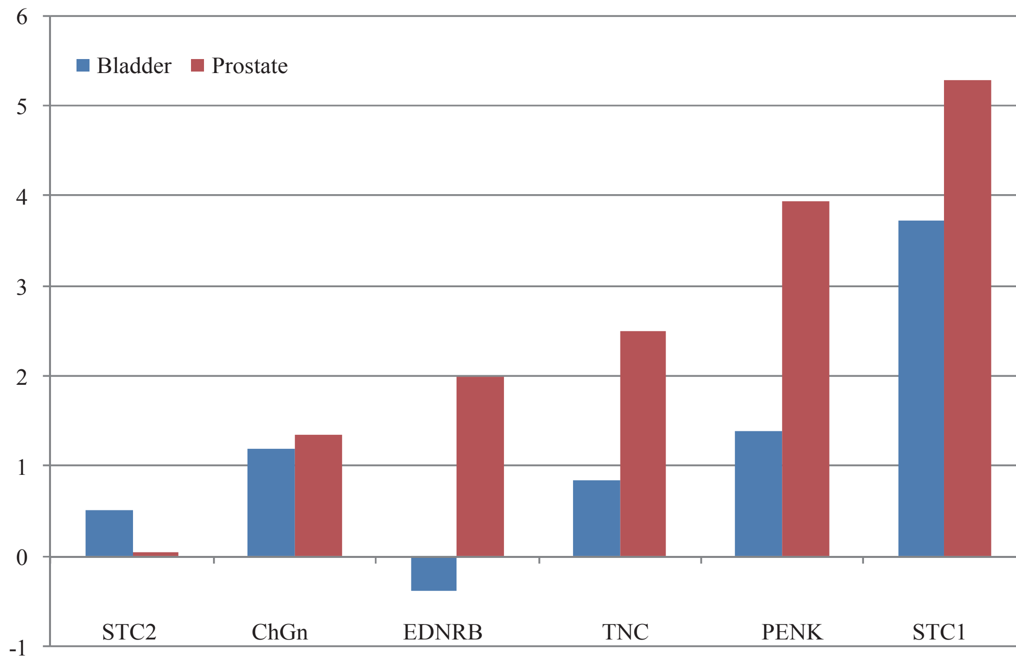

Fig. 6. Bladder stromal induction of NCCIT. The histogram shows prostate-induced vs. bladder-induced signal values for the genes listed.

1.10. NCCIT induction of NP stromal cells

Taranger et al. [54] reported that extract prepared from NCCIT cells when added to permeabilized kidney epithelial 293T cells caused up-regulation of NCCIT genes such as OCT4, NANOG, SOX2 and down-regulation of 293T genes with the recipient cells adopting a pluripotent cell phenotype. These gene expression changes, according to the authors, may be due to demethylation of gene promoters and chromatin remodeling. In light of these findings, could NCCIT cells also affect stromal cells through their secreted factors? With regard to NCCIT influence on stromal cells, we examined not only mRNA expression but also that of microRNA (miRNA) [55]. The importance of miRNA in the cancer process has recently been recognized with the discovery of miRNA functioning as oncogenes or tumor suppressors through their action on potential target mRNAs [56,57]. In prostate cancer, miRNA expression could be regulated by androgen signaling and may contribute to the development of hormone refractory disease [58]. In structure, miRNAs are small $\sim$20 nucleotide long non-coding RNAs that could recognize specific mRNA(s) through complementary binding to their 3' untranslated region. The binding leads to RNA degradation and translation inhibition with loss of protein expression.

For miRNA expression, the following cultures were prepared: NCCIT alone, NP stromal alone, CP stromal alone, NCCIT and NP stromal, NP stromal and NCCIT, NCCIT and CP stromal, CP stromal and NCCIT, with the underlined cell types in the co-cultures analyzed. MicroRNA expression was determined by the Agilent Human miRNA Microarray, which contains probesets for 723 human and 76 human viral miRNA from the Sanger Database v.10.1. Probe RNA was labeled by cyanine 3-pCp with T4 RNA ligase. CP stromal cells were established from collagenase digestion of a Gleason 4 + 4 tumor specimen. Cultured NP stromal cells were derived from a non-cancer specimen. Our data showed that NP and CP stromal cells express different miRNA (Fig. 7) in addition to the difference in mRNA expression as described above. NCCIT showed its own characteristic miRNA expression (Fig. 7), which was unaffected by either NP or CP stromal influence as shown in the data display.

In contrast, NCCIT had different effects on NP and CP stromal cells. Whereas CP stromal miRNA expression was not significantly altered by NCCIT, that of NP stromal cells was. The expression pattern of NP stromal cells treated by NCCIT resembled that of CP stromal cells. The differentially expressed miRNAs in CP vs. NP stromal cells include hsa-miR-21, miR-923, miR-125b, miR-29a, miR-22, and miR-23a.

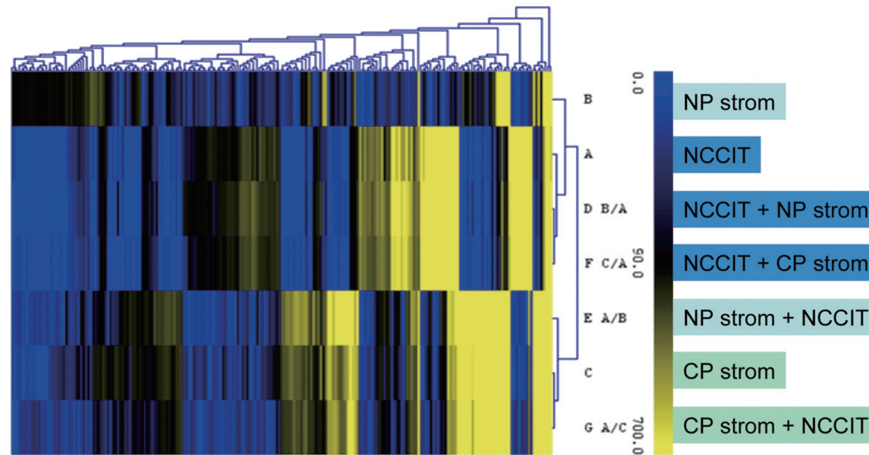

Fig. 7. Stromal cell miRNA expression. The heatmap shows clustering of miRNA expression for NP strom (lane marked B), CP strom (C), NCCIT (A), NCCIT induced by NP strom (D) or CP strom (F), NP stroma induced by NCCIT (E), CP strom induced by NCCIT (G).

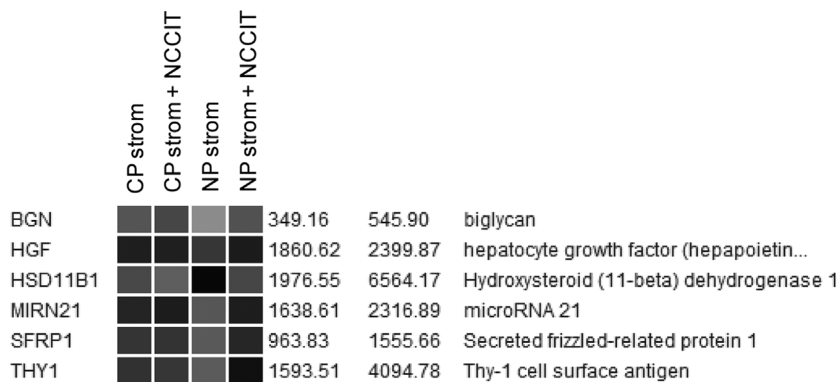

Fig. 8. NCCIT induction of stromal cells. The array signal display on a grayscale shows the increase in expression of BGN, HGF, MIRN21, SFRP1, THY1/CD90, and decrease in expression of HSD11B1 in NP stromal cells after co-culture with NCCIT.

NP stromal + NCCIT was also analyzed by Affymetrix GeneChips, where increased signal values were found for THY1, MIRN21, HGF, SFRP1, BGN, and decreased signal value was found for HSD11B1, for example (Fig. 8). The differential expression of SFRP1, BGN, IGFBP5 between (cultured) NP and CP stromal cells was also reported by Joesting et al. [59]. The signal values for these genes in CP stromal + NCCIT were not significantly different from those in CP stromal, showing that NCCIT had minimal effect on gene expression (for both mRNA and miRNA) in CP stromal cells. MIRN21 is the polyadenylated, capped transcript that encodes miR-21 [60]. Hence, both MIRN21 mRNA and miR-21 were detected by the corresponding arrays in NP stromal cells after NCCIT induction. miR-21 has been reported to have antiapoptotic activity [61]. HGF (hepatocyte growth factor) is an important signaling molecule in epithelial differentiation. In embryogenesis, HGF is produced by the undifferentiated mesenchyme, and its synthesis is less pronounced in adult tissue [62]. Note the $>$ 10-fold increase in THY1/CD90 in the NP stromal + NCCIT dataset compared to 3-fold increase in the CP stromal and CP stromal + NCCIT datasets over NP stromal. This increased CD90 expression is reflective of CD90 immunostaining of the tumor-associated stroma. Thus, NCCIT factors could up-regulate CD90 expression in prostate stromal cells as well as cause genome-wide expression changes.

1.11. Unanswered questions

These studies illustrate that interaction between stromal and epithelial cells in cancer is abnormal based on the differential gene expression of cancer cells and tumor-associated stromal cells from their respective normal counterpart. If NP stromal cells could induce

normal epithelial differentiation then would CP stromal cells induce differentiation that results in a cell type with cancer-like gene expression given that the organ-specific stromal genes are down-regulated in cancer? In addition, is the lack of basal cells in tumor glands also a result of defective CP stromal signaling?

Is there variability in gene expression among CP stromal cells from different tumors? Since prostate tumors can be distinguished by Gleason grades and also by their composition of tumor cell types (e.g., $CD10^{-}$ vs. $CD10^{+}$) [6,63], could the associated stromal cells be distinguished by gene expression as well?

Does stem cell response require continuous stromal influence? Thus, in the absence of stromal factors, would induced NCCIT cells, for example, revert? It is likely they do not, and the induced state would be maintained without continuous stromal stimulation.

Could stromal induction be exclusive or cumulative? If stem cells are first treated with CP followed by NP stromal, or first with NP followed by CP stromal, would the gene expression changes be a summation of both induced datasets? One outcome would be that a second induction produces no further significant changes, i.e., NCCIT, once induced, could no longer respond to another set of signaling molecules. This concerns whether the cancer process could be reversed if normal signaling is restored.

Is there a hierarchy of stromal induction such that when both NP and CP stromal influences (as in areas where tumor tissue abuts non-cancer) are applied only one response is observed? One possible outcome is that NP stromal induction overrides that of CP stromal, such that the normal influence is dominant whereas the non-normal is recessive. Similarly, experiments can be carried out to compare prostate stromal and bladder stromal with regard to "dominance" or "recessiveness" in response of stem cells to two competing sets of signaling. A likely result is that prostate and bladder stromal signaling are mutually exclusive such that a stem cell responds to either prostate or bladder signaling but not both. Answers to these questions would have implication in how organ development proceeds.

Are there other cell types susceptible to stromal induction? Are primitive stem cells such as NCCIT unique in their response to stromal signaling? For prostate, cell lines that have stem cell expression signature (e.g., CD44, CD49b, CD49f, CD133) as well as isolated adult prostate stem cells are potential candidates for testing.

The above questions could equally apply to NCCIT induction of NP stromal cells. Is continuous NCCIT induction required to maintain the CP stromal-like expression in treated NP stromal cells? In addition, could NP stromal-induced NCCIT (after purging of uninduced cells) affect NP stromal miRNA/mRNA expression? Since the treated cells have lost their stem cell characteristics, could they still alter expression in NP stromal cells? Is the stromal inductive property unique to NCCIT? Could ES cells produce a similar alteration? A prostate cancer cell line, CL1 [64], with stem-cell markers could be tested to see if cancer-specific factors are responsible.

What are these cell-cell signaling factors? We have shown that stromal cell conditioned media could also induce EC cells, these factors could feasibly be identified from these media preparations of NP stromal cells. At near confluence, stromal cells are rinsed with media, and the culture media is changed to serum-free media for 24h. Within this time span, there is minimal induction of serum deficiency shock proteins. Serum-free treatment is called for because the fetal bovine serum supplement would overwhelm proteomic analysis. Media from several cell cultures are combined, and made cell-free. Proteins in the media can be concentrated by using filtration devices. After concentration, different amounts of the protein preparation are added to stem cells. These experiments will show if the stromal activity is concentration dependent, and that protease treatment would abolish the activity. Similarly, NCCIT factors can be studied in this manner. Are these factors specific to cancer (i.e., absent in ES cells)? The collagenase tissue digestion media preparations [16] of tumors with Gleason pattern 3, Gleason pattern 4, xenografts (representative of adenocarcinoma and small cell cancer) could all be tested on NP stromal cells.

2. Conclusion

In summary, stromal cells have the ability to alter the gene expression of (EC) stem cells through secreted factors leading to phenotypic changes in the responding cells. Stromal induction, furthermore, shows organ specificity. Factors derived from EC cells appeared to convert gene expression of stromal cells to a state similar to that of tumor-associated stromal cells. Extensive gene expression changes could thus result from intercellular signaling between two human cell types. This signaling could produce differential response depending on the cell types involved. The tumor-associated stromal cells constitute a major cell type of the tumor microenvironment, and their contribution to carcino-

genesis is still undefined. With advances in molecular analysis technologies, we may be able to uncover their precise functioning.

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