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Embryonal carcinoma cell induction of miRNA and mRNA changes in co-cultured prostate stromal fibromuscular cells

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Abstract

The prostate stromal mesenchyme controls organ-specific development. In cancer, the stromal compartment shows altered gene expression compared to non-cancer. The lineage relationship between cancer-associated stromal cells and normal tissue stromal cells is not known. Nor is the cause underlying the expression difference. Previously, the embryonal carcinoma (EC) cell line, NCCIT, was used by us to study the stromal induction property. In the current study, stromal cells from non-cancer (NP) and cancer (CP) were isolated from tissue specimens and co-cultured with NCCIT cells in a trans-well format to preclude heterotypic cell contact. After 3 days, the stromal cells were analyzed by gene arrays for microRNA (miRNA) and mRNA expression. In co-culture, NCCIT cells were found to alter the miRNA and mRNA expression of NP stromal cells to one like that of CP stromal cells. In contrast, NCCIT had no significant effect on the gene expression of CP stromal cells. We conclude that the gene expression changes in stromal cells can be induced by diffusible factors synthesized by EC cells, and suggest that cancer-associated stromal cells represent a more primitive or less differentiated stromal cell type.

In the prostate, stromal mesenchyme smooth muscle cells mediate organ-specific epithelial differentiation and function (Marker et al., 2003). A bladder mesenchyme will induce bladder differentiation even with stem/progenitor cells isolated from the prostate, and a prostate mesenchyme will induce prostate differentiation with stem cells isolated from the bladder (Aboseif et al., 1999). The inductive property of prostate stromal cells was shown

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recently by us using a co-culture system. An embryonal carcinoma (EC) cell line, NCCIT (Damjanov et al., 1993), responded to prostate stromal signaling by undergoing differentiation with expression loss of stem cell markers, expression gain of prostatic markers, reduction in cell proliferation and change in morphology (Pascal et al., 2009b). NCCIT showed plasticity in response in that bladder stromal cells induced expression of a marker profile more of bladder cells (Pascal et al., 2009b). What mediates this stromal signaling is currently unknown. One possibility are genes differentially expressed between prostate and bladder stromal cells that encode secreted proteins or hormones like PENK (proenkephalin) with a signaling function (Goo et al., 2005; 2009).

In prostate cancer, in addition to the gene expression difference between cancer cells and their normal counterpart luminal cells, one was found between cancer-associated stromal cells and their normal counterpart (Pascal et al., 2009a). The cancer-associated stromal cells are characterized by increased expression of CD90 or THY1, and can be isolated from tissue specimens by the use of CD90 antibodies (True et al., 2010). These CD90⁺ stromal cells were shown to have down-regulated expression of genes involved in smooth muscle cell differentiation and those that are expressed in the prostate and not the bladder (i.e., organ-restricted, Pascal et al., 2009a). What is the origin of the prostate cancer-associated stromal cells? One possibility is that multipotent mesenchymal stromal cells (MSC) are recruited to constitute the tumor stroma (Santamaria-Martinez et al., 2009). Although CD90 is a marker of MSC, the lower expression of other MSC markers CD13, and POU5F1/OCT4 in cultured cancer-associated stromal cells, and the similar expression of MSC markers CD29, CD44, CD105, CD166 in cultured normal tissue stromal cells suggest this is unlikely (Zhao and Peehl, 2009). Another possibility is epithelial-mesenchymal transition (EMT) of malignant epithelial cells (Gonzalez-Moreno et al., 2010). Since post-mitotic luminal-like cancer cells cannot be cultured, it will be difficult to experimentally show the EMT process. Even if not derived from MSC, the CD90⁺ cancer-associated stromal cells could still represent a more primitive, less differentiated cell type in the stromal lineage.

In our co-culture study of NCCIT and stromal cells, the inductive effect of stromal cells on NCCIT was examined. However, there might also be an effect of NCCIT on the stromal cells as cell-cell interaction and signaling could well be bidirectional. In this study, we examined the influence of NCCIT cells on both normal tissue stromal and tumor-associated stromal cells. Prostate cancer-associated stromal cells can be isolated from tumor tissue specimens and normal tissue stromal cells from non-cancer specimens after digestion with collagenase. Both stromal cell types can be grown in vitro in serum-supplemented media. Cultured stromal cells derived from tumor and normal tissue appear to retain their gene expression difference (Joesting et al., 2005; Zhao et al., 2007). Our results showed that expression of both gene-encoding mRNA and noncoding microRNA (miRNA) in stromal cells derived from normal tissue was altered by secreted factors from NCCIT cells to resemble that of cancer-associated stromal cells. Taranger et al. (2005) reported that extract prepared from NCCIT when added to permeabilized kidney epithelial 293T cells caused up-regulation of stem cell genes such as POU5F1/OCT4, NANOG, SOX2, and down-regulation of 293T genes with the recipient cells acquiring a pluripotent cell phenotype. According to these researchers, the expression changes may result from demethylation of gene promoters and chromatin remodeling induced by the introduced NCCIT factors. EC cells are very similar to embryonic stem (ES) cells in gene expression (Sperger et al., 2003). NCCIT expresses stem cell markers including CD90, SOX2, NANOG, POU5F1, TDGF1 (Pascal et al., 2009b). Rather than introducing NCCIT extract directly into stromal cells, we chose to investigate factors secreted by NCCIT as this is a more natural way of intercellular communication. The effect of NCCIT factors on stromal cells was monitored via gene expression changes analyzed by DNA microarrays. This work provides experimental

evidence for the possible genesis of the prostate tumor-associated stromal cells and lineage relationship to normal tissue stromal cells.

Materials and Methods

Cells, tissue specimens and cell culture

The EC cell line, NCCIT, was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI1640 (Cambrex BioScience, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS). Isolation of stromal cells from normal prostate (NP) and prostate cancer (CP) tissue was carried out as described in a previous report (Pascal et al., 2009a). All excess tissue samples from surgically resected glands were approved for research purposes by the University of Washington Institutional Review Board. Approximately 0.3 to 0.7 g of tissue samples were processed to obtain cells for in vitro culture. Corresponding frozen sections were histologically assessed to confirm the phenotype of the selected specimens. Immunohistostaining of frozen sections with CD90 antibody (clone 5E10, BD-PharMingen, San Diego, CA) was done as before (Liu et al., 2004; True et al., 2010). The tumor samples used in this study were obtained from 08-032 CP (Gleason 4+4) and 09-124 CP (Gleason 4+3). The NP samples were obtained from the non-cancer part of surgically resected prostates. Western blot analysis was used to show CP as CD90^{hi}TIMP1^{lo} and NP as CD90^{lo}TIMP1^{hi} (Pascal et al., 2009a). Other gene expression datasets of 3 cultured CP and 7 cultured NP stromal samples were available for gene expression comparison.

Tissue samples were minced and digested by overnight incubation with 0.2% collagenase type I (Invitrogen, Carlsbad, CA) in RPMI1640, 5% FBS, 10^{-8} M dihydrotestosterone (DHT) on magnetic stirrer at room temperature. The resultant cell suspensions were filtered through 70- μ m Falcon cell strainers, diluted with equal volume of Hanks balanced salt solution (HBSS), and aspirated with 18-gauge needles. Cells were pelleted by centrifugation and partitioned into stromal and epithelial fractions on discontinuous Percoll density gradients (Amersham Pharmacia, Piscataway, NJ; Kassen et al., 1996; Liu et al., 1997). Stromal cells were collected, sorted with anti-CD90 for CP and anti-CD49a for NP. The sorted cells were cultured in RPMI1640, 10% FBS, gentamycin (Liu et al., 2000). Stromal cells were grown for several passages, and their identity was checked by RT-PCR analysis and Affymetrix GeneChip arrays as described (Goo et al., 2009). Serial culture was done to ensure elimination of any epithelial elements, which have a lower plating efficiency, and to produce enough cells for multiple replicate experimental conditions. Also, if the stromal cells were not adapted to culture, the (culture-adapted) NCCIT cells would overwhelm the stromal cells during the co-culture. Cultured stromal cells were harvested by trypsin digestion and placed in co-culture with NCCIT cells (Pascal et al., 2009b).

Co-culture experiments

For co-culture, inserts with 0.4- μ m polycarbonate membrane (BD Falcon, Franklin Lakes, NJ) to preclude heterotypic cell contact were used. Both prostate stromal and NCCIT cells were seeded at 10^4 /ml in RPMI1640, 10% FBS, 10^{-8} M DHT in 6-well plates. After overnight, the second cell type was plated on inserts to initiate the co-culture. The following test conditions were prepared: NCCIT, NP strom, CP strom, NCCIT + NP strom, NP strom + NCCIT, NCCIT + CP strom, CP strom + NCCIT with the first named cell types in the co-cultures to be analyzed by gene arrays. The cultures were maintained for 3 d based on our previous time course study in which stromal induction of NCCIT cells took no longer than 3 d (Pascal et al., 2009b). Cells in the wells to be analyzed were harvested for RNA isolation. Cells in the inserts were not harvested for analysis.

RNA isolation, and DNA microarray analysis

Total RNA containing both mRNA and miRNA was isolated using mirVana (Applied Biosystems/Ambion, Austin, TX). RNA concentration was determined by ND1000 spectrophotometer (NanoDrop, Wilmington, DE), and RNA quality was checked by Agilent 2100 Bioanalyzer RNA Nano Labchips (Agilent Technologies, Santa Clara, CA). Two replicates of each experimental condition were analyzed by both Agilent miRNA Human Microarray (miRNA V2 Oligo, Agilent Technologies), which contained oligonucleotide probesets for 723 human and 76 human viral miRNA culled from the Sanger Database v. 10.1, and Affymetrix Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA), which contained probesets representing 54,675 mRNA genes, splice variants, and ESTs.

For miRNA in culture media, 3.5 vol QIAzol reagent (Qiagen miRNeasy, Valencia, CA) was added to media plus 0.1 μ M spiked-in miRNA (miSPIKE, IDT, Coralville, IA) to monitor recovery, and 0.7 vol chloroform. After vigorous mixing, centrifugation at 12,000g, 15 min, the aqueous phase was collected and 1.5 vol ethanol added. This preparation was applied to silica membrane column for RNA isolation.

Array hybridization

For miRNA expression analysis, 100 ng RNA was dephosphorylated with calf intestine alkaline phosphatase, heat denatured in the presence of dimethyl sulfoxide, and labeled by cyanine 3-cytidine bisphosphate (3-pCp) with T4 RNA ligase. Unincorporated cyanine dye molecules were removed by MicroBioSpin 6 (BioRad, Hercules, CA). The labeled RNA was added to Agilent arrays in rotating hybridization oven at 10 rpm, 55° for 20 h. The arrays were washed in Agilent GE Wash Buffer 1/Triton X102 and Wash Buffer 2/Triton X102. After washing, the array slides were scanned at 5- μ m resolution using ScanArray Express (PerkinElmer, Waltham, MA). The resulting images were quantified with Agilent Feature Extraction.

For mRNA expression of stromal cells, 200 ng RNA was reverse transcribed with poly (dT) primer/T7 promoter, and the cDNA was made double-stranded. In vitro cDNA transcription was performed to produce unlabeled cRNA. Next, first-strand cDNA was again produced with random primer/T7 promoter followed by in vitro transcription to produce biotinylated cRNA probes. The GeneChips were prepared, hybridized, and scanned according to the protocols provided by Affymetrix (Oudes et al., 2006).

Microarray data analysis

The array results were normalized with global mean, and differentially expressed miRNA was identified by a standard protocol developed for mRNA gene arrays (Irizarry et al., 2003; Oudes et al., 2006). To promote data reliability, miRNA species with hybridization intensities less than 0.75X global mean intensity were excluded from analysis. Clustering was performed with Hierarchical Clustering algorithm provided by MultiExperiment Viewer MeV4.0 (<http://www.tm4.org/mev/>). Data analysis of mRNA arrays was described previously (Pascal *et al*, 2009b). 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 by the standard robust multi-array average method (Irizarry et al., 2003), implemented in the in-house analysis pipeline SBEAMS (Marzolf et al., 2006). In-house designed analysis software was used to group genes into clusters based on fold-change and normalized intensity, and then to cross-compare these datasets. The principal components analysis of transcriptome datasets was described in full by Pascal et al. (2009b).

PCR analysis

Quantitative PCR reactions were carried out with a kit for miRNA-210, which had been quality controlled by the supplier (Applied Biosystems). The analysis was conducted according to their Human TaqMan miRNA assay. RNU66 snoRNA was used as reaction control. All reactions were carried out in triplicate using Applied Biosystems 7300 Real Time PCR. The isolated RNA was treated with DNase using DNA-Free (Ambion) before PCR.

Results

Prostate cancer-associated stromal cells

Fibromuscular cells of the prostate stroma can be immunostained for a number of CD cell surface antigens: CD49a, CD56, CD184 (Goo et al., 2005). Unlike normal tissue stroma, the prostate cancer stroma was strongly stained by CD90 or THY1 (Fig. 1A). CD90 expression was seen in all tumors studied (True et al., 2010). The CD90 immunohistochemistry data are archived in our public database UESC (<http://scgap.systemsbio.net/data/>, Pascal et al., 2007). The figure shows that the CP stroma (right half of image) is comprised of a single CD90⁺ cell population, whereas the NP stroma (left half of image) is comprised of CD90⁺ cells plus a small percentage of CD90⁺ cells adjacent to the basal epithelium. It remains to be determined if these NP CD90⁺ cells are similar to the CP CD90⁺ cells. We compared the transcriptome of CD90⁺ CP stromal cells (Pascal et al., 2009a) and that of CD49a⁺ NP stromal cells (Oudes et al., 2006). On a principal components analysis plot defined by 4 cell types of the prostate: CD26⁺ luminal, CD104⁺ basal, CD49a⁺ stromal, CD31⁺ endothelial (Pascal et al., 2009b), the gene expression difference between NP stromal and CP stromal (two examples 1 and 2) was larger than that between luminal and Gleason pattern 3 (G3) cancer cells (Fig. 1B). This was indicated by the distance of separation between the respective transcriptome dataset nodes. On this plot, cell types with similar gene expression would cluster near each other; for example, the CD90⁺ CP stromal cells from different patients, or multiple cultures of NCCIT (not shown).

Co-culture of prostate stromal cells with NCCIT to study intercellular communication

Previously, the influence of stromal cells on NCCIT was examined by co-culture (Pascal et al., 2009b). Here, the reciprocal influence of NCCIT on stromal cells was examined. Stromal cells were isolated from tissue specimens and adapted for cell culture. For convenience, the cultured CD90-sorted cancer-associated stromal cells were labeled as CP strom while the CD49a-sorted normal tissue stromal cells were labeled as NP strom. The cultured stromal fibroblastic cells were checked by morphology, as well as by DNA microarray analysis. Our mRNA array data showed that cultures initiated from different specimens maintained a very similar gene expression profile with a correlation efficiency $R=0.99$ (Goo et al., 2009). Stromal cells were co-cultured with NCCIT in a ratio of 1:1. Heterotypic cell contact was prevented by a membrane barrier, which only allowed diffusible molecules to pass between the culture compartments. Co-cultures lasted for 3 d.

Fig. 2 shows phase contrast images of the fibroblastic morphology of CP strom and NP strom cells under the experimental conditions used. There was no discernible gross morphology difference between the two, or after their co-culture with NCCIT. The stromal cells did not adopt a stem cell phenotype. This was in contrast to NCCIT cells after co-culture. Morphological change in the stromal treated-NCCIT cells was observed after 3 d. As previously described (Pascal et al., 2009b), robust colony growth typical of EC cells was greatly diminished together with a reduced cell density, as shown by the gaps among colonies, due to signaling influence from stromal cells. This influence was different between CP and NP stroma (Pascal et al., in preparation). For example, PENK was not induced by

CP strom (since CP stromal cells, unlike NP stromal cells, were negative for PENK expression).

NP strom miRNA expression is altered by NCCIT influence

Although no morphological change was observed, the co-cultured stromal cells were analyzed for gene expression. First, miRNA expression was determined by Agilent arrays. The mean expression value of each condition was subjected to unsupervised hierarchical clustering and tree-view analysis. For differential expression, only those miRNA with hybridization intensity values ≥ 300 and a 3-fold or higher difference in expression were included. The overall miRNA expression scored by the Agilent arrays could be assorted into three patterns based on the heatmap image display: 1) NP strom; 2) NCCIT, NCCIT + NP strom, NCCIT + CP strom; and 3) NP strom + NCCIT, CP strom, CP strom + NCCIT (Fig. 3). Individually, NP strom, CP strom, NCCIT showed distinct miRNA expression. NCCIT, being a stem cell type, expressed miRNA associated with stem cells such as members of the miR302 family (Suh et al., 2004). Given the difference in mRNA expression, it was not entirely unexpected that the miRNA expression was different between NP strom and CP strom since miRNA may have a role in the regulation of mRNA expression. Thus, prostate cancer-associated stromal cells differed from their normal counterpart in both mRNA and miRNA expression. A number of the CP strom miRNA showed down-regulation compared to NP strom (entries in yellow row C vs. row B in Fig. 3), while less miRNA showed up-regulation in NP strom compared to CP strom. There was limited overlap between NCCIT and CP strom (row A vs. row C in Fig. 3) with several that were not detected in NP strom (marked by arrow in Fig. 3).

The more notable result was the change by NCCIT co-culture of NP strom miRNA expression to a pattern similar to that of CP strom as indicated by the visual comparison of heatmap rows B, E and C in Fig. 3. In contrast, NCCIT co-culture showed minimal effect on CP strom miRNA expression (compare rows C and G in Fig. 3). This same result was obtained with CP strom derived from two patients' tissue specimen. Thus, NCCIT showed different effects on the two stromal cell types. The top 4 differentially expressed miRNA between CP strom and NP strom were let-7f (15X), miR-29b (13X), miR-23a (12X), and miR-21 (9X) with miR-21 being the most abundant based on array signal intensity values. Increased miR-21 expression has been reported for many tumor types, and may be associated with cellular dedifferentiation (Krichevsky and Gabriely, 2009). In the Agilent array, there are two probesets each for miR-21 and the less abundantly expressed related miR-21*. These produced similar values in the conditions studies (Fig. 4). Unlike miR-21, miR-21* showed a lower level in CP strom, and a decrease in NP strom + NCCIT. Other overexpressed miRNA in CP strom were miR-125b, miR-199a-5p, miR-29a, let-7b, miR-130a and miR-100. An example with lowered expression, by 3.5X, in CP strom was miR-923. A listing of the expression levels as measured by signal values for individual miRNA in each co-culture condition is available in the supplementary data (Additional File 1). In general, the NP strom to CP strom conversion was qualitative (miRNA species) as well as quantitative (based on array signal values).

While an assessment of data quality in mRNA arrays based on correlation between array signals and immunohistochemistry of cell type-specific CD antigens had been reported by us (Pascal et al., 2008), a similar one for miRNA arrays was not possible. Instead, quantitative polymerase chain reaction (qPCR) was carried out on miR-210 for this purpose. Expression of miR-210 was being studied in our work on hypoxia and prostate tumor biology (AJW and HR-B). Developing a reliable qPCR assay for any miRNA is time consuming, but the miR-210 assay had been established. The array values for miR-210 were shown to correspond well with the quantitative measures of the PCR reaction products for the various

experimental conditions (Fig. 5). The average Ct values obtained from the replicate reactions were as follows: 24.78 for NCCIT, 25.82 for NP strom, 27.70 for CP strom, 22.23 for NCCIT + NP strom, 24.63 for NP strom + NCCIT, 23.75 for NCCIT + CP strom, and 25.80 for CP strom + NCCIT to correspond with the array signal values given in the figure.

mRNA expression changes in NP stromal cells after NCCIT induction

Second, NP strom + NCCIT was analyzed by Affymetrix GeneChips to determine if mRNA expression changes accompanied those in miRNA expression using the same RNA preparations. By dataset comparison, increased array signal values were found for CD90/THY1, MIRN21, HGF, SFRP1, BGN, and decreased array signal values were found for IGFBP5, HSD11B1 as shown in Fig. 6A. A listing of other genes is provided in the supplementary data (Additional File 2, up-regulated; Additional File 3, down-regulated). Differential expression of SFRP1, BGN, IGFBP5 between NP strom and CP strom was also reported by Joesting et al. (2005) for cultured NP and CP stromal cells. In contrast, the signal values for these genes in CP strom + NCCIT were not significantly different from those in CP strom showing that NCCIT factors had minimal effect on gene expression for both miRNA and mRNA in CP stromal cells. MIRN21 is the polyadenylated, capped transcript that is processed into mature miR-21 (Cai et al., 2004). Thus, both MIRN21 mRNA and miR-21 were detected as up-regulated by the corresponding arrays in NP strom after NCCIT co-culture. For CD90, a >10-fold increase (signal values 4911 vs. 335) was found in NP strom + NCCIT compared to a 3-fold difference (signal values 1279 vs. 335) between CP strom and NP strom. This increased CD90 expression correlated well to the strong CD90 immunostaining of tumor-associated stroma. CD90 was expressed in NP stromal cells as indicated by the signal value of 335 when placed in culture, and this was confirmed by flow cytometry analysis (data not shown).

Using our principal components analysis of cell-type transcriptome datasets (Pascal et al., 2009b), the conversion of NP strom mRNA expression by NCCIT influence to resemble that of CP strom is shown clearly in Fig. 6B as a clustering of NP strom + NCCIT with CP strom and CP strom + NCCIT. In the display, the gene expression difference between cultured stromal cells, labeled NP strom, and sorted stromal cells, labeled stromal, is also shown (Pascal et al., 2009b). Note the individual distinct transcriptomes of luminal, stromal, basal, endothelial, and NCCIT as indicated by the placement of their transcriptome dataset points in this 3-dimensional space representation.

Secreted miRNA can be detected in culture media

Given the report (Yuan et al., 2009) that ES cells could transfer miRNA to recipient cells, these small RNA molecules might be part of the NCCIT signaling to NP strom in addition to secreted protein molecules. Therefore, the culture media of the various co-cultures was screened for miRNA. The culture media miRNA expression could be assorted into two patterns based on the heatmap display – one for NCCIT and one for the rest (Fig. 7, with cytoplasmic miRNA expression for the different cultures included for comparison). A possible source of interference in this analysis would be any miRNA present in the FBS media supplement to which the Agilent probesets might cross-hybridize. However, since all test conditions used the same media preparation, differential expression of miRNA in the culture media could only be caused by differential uptake or release of miRNA from the cells. Table 1 shows the decrease in levels detected for several miRNA in the media of NCCIT compared to those of NCCIT + strom. For example, the stem cell miR-302a showed a 6.5X decrease after co-culture with CP strom (i.e., column labeled NCCIT vs. column labeled NCCIT + CP), and a 3.6X decrease after co-culture with NP strom. Similar values (i.e., under the 3-fold threshold of significant change) were seen in the co-cultures of NP + NCCIT and CP + NCCIT. Based on the data display in Fig. 7, the stromal cells appeared to

contribute relatively fewer stromal-specific miRNA to the culture media pool. In the media data, the array signal values would be expected to be roughly similar, and were, for all the co-cultures (NCCIT + CP strom, NCCIT + NP strom, NP strom + NCCIT, CP strom + NCCIT) in contrast to the monocultures (NCCIT, NP strom, CP strom), showing that these small RNA molecules could freely diffuse between the two growth compartments.

Cytoplasmic miRNA expression in NCCIT is not affected by stromal cells at 3 d

In contrast to the media miRNA profiles, expression of NCCIT cytoplasmic miRNA was not significantly affected by the presence of stromal cells at 3 d (compare row A with rows D and F in Fig. 3). Of the highly expressed ones, miR-367, miR-302b, miR-302a, miR-302d, miR-302c are known to be synthesized from a gene cluster on chromosome 4 (Barroso-del Jesus et al., 2009). This cluster is specifically expressed in both EC and ES cells, and not in any adult cell lines (Suh et al., 2004). Its transcription could be controlled by stem cell factors POU5F1/OCT4, NANOG, SOX2. Ectopic expression of this miR-302-367 cluster can reprogram human cell lines into stem-like cells (Lin et al., 2008). These miRNA were not detected in the stromal cells (signal values <300), and induction by NCCIT factors did not up-regulate their expression in either NP strom or CP strom (Additional File 4, first table). Other highly expressed miRNA in NCCIT: miR-19b, miR-130a, miR-20a, miR-21 were probably not stem specific as they were also detected in the stromal cells. Of the less abundant ones, miR-371-5p was reported in stem cells (Suh et al., 2004). Others (miR-574-5p, miR-106a, miR-17*) with low signal values in stromal cells could also be specific to NCCIT (Additional File 4). Untreated NCCIT expressed a total of 140 miRNA with values >300, and only a few changes in their expression were detected in NCCIT cells after co-culture with stromal cells (Fig. 3 and Additional File 4, second table) for 3 d. Thus, while the cytoplasmic pool of miRNA of stromal-treated NCCIT cells appeared to remain unchanged at 3 d, the exported pool was changed. A longer half-life of intracellular miRNA (i.e., >3 d) could be the reason.

Discussion

The cancer-associated stromal cells constitute an important cell type of the tumor microenvironment. In prostate cancer, the tumor stroma can be identified by strong CD90 immunostaining. Gene expression analysis showed differences from normal tissue stromal cells. This suggests that these cells may have an altered biological function. We postulated that CP stromal cells could represent a less differentiated cell type defective in stromal inductive signaling (Pascal et al., 2009a). With regard to their genesis, these experimental data provide a possible mechanism through which these cells could arise. This stromal phenotype could result from stem cell influence. In normal tissue, CD90⁺ stromal cells are localized adjacent to the basal epithelium of individual prostatic acini. The basal epithelium is thought to harbor the stem/progenitor cell population responsible for prostate regeneration, for example, after hormone removal and readministration (Bonkhoff and Remberger, 1996). Whether such a cell population, which can be isolated from this compartment using a stem cell marker (Pascal et al., 2007), can influence stromal cell differentiation remains to be determined since successful propagation of these cells in vitro is required for the co-culture experiments. We are beginning to sort these normal prostate tissue CD90⁺ cells for transcriptome analysis and cell culture.

With regard to NCCIT influence on stromal cells, we examined expression of not only mRNA but also miRNA. The importance of miRNA has been recognized with the discovery of miRNA possibly functioning as oncogenes or tumor suppressors through their regulatory action on target mRNAs (Cho, 2007; Papagiannakopoulos and Kosik, 2008). In structure, miRNA are small ~20 nucleotides long molecules that could interact with specific mRNA(s) through complementary binding in the 3' untranslated region (UTR). This binding leads to

RNA degradation and translation inhibition with loss of protein expression. In prostate cancer, miRNA expression could be regulated by androgen signaling and may contribute to the development of hormone refractory disease (Shi et al., 2008). Stromal cells are the mediators of androgen effect in the prostate (Cunha, 2008), and stromal miRNA could conceivably play a role. To functionally test these miRNA would require a systematic study to target each, either individually or simultaneously, to examine the effect on stromal cell biology.

Unlike the case for miRNA, expression difference in mRNA between CP and NP stromal cells has been well characterized by several research groups. More importantly, we showed here that NP stromal cells could acquire the expression pattern of CP stromal cells upon induction by diffusible factors synthesized by NCCIT cells. The NP to CP conversion was based on the expression of many genes as illustrated by the principal components analysis plot. Reverse transcriptase-PCR analysis further verified this for single selected genes like MMP1 and TIMP3 (unpublished data). TIMP3 was higher and MMP1 lower in NP strom compared to CP strom. After induction, TIMP3 was down-regulated and MMP1 up-regulated in the treated NP strom to the pattern found in CP strom. One of the notable up-regulated genes was hepatocyte growth factor (HGF). HGF is an important signaling molecule in stromal-epithelial interaction. It is produced by the undifferentiated mesenchyme during embryogenesis, and its synthesis is less pronounced in adult tissue (van der Voort et al., 2000). Increased HGF expression could also be indicative that cancer-associated stromal cells are less differentiated than normal tissue stromal cells. Thus, diffusible factors derived from NCCIT could cause genome-wide expression changes in a span of 3 days. This result suggested an alternative to the origin of CP stromal cells from epithelial-mesenchymal transition of malignant epithelial cells or that from recruitment of mesenchymal stem cells.

In development, stromal cells orchestrate organ-specific epithelial differentiation (Cunha, 2008). A defective stromal signaling, due to expression alteration, could instead lead to cancer differentiation (Baker et al., 2009). Knowing the molecules responsible for the generation of cancer-associated stromal cells could be the key. Aside from secreted proteins, these factors could be secreted miRNA released in membrane-bound microvesicles (Yuan et al., 2009). Whether it is protein or miRNA, the biochemical identification of the NCCIT signaling factor or factors poses a great experimental challenge since there are many candidates in the differentially expressed miRNA and mRNA. One may well need to inhibit multiple targets simultaneously to bring about such a change in phenotype.

An important aspect of work involving cultured cells to consider is gene expression changes induced as a result of cell culture. We have shown that cultured stromal cells differed from sorted stromal cells in the expression of multiple genes (Liu et al., 2000; Pascal et al., 2009a), and this is reflected in the principal components analysis of the transcriptomes in Fig. 6B. A major category of genes are those involved in cellular replication. However, one cannot perform these cell-cell interaction experiments without cell growth, and indeed one might not be able to observe gene expression changes in affected cells without cellular division. Differentiation in tissue involves asymmetric division of stem/progenitor cells in which one daughter cell is committed to becoming a functional cell type with the attendant gene expression changes. It may also be necessary to determine the miRNA expression of sorted CP stromal vs. NP stromal cells along with their respective mRNA expression to determine the effect of cell culture on miRNA expression as well. However, unlike in mRNA profiling, miRNA profiling of sorted cells is technically more demanding because higher numbers of cells, hence, larger (tumor) tissue specimens are required. While amplification technologies are available for mRNA similar ones are lacking for miRNA.

In summary, we found that factors produced by stem cells as represented by NCCIT can alter gene expression of prostate stromal cells in a specific way to that of prostate cancer-associated stromal cells. This expression change involved both protein coding and non-coding RNA molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

CP stromal cells. A: In tissue section 99-068D, the tumor in the right half of the field is outlined in red and stained strongly by CD90. The small cancer glands are surrounded by CD90⁺ stromal cells (magnification 40X). In normal glands, stromal CD90 staining is confined to cells adjacent to the basal epithelium (black arrows). B: The principal components analysis plot shows the separation between sorted NP stromal (labeled as NP

strom) and CP stromal cells (labeled as CP strom 1 and CP strom 2 from two cases), luminal and G3 cancer cells based on their transcriptome datasets. This analytic space is defined by the transcriptomes of prostate luminal, stromal, basal, and endothelial cells, and can be rotated for different aspects of view.

Fig. 2.

Cultured cell morphology. The micrographs show phase contrast images of NP strom, CP strom and NCCIT cells in culture (magnification 200X). Note the increase in space between the NCCIT colonies (dark lacunae) as an indication of lower cell density compared to untreated NCCIT (see main text for an explanation). In contrast, the stromal morphology was not appreciably altered by co-culture with NCCIT.

Fig. 3. MicroRNA expression profiles of stromal/NCCIT interaction. Shown are the hierarchical clustering and tree-view analysis of relative expression levels for miRNA in the different cell cultures arranged in the order written on the right. Array signal intensities are represented in color, blue hues for low and yellow for high. Arrow indicates miRNA shared between CP strom and NCCIT.

Fig. 4. MicroRNA-21 expression pattern. The histogram shows array signal values, which are listed in the table below, scored by miR-21 probesets in the different cultures. Array signal intensity values are indicated on the y-axis. The display gives a visual representation of the data. The fold-change between NP strom and NP strom + NCCIT is relatively constant for the two probesets. MicroRNA-21* was only detected (>300) in NP strom.

Fig. 5. MicroRNA-210 expression analysis by PCR. The relative product levels are indicated on the y-axis. Array signal values for each condition are indicated next to the culture identifiers.

Fig. 6. Stromal mRNA expression in co-culture with NCCIT. A: Array signal values are shown for the genes listed on the *x*-axis with array signal intensity values on the *y*-axis. The first 5 examples show increased expression in NP strom after NCCIT signaling while the next 2 examples show decreased expression. Expression levels of these genes in CP strom were not much altered by NCCIT. THY1 is CD90. B: A principal components analysis plot shows the “migration” of NP strom + NCCIT transcriptome towards the CP strom transcriptome, and

little change of CP strom + NCCIT compared to CP strom. The NCCIT transcriptome is distinct from the others. Note the difference shown by sorted stromal cells (labeled stromal) and cultured stromal cells (labeled NP strom).

Fig. 7. MicroRNA in media of co-culture. Expression patterns of the different cultures for cytoplasmic (left half) and culture media (right half) are compared. Three overall patterns are seen in the cytoplasmic data: [NP + NCCIT, CP, CP + NCCIT], [NCCIT, NCCIT + NP, NCCIT + CP], and [NP strom]. Two general patterns are seen in the culture media data: [NCCIT] and [all others]. Cell types underlined are the ones analyzed by arrays. Red hues represent high signal values and green low signal values.

Expression pattern of selected miRNA. The table summarizes differential miRNA expression in culture media after 3 d. Note decreased extracellular levels of these selected NCCIT miRNA in the presence of stromal cells.

Description	cell lysate						media							
	NP_NCCIT	CP	CP+NCCIT	NCCIT	NCCIT+NP	NCCIT-CP	NP	NCCIT	NP	CP	NCCIT+CP	NCCIT+NP	NP_NCCIT	CP+NCCIT
hsa-miR-302a	38	24	51	35720	27381	16189	22	452	69	49	70	125	93	97
hsa-miR-367	34	13	19	54912	42835	25130	30	566	69	71	89	129	98	143
hsa-miR-367	33	53	56	46219	36191	22472	5	508	59	53	84	182	93	135
hsa-miR-433-5p	7	45	5	53	13	23	21	540	944	1338	302	468	625	718
hsa-miR-188-5p	36	26	21	61	3	25	48	621	351	530	233	335	299	460
hsa-miR-134	39	47	46	67	15	23	57	956	323	346	321	383	322	414
hsa-miR-135a*	7	41	30	61	7	8	24	728	137	103	256	276	209	255
hsa-miR-575	44	87	25	89	30	29	62	601	88	85	211	224	183	206
hsa-miR-150*	55	85	31	495	63	49	113	1252	311	292	466	471	471	618
hsa-miR-939	74	61	40	202	137	79	148	1013	213	175	405	447	455	545
hsa-miR-940	243	126	102	709	447	282	108	1441	627	427	859	951	935	1169
hsa-miR-17	31	29	41	972	1190	571	72	123	44	74	47	94	57	64
hsa-miR-20b	63	83	27	6407	6279	3325	58	512	64	49	77	121	97	116