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Bladder expression of CD cell surface antigens and cell-type-specific transcriptomes

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Abstract

Many cell types have no known functional attributes. In the bladder and prostate, basal epithelial and stromal cells appear similar in cytomorphology and share several cell surface markers. Their total gene expression (transcriptome) should provide a clear measure of the extent to which they are alike functionally. Since urologic stromal cells are known to mediate organ-specific tissue formation, these cells in cancers might exhibit aberrant gene expression affecting their function. For transcriptomes, cluster designation (CD) antigens have been identified for cell sorting. The sorted cell populations can be analyzed by DNA microarrays. Various bladder cell types have unique complements of CD molecules. CD9⁺ urothelial, CD104⁺ basal and CD13⁺ stromal cells of the lamina propria were therefore analyzed, as were CD9⁺ cancer and CD13⁺ cancer-associated stromal cells. The transcriptome datasets were compared by principal components analysis for relatedness between cell types; those with similarity in gene expression indicated similar function. Although bladder and prostate basal cells shared CD markers such as CD104, CD44 and CD49f, they differed in overall gene expression. Basal cells also lacked stem cell gene expression. The bladder luminal and stromal transcriptomes were distinct from their prostate counterparts. In bladder cancer, not only the urothelial but also the stromal cells showed gene expression alteration. The cancer process in both might thus involve defective stromal signaling. These cell-type transcriptomes provide a means to monitor in vitro models in which various CD-isolated cell

types can be combined to study bladder differentiation and bladder tumor development based on cell-cell interaction.

Keywords

Bladder cell; CD phenotypes; Cell transcriptomes; Bladder cancer; Gene expression; Cancer-associated stromal cells

Introduction

The urinary bladder is composed of a urothelium or transitional cell epithelium overlying the lamina propria, muscularis mucosa, submucosa of connective tissue and fascicles of the muscularis propria (Hu et al. 2002). In addition to the superficial umbrella/luminal cells, the multilayered urothelium contains intermediate and basal cells. Although their lineage relationship is not known, basal cells are candidate progenitors of functional luminal cells as has been postulated for prostate basal cells (Bonkhoff and Remberger 1996). Subjacent to the urothelium is the so-called cluster designation 13 (CD13; alanyl aminopeptidase)-positive superficial lamina propria layer of 10–20 cells in width (Goo et al. 2005). These CD13⁺ stromal cells are probably the equivalent of the prostatic stromal cells lying next to the glandular epithelium, although the latter are CD13-negative. To determine their cell-type-specific gene expression or transcriptome, antibodies to CD cell surface antigens have been used to phenotype the component cell types. The appropriate CD antibodies can then be used to isolate individual bladder cell populations after tissue digestion by collagenase. We have previously used this approach to determine the transcriptomes of CD26⁺ luminal, CD104⁺ basal and CD49a⁺ stromal cells of the prostate (Oudes et al. 2006). Whereas the luminal cells of these two urologic organs are morphologically distinguishable and have well-defined functions, the stromal and basal cells are not and their biological functions are largely not as well known. Such cell-type-specific transcriptomes are also useful tools to monitor bladder cell differentiation within in vitro models (Scriven et al. 1997; Pascal et al. 2009b).

Stromal signaling plays an important role in bladder development with the stromal cell type determining the identity of the resultant tissue (Cunha et al. 1992; Aboseif et al. 1999). Cancer can be regarded as a disease in tissue formation (Soto and Sonnenschein 2011). Therefore, is stromal signaling abnormal in bladder tumors? Gene expression differences have previously been found between cancer and normal urothelium (Aaboe et al. 2005). Whether the differential gene expression in bladder tumors also involves the stromal compartment, as in the prostate (Pascal et al. 2009a), has not been examined. Accordingly, urothelial cancer and cancer-associated stromal cells have been sorted separately for array analysis and the transcriptomes obtained have been compared with those of the respective benign cell types.

Materials and methods

Bladder tissue

Excess human tissue specimens were obtained from cancer patients treated at the Department of Urology with written informed consent from all participants under the guidelines and approval of the Institutional Review Board of the University of Washington. De-identified bladder tissue specimens were obtained from surgeries. These were processed for research as rapidly as possible to minimize RNA degradation. Regions of bladder mucosae and wall that appeared grossly normal both visually and by palpation were identified and histologically confirmed as non-neoplastic urinary bladder. The tissue

retrieval used in our studies was as follows. An approximately 2×2 cm portion of bladder wall with minimal perivesicle fat was excised and cut into five pieces. The two most lateral pieces of tissue were placed into cryomolds containing OCT and stored at −80°. The middle piece of the tissue was apportioned into two pieces, one enriched for mucosa and the second for muscularis propria. These were placed into cryotubes and stored. The remaining two pieces were fixed in buffered formaldehyde and processed for paraffin embedding and histological characterization by using hematoxylin/eosin. These tissue samples, being representative of the former three pieces of tissue, were the basis for the cellular characterization of the other samples in order to verify that the tissue macroscopically selected was indeed normal non-neoplastic bladder. Tumor specimens, verified by histostaining, were also obtained and characterized in this fashion. The pathologic features of the tumors used for cell sorting were: 04–078, invasive high-grade urothelial carcinoma in right upper lateral wall with tumor in the muscularis propria and perivesicle fat, no evidence of spread to lymph node and ureter; 07–008, poorly differentiated tumor cells with spindle cell component at right ureteral-vesicle orifice, partially invasive of muscularis propria without transmural invasion into perivesicle fat, no evidence of local spread; 07–068, tumor in the left inferior lateral-to-midline region with invasion deep into muscularis propria but no involvement of the lateral soft-tissue margin and no evidence of local spread.

CD immunohistochemistry

For immunohistochemistry with antibodies to CD cell surface molecules, serial 5-μm-thick frozen sections were fixed in cold acetone and processed for staining by using a three-step indirect avidin-biotin-peroxidase procedure (Liu and True 2002). All CD antibodies (>200) were obtained from BD PharMingen (San Diego, Calif., USA). The immunostained sections were imaged with an Olympus BX41 microscope (Olympus, Melville, N.Y.) equipped with a MircoFire digital camera (Optronics, Goleta, Calif., USA). Images were processed with Photoshop CS (Adobe Systems, San Jose, Calif., USA).

Cell sorting

For magnetic cell sorting (MACS) of bladder cell populations, 0.5 g specimens were collected in sterile tubes, rinsed with Hank's balanced salt solution (HBSS) and minced for collagenase digestion. The resultant cells were partitioned on Percoll density gradient as described (Liu et al. 1997; Oudes et al. 2006). Cells in either the epithelial or the stromal fraction were labeled with R-phycoerythrin (PE)-conjugated CD antibodies. The targeted cell populations were CD9⁺ urothelial, CD104⁺ basal urothelial, CD13⁺ superficial lamina propria, CD9⁺ cancer cells and CD13⁺ tumor-associated stromal cells. PE-CD9 (antibody clone M-L13), PE-CD13 (clone L138) and PE-CD104 (clone 439-9B) were used at 1:20–1:60. The labeled cells were resuspended in phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA) and 15 μl paramagnetic microbead-conjugated anti-PE (Miltenyi Biotec, Auburn, Calif., USA) were added for 15 min. Afterwards, the PE-positive and PE-negative cells were fractionated by AutoMACS (Miltenyi Biotec). Aliquots of the sorted fractions were analyzed by fluorescence-activated cell sorting (BD, Mountain View, Calif., USA) to assess sort efficiency. The sorted cells were pelleted and lysed in RNeasy lysis buffer (Qiagen, Crawley, UK) to assess sort efficiency. The sorted cells were pelleted and lysed in RNeasy lysis buffer (Qiagen, Crawley, UK) to assess sort efficiency. The sorted cells were pelleted and lysed in RNeasy lysis buffer (Qiagen, Crawley, UK) to assess sort efficiency. Only RNA samples that were of sufficient concentration and showed no degradation were used for array hybridization.

DNA microarray analysis

For analysis, the quality and concentration of RNA were determined by an Agilent 2100 Bioanalyzer and RNA Pico Labchip (Agilent Technologies, Santa Clara, Calif., USA). The Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, Calif., USA) were used for expression profiling, as all previous datasets were obtained with this platform. The U133 array contained probe sets representing 54,675 genes, splice variants and expressed

sequence tags. The GeneChips were prepared, hybridized and scanned according to protocols provided by Affymetrix (Oudes et al. 2006). In brief, 200 ng RNA was reverse-transcribed with poly (dT)/T7 promoter primer and the cDNA was made double-stranded. In vitro transcription was performed with biotinylated ribonucleotides and the biotin-labeled cRNA was applied to the GeneChips. The chips were washed and stained with streptavidin-PE by using an FS-450 fluidics station (Affymetrix). Data were collected with Affymetrix GeneChip Scanner 3000. All transcriptome data-sets were deposited in our public database (<http://scgap.systemsbiology.net/>) for data query (Pascal et al. 2007). The microarray data were MIAME-compliant and the raw data were deposited in GEO (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE30522.

Array dataset analysis

For differential gene expression, datasets were analyzed by HTself, a statistical method designed for low replication micro-arrays (Vêncio and Koide 2005). In HTself, all possible combinations of pair-wise comparisons among experiments were taken to create sets of ratios. 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 using the standard robust multi-array average method (Irizarry et al. 2003), implemented in the analysis pipeline SBEAMS (Marzolf et al. 2006). The strength of differential expression between any pair of experiments was estimated by $M_f = \log_2(\text{ratio}) = X_f - X_a$, where a represented one particular cell type and f represented another in the set CD104⁺ basal, CD9⁺ urothelial, CD13⁺ stromal, CD9⁺ cancer and CD13⁺ cancer-associated stromal cells. To estimate reliability, HTself used virtual self-self experiments to derive intensity-dependent cutoffs. Accordingly, a probeset was considered to be significantly differentially expressed if at least 80% of its log-ratio combinations were outside the 99.9% credibility intensity-dependent cutoff. Moreover, an average greater than eight-fold difference in the expression level was chosen. The computational analysis results were verified by dataset query of identified differentially expressed genes.

In the principal components analysis (PCA) of the bladder-cell-type transcriptome datasets, a gene expression subspace based on the transcriptomes of four cell types isolated from the prostate, namely CD104⁺ basal (B), CD26⁺ luminal (L), CD49a⁺ stromal (S) and CD31⁺ endothelial (E), was utilized. The three-dimensional (3D) coordinate system was obtained by performing the usual PCA, by defining the rotation matrix related to the top three principal components and by applying it to all datasets to create a subspace that highlighted the expression particularities of each prostate cell type (Pascal et al. 2009b). The rotation matrix was obtained by using averages of X_B , X_L , X_S and X_E and these were plotted as projections in the principal components space. Other transcriptomes were next projected into this PCA-generated subspace to visualize the spatial relationship of the individual datapoints. This plot could be rotated to provide multiple points of perspective (<http://labpib.openwetware.org/PCA.html>). The separation between any two datapoints, A and B, was calculated by the following formula: $\Delta = \text{square root of } [(A_1 - B_1)^2 + (A_2 - B_2)^2 + (A_3 - B_3)^2]$, where A1-3 and B1-3 are the coordinate values along the three principal components axes. Transcriptomes of stem cells were represented by those of the cultured embryonic carcinoma (EC) cell line NCCIT (American Type Culture Collection, Manassas, Va, USA; Pascal et al. 2009b) and the embryonic stem (ES) cell line H1/WA01 (WiCell Research Institute, Madison, Wis., USA; Ware et al. 2006).

Results

Bladder cell CD phenotypes

The major cell types of the bladder were found to express individual complements of CD antigens. Examples of bladder CD immunohistochemistry are shown in Fig. 1 for CD46, CD105, CD97, CD74, CD100 and CD49f and all data images are available at <http://scgap.systemsbiology.net/data/>. The urothelial cells stained strongly for CD46 (complement regulatory protein), which was also detected in blood vessels. The basal cells had a number of CD molecules (e.g., CD44, CD49f, CD104) in common with the basal cells of the prostatic epithelium, with basal staining for CD49f (integrin $\alpha 6$) being shown in Fig. 1i-l. No staining pattern of the available CD antibodies could distinguish the intermediate cells from the others. The lamina propria was partitioned into a CD13-positive layer subjacent to the urothelium and a CD13-negative remainder (Goo et al. 2005). Unlike prostate stromal cells, the bladder stromal cells were negative for CD56 (neural cell adhesion molecule 1). Other significant cell types, i.e., white blood cells, nerve sheath cells and endothelial cells, were also detected by these CD antibodies (e.g., Fig. 1l). The cell-type-specific CD reactivities are summarized in Fig. 2. For the analysis of cell-type-specific gene expression, CD9 antibody was used for the sorting of urothelial cells, CD104 (integrin $\beta 4$) for basal cells and CD13 for stromal cells processed from benign tissue (normal bladder; NB) specimens; CD9 and CD13 were used to sort cancer cells and cancer-associated stromal cells, respectively, from cancer tissue (CB) specimens.

Bladder-cell-type transcriptomes

The Affymetrix U133 array-derived 07-015NB CD9⁺ urothelial, 06-125NB CD13⁺ stromal and 06-125NB CD104⁺ basal transcriptome datasets were plotted in a PCA space defined by prostate cell transcriptomes (Fig. 3a). This PCA plot could be viewed as a cell differentiation display of various somatic cell types in relation to each other and to stem cells as represented by ES and EC cells. In this 3D display, the stem cells were placed in an interior position, with the differentiated cell types lying toward the periphery. The separation between datapoints (Δ , Table 1) was used as a measure of relatedness between cell types; the smaller the distance, the more related, as shown by ES vs EC cells ($\Delta=36.37$) vis-à-vis prostate stromal vs luminal cells ($\Delta=157.2$), prostate stromal vs basal cells ($\Delta=141.94$), or prostate luminal vs basal cells ($\Delta=114.96$). The separation between luminal and basal cells was significant, despite both being epithelial. If basal cells were the precursor of luminal cells (see below), this distance was larger than luminal vs ES cells ($\Delta=64.06$) or basal vs ES cells ($\Delta=91.32$). Human ES cells have been experimentally shown to differentiate into luminal cells (Taylor et al. 2006). The datapoint placements indicated that the bladder transcriptomes were distinct from each other and from the corresponding prostate cell types. A closer relatedness was seen between the respective stromal and basal cell types of these two organs than the luminal cell type as indicated by stromal cells $\Delta=78.94$ and basal cells $\Delta=81.75$ vs luminal cells $\Delta=152.43$.

For luminal cells, the transcriptomes reflected the dissimilar functions between bladder and prostate: barrier vs secretion. The urothelial cell datapoint was also plotted near that of the endothelial cells ($\Delta=61.43$) to highlight the shared functional property that urothelial cells line the bladder, whereas endothelial cells line the blood vessels. For basal cells, the transcriptome data showed that the CD104⁺ bladder basal cells were also distinct from the CD104⁺ prostate basal cells, despite their shared CD molecules. In Fig. 3b, four replicate datasets of CD104-sorted prostate basal cells (red cubes) and the composite representative (dark blue cube labeled B) are shown. The bladder basal cell transcriptome obtained from 06-125NB (black cube) was plotted distal to the prostate B transcriptome. This result indicated that, based on overall gene expression, bladder basal cells were functionally

different from prostate basal cells. Furthermore, both basal cell types were unlike stem cells (ES, yellow cube) with regard to gene expression (bladder *vs* ES: $\Delta=94.34$; prostate *vs* ES: $\Delta=91.32$). Hence, basal cells expressed few stem cell genes despite being positive for stem cell CD molecules such as CD49f. By dataset query, both ES and EC cells showed low expression of the basal CD104 (data not shown). For stromal cells, the differential gene expression between bladder and prostate identified the organ-restricted (i.e., prostate-not-bladder and bladder-not-prostate) stromal genes. Many of these genes overlapped with those determined from these cells in culture by array analysis and validated by reverse transcription with the polymerase chain reaction (Goo et al. 2005; Pascal et al. 2009a). Hence, cell culturing appeared not to affect the expression of the organ-restricted stromal genes and the stromal cells maintained their organ identity.

Bladder cancer gene expression

For bladder cancer, CD9⁺ cells were sorted from specimen 07-008CB (2.6g). The differentially expressed genes between the bladder cancer cells (labeled as CBepi) and CD9⁺ urothelial cells (labeled as NBepi) were identified by HTself. *XIST*, *X*(inactive)-specific transcript (non-protein coding), showed one of the highest fold ($>65\times$ in array signal intensity values) increases in the cancer cells. The dataset query display in Fig. 4 (top) presents gene expression levels, indicated by array signal values for all *XIST* probesets, on a gray scale. To calculate the fold of differential expression between CB and NB, the Affymetrix array hybridization signal intensity values were retrieved by clicking on the individual gray data boxes. A second dataset query result (Fig. 4, bottom; abbreviated and full names of genes are given) shows the expression levels of selected genes in CBepi *vs* NBepi. For comparison, the known prostate cancer genes of AGR2, AMACR, CRISP3, ERG, HPN and PCA3 (Pascal et al. 2009c) were detected at or below the background (signal values of 50–100). The AGR2 signal in NB was verified by immunostaining (data not shown). Thus, bladder cancer gene expression was distinct from that of prostate cancer. In addition, expression of the high abundance class prostate genes ACP, AZGP1, KLK2 and KLK3 (prostate-specific antigen) was absent in bladder cancer cells. Luminal cytokeratin KRT18 but not basal KRT5 was expressed by the CD9⁺ urothelial cells. In validation of the CD immunohistochemistry results (Fig. 1), the bladder cells were scored as positive by array analysis for CD9, CD24, CD46, CD63, CD71 (transferrin receptor), CD74 and CD100 (semaphorin 4D) but not prostate luminal CD26 (dipeptidyl-peptidase 4). Hence, the CD expression results were concordant between immunohistochemistry and array analysis as was previously reported for the prostate (Pascal et al. 2008). Strong CD46 and CD26 immunostaining distinguished bladder and prostate cells, respectively, in both benign tissue and cancer. The 07-008CBepi showed an increased signal for CD10 (membrane metallo-endopeptidase) and a decreased signal for CD24 compared with NBepi. Figure 5 presents the CD immunohistochemistry of 04-078CB, which contained infiltrating tumor cells in the underlying tissue. The cancer cells were variably positive (patchy) for CD10 and intensely positive for CD24 and CD46. Differential expression of CD10 and CD24 had also been found in prostate cancer (Liu et al. 2004).

Stromal cells associated with the bladder cancer (labeled as CBstrom) were sorted by CD13 from specimen 07-068CB (1g). In addition to the Percoll density gradient partition of the epi and strom elements, CD9 was used to remove any residual epi cells in the strom fraction before sorting by CD13. Dataset query result (Fig. 6) showed that epithelial genes such as CD74 (Fig. 1e, f) were not detected in the strom datasets. Like the cancer cells, the CBstrom cells showed altered gene expression from their normal counterpart, NBstrom. Figure 7a shows the PCA placements of the CBstrom and NBstrom datasets and those of the CBepi and NBepi datasets. The larger separation between the strom datapoints ($\Delta=44.05$) than that between the epi datapoints ($\Delta=14.21$) indicated more gene expression changes in the

stromal compartment (07-068CB) than the epithelial compartment (07-008CB). In Fig. 7b, the CBstrom cells showed lower expression of bladder-restricted stromal genes such as HSD17B2 [hydroxysteroid (17- β) dehydrogenase], SALL1 [sal-like (*Drosophila*)], TRPA1 (transient receptor potential cation channel) and IL24 (interleukin 24). Expression of non-bladder-restricted genes such as STC2 (stanniocalcin) and VIM (vimentin) was equivalent in CB and NB. These cells had no signals for prostate stromal genes such as CXCL13, CNTN1, MAOB, PAGE4, PENK and SPOCK3 (Pascal et al. 2009a; query results not shown).

Discussion

The major cell types of the urinary bladder can be phenotyped by CD antigens. This permits their isolation (CD9⁺ urothelial, CD13⁺ stromal, CD104⁺ basal) for transcriptome determination by DNA microarrays. For analysis, these transcriptomes have been projected into a PCA space defined by the transcriptomes of prostate cell types and stem cells obtained previously. As expected, the transcriptomes of CD9⁺ urothelial and CD26⁺ prostate luminal cells are unlike each other, since these two organs serve different functions. Although morphologically indistinguishable, the CD13⁺ bladder and CD49a⁺ prostate stromal cells also differ in their gene expression. The differentially expressed genes are perhaps the basis of organ-specific inductive stromal signaling (Cunha et al. 1992). This signaling involves secreted molecules and heterotypic cell contact. Likewise, the CD104⁺ bladder and prostate basal cells differ in their gene expression. The precise function of basal cells is unknown but them possibly being tissue progenitor cells is problematic because of, on the one hand, the large differential gene expression between bladder and prostate basal cells (one would expect a more similar gene expression if they served a similar function) and, on the other, the absence of the stem cell gene signature. This does not rule out that a small subpopulation could constitute the tissue progenitors. Basal cells have recently been reported to be the cell type of origin for prostate cancer (Goldstein et al. 2010). However, none of the cancer cell types, including some considered to represent the so-called cancer stem cell type, profiled by array analysis shows much basal cell gene expression (Pascal et al. 2011b). Like prostate cancer, the bladder cancer profiled here (07-008CB) is luminal in expression signature, by cytokeratins KRT18⁺/KRT5⁻. Basal cells, unlike luminal cells, can be readily propagated *ex vivo*; this might explain them being experimentally transformed into cancer cells by oncogene introduction in vitro (Goldstein et al. 2010). The differential gene expression of all three cell types between bladder and prostate suggests that they have organ-specific functions. The cell-type transcriptomes allow a bladder PCA subspace to be defined so that other cell types in the urinary tract can be comparatively analyzed when they become available.

The bladder-cell-type-specific transcriptomes are also useful in the study of cellular differentiation from stem cells induced by cell-cell interaction and signaling molecules. The biology of bladder development has been studied by tissue recombination in rodents (Staack et al. 2005) in which the implanted mesenchyme determines the nature of the developed epithelium regardless of whether the tissue stem cells have been obtained from the bladder or the prostate (Aboseif et al. 1999; Li et al. 2000). A stem cell population in tissue is essential for normal repair and renewal and these cells can in practice be harvested for organ regeneration. In addition to tissue stem cells, ES cells have been induced by fetal bladder mesenchyme to undergo urothelial differentiation in vivo (Oottamasathien et al. 2007). We have used an in vitro co-culture system to study this induction process, in which bladder stromal cells cause the EC cell line NCCIT to differentiate by secreted molecules. This bladder stromal induction is different from prostate stromal induction (Pascal et al. 2009b) and the cell-type transcriptomes are instrumental in the identification of the cell types resulting from such a co-culture.

Bladder tissue engineering will profit from a molecular understanding of cellular differentiation. Ideally, a neobladder should be generated from (cultured) autologous cells. In tissue engineering, a biodegradable material made of small intestine submucosa or bladder acellular matrix is used to provide a scaffold on which urothelial and smooth muscle cells can grow to produce a functional bladder (Kanematsu et al. 2007). A more appropriate model has been constituted by seeding urothelial cells on de-epithelialized stroma (i.e., to provide the appropriate stromal signaling). The resultant urothelial structure becomes polarized with distinguishable basal, intermediate and superficial cells. The surface luminal cells appear to possess a characteristic asymmetric unit membrane (Scriven et al. 1997). Gene expression of these in-vitro-developed cell types should be compared with that of the sorted bladder cell types to gauge the extent of expression match as a measure of functional differentiation.

The cell transcriptome datasets have allowed us to compare the gene expression of bladder and prostate cancer for common molecular defects in these two urologic malignancies. Overall, little overlap occurs between the cases analyzed as indicated by the PCA plot. However, as more cancer transcriptomes become available, some common genes might be found, for example, in the differential cancer expression of CD molecules, such as CD10 (Murali and Delprado 2005) and CD24 (Choi et al. 2007). Bladder cancer case 07-008 is CD10^{hi}CD24^{lo}, whereas case 04-078 is CD10^{lo}CD24^{hi}. CD10 expression in prostate cancer is associated with unfavorable outcomes (Fleischmann et al. 2008), whereas that in bladder cancer is associated with favorable outcomes (Seiler et al. 2012). CD24 is also a marker in several other cancers (Lee et al. 2009) and has prognostic value in epithelial ovarian cancer (Kristiansen et al. 2002). The non-coding *XIST* has also been reported in breast cancer cells (Sirchia et al. 2009). We hope to test differentially expressed markers by using the bladder cancer tissue microarrays reported by Seiler et al. (2012).

The cancer cells profiled here appear mostly to retain their organ identity with only ~100 genes being differentially expressed from their respective normal counterpart. In addition, gene expression alteration has been found in the cancer-associated stromal cells. One change involves the down-regulation of the so-called organ-restricted genes. We postulate that this renders these cells defective in stromal signaling, as has been shown for prostate-cancer-associated stromal cells in co-culture with NCCIT cells (Pascal et al. 2011a). The prostate-cancer-associated stromal cells appear to represent a less differentiated cell type in the stromal lineage, as factors from NCCIT cells can alter the gene expression of normal tissue stromal cells to that of cancer-associated stromal cells (Vêncio et al. 2011). Whether bladder stromal cells can be similarly affected in co-culture with NCCIT remains to be investigated. The extensively altered gene expression in cancer-associated stromal cells suggests the possibility that defective stromal signaling plays a role in the cancer process, in addition to gene mutations.

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Abbreviations

CB	Bladder cancer
CD	Cluster designation

EC	Embryonal carcinoma
ES	Embryonic stem
MACS	Magnetic cell sorting
MIAME	Minimum information about a microarray experiment
NB	Normal bladder
PCA	Principal components analysis

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Fig. 1.

Bladder immunostaining for cluster designation (CD). **a, b** CD46 stains the entire urothelium (basal, intermediate and superficial cells) in specimen 03-043B1. **c** CD105 stains stromal cells underneath the urothelium (and blood vessels) in 03-043B1. **d** CD97 stains the muscle bundles in 03-024A1. **e, f** CD74 stains the urothelium in 03-035A1. **g, h** CD100 stains the urothelium in 03-035A1. **i-l** CD49f stains basal urothelial cells with the staining intensity diminishing toward the luminal surface; capillaries and nerve elements are also positive in 03-043B1. Bars 100 μ m (**a, c, g, i**), 40 μ m (**b, f, h, j, l**), 20 μ m (**d, e, j, l**), 10 μ m (**k**)

Fig. 2. Bladder CD reactivity. The various identifiable cell types are listed *left*. Only the informative CD molecules are included. For each *color* representation of immunostaining, a *lighter hue* is used to indicate weaker reactivity. The *gray scale* is used to indicate abundance of particular white blood cell types (CD molecules were first identified on these cells)

Fig. 3. Bladder-cell-type transcriptomes. **a** 3D projection of bladder CD104 basal (06-125, *black cube*), CD13 stromal (06-125, *light blue cube*) and CD9 urothelial (07-015, *green cube*) cells with respect to those of prostate cell transcriptomes (*red cubes*) for stromal (*S*), luminal (*L*), endothelial (*E*) and basal (*B*) cells in a principal components analysis-derived plot. Datasets of embryonic stem (*ES*, *yellow cube*) and embryonal carcinoma (*EC*, *dark blue cube*) cells are included. Like the prostate cell types, the bladder cell types are also plotted on the periphery in relation to the stem cell types (*PC1*, *PC2*, *PC3* principal components axes). **b** The 06-125 CD104⁺ bladder basal cell transcriptome (*black cube*) is compared with the CD104⁺ prostate basal cell transcriptomes (four replicates, *red cubes*)

Fig. 4.

Bladder cancer gene expression. *Top* Gene expression levels (represented on a gray scale) of X(inactive)-specific transcript (*XIST*) in bladder cancer (07-008CB) and non-cancer/normal (07-015NB) cells. All *XIST* probesets are listed. *Bottom* Selected genes for interrogation in CB and NB are listed in the *first column* (abbreviations are explained *right*). Known prostate genes (*ACPP*, *AZGP1*, *DPP4*, *KLK2*, *KLK3*) and prostate cancer genes (*AGR2*, *AMACR*, *CRISP3*, *HPN*, *PCA3*) have low signal values in the bladder datasets

Fig. 5. Bladder cancer CD staining. Specimen 04-078B1 shows tumor cell infiltration in the lamina propria (a); hematoxylin and eosin staining. The cancer cells are positive for CD24 (b) and CD46 (c), weakly positive for CD10 (d). Bar 100 μ m

Fig. 6. Bladder tumor cell transcriptome. Dataset query (*top*) showing selected genes with higher signal levels in CBepi than NBepi and their generally low levels in the stromal compartment (NBstrom and CBstrom). CD74 was detected by immunohistochemistry on the superficial umbrella cells (see Fig. 1e, f). The signal values are also plotted in histogram format (*bottom*)

Fig. 7. Cancer-associated stromal cells. **a** Display showing the transcriptomes of the bladder tumor cell types (urothelial and cancer-associated stromal) and their respective normal counterparts. Note the wider separation between CBstrom and NBstrom than that between CBepi and NBepi. **b** Dataset query showing the down-regulation of genes (IL24, HSD17B2, SALL1, TRPA1) previously identified as differentially expressed between bladder and prostate in CBstrom (07-068) vs NBstrom (06-125). Stromal genes not restricted to bladder, namely STC2 and VIM, are shown for comparison

Principal components distance matrix. Separation between individual data point pairs was calculated for the cell types listed (*P* prostate, *B* bladder, *ES* embryonic stem cells, *EC* embryonal carcinoma cells) and tabulated

Table 1

	P luminal	P basal	endothelial	P stromal	B stromal	B urothelial	B basal	ES	EC
P luminal		114.96	159.12	157.2	127.55	152.43	107.83	64.06	66.35
P basal	114.96		132.2	141.94	132.61	168.07	81.75	91.32	104.82
endothelial	159.12	132.2		155.09	89.79	61.43	63.41	133.2	107.68
P stromal	157.2	141.94	155.09		78.94	155.42	150.24	94.22	107.13
B stromal	127.55	132.61	89.79	78.94		77.06	96.46	79.58	61.46
B urothelial	152.43	168.07	61.43	155.42	77.06		95.4	132.73	98.92
B basal	107.83	81.75	63.41	150.24	96.46	95.4		94.34	75.81
ES	64.06	91.32	133.2	94.22	79.58	132.73	94.34		36.37
EC	66.35	104.82	107.68	107.13	61.46	98.92	75.81	36.37	