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Research Article



The Cancer Stem Cell Concept as Applied to Prostate Cancer

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Abstract

The cancer stem cell (CSC) hypothesis proposes that rare tumor-initiating cells with stem-like properties drive cancer progression and metastasis. Through comprehensive analysis of prostate cancer patient-derived xenografts (PDX), we demonstrate that all cancer cell types—not exclusively stem-like cells—can initiate tumors in mice, contradicting a core CSC tenet. CD44, commonly used to identify CSCs, proved inconsistent in prostate cancer. However, stem-like cancer cells do exist, characterized by expression of stem cell transcription factors (scTF LIN28A, NANOG, POU5F1, SOX2) and low β -2 microglobulin (scTF⁺B2M^{lo}). We show that differentiated adenocarcinomas can be experimentally reprogrammed to stem-like small cell carcinomas through scTF expression, while stromal signaling molecules like proenkephalin (PENK) induce differentiation. These findings reveal that prostate cancer progression involves dynamic dedifferentiation due to loss of stromal signaling rather than clonal expansion of rare CSC. Cancer cells exhibit remarkable plasticity, undergoing reversible differentiation-dedifferentiation cycles independent of mutation burden, suggesting differentiation therapy as a promising treatment strategy.

Introduction

The cancer stem cell hypothesis posits that a population of cancer cells with stem cell properties is capable of starting, promoting tumor growth, and metastatic spread. Like stem cells, these cancer cells can undergo differentiation into other cancer cell types, producing tumor heterogeneity. Furthermore, cancer stem cells (CSC) are rare, with a frequency of about 10^{-4} [1]. We will review based on research in prostate cancer if the CSC description of human solid tumors is applicable point by point.

Tumor initiation and growth

Xenotransplantation and serial passaging in mice form the basis for demonstrating the existence of CSC, since only they can engraft and propagate tumor growth *in vivo*. In blood cancer, cell sorting was used to isolate leukemia stem cells (LSC) by targeting the cell surface molecules CD34 and CD38 in samples of acute myeloid leukemia [2]. The sorted CD34⁺CD38⁻ cells were able to form tumors in mice, whereas those of CD34⁺CD38⁺ and CD34⁻ were not. The formed tumors were serially transplanted to show self-renewal. Furthermore, the passaged tumors contained not only CD34⁺CD38⁻ but also CD34⁺CD38⁺ and CD34⁻ cells. It would appear that these LSC could undergo spontaneous differentiation to produce non-stem progeny, or perhaps under the influence of murine

factors at the implantation sites. These results showed that LSC shared properties with hematopoietic stem cells in expressing specific cell surface antigens and undergoing differentiation. In solid tumors, sorted CD44⁺CD24⁻ breast cancer cells were also shown to possess tumor-forming capability in contrast to CD44⁺CD24⁺ or CD44⁻ cells, and at an efficiency of 100-fold less in the number of cells needed [3]. The serially transplantable tumors in mouse mammary pads were shown to contain the three distinguishable cell types. The resultant cancer cells were postulated to derive from a CD44 precursor. Such findings were extended to brain tumor cells isolated by the stem cell surface marker CD133. Implanted CD133⁺ tumor cells in the mouse brain were shown to produce both CD133⁺ and CD133⁻ progeny [4,5]. Studies from several other tumor types reported similar data.

For prostate cancer, a family of cancer-derived xenografts (PDX) was obtained and characterized [6]. To establish each PDX, tumor samples of ~20 mg free of other cells, fat, and necrotic tissue were implanted subcutaneously. Tumor growth in mice was monitored over 18 months. A line was deemed established if it survived three passages and was then maintained by continuous passaging in mice. The first generated cohort was derived from >250 donations, which included primary tumors, metastases to the bladder, adrenal gland, local and distant lymph nodes, bone, liver, and bowel.

The tally of twenty-six individual lines represented a take rate of 10%, well above that of 10^{-4} reported for CSC. One could argue that a greater number of tumor cells were implanted to begin with to produce the higher success rate. In success and failure, roughly the same number of cells were implanted. No association between take rate and sample procurement from either surgery or rapid autopsy, or the anatomical sites was found. Initial tumor growth lasted 4-12 months, and a 50% - 80% secondary take rate. Tumor size could achieve 1 g in 4-16 weeks. The different PDX lines varied in growth rates. Castration resistant (CR) variants were obtained by passaging in castrated mice. These LuCaP PDX include androgen receptor (AR)-positive, well-differentiated and less differentiated adenocarcinomas, plus AR-negative neuroendocrine small cell carcinoma. AR expression is associated with differentiation. These PDX retained the genomic and expression signatures such as AR amplification, AR mutation, AR splice variants, loss of PTEN and RB1, and TMPRSS2-ERG rearrangement of the original resected tumors. Some differences were seen in lines derived from several metastases of the same patient. These characteristics were maintained throughout the prolonged growth of >100 passages to date. Transcriptome analysis of LuCaP tumors over selected passages (p) - p40-p59 for AR⁻ LuCaP 49, p64-p99 for AR⁺ LuCaP 35 - showed no detectable changes in overall gene expression [7]. Against the CSC core postulate, all prostate cancer cell types can initiate growth in mice rather than just CSC. Importantly, each LuCaP line, AR⁺ or AR⁻, retained its unique identity with no other cell types emerging over time. We suggest that this approach of generating multiple PDX could be replicated for other solid tumor types.

The CSC marker CD44

As described above, CD44 was used to identify breast cancer CSC, and has also been used as a distinguishing marker in pancreatic, colorectal, head and neck cancers [8-10]. CD44⁺ PC3 prostate cancer cells grown as xenografts were found to be enriched in tumorigenic and metastatic capabilities [11]. We generated a prostate CD map by immunohistochemistry using the CD antibodies available commercially [12,13]. For those associated with CSC, CD24, CD34, CD38, CD44, and CD133 all showed differential expression between normal/benign and cancer, as well as among cancer cells in culture. CD24 showed increased expression by cancer cells from low to high grade [13,14]. CD34 was detected mainly in infiltrating white blood cells. CD38 showed decreased expression in cancer cells. CD44 expression was restricted to basal epithelial cells and a few cancer cell lines [15]. Notably, the expression level of CD44 was not appreciably high in stem cells [7]. Transcriptome dataset query showed a >50-fold lower expression in embryonic stem (ES), embryonal carcinoma (EC), induced pluripotent (iPS) cells, and small cell carcinoma line LuCP49 than cancer cell lines DU145, PC3, and

CL1 [7]. CD133 expression was detected in stem-like cancer cells but not in those of primary tumors [7]. The generated CD map allowed CD antibody-mediated sorting of prostate cell types - CD26⁺ luminal (L), CD104⁺ basal (B), CD49a⁺ stromal (S), plus CD31⁺ endothelial (E) for transcriptomics and cell-to-cell interaction analysis [16]. Together with those of ES, EC, and iPS, a prostate principal components analysis (PCA) space was generated to visualize cellular differentiation keyed on gene expression [17]. As expected, the four differentiated cell types, L, B, S, and E, were distinct in gene expression to reflect their own functional property. Their individual placements in the PCA space were widely separated on the periphery of the centrally placed stem cell types ES, EC, and iPS (Figure 1). The separation between any two cell types represented by their respective transcriptome datapoints, measurable by a value Δ [17,18], corresponds to the degree of differential gene expression, and thus lineage relatedness. The PCA plot provides a visual tool to track cellular differentiation. The transcriptome of CD44⁺ basal cells, like those of the other differentiated cell types, was distal to that of the stem cells, hence not like stem cells. When the transcriptome datapoints of prostate cancer cell types (including CD44⁺ PC3, DU145, and CL1) were displayed in PCA (Figure 1), none were close to the basal cell datapoint. One can conclude that CD44 is not a particularly consistent marker associated with prostate cancer cells. Parenthetically, CD104⁺ basal cells of the bladder

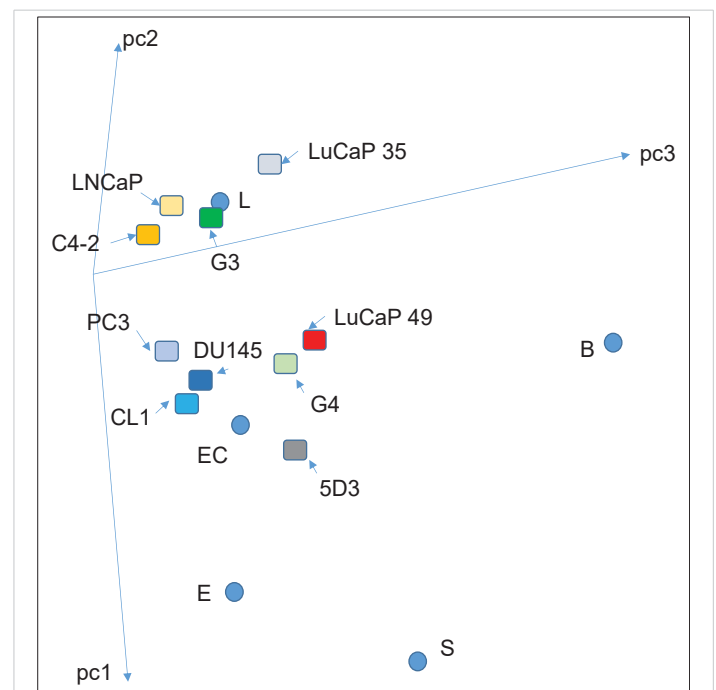
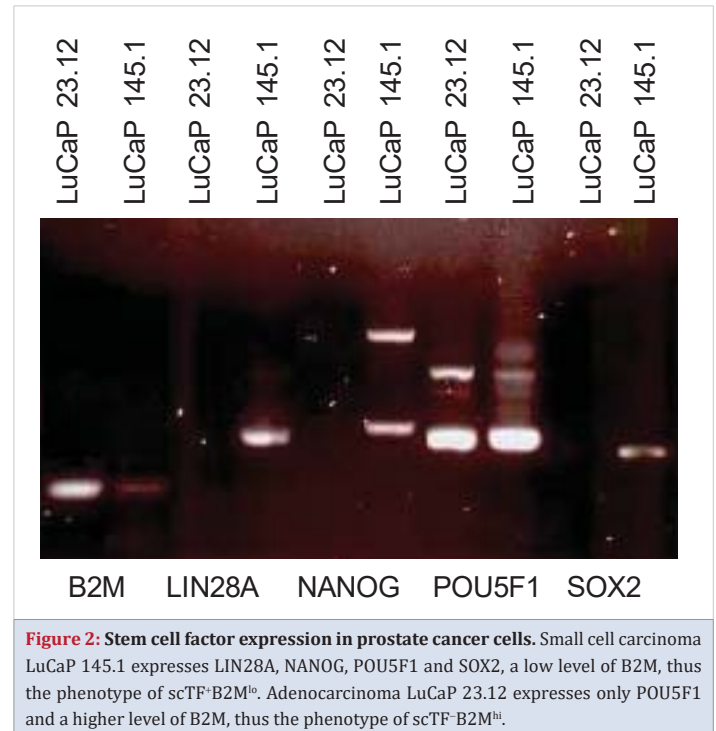


Figure 1: Prostate cancer cell types. In the PCA space, transcriptomes of the cancer cell types are displayed. One grouping is around the luminal cell datapoint (L) - LNCaP, C4-2, LuCaP 35 and G3. The other grouping is around the stem cell datapoint (EC) - PC3, DU145, CL1, LuCaP 49 and G4. 5D3 represents a possible prostate progenitor cell population sorted by antibody to ABCG2. No cancer cell type is close to the basal cell datapoint (B) even for the CD44⁺ PC3 or CL1. C4-2 and CL1 are derivatives of LNCaP through growth without androgen. The lines labeled pc1, pc2 and pc3 are the principal components axes of the 3D plot (adapted from ref. 7).

urothelium were also shown to possess no stem cell gene signature, and their gene expression was different from that of prostate basal cells, hence functionally distinct in the two organs [19]. Overall, the distribution of cancer cell datapoints revealed two separate groupings: either luminal-like around the L datapoint or less luminal-like, more stem-like around the ES, EC, and iPS datapoints. Included in this PCA display was the datapoint for a small CD44⁺ subpopulation (<1%) in the basal epithelium sortable by side population (SP) flow cytometry of cells capable of excluding Hoechst 33342 dye [20], or antibody to ABCG2, the stem cell membrane pump responsible for dye efflux. These cells were first selected by CD44 to exclude CD31⁺ABCG2⁺CD44⁻ endothelial cells [19]. Of note, CD31⁺ endothelial cells were also positive for CD133, hence not exclusively a stem cell marker [7,19]. The placement of the ABCG2⁺ cell datapoint near those of stem cells suggested a possibility of their representing an organ progenitor population [21]. Some cancer cells might derive from these CD44⁺ABCG2⁺ cells such as the CD44⁺ cell lines PC3, DU145, CL1. CL1 was, however, derived from the CD44⁻ luminal-like LNCaP via growth in androgen-free media [22,23].

Gene expression of stem-like prostate cancer cells

Given that markers like CD44 are not specific to stem cells, what makes CSC stem-like? Transcriptomics has distinguished luminal-like adenocarcinoma vs. more stem-like non-adenocarcinoma and small cell carcinoma. A majority of the LuCaP PDX lines are luminal-like based on transcriptome, even though many of them were established from metastatic lesions of advanced diseases. For example, LuCaP 35 from a distant lymph node metastasis is more luminal-like than Gleason pattern 4 (G4) cancer cells of histologically non-glandular primary tumors. The basis that small cell carcinoma cells are stem-like is their expression of stem cell transcription factors (scTF) LIN28A, NANOG, POU5F1, and SOX2. This quartet of scTF could be used to convert adult cells into iPS cells with a resultant transcriptome like that of ES cells [24,25]. Figure 2 shows the presence of these scTF transcripts in small cell carcinoma LuCaP 145.1 vs. adenocarcinoma LuCaP 23.12. Of the four, only POU5F1 was present in LuCaP 23.12. SOX2 expression is likely responsible for the neuroendocrine phenotype of small cell carcinoma, as SOX2 can singly convert fibroblasts to induced neuronal stem cells [26]. Neuroendocrine genes, chromogranin A, enolase, and AURKA were also found in stem cells. The absence of stem cell RB1 in small cell carcinoma was a notable difference [27]. SOX2 was the sole scTF of four expressed by small cell carcinoma LuCaP 49 [7]. These scTFs were found expressed by other small cell carcinoma lines, LuCaP 93, LuCaP 145.2, non-adenocarcinoma lines, LuCaP 173.1, and LuCaP 173.2A [28]. LuCaP 77 showed expression of POU5F1 and LIN28A. The conversion from LuCaP 73 to LuCaP 73CR showed increased expression of LIN28A [27]. It is possible



that scTF genes are activated sequentially in the progression from adenocarcinoma to non-adenocarcinoma and small cell carcinoma, as suggested by their expression levels in these different LuCaP cells [27,28]. Nevertheless, no scTF was detected in the transcriptomes of Gleason pattern 3 (G3) and G4 tumor cells [29]. CD133⁻ adenocarcinoma cells showed signals for differentiation-associated AR, KLK3 (PSA), +/-CD10 (MME) but absent in CD133⁺ small cell carcinoma cells [7]. Significant is the differential expression levels of β -2 microglobulin (B2M), where it was found at a 10-fold lower level in stem cells (ES, EC, iPS), LuCaP 145.1 than differentiated G3 cancer cells, adenocarcinoma LuCaP 35, stromal cells of normal prostate (NPstrom) and stromal cells associated with cancer (CPstrom) through transcriptome dataset query of DNA microarray signal intensity values [27]. In a sense, stem and non-stem cells can be operationally phenotyped as scTF⁺B2M^{lo} and scTF⁻B2M^{hi}, respectively.

Molecular mechanism in the emergence of stem-like prostate cancer cells

Is the expression of scTF responsible for CSC? The presence of prostate cancer-specific TMPRSS2-ERG fusion in both adenocarcinoma and small cell carcinoma of the same tumor cases provides an argument for a direct lineage between the two tumor types [30]. Cases under active surveillance have been reported to show disease progression with adverse pathology, from a glandular to non-glandular histology with diminution or loss of luminal secretion [31]. Prostate cancer, like cancer in general, exhibits dedifferentiation in progression from lower to higher grades. Could scTF activation convert differentiated adenocarcinoma cells into undifferentiated

small cell carcinoma cells? A few LuCaP xenograft lines could be forced to grow *in vitro*, but a majority could not. A method used in propagating ES and iPS cells *in vitro* was found to be applicable in adapting LuCaP lines to grow in culture [26,32]. This involves placing freshly prepared single LuCaP cells by collagenase digestion of minced tumor pieces in the presence of irradiated mouse embryonic fibroblasts (MEF). MEF can be harvested directly from mouse embryos or obtained from commercial vendors. In this way, LuCaP cells proliferate, can be passaged by trypsin treatment, and replated on MEF (irradiated MEF cannot be passaged). The *in vitro* grown LuCaP cells can be stored frozen in liquid N₂ using a cooling protocol used in stem cell research [32]. The AR⁺ adenocarcinoma LuCaP 70CR, LuCaP 73CR, LuCaP 86.2, LuCaP 92, LuCaP 105CR were infected by lentiviral vectors containing LIN28A, NANOG, POU5F1, SOX2 gene cassettes [28]. In three weeks, the culture morphology changed, in which small, darker appearing (under light microscopy) cells took over, which differed from that of mock-infected cells. This cell appearance resembled that of cultured non-adenocarcinoma PC3 cells. Transcriptome analysis of LuCaP 70CR* [* to denote reprogrammed or induced pluripotent cancer (iPC) cells] showed a large difference with that of parental LuCaP 70CR (Figure 3). Our PCA software allows new transcriptome datasets to be displayed with respect to those of CD26⁺ luminal, CD49a⁺ stromal, CD104⁺ basal, CD31⁺ endothelial, ES, EC, iPS and others for lineage relationship. In LuCaP 70CR*, genes associated with differentiation, such as anterior gradient 2 (AGR2), PSA/KLK3, were down-regulated, as well as B2M. Signal values for LIN28A, NANOG, POU5F1, and SOX2 were increased. The value between the datapoints LuCaP 70CR* and LuCaP 70CR was 91.72, comparable to that between CPstrom and its derived iPS cells (NPstrom cells are refractory to reprogramming for the reason given below)

[25]. Placement of the LuCaP 70CR* datapoint was distal to those of all the prostate cell types included, and separate from those of stem cells. LuCaP 70CR* was closest to LuCaP 145.1 ($\Delta = 45.85$; compared to $\Delta = 61.83$ with EC, $\Delta = 78.04$ with ES). Also shown are the small cell carcinoma LuCaP 145.1 and LuCaP 49 with gene expression difference of $\Delta = 58.95$, which reflected the existence of subtypes as found in clinical specimens [33]. This measure was similar to $\Delta = 41.42$ between adenocarcinoma LuCaP 70CR and LuCaP 35. The reprogramming of differentiated cancer cells demonstrated that scTF were involved in prostate cancer dedifferentiation, and that adenocarcinoma could be converted to small cell carcinoma-like and stem-like with attendant changes in culture morphology and cell appearance, plus down-regulation of B2M. Since these initial experiments, scTF plasmid vectors of lower biosafety concern were generated that produced equivalent efficiency in reprogramming [27]. These vectors can be readily produced in large quantities rather than by the complicated route in preparing lentiviral vectors. Full-length scTF cDNA cloned from LuCaP 145.1 functioned as that cloned from ES cells to produce reprogrammed cells. Transfection of scTF into C4-2B, LNCaP, PC3, and human kidney fibroblasts HK293F produced stem-like derivatives with decreased B2M and culture morphology change; transfection with vectors containing other gene constructs did not [27]. Thus, scTF expression in adenocarcinoma leads to dedifferentiated cancer cells. The resultant stem-like cancer cells become less epithelial-like with loss of epithelial cell density ($\rho = 1.07$) to one of stromal cells ($\rho = 1.035$), shown by the banding of LuCaP 145.1 cells in a density gradient [27,34]. Association between expression of these four scTF and increased prostate cancer aggressiveness from adenocarcinoma to small cell carcinoma has been documented in the literature [35-38]. CSCs do exist in prostate cancer despite not identifiable by CD44 reactivity.

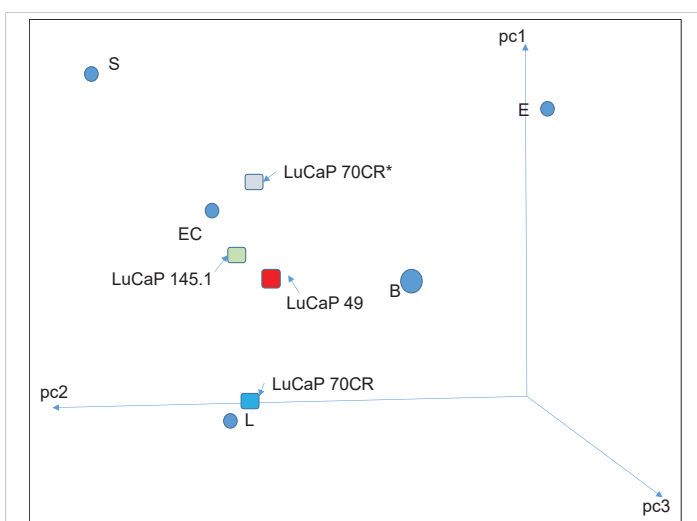


Figure 3: Reprogramming of adenocarcinoma LuCaP 70CR. The PCA display shows the gene expression change after transfection of scTF into LuCaP 70CR. The transcriptome datapoint of the resultant derivative, LuCaP 70CR*, is now localized to the stem-like grouping (adapted from ref. 25).

Differentiation of prostate CSC

Can prostate CSC undergo differentiation to produce other cancer cell types? In embryonic development of the prostate and bladder, the stromal mesenchyme controls the formation of these two organs. Organ stem or progenitor cells respond to stromal instruction by undergoing epithelial differentiation to produce prostate or bladder tissue accordingly [39]. This stromal induction is retained in the developed organs for tissue renewal and repair. By comparative transcriptomics between CD49a⁺ prostate stromal cells and CD13⁺ bladder stromal cells (localized adjacent to the basal urothelium), genes encoding secreted protein molecules were identified [40]. The experimental rationale was that these genes encode candidate organ-specific signaling hormone molecules. Among the identified were proenkephalin (PENK) specific to the prostate, stanniocalcins (STC1, SCT2) with differential expression in prostate vs. bladder. Bladder-specific secreted molecules were likewise identified. Prostate stromal expression of PENK

was verified by immunohistochemistry using a polyclonal antibody generated via a selected peptide sequence. PENK was found to be a marker for also smooth muscle cells of the bladder muscularis and blood vessel walls. Prostate stromal cells are characterized as smooth muscle cells positive for actin (ACTA2), desmin (DES), caldesmon (CALD1), calponin (CNN1) [41,42]. Significantly, PENK was absent in CPstrom. One can conclude that this secreted molecule is no longer expressed by stromal cells associated with tumor cells. On the other hand, CPstrom differ from NPstrom by intense immunostaining for CD90, which was used to isolate these cells from tumor samples [43]. Transcriptomics showed the absence of PENK transcripts in CD90⁺ CPstrom [42]. Unlike PENK, STC1 was also expressed by luminal cells, but its transcript level was down-regulated in cancer cells and xenografts representing advanced diseases [34]. These results suggest that stromal influence through intercellular communication is either lost or diminished in tumors. Lack of differentiation signaling over time could lead to less differentiated and more stem-like cancer cells.

We designed a simple assay to test the functional property of stromal cells. Cultured CD49a⁺ NPstrom and CD13⁺ bladder stromal cells (NBstrom) or their respective cell-free conditioned media containing secreted molecules were added to an EC cell line, NCCIT, employed to serve as stem cells (ES cells were not allowed administratively for research) [17]. The stem cell marker alkaline phosphatase (ALP) was used to monitor the co-culture in a time course of 7 d. At selected time points, treated NCCIT cells were analyzed by DNA microarrays. In time, the colony morphology changed from that of stem cells to that of fibroblast-like with loss of ALP staining and decreased proliferation. In PCA, the transcriptome datapoints “migrated” from that of stem cells to cultured stromal cells (not sorted stromal cells due to proliferation genes activated when cells were placed in culture). Dataset query showed increases in the expression of NPstrom markers STC1, PENK, TNC, EDNRB, CNTN1, MMP3, verified by reverse transcriptase-polymerase chain reaction (RT-PCR). That the EC cells were induced to differentiate was evidenced by down-regulation of scTF and up-regulation of B2M. Plasticity in EC response was shown by co-culture with NBstrom. Down-regulation of scTF and up-regulation of B2M were similarly found but with increased expression of NBstrom genes such as GFRA1 and not that of NPstrom genes PENK, CNN1. The different levels of STC1 and STC2 between NPstrom and NBstrom were also replicated in the treated EC cells. In addition, CD90⁺PENK-CPstrom isolated from a G3 tumor induced down-regulation of scTF and up-regulation of B2M but without induction of PENK [44]. In essence, the stromal cells dictated how stem cells respond. Each response was specific to NPstrom, NBstrom or CPstrom. We postulate that such intercellular communication exists in every organ that requires tissue renewal. It remains

to be studied for CPstrom isolated from G4 (nonglandular) or Gleason pattern 5 (G5, single cells) tumors as these sample types are now rarer especially in the era of robotic surgery. Since stem-like prostate cancer cells are scTF⁺B2M^{lo} could they also respond to stromal signaling trackable by down-regulation of scTF and up-regulation of B2M?

Small cell carcinoma LuCaP 145.1 adapted to *in vitro* growth was transfected by a plasmid vector containing the PENK gene cassette (pPENK1 vs. control vector). At 3 days, the cells were analyzed (a longer time course was not attempted because the MEF feeder cells were killed by the drug selection; without them, LuCaP cells could not survive) [30]. The RT-PCR result showed down-regulation of scTF and up-regulation of B2M to indicate that the LuCaP 145.1/PENK cells were undergoing differentiation. Of the four scTF monitored, expression of POU5F1 was less affected due to this factor being expressed by many LuCaP lines of adenocarcinoma and non-adenocarcinoma (*cf.* Figure 2). Moreover, differentiation through PENK and dedifferentiation through scTF can both take place in cancer cells. scTF⁻B2M^{hi} adenocarcinoma (AR⁺PSA⁺) LNCaP prostate cancer cells were first reprogrammed by scTF transfection using our constructed plasmids pLP4 (LIN28A-POU5F1)-*neo* and pSN2 (SOX2-NANOG)-*neo* [28] to obtain neo^RscTF⁺B2M^{lo} LNCaP*. The cell appearance was changed, as occurred with scTF reprogrammed LuCaP adenocarcinoma lines. One clone, LNCaP*-2, was then transfected with pPENK1-*bcr*. The resultant neo^Rbsr^RLNCaP*-2/PENK cells lost the stem-like appearance, replaced by one resembling that of LNCaP transfected by pPENK (Figure 4). Thus, cancer cells can undergo dedifferentiation from scTF⁻B2M^{hi} to scTF⁺B2M^{lo}, and re-differentiation back to scTF⁻B2M^{hi}. These various phenotypes were maintained in serial passages as the plasmid sequences were incorporated into the genome. Thus, PENK can antagonize multiple stem cell transcription factors simultaneously. Being aneuploid with multiple known DNA mutations did not appear to hinder differentiation in LNCaP. One can perform a systematic investigation to see whether this applies to all the available prostate cancer cell lines or even cell lines of other tumor types. Since PENK acts on scTF, other tumor cell types with expression of scTF would also likely respond to PENK. For example, PENK could be tested on lung small cell cancer cells if they are shown to be stem-like with the scTF⁺B2M^{lo} phenotype. Furthermore, identified NBstrom signaling molecules could likewise induce stem-like prostate cancer cells to undergo differentiation, as shown by their effect on NCCIT. This would demonstrate that stem-like cancer cells also possess response plasticity of normal stem cells. We postulate that signaling molecules could be identified in all organs, and tumor progression in these organs would follow the pathway of dedifferentiation from an initial differentiated cancer phenotype to more stem-like phenotypes. The following research could be carried out to

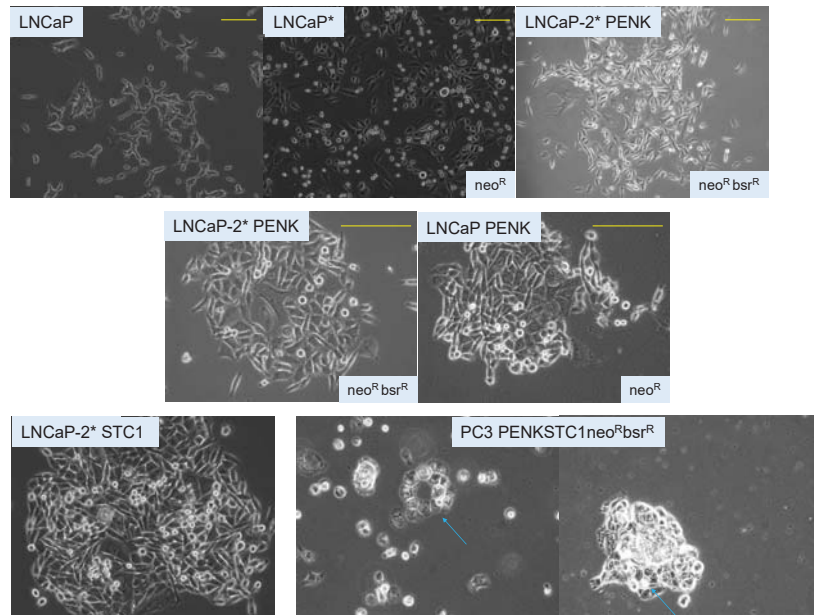


Figure 4: Prostate cancer cell dedifferentiation and differentiation. The top five panels show LNCaP, LNCaP+scTF=LNCaP-2*, LNCaP-2+PENK. Gene expression changes as monitored by transcriptome analysis are accompanied by changes in cell appearance. The bottom three panels show LNCaP-2* transfected by STC1 and the more stem-like PC3 by PENK and STC1. Gland-like cell clusters (blue arrows) with a central lumen can be seen in PC3/PENKSTC1. neo^R and bsr^R indicate drug resistance in the selection of transfected cells.

show whether the mouse equivalent of Penk has any effect on LuCaP 145.1; whether stem-like cancer cells can be induced to differentiate into pseudo-normal or non-prostatic cell types, depending on the signaling applied [45-49].

Like PENK in the embryonic mesenchyme [50,51], the STC1 and STC2 proteins have a similar function in organ development. STC1 and STC2 regulate phosphate transport across the epithelia and promote cellular maturation [51,52]. STC1 was induced in NPstrom-treated NCCIT cells at an earlier point than PENK [17]. Unlike PENK, it is not silenced in primary prostate cancer with expression in both cancer epithelial and cancer-associated stromal cells [34]. Its expression was found to decrease in disease progression, with cancer cell and xenograft lines showing reduced levels [34]. The effect of PENK on prostate cancer cell morphology is shown above. The PENK-transfected LNCaP cells appeared in close contact with each other, unlike untransfected cells. A similar appearance change was seen when LNCaP cells were transfected with STC1 [53]. When both PENK and STC1 were transfected into the more stem-like PC3 cells, organoid-like cell clusters were obtained. A gland-like structure with cells around a central luminal space was found (Figure 4) [53,54]. A follow-up study could probe the synergistic effect of PENK + STC1 on a panel of LuCaP adenocarcinoma, non-adenocarcinoma, and small cell carcinoma lines, each with its unique genomic alterations. In addition, STC2 could be included using an expression plasmid gene copy number of 2:1 STC1:STC2 to mimic their stromal cell expression levels [17,40]. The transfections could start with STC1 and STC2, to

be followed by PENK. Changes in cell shape are monitored for correspondence to the expression of tight junction proteins. These protein molecules, located between functional epithelial cells, provide apicobasal polarity for polarized secretion. Barrier integrity is maintained by transmembrane proteins such as claudins, occludins, and junctional adhesion molecules [55]. Abnormal expression of tight junction proteins can be found in different solid tumor types [56,57]. Loss of claudin is linked to castration-resistant prostate cancer [58]. Restoring the expression of claudin and others through PENK and STC could constitute a form of effective cancer differentiation treatment. The influence of all secreted stromal factors found in conditioned media could be tested, as was studied with NCCIT. Response plasticity of stem-like prostate cancer cells can be tested by using NBstrom and CPstrom; transcriptomics is used to identify differential gene expression in the resultant cells. An interesting experiment is to prepare CPstrom from G3 vs. G4 or G5 tumors for incubation with NCCIT, followed by adenocarcinoma and non-adenocarcinoma cells to determine the specific defects in mesenchymal-epithelial interaction of these tumor grades. Thus, prostate CSC can differentiate in response to secreted factors from stromal cells. Maintenance of gene expression over multiple passages in mice indicates that CSCs do not undergo spontaneous differentiation or are induced by host factors.

Accumulation of DNA mutations

As alluded to above, is the aggressiveness of CSC attributed to accumulated mutations in the genome over the disease

course? Exome sequencing showed no such pattern of a high number of DNA mutations for scTF⁺B2M^{lo} small cell carcinoma [45]. DNA mutations detected in the LuCaP lines studied presented the following order in decreasing numbers: adenocarcinomas LuCaP 58/liver metastasis > LuCaP 73/primary > LuCaP 147/node metastasis > small cell carcinoma LuCaP 145.2/node metastasis (a sister clone of LuCaP 145.1/liver metastasis). One hypermutated genome was found to contain ten times more single-nucleotide variations. No substantially different patterns of structural alterations, gain or loss in chromosome copy numbers, interchromosomal translocations, intrachromosomal rearrangements among tumors of similar grade and stage, presence of Tmprss2-ERG fusion were found. No trend of more changes with higher disease grade or stage was detected [46]. These findings contrast the mutational basis of carcinogenesis and one of the key features of the CSC postulate. Similar findings were found among types of breast cancer [47]. For small cell carcinoma of the bladder and lung, genomic changes were specific to the organ rather than to the cancer type [48]. The transition from urothelial carcinoma to small cell carcinoma also appeared to involve dedifferentiation. As described above, there are more gene expression changes between CPstrom and NPstrom than between G3 cancer and luminal epithelial cells, yet no significant mutations were detected in tumor-associated stromal cells [49]. In other words, there is no correlation between gene expression changes in cancer and the degree of genomic changes.

Loss of stromal signaling underlying cancer dedifferentiation

What is the lineage relationship between CPstrom and NPstrom? We found that the interaction between NPstrom and NCCIT was bidirectional. The effect of stem cell-secreted factors on stromal cells was monitored by transcriptomics. PCA of the transcriptome datasets showed that the datapoint of NPstrom+NCCIT “migrated” toward that of CPstrom. The change was also evident in the expression of microRNA (miRNA) [59]. mRNA transcripts of CD90, MiRN21, HGF, SFRP1, and BGN were increased in correspondence with their higher levels in CPstrom. HGF, a mediator of stromal-epithelial interaction, is expressed by undifferentiated mesenchyme in embryogenesis [60]. Increased miRNA species included miR-21 (processed from MiRN21), let-7f, miR-23a, miR29b. miR-21 is associated with dedifferentiation [61]. On the other hand, NCCIT factors showed minimal effect on CPstrom (datapoints CPstrom+NCCIT=CPstrom). Table 1 lists the candidate genes encoding secreted proteins from NCCIT for converting NPstrom to CPstrom. As a result, a less differentiated stroma could be the underlying cause for the emergence of cancer cells. One research approach could employ partitioning of NCCIT conditioned media into molecular weight fractions, each of which are added to NPstrom to narrow down the

Table 1: Stem cell secreted proteins. Listed are genes encoding proteins secreted by NCCIT in culture. “2x” indicates higher expression than the others according to DNA microarray signal intensity values.

Stem cell secretory protein					
GPC4	2x	CPVL		CXCL14	C5orf26
CR1L		IGF1R	2x	PCSK9	MASP2
APOE	2x	RARRES2		CLYBL	CHRDL1
GDF3		AXIN2		RBM35A	2x MKRN1
MDK		NASP		DNAJC19	2x AMT
RET		MFI2		CKMT1B	LOC38985
KAL1		ZNF678	2x	NAALAD2	PROK2
SCG5		NRTN		OLFML3	ALKBH7
INHBE		FUCA1		NIPSNAP1	FLJ14712
CTSL2		FAAH2		PLAC1	
ALPL	2x	PGA5		TMEM177	
GPC3		B3GNT7	2x	COCH	
COL2A1	2x	FAM24B		C6orf166	
FLJ22662		SMPDL3B		MAP3K9	
APOC1		C12orf39		C18orf54	

pool of candidates. Thus, by total gene expression, CPstrom represents an under-differentiated version of NPstrom missing PENK and other possibly key signaling molecules to instruct epithelial maturation. Of note, due to the antagonistic effect of PENK on scTF PENK-positive NPstrom could not be reprogrammed whereas PENK-negative CPstrom could [25].

As NPstrom is being replaced by CPstrom in tumor foci, NPstrom in the non-involved part of the gland is being ablated by cancer-secreted AGR2. Secreted Agr2 in lower vertebrates is involved in limb regeneration [62]. Receptor binding in wound tissue initiates removal of cells and differentiation of stem/progenitor cells. In prostate cancer, the secreted AGR2 affects NPstrom through induction of programmed cell death (PCD) [63], thus depleting further functional stromal signaling. As a consequence, epithelial differentiation is compromised leading to less luminal-like more stem-like abnormal cells over time. Down-regulation of STC1 in cancer cells may also be a contributing factor. Further research will attempt to better understand prostate cellular differentiation, how PENK and STC proteins function in concert, and how their genes are activated.

Metastatic capability - role of cancer-secreted AGR2

The AGR2 protein exists in two forms: intracellular iAGR2 and extracellular eAGR2. In normal cells, the form is iAGR2 localized to the endoplasmic reticulum, while in cancer cells, overexpression leads to a portion being secreted and localized to the cell surface, hence eAGR2 [64,65]. In prostate cancer, the specific phenotype of AGR2^{hi}CD10^{lo} has a 9-fold survival advantage over that of AGR2^{lo}CD10^{hi} [66]. CD10 functions in allowing escape of cancer cells from the glandular capsule, as cancer cells in a majority of local metastases are CD10^{hi}AGR2^{-/lo} [67,68]. In addition, AGR2 expression is associated with cancer differentiation, and transcriptomes of AGR2^{hi} cancer cells like

LNCaP, LuCaP 35, established from lymph node metastases show them to be luminal-like [7]. However, immunostaining showed that most distant metastases in bone, lung, and liver had high expression of AGR2 (and low expression of CD10) [66]. LuCaP lines established from these lesions are luminal-like, not more stem-like, as one would expect if metastatic capability is restricted to CSC [7]. For extracapsular cancer cells, AGR2 is needed for dissemination throughout the body. In the absence of androgen in culture, CD10⁺AGR2⁻ LNCaP was converted to more stem-like CD10⁻AGR2⁺ CL1 (*cf.* Figure 1) [69], and this derivative acquired metastatic capability [22]. Dormancy in prostate cancer, in which recurrence occurs after a latent period post-treatment [70,71] may be explained by the need for a gene expression change from AGR2^{-/lo} to AGR⁺ in tumor cells. These dormant cancer cells were PSA⁺ (i.e., differentiated) as RT-PCR of PSA was used to detect them. Other results showed that benign non-metastatic rat mammary tumor cells transfected by human AGR2 produced lung metastasis in syngeneic hosts [72]. Inhibition of AGR2 in mouse models could prevent cancer metastasis [73]. Part of the metastatic process may involve cancer-secreted AGR2 to induce PCD of susceptible cells, allowing colonization of migrating cancer cells in their place. In contrast, small cell carcinoma lesions in metastases and the LuCaP lines established from them were not stained for AGR2 [66]. Acquisition of stem-like characteristics (AGR2⁻CD10⁻scTF⁺B2M^{lo}) is not a requisite for dissemination. At present, we lack experimental proof that AGR2-negative small cell carcinoma cannot metastasize. In general, AGR2⁺ tumor cells are found in many solid tumor types [74]. A cohort of 12,434 tumors from 134 categories was immunostained, with the result that a majority were positive for AGR2. Non-epithelial neoplasms rarely showed expression. Besides prostate, the cases probed were female genital tract adenocarcinoma, breast cancer subtypes, adenocarcinoma of the gastrointestinal tract, pancreatic cancer, lung cancer, and urothelial carcinoma. Since eAGR2 is cancer-specific, curative treatment and a cancer vaccine based on targeting eAGR2 could be envisaged, especially since non-cancer cells express iAGR2 in the cell interior and are therefore invisible to anti-AGR2 antibodies [75]. Thus, AGR2 expression can be switched on and off during the prostate cancer disease course. The involvement of AGR2 and CD10 in metastatic spread of prostate cancer, bladder cancer, and lung cancer is complex and entails interaction with different other proteins, as detailed previously [32]. Like AGR2 in cancer, CD10 can exist in two forms - intracellular iCD10 [76], where it interacts with heat shock proteins in the cytoplasm [77], and extracellular eCD10, the common form of normal cells.

Conclusion

We postulate that cancer in the prostate arises as a result of defective epithelial differentiation. The tissue renewal process

is controlled by CD49a⁺ stromal smooth muscle cells through, in part, signaling molecules such as PENK and STC [32,53]. The defect occurs when smooth muscle cell differentiation is arrested at an earlier stage, as represented by CD90 CPstrom. These cells fail to synthesize PENK, among others. Without it, the maturation of epithelial cells is stalled. The resultant neoplastic cells overexpress AGR2, [78] and cancer-secreted AGR2 further depletes functional stromal cells through PCD. As the disease progresses, the tumor epithelial cells begin to lose expression of STC1 (and STC2) and perhaps other genes associated with differentiation [34], eventually becoming stem-like. Reprogramming of adenocarcinoma to stem-like small cell carcinoma-like indicates that scTF could be reactivated in the absence of differentiation, or not inactivated in developing epithelial cells without STC1 and PENK. Forced expression of STC1 and PENK in cancer cells could produce organized glandular structures.

Successful mouse implantation to establish the family of LuCaP lines contradicts the supposition that only stem-like human tumors could initiate growth *in vivo*. All established tumor types can be maintained over multiple passages without detectable changes in gene expression and individual growth characteristics. This shows that stem-like cancer cells do not undergo differentiation spontaneously to yield non-stem-like progenies. Rather, stem-like cancer cells respond to hormone molecules secreted by stromal cells with down-regulation of scTF and up-regulation of B2M, with attendant changes in cell shape. This response is indifferent to the background levels of DNA mutations or aneuploidy in the tumor cells. Prostate cancer cells can readily undergo differentiation and dedifferentiation, suggesting that disease progression could be reversed.

Prostate cancer metastasis consists of two separate phases. First, CD10 is responsible for extracapsular escape. This is reflected in the association of poor patient outcome with CD10 expression in tumor cells. Second, AGR2 is needed for the spread throughout the body. This is manifested by the presence of luminal-like AGR2-positive instead of strictly stem-like tumor cells in distant metastases. Acquisition of stemness is, therefore, not a precondition for cancer spread. Emergence of stem-like cancer cells is due to suppression of differentiation through absence of signaling. Application of anti-androgen in treatment provides supporting evidence since testosterone is required for differentiation of this hormone-dependent organ [79]. This biology of prostate cancer differentiation could be equally applicable to describe other solid tumor types, in particular breast cancer in another hormone-dependent organ [80]. The proper signaling molecules await identification. Research effort could be spent on establishing multiple breast cancer xenografts [81] using the methodology developed in the establishment of LuCaP lines. We will use the experimental procedures to study the identified bladder

stromal factors on urothelial cell differentiation. In summary, our work in prostate cancer has shown that CSC exist, and that their stem-like characteristics are due to dedifferentiation/re-activation of scTF or arrested differentiation because of faulty intercellular communication. Accumulation of DNA mutations and acquisition of metastatic capability are not specific to CSC. The reliance of mouse implantation and certain markers to define CSC (more appropriately, stem-like cancer cells) is problematic.

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