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13. ABSTRACT ***(Maximum 200 Words)***

The first aim is to identify prostatic stem cells using an intraprostatic transplantation approach using GFP-tagged prostatic basal and luminal cell lines that we established. We found that both basal and luminal cells engraft and that engrafted basal cells give rise to luminal cells. The second aim studies the characteristics of prostatic tumors that arise after *ras* transformation of basal and luminal cells. We show that the transformed basal and luminal cell lines form subcutaneous tumors and that the rate of tumor growth is enhanced by co-inoculation with normal prostatic smooth muscle cells. This indicates that smooth muscle cells may contribute to the progression of prostatic tumors and that paracrine signaling between basal and smooth muscle cells may be involved in tumorigenesis. We also show that intraprostatic inoculation of transformed basal cells gives rise to tumors that contain luminal cells. This is significant as it may explain why most prostatic tumors have a luminal phenotype. Prostatic stem cells are considered to be of basal origin and stem cells are the likely targets of transformation leading to prostate cancer. Our results may explain this paradox as transformed basal cells may give rise to transformed luminal cells that may have a growth advantage. Hence by the time the cancer is clinically manifest most of the cells in the tumor may be luminal in nature.

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Introduction

The first aim of this proposal is to identify prostatic stem cells using a novel in vivo approach using prostatic basal and luminal cell lines that we have established. We have used one of the important properties of stem cells, their ability to self renew, to determine that our cell lines engraft in vivo and give rise to normal prostatic tissue. The second aim is to study the characteristics of prostatic tumors that arise after transformation of basal and luminal cells. The cells lines have been transformed with the *ras* oncogene and the composition and characteristics of the tumors are being examined. We are especially interested to determine whether transformed basal cells can give rise to luminal cells as most prostatic tumors have a luminal phenotype. As the stem cell compartment is considered to reside in the basal layer [1-3] and as tumors may originate from the transformation of stem cells [4] it is puzzling that most prostatic tumors express a luminal phenotype. The experiments using *ras* transformed basal cells are designed to explore the possibility that transformed basal cells may give rise to tumors with a luminal phenotype.

Body

Task 4. Generation of a GFP-tagged ras-oncogene expression vector

We have previously established basal and luminal prostatic cell lines that are non-tumorigenic [5, 6] and that are GFP-tagged and can incorporate into normal prostatic epithelium. The basal cell line can incorporate into normal prostate epithelium in vivo and also gives rise to normal luminal cells. These basal and luminal epithelial cells lines were transfected with expression vector pTracer-SV40 which has a GFP tag and into which we cloned a N-ras proto-oncogene, with a site-directed V12 mutation, creating vector pTracer-SV40-N-RasV12. The *ras* oncogene was amplified from vector pS65-N-rasV12 (obtained from Dr Angel Pellicer in the Department of Pathology at NYU Medical Center) using the following primers: 5′ GAA TCC AGG ATG GCC ATG ACT GAG TAC AAA CTG GTG GTG and 3′ GCG GCC GCT TAC ATC ACC ACA CAT GGC AAT CC. The PCR product was gel purified and cloned into PCR Blunt. The plasmid was amplified in One Shot Competent Cells and the N-RasV12 insert was excised with EcoR1 and Not1, gel purified and subcloned into the pTracerSV40. The orientation and fidelity of the insert was validated and basal and luminal cells were transfected with the construct.

Task 5. Transfection of prostate epithelial cell lines with the GFP-tagged ras construct

Transfection was performed on cells seeded at 0.5 x 10^5 cells/ml using Fugene and transfected cells were selected using Zeocin. Zeocin was used as our cells are resistant to Neomycin as they were derived from p53 null mice in which the p53 gene is disrupted by a neomycin cassette. Antibiotic-resistant clones were selected. The expression of GFP was determined by fluorescent microscopy and the expression of the N-RasV12 gene was verified by RT-PCR and by Western blot using antibodies to *ras*.

Three tumorigenic epithelial cell lines were derived by N-RasV12 transformation of the basal and luminal cell lines. One was basal in origin and two were of luminal origin. A third transformed luminal cell line was obtained by passaging normal luminal cells through athymic

mice. Although the luminal cell line was non-tumorigenic when inoculated subcutaneously, in one animal a small subcutaneous tumor developed and a cell line was derived from this tumor. The *ras*-transformed basal cell line expressed only basal cytokeratins (CK 5/14) and the three transformed luminal cell lines expressed only luminal cytokeratins (CK 8/18) in vitro.

We had previously planned to transform an additional cell line that expressed both basal and luminal cytokeratins (line 12′). As mentioned in my previous progress report we had problems with the stability of this line in that when it was cloned the cells were heterogeneous in their expression of basal and luminal cytokeratins. We therefore proceeded with experiments using the *ras* transfected GFP-expressing basal and luminal cell lines.

Task 6. Assessing the tumorigenicity of *ras* transformed basal and luminal cell lines.

Growth of transformed cell lines in vitro

The transformed cell lines contained more non-adherent and loosely attached cells than their normal counterparts when cultured in vitro. Both the normal and *ras*-transformed basal cell lines expressed androgen receptors and dihydrotestosterone (DHT) stimulated growth of both cell lines although the transformed cells were more sensitive to DHT than its parental line with growth being stimulated by 20-40% by 10^{-8} – 10^{-12} M DHT. The luminal cell lines did not respond to DHT.

As the ability of cells to grow in an anchorage independent manner is a characteristic of transformed cells we determined their colony forming ability when seeded in soft agar (0.33%). Both the parental and *ras*-transformed basal cell lines formed colonies in soft agar with a low, comparable efficiency of 1%. The parental luminal population did not form colonies in agar whereas the three transformed luminal cell lines formed colonies with an efficiency of 11-13%.

Growth of transformed cell lines in vivo

The ability of the cell lines to form tumors when inoculated subcutaneously (5 x 10^6 cells/100µl) into athymic mice was examined. Athymic mice were used as GFP can elicit an immune response in syngeneic hosts [7]. All four transformed cell lines formed progressively growing subcutaneous tumors (Fig. 1). The rate of growth of tumors varied between the lines with the spontaneously transformed luminal cell line (TE-L-4) growing most rapidly while tumors from one of the *ras*-transformed luminal cell lines (TE-L-1) grew most slowly.

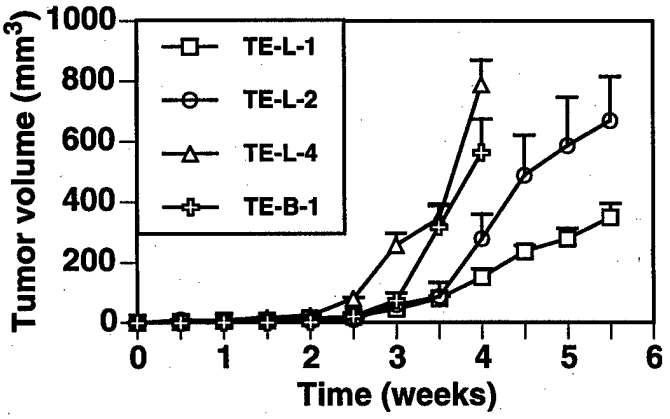

Figure 1. Growth of subcutaneous tumors in athymic mice. Transformed cell lines, TE-B-1, TE-L-1, TE-L-2 and TE-L-4 were inoculated at 5x10^6 cells/100 µl in the subscapular region of 6 week-old male athymic mice. Tumor size was measured twice a week.

As stromal/epithelial interactions are relevant in the growth and behavior of normal and abnormal cell proliferation we determined the effect of prostate smooth muscle (SM) cells on tumor cell growth. We used a prostatic SM cell line that we established as part of a panel of murine prostatic cell lines [5]. The *ras*-transformed basal cell line (TE-B-1) was inoculated alone (5×10^6 cells/100 µl) or with the normal SM cell lines (PSMC1 or PSMC2; 2.5×10^6 cells/100 µl). Growth of tumors from the *ras*-transformed basal cell line was increased by both SM cell lines PSMC1 and PSMC2 cell lines ($p<0.05$ and <0.01; Fig. 2).

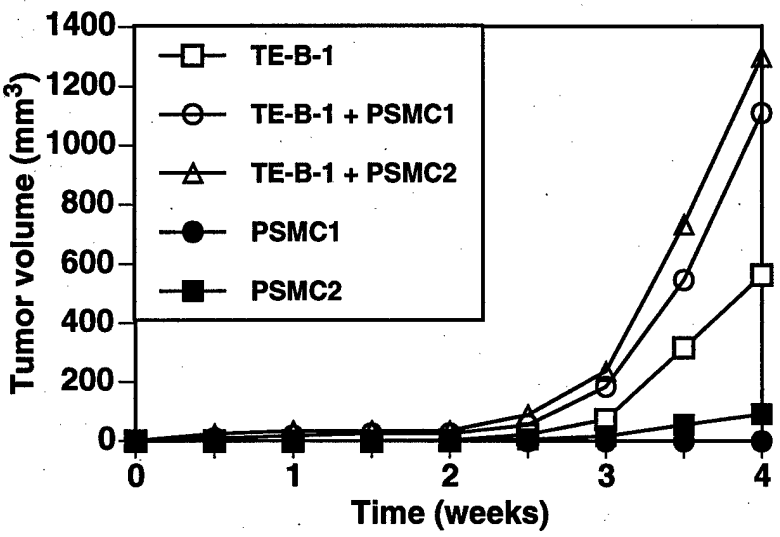

Figure 2. Growth of subcutaneous tumors in the absence and presence of normal murine prostatic smooth muscle cells (SMC). The transformed *ras* basal cell line, TE-B-1 (B), was inoculated subcutaneously alone ($5x10^6$ cells/100µl), or in combination with prostatic SMC (PSCM1 and PSCM2) ($5x10^6$ basal cells with $2.5x10^6$ normal SMC/100 µl). Mice were examined biweekly for evidence of tumor growth and tumor volume was measured.

This indicates that the prostatic stroma may aid in tumor development. Paracrine signaling between tumor cells and fibroblasts has been shown to be relevant in carcinoma formation [8] and our results indicate that in prostatic carcinoma the adjacent SM cells may also aid in tumor progression.

The tumors arising from each transformed line inoculated alone were examined histologically. Tumors obtained from TE-B-1 had a uniform epithelial morphology (Fig. 3A, page 9). They expressed both GFP and basal cytokeratins at a low level, indicating their basal origin. They did not express luminal cytokeratins. Tumors arising from TE-L-1 and TE-L-2 were poorly differentiated with irregular epithelial cords interspersed with elongated stromal-like cells (Fig. 3B and C, page 9). The phenotype of tumors formed by the spontaneously transformed luminal line TE-L-4 was distinct in that they contained duct-like structures that were lined with cuboidal epithelial cells that strongly expressed luminal cytokeratins 8 and 18 (Fig. 3D and E, page 9). Tumors from lines TE-L-1 and 2 did not express either basal or luminal cytokeratins. All tumors obtained from transformed luminal cells contained GFP-expressing cells, although GFP expression was at a significantly lower level than found when the transformed cell lines were cultured in vitro.

We also did some preliminary orthotopic experiments in which we inoculated the *ras*-transformed basal cell line into the murine dorsolateral prostate (0.5×10^6 cells/lobe/10µl) to determine the effect of intraprostatic inoculation on tumor growth and behavior. Tumors (Fig. 4A, page 9) were removed, fixed and examined for cytokeratin expression and, in contrast to the subcutaneous tumors, were found to express both basal (Fig. 4B, page 9) and luminal cytokeratins (Fig. 4C, page 9). This result indicates that transformed basal cells give rise to

luminal cells when inoculated into a prostatic environment, confirming that luminal tumors may arise from transformed basal cells. Examination of the tumors using a wide-spectrum anti-cytokeratin antibody showed that the majority of the cells were epithelial in origin (Fig. 4E, page 9). Furthermore, the TE-B-1 origin of the cells in all tumors was confirmed by detecting GFP expression by immunohistochemistry. It could be shown that the majority of the cells within subcutaneous and orthotopic tumors arising from inoculation of TE-B-1 cells expressed GFP. Since only the transformed epithelial cells expressed GFP, this result indicates that host cells did not contribute significantly to the tumor mass.

Part of this work has recently been published in the journal The Prostate [9].

Key research accomplishments

1) A GFP-tagged *ras*-transformed basal cell line and two GFP-tagged *ras*-transformed and one spontaneously transformed luminal cell lines have been generated.
2) The transformed cell lines are tumorigenic in vivo.
3) Normal prostatic smooth muscle cells promote the growth of *ras*-transformed basal cells in vivo.
4) Intraprostatic inoculation of GFP-tagged *ras*-transformed basal cells gives tumors that contain luminal cells indicating that transformed basal cells can give rise to luminal cells in vivo.

Reportable outcomes

We have developed a panel of GFP-tagged *ras*-transformed basal and luminal cell lines that are tumorigenic in vivo. These cell lines are available for distribution.

Part of this work has recently been published in The Prostate [9].

Conclusions

We have generated a unique panel of cells that represent both normal basal and luminal prostatic cells and their transformed counterparts that will be of use to study a variety of aspects of prostate cancer. The cell lines have been GFP-tagged which aids in their localization and identification in in vivo experiments. In addition we find that normal prostatic smooth muscle cells promote the growth of tumors arising from the transformed basal cells. This indicates that smooth muscle cells may contribute to the progression of prostatic tumors and that paracrine signaling between basal and smooth muscle cells may be involved in this process.

We have also shown that transformed basal cells can give rise to tumors that contain luminal cells when inoculated intraprostatically. This is an interesting finding. The prostatic stem cells are considered to reside in the basal layer and stem cells are the likely targets of transformation leading to prostatic carcinoma. Yet most prostatic tumors have a luminal phenotype. Our results may explain this paradox as transformed basal cells may give rise to transformed luminal cells. It is possible that the luminal cells may have a growth advantage and hence by the time prostate cancer is clinically manifest all of the cells within a tumor may have a luminal phenotype. We plan to test this hypothesis by passaging tumors through animals and determining if they progressively become more luminal in nature. We also plan to isolate basal and luminal cells from the tumors and re-inoculate them in vivo to determine if their growth properties differ.

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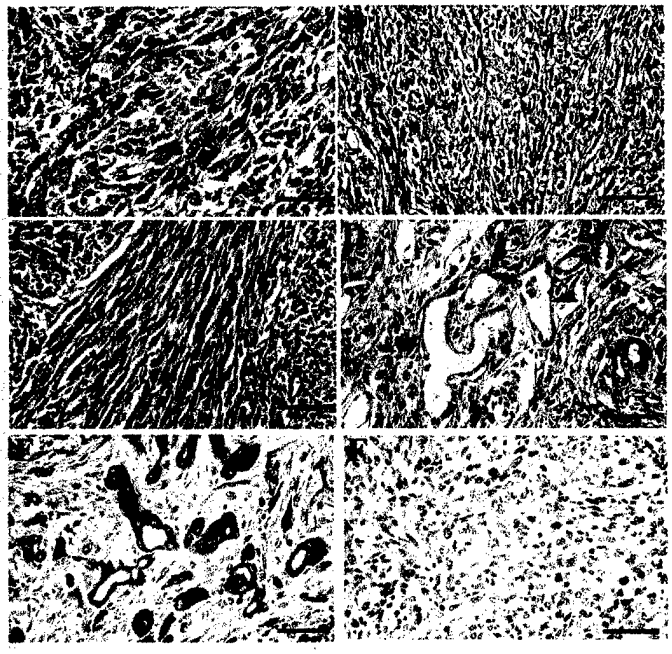

Figure 3. Histology of subcutaneous tumors arising from transformed epithelial cell lines. A - Paraffin-section of a tumor arising from the transformed basal line, TE-B-1, showing uniform epithelial morphology. B and C - Paraffin-sections of tumors arising from transformed luminal lines TE-L-1 (B) and TE-L-2 (C) showing poorly differentiated tissue interspersed with irregular epithelial cords and elongated stromal-like cells. D and E - Paraffin sections of a tumor arising from TE-L-4 cells, showing ducts lined with epithelial cells (D - arrows), that strongly expressed luminal cytokeratin, CK 8 (E). Use of normal rabbit or mouse IgG indicated that the cytokeratin staining was specific (F). Bars = 100 mm.

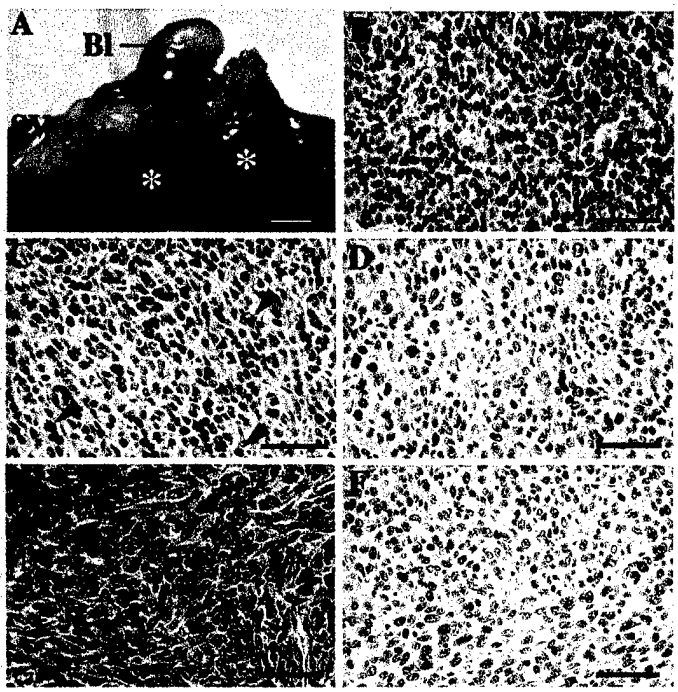

Figure 4. Morphology and cytokeratin expression of intraprostatic tumors arising from the transformed basal cell line, TE-B-1. A - Gross morphology of intraprostatic tumors (*) growing in both lobes of the dorsolateral prostate. SV = seminal vesicle; Bl = bladder. Bar = 0.5 mm. Intraprostatic tumors arising from the transformed basal cell line were removed from sacrificed mice, fixed in 70% ethanol, embedded in paraffin and sectioned. Tissue sections were examined immuno-histologically using antibodies to basal (CK14) and luminal (CK 8) cytokeratins. Tumors arising from line TE-B-1 were found to contain cells expressing basal cytokeratins (B) and scattered luminal cytokeratins (C - arrows). Use of a wide spectrum anti-cytokeratin antibody confirmed that all the cells were epithelial in origin (E). Use of normal mouse or rabbit IgG indicated that the cytokeratin staining was specific (D and F). Bars = 100 mm.