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### 13. ABSTRACT (Maximum 200 Words)
This report summarizes the second year of activity on this study. The long term goal of this project is to better understand why some prostate tumors grow aggressively while others are extremely slow growing lesions. The objective of the proposed research is to establish immortalized stromal cell lines derived from normal human prostate and from human prostate cancer and to use these cells to investigate the role of IGFs in prostate cancer growth. In this year we have focussed on using the molecular tools made in year one to generate new immortalized stromal cell lines and to examine the effects of IGF signaling on malignant and pre-malignant epithelial cells. Data have been generated, which at this time must be considered preliminary, which suggest a role for IGF signaling in malignant progression as hypothesized in the original application. No major technical obstacles have cropped up. The project is close to its timeline predicted in the accepted statement of work.

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Introduction

The long term goal of this project is to better understand why some prostate tumors grow aggressively while others are extremely slow growing lesions. The objective of the proposed research is to establish immortalized stromal cell lines derived from normal human prostate and from human prostate cancer and to use these cells to investigate the role of IGFs in prostate cancer growth. The central hypothesis on which this proposal is based is that prostate cancer progression is regulated, at least in part, by paracrine interactions between the prostatic stroma and the tumor. The first specific aim will generate immortalized cell lines with which to pursue mechanistic studies. The hypothesis is that fibroblastic cells immortalized by the insertion of a telomerase (hTERT) construct will behave in the same way in bioassays of their tumor-promoting activity as do the primary cell cultures from which they are derived. The rationale for these experiments is based upon observations by the PI and others on the role of stromal cells as promoters of carcinogenesis. The hypothesis of the second specific aim is that IGF family ligands act in a paracrine manner to elicit proliferation and/or tumorigenesis in human prostate cancer. The rationale for this specific aim is based on a variety of published observations connecting local and systemic levels of IGFs with prostatic growth and malignancy. The third specific aim will examine gene regulation in epithelial cells caused by changes in IGFs in the local microenvironment. The hypothesis is that changes in epithelial behavior are reflected in gene expression, the rationale is to identify gene products which might be targets for therapeutic intervention.
Statement of Work
Paracrine Regulation of Prostatic Carcinogenesis

Task 1
Establish and characterize immortalized normal and carcinoma associated human prostatic fibroblast lines.

a. Establish retroviral expression of hTERT in LZRS/Phoenix A cells (month 1)
   Transfection of LZRS construct into Phoenix A packaging cells. Selection of stable transfecants.

b. Infect fibroblasts and select based upon reporter gene expression (months 2-4)
   Infection of fibroblasts, FACS sorting for expression of GFP reporter

   c. Screen hTERT expressing cells for malignant transformation (months 3-9)
      Graft to athymic mouse hosts for 3 months, histopathological examination of recovered grafts (total 36 mice).

   d. Establish cell activity in tissue recombination bioassays (months 3-9)
      Recombine fibroblast cell lines with BPH-1 reporter cells. Graft to athymic mouse hosts, examine recovered grafts to determine biological effects (total 36 mice).

This task will produce immortal fibroblastic cells representative of both normal and malignant human prostate.

Task 2
Investigate the role of insulin-like growth factors in prostate tumor progression and proliferation.

   a. Generate LZRS constructs containing IGF-1, IGF-2 and IGFBP-3 and EYFP reporter (months 6-12)
      The constructs will be made from already existing pieces

   b. Establish retroviral expression of IGF family members in LZRS/Phoenix A cells (months 9-15)
      Transfect LZRS constructs into Phoenix A packaging cells. Select stable transfecants

   c. Infect immortalized stromal cells with the IGF family-expressing retroviruses (months 10-18)

   d. Select fibroblasts expressing EYFP reporter (months 11-19)
      FACS sorting for the EYFP reporter

   e. Screen infected cells for malignant transformation (months 12-22)
      Graft to athymic mouse hosts for 3 months, histopathological examination of recovered grafts (36 mice).

   f. Assess biological activity of IGF family-expressing cells in vitro (months 16-26)
      In vitro conditioned medium experiments

   g. Assess biological activity of IGF family-expressing cells in vivo (months 16-30)
Recombine with BPH-1 cells, graft to nude mice, after three months recover grafts and undertake histopathological analysis (138 mice).

This task will provide a series of stromal cell lines expressing IGF-1, IGF-2 or IGFBP-3. These will be matched with cells which do not express these proteins. It will provide information on the role of IGF family members as mediators of prostatic carcinogenesis in vivo.

Task 3
Investigate changes in epithelial gene expression elicited by IGF family members in the stroma.

a. Make and graft tissue recombinants (months 24-32)
Recombine representative cell lines from specific aim 2 with BPH-1 cells. Graft and harvest grafts after three months.

b. Prepare RNA, make cDNA, hybridize to arrays (months 27-35)
Dissociate harvested grafts, sort cells. Prepare RNA from the epithelial cell population.

c. Analyze array data (months 28-36)

This task will provide data on the changes of gene expression induced in human prostatic epithelial cells growing in vivo by local changes in IGF ligand availability.
Work Ongoing and Completed

Task 1a. hTERT constructs have been made and inserted into LZRS retrovirus. The sequence of the construct has been checked. The ability of the retrovirus to infect human primary prostatic cell cultures has been confirmed. A variation of the proposed methodology was used in that a construct containing puromycin resistance was used in place of the selectable EGFP marker. This modification makes cell selection more rapid and cost effective.

Task 1b. A series of normal and cancer associated prostatic stromal cell lines have been infected with the hTERT construct and puromycine selected (fig1). A real-time RT-PCR assay has been established to monitor and quantitate hTERT expression in the infected cell lines. As a check on protein expression a Western blot assay for hTERT expression is also being optimized. These methods are more straightforward and cost effective than the more traditional TRAP assay.

Task 1c. Screening for malignant transformation caused by the specific retroviral insertion point is ongoing. As noted in the original application this is more of a theoretical than a practical concern, however this is an aspect of retroviral immortalization which must be formally tested before further experiments can be performed. To this point no malignant transformation has been observed.

Task 1d. Testing of the CAF/normal fibroblastic activity of the hTERT immortalized cells in a BPH-1 tissue recombination bioassay is also ongoing. This process is somewhat delayed as tissue recombinations cannot be performed with specific stromal cell strains until malignant transformation testing of the stromal cells grown alone proves negative (task 1c). This backlog is easing as more cells strains pass through this barrier.

![Figure 1: A. Human stromal cells from patient 807 3.2 after infection with pBabeTert and selection with puromycin. B. Human stromal cells from patient 813 after infection with pBabeTert and selection with puromycin.](image-url)
Task 2a. LZRS constructs for expression of IGF-1, IGF-2 and IGF-BP3 have been generated. The constructs are fully sequenced and validated.

Task 2b. Retroviral expression of the IGF-family LZRS retroviruses in PHNX cells has been successfully achieved. Transfected cells show expression of EGFP as expected.

Task 2c. Infection of human prostatic stromal cells with all three IGF family members has been achieved in limited numbers of cells strains at this point.

Task 2d. Cell selection using FACS sorting is proceeding. Expression of GFP is confirmed in many cells. Cell sorting allows successful separation of expressing and non-expressing cells. Post selection culture demonstrates expression of GFP in all cells.

Task 2f. Infection of the human prostatic epithelial cell line BPH1 caftd3 with the IGF1 virus from task 2a allows secretion of IGF1 in the media for an autocrine/paracrine effect. Different clones have been obtained with different level of synthesis and production of IGF1 (figure 2). It was noted that the cells that express IGF1 do not form junctions with each other while the cells that do not express IGF1 or just have the empty vector assemble in a characteristic cobblestone pattern. The junction protein E-cadherin is localized in the cytoplasm in the cells that express IGF1 while E. Cadherin is at the plasma membrane in the control cells infected with an empty vector. A similar experiment performed using the parental BPH-1 cells (which are normally non-tumorigenic) gave essentially the same result. Those cells are being tested in vivo in xenograft experiment. Preliminary results show a bigger size of the graft recovered 4 weeks after grafting for the highest expressing IGF1 clone of BPH1 CAFTD3 cells, parallel experiments using the IGF1-expressing parental BPH-1 cells are underway.

Since this grant was originally written a new methodology (RNA interference) for suppressing gene expression and thus studying protein function has become available. As an alternative method to decrease IGF signaling we have generated a retroviral vector for RNA interference of IGFR1. The vector was designed (figure 3) in the pSuper retro backbone (Oligoengine), and BPH1 CAFTD3 cells were infected and selected for puromycin resistance. The abundance of the messenger RNA for IGFR1 was measured by realtime RT-PCR and the plasma membrane expression of the receptor was assessed by Immunofluorescence. Tissue recombination xenografting with rat UGM of those epithelial cells resulted in a lower invasion of the adjacent renal tissue by the epithelial cells after 4 weeks in the mice.
Cells  Conditioned media

Cells: C4 E4 C8 EV  C4 E4 C8 EV

Figure 2:
A. Western blot with an anti IGF1 antibody (Santa Cruz) demonstrating different expression levels of IGF1 in 3 clones of BPH1 caftd3 cells infected with a retroviral IGF1 expression vector (C4, E4 and C8) and the empty vector (EV).

B. Phase contrast photo micrograph at the 10X power showing the 4 clones of BPH1 CAFTD 3 cells expressing different levels of IGF1, seeded at low density.

C. Immunofluorescence anti E-Cadherin (Transduction Laboratories) in the same 4 clones seeded at high density. The secondary antibody is an anti mouse FITC conjugated. Pictures taken with a 40X objective.
Figure 3:
A. Immunofluorescence anti IGFR1 (US Biologicals) in BPH-1 cells (control) and BPH-1 cells infected by a pSuper retro SI-IGFR1 retrovirus and selected for puromycin resistance. Composite picture of phase contrast photomicrograph (gray) and FITC labeled secondary antibody (green).

B. Realtime RT-PCR, showing the mRNA levels of IGFR1 relative to the actin cDNA illustrate suppression of IGFR1 message by siRNA.

C. Immunohistochemistry of a tissue recombination between rat UGM stromal cells and human BPH1 CAFTD3 cells infected or not by the pSuper retro siIGFR1. T-antigen visualized in (brown) and counterstained with hematoxylin. In the control xenograft (left), some T-antigen expressing epithelial cells are found invading the renal tissue. In contrast no invasion is seen in the xenograft of epithelial cells infected with the retrovirus siIGFR1 (right).
Task 3. a. The xenograft of stromal cells from task 1 with BPH-1 cells is underway. This work was predicted to start around the beginning of year three. While the materials were on hand to start somewhat ahead of schedule this did not occur because the Vanderbilt Microarray Shared Resource is in the process of switching from 11k cDNA arrays to 29k oligoarrays. These are made of short oligonucleotides instead of big cDNAs, insuring a more specific hybridization. This is a much better annotated and much more cost effective array providing data on approximately 4.5x more unique genes for only marginally greater cost than the 11k chips.

**Key Research Accomplishments**

- Establishing and validating of biochemically selectable retroviral vectors for the introduction of hTERT into primary cell cultures of human prostatic stromal cells.
- Establishing and validating of optically selectable retroviral vectors for the introduction of IGF-1, IGF-2 and IGFBP-3 into primary cell cultures of human prostatic stromal and epithelial cells.
- Confirmation of expression of introduced genes of interest in infected cell cultures: apparition of a phenotype associated with IGF-1 expression in the cells: labile tight junction in BPH-1 CAFTD cells.
- Introduction of RNA interference technology in cell culture using retroviral vectors and validation in xenografting experiments: apparition of a phenotype associated with a decreased expression of the IGF receptor 1: decreased invasion in BPH-1 CAFTD cells.
- Taken together these last two point suggest that overexpression of IGF signaling in epithelial cells leads to increased invasive activity while loss of such signaling leads to decreased invasion in an in vivo model. Studies now underway will determine whether paracrine signaling provides a mechanism for such signaling to occur.

**Reportable Outcomes.**

**Book Chapter**


**Poster presentation.**

Conclusions.

This work is proceeding on the predicted timeline. No major hurdles have been encountered. The second year of this project, as described in the approved Statement of Work, was aimed at generating experimental data for further analysis in the conclusion of this project. These data are unveiling unsuspected aspects of IGF signaling: control of invasion.