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14. ABSTRACT Rate of prostate cancer progression is affected by the reactive stroma microenvironment. Our previous studies have shown that reactive stroma is recruited to the cancer foci and functions to promote angiogenesis and tumorigenesis via TGF- β regulated pathways. The objectives of the proposed research is to assess the origin / ontogeny of reactive stroma in cancer and the fundamental mechanisms of recruitment / activation in prostate cancer. To date, we have addressed studies proposed for Tasks 1 and 2. We have developed an <i>in vivo</i> matrix trapping approach to isolate, characterize and culture reactive stromal cells recruited to Matrigel plugs. We have also developed a three dimensional co-culture model that permits co-culture of prostate carcinoma cell spheroids with prostate stromal cells. We have initiated studies to re-introduce reactive stroma progenitors to the circulation of mice that receive xenograft implants of prostate cancer cells. These studies will allow us to dissect key mechanisms that mediate recruitment of reactive stroma to the tumor microenvironment and to target these mechanisms in order to inhibit tumorigenesis.					
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W81XWH-08-1-0144 "Targeting the Reactive Stroma Niche in Prostate Cancer"

Introduction:

The tumor microenvironment reactive stroma niche in prostate cancer remains poorly understood, yet the biology of this stroma has potent tumor regulatory functions. The origin of reactive stroma and the mechanisms through which reactive stroma regulates carcinoma progression are not known. Accordingly, the purpose of this project is to deduce the origin and ontogeny of reactive stroma progenitor cells in prostate cancer. It is our hypothesis that reactive stroma is recruited from both local and circulating progenitor cells. The goal of this research is to establish whether reactive stroma recruitment to the cancer foci can be used as a novel therapeutic targeting point. The long-range goal is to target reactive stroma recruitment as a novel therapeutic for the treatment of prostate cancer in order to disrupt the biology of the reactive stroma niche and therefore alter the progression of the cancer.

Body:

Task 1. Task 1 is to complete Specific Aim 1 studies: To determine mechanisms of local reactive stroma recruitment using a novel matrix trapping procedure and to assess the role of TGF- β in driving both recruitment and activation to reactive stroma phenotype.

The purpose of this Aim is to refine the matrix trapping procedure outlined in the application in order to define the population of cells recruited to reactive stroma. In addition, we have also worked to establish a novel co-culture model as proposed for this Aim. The co-culture model will be used to deduce the mechanisms of progenitor or reactive stroma cell interactions with human prostate cancer cells.

Matrix trapping studies have used EHS Matrigel plugs inoculated subcutaneous into the flanks of NCr *nu-nu* (nude) mice. The plugs have been prepared with either control media or

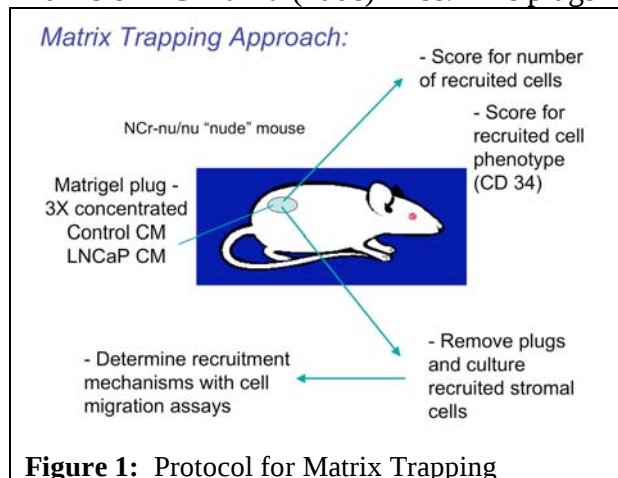


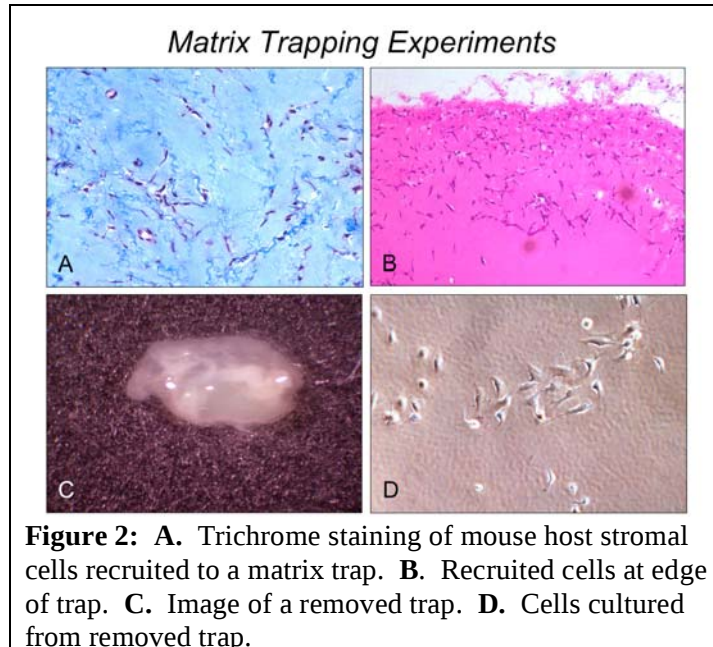
Figure 1: Protocol for Matrix Trapping

LNCaP (human prostate cancer cell) conditioned media. Our previous studies have shown that xenografts prepared with LNCaP carcinoma cells and Matrigel will recruit mouse host reactive stroma cells and that this recruitment is important for angiogenesis and xenograft survival (1-3). Matrigel plugs were removed and analyzed by light microscopy and immunohistochemistry. In addition, Matrigel plugs were removed, diced into small pieces and placed in culture in order to propagate recruited cells in vitro for further study. Figure 1 shows the matrix trapping protocol. Figure 2

shows that mouse host stromal cells are recruited to the Matrigel plugs and are observed as early as 7 days (Figure 2A). Figure 2B shows the edge of a Matrigel plug with recruited stromal cells invading the periphery of the plug. Figure 2C shows an image of a removed Matrigel plug prior to processing and Figure 2C shows that we have been successful in culturing stromal cells

derived from Matrigel plugs. Cells migrated from the diced pieces of plugs and proliferated in vitro. These cells are positive for vimentin as shown in Figure 3.

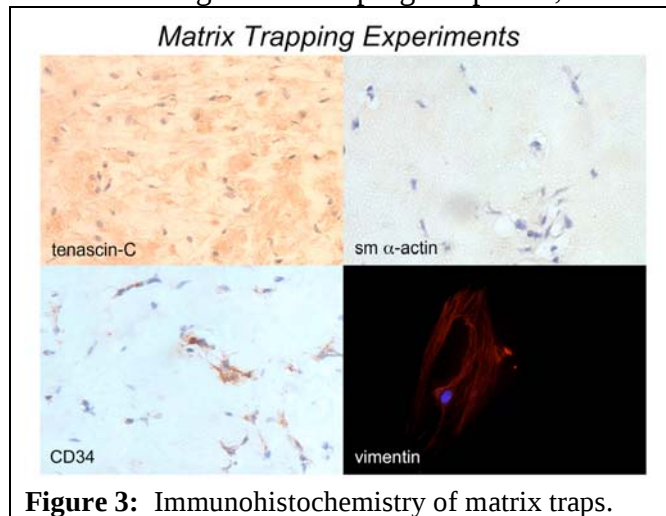
Preliminary studies have evaluated Matrigel plugs using immunohistochemistry for tenascin-C, CD34 and smooth muscle α -actin as shown in Figure 3. Of interest, plugs showed



considerable staining for tenascin-C although we do not yet know whether is due to endogenous tenascin-C in the Matrigel or tenascin-C synthesized by the recruited reactive stromal cells. Recruited cells were not positive for smooth muscle α -actin (Figure 3B), as we had originally suspected, since reactive stroma usually exhibits a myofibroblast phenotype. However, some recruited cells were positive for CD34 (Figure 3C). In vitro, some cells were positive for vimentin (Figure 3D). This is of interest to us, since we have recently shown that some reactive stroma in human prostate cancer specimens is dual positive for both vimentin and CD34 (Figure 4). CD34 is

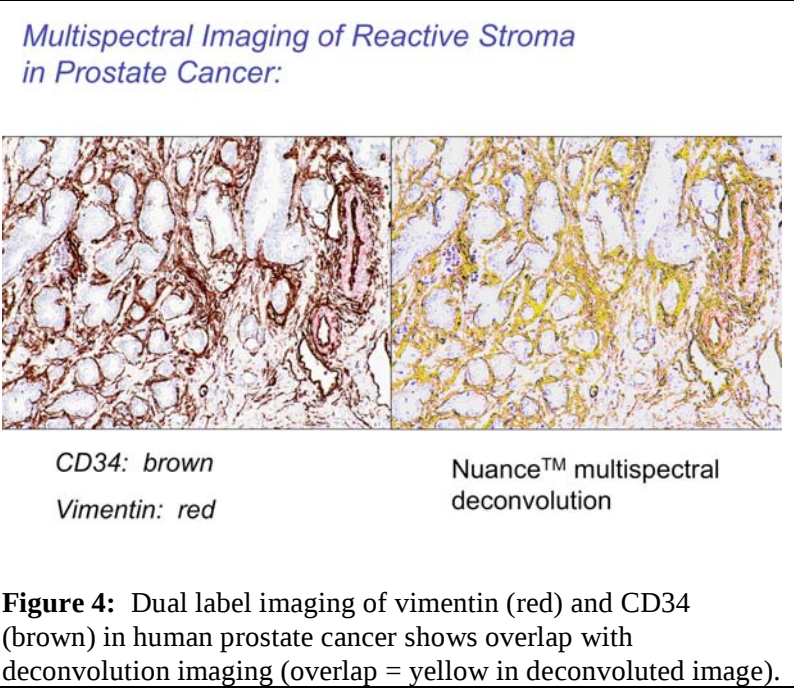
a marker of progenitor status for several cell lineages and is not restricted to cells of the hematopoietic series as once believed. Our preliminary data so far for Aim 1 studies suggests that CD34 positive cells are recruited and that these differentiate to vimentin positive reactive stroma over time. We have been successful in culturing these cells and will be able to use the procedures developed to probe for specific biological mechanisms of cell fate determination and recruitment mechanisms as proposed.

During this initial progress period, we have also worked to develop a co-culture method



in order to evaluate the biological interactions and mechanisms involved with carcinoma cells and recruited reactive stromal cells. There are many advantages to an in vitro approach such as easy manipulation of cells, defined media conditions, and the ability to sort out cells at the end of an experiment to evaluate gene expression patterns in a cell type specific manner. Figure 5A shows that we have been able to culture LNCaP human prostate cancer cells as spheroids using soft agar culture conditions and maintain expression of a TGF- β 1 transgene (Figure 5B).

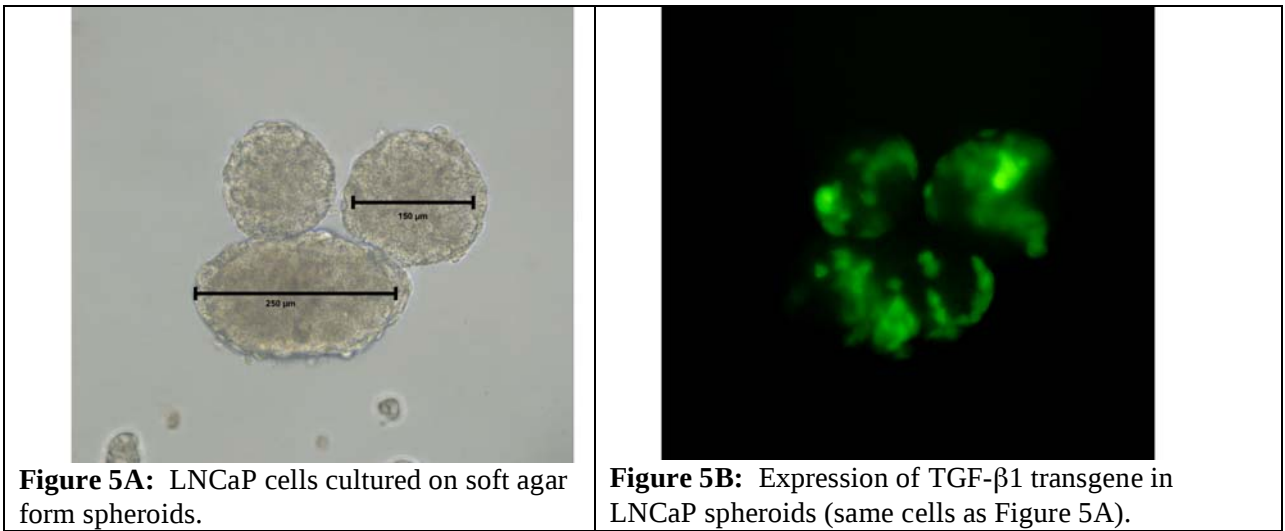
Spheroids of approximately 150 μm were collected and co-cultured with human prostate stromal cells in vitro. Figure 6 shows these co-cultures in fully defined media conditions. Briefly,



human prostate stromal cells were cultured on glass coverslips or slides that had been pre-coated with collagen type I matrix. We were successful in culturing these cells in fully defined, serum-free conditions using DMEM media and 0.5% ultrapure BSA only. The 150 μm diameter LNCaP spheroids were added to these stromal cultures as a layer of spheroids mixed with collagen type I gel on top of the stromal cell monolayers. Images of this are shown in Figure 6. The LNCaP / stromal cell co-cultures were continued in serum free media for up to 7 days. We have not

yet determined the overall effects of overexpression of TGF- β 1 in this model, however in our preliminary studies to date, there appears to be larger LNCaP spheroids in these co-cultures (Figure 6B). Overall, this data is important, as this method will allow us to examine co-cultures of recruited reactive stromal cells together with spheroids of human LNCaP carcinoma cells in fully defined media conditions where growth factors or agents that inhibit certain signaling pathways can be added in order to dissect mechanisms of interactions as planned.

In summary, we have made considerable progress on Aim 1 goals and anticipate completing these studies in year 2 of the project. No significant changes in the nature or scope of the plans in the Statement of Work are expected for Task 1.



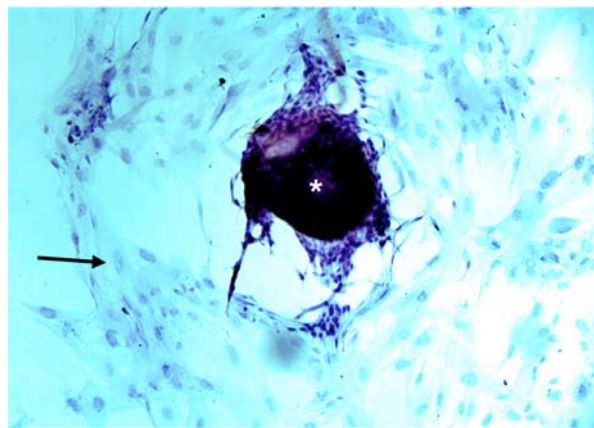


Figure 6A. Control co-cultures. Arrow shows co-cultured stromal cells

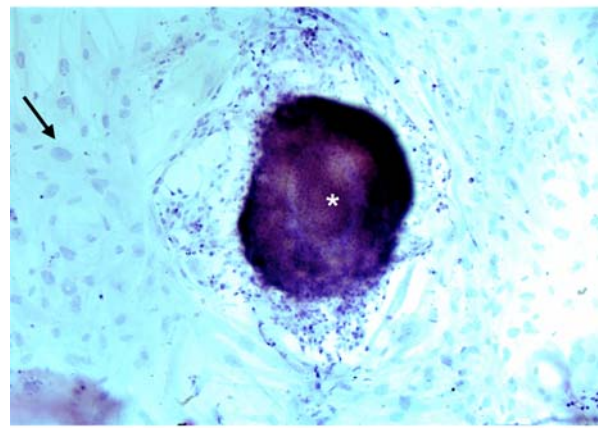


Figure 6B. Co-cultures - LNCaP expressing HA-TGF- β 1. Arrow shows co-cultured stromal cells.

Task 2. Task 2 is to complete Specific Aim 2 studies: To determine the mechanisms of reactive stroma recruitment from marrow-derived circulating progenitors and to assess the role of TGF- β in active recruitment and induced differentiation to reactive stroma.

We have initiated preliminary studies regarding adoptive transfer of marrow derived suspected progenitor cells into nude mice bearing xenografted LNCaP / Matrigel combinations as proposed. To date, we have completed three adoptive transfer experiments using human bone marrow cells (CD34 positive and CD14 positive) as part of another funded study. Even though these experiments were not part of this project, we have gained considerable experience at successfully completing adoptive transfer of blood borne cells into the tail vein of nude mice. We have also successfully isolated circulating progenitor fibrocyte cells (pro-fibrocytes) from mouse peripheral blood and have cultured these. To date, we have been successful two of the six times we have attempted these studies. Hence, we are still trying to delineate the most optimal culture media and cell seeding conditions for culture and preparation of these cells. We are making progress and do not expect any further delays in the proposed pro-fibrocyte isolation studies or adoptive transfer of cells to mice bearing LNCaP xenograft tumors.

In addition, we have conducted many bone marrow transplants using engineered mice as marrow donors with transplants into irradiated NCr *nu-nu* mice. Again, this was funded as part of another study. These studies are important since they can be used as a backup approach should we have difficulties with the adoptive transfer experiments.

No significant changes in the nature or scope of the plans in the Statement of Work are expected for Task 2.

Task 3. Task 3 will be to complete Specific Aim 3 studies: To use a drug-inducible expression system to assess whether progenitor cells can be targeted to deliver drug-induced gene expression at sites of recruitment / activation of reactive stroma and whether this approach can uncouple key recruitment pathways.

We have not yet initiated studies specifically pertaining to Aim 3. Once the fibrocyte isolation procedures are fully implemented as part of Aim 2 studies, we will work on engineering cells with drug-inducible gene expression as proposed.

No significant changes in the nature or scope of the plans in the Statement of Work are expected for Task 3.

Key Research Accomplishments:

- Development of an *in vivo* matrix trapping procedure that allows for phenotype characterization of recruited reactive stroma *in situ* and for isolation of these cells for cell culture.
- Development of an *in vitro* co-culture method that uses LNCaP spheroids combined with prostate stromal cells in fully defined and serum-free culture media.
- Development of protocols to isolate and either culture or prepare for injection of circulating pro-fibrocyte cells as suspected progenitors to reactive stroma.

Reportable Outcomes:

- Presentation of this data at several seminars and national meetings by Dr. Rowley.
- Some of this data was used to support the proposed use of the co-culture methodology in two other research proposals. One proposal to the NIH focuses on the role of TGF- β in regulating reactive stroma fate determination and gene expression profiling and one proposal to the DOD to evaluate the role of WFDC1/ps20 in reactive stroma cell fate determination.

Conclusions:

To date, we have made considerable progress on Task 1 (Specific Aim 1) and reasonable progress on Task 2 (Specific Aim 2). Task 3 has not yet initiated. We are poised to now complete the experimental aspects of Aims 1 and 2. Importantly, this work has led to development of a novel co-culture method that recombines human prostate carcinoma cell spheroids with human prostate stromal cells in a collagen I three dimensional culture model that is maintained in serum-free conditions. Moreover, we have nearly mastered procedures to inject tail veins with living cells in order to restore marrow function from marrow ablated mice or to insert circulating cells back into the bloodstream in experimental mice. Expertise in these procedures are necessary in order to complete the study as proposed. We are highly experienced at engineering prostate stromal cells for constitutive or induced gene expression using a number of approaches in previous studies (1, 2, 4, 5). Hence, we expect to be able to complete the engineering of drug-inducible gene expression as proposed in Aim 3. We believe these studies will allow us to pinpoint the cell type that is recruited to reactive stroma, to understand the fate determination of this cell type and assess the importance of TGF- β signaling to this recruitment,

and to target this cell with inducible gene expression as a proof of concept that targeting the reactive stroma microenvironment is possible. It is anticipated that these studies will allow us to develop critical pre-clinical data from which to base a strategic approach aimed at targeting the reactive stroma in human patients.

To address the "so what" aspect of required information, it is important to understand the potential benefits of targeting the reactive stroma niche. The reactive stroma is arguably a normal tissue response to the development of cancer in an epithelium. Although, it should be pointed out that this is the presumption at this point and we have not yet established that the reactive stroma response is "normal" per se. However, the reactive stroma response is very predictable in most epithelial cancers where studied, and a relatively common phenotype has been predicted and shown by us and others (6-8). If a common phenotype and biology of reactive stroma recruitment is observed in different epithelial cancers and if a novel approach is developed that targets this pathway, then it is possible that this approach could be useful in many different epithelial cancers. Hence, it is important to delineate specific mechanisms and signaling pathways in order to better understand the biology of this recruitment and to generate model systems to evaluate the targeting of these pathways as a potential new approach to target cancer via the reactive stroma compartment.

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