

AD _____

Award Number: W81XWH-06-1-0101

TITLE: Cells with Unique Properties in Prostate Cancer-Associated Stroma Are Mesenchymal Stem Cells

PRINCIPAL INVESTIGATOR: Donna M. Peehl, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, CA 94305

REPORT DATE: June 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-06-2007		2. REPORT TYPE Final		3. DATES COVERED 15 Nov 2005 – 14 May 2007	
4. TITLE AND SUBTITLE Cells with Unique Properties in Prostate Cancer-Associated Stroma Are Mesenchymal Stem Cells				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0101	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Donna M. Peehl, Ph.D. Email: dpeehl@stanford.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Stanford University Stanford, CA 94305				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES .					
14. ABSTRACT Our objective was to explore the hypothesis that prostate cancer-associated stromal cells are derived, at least in part, from mesenchymal stem cells. Our objective was to show that cancer-derived stromal cells capable of anchorage-independent growth have characteristics of stem cells and convert nontumorigenic prostatic epithelial cells to tumorigenicity. We could not identify cells capable of anchorage-independent growth, so we sought other stem cell markers. We pinpointed CD90 as a marker associated with mesenchymal stem cells that is overexpressed in cancer-derived stromal cells. In the no-cost extension period that we have requested, we will determine whether CD90-positive cells are capable of multilineage differentiation, and transforming nontumorigenic prostatic epithelial cells to tumorigenicity. Our studies also continue to point to high expression of transforming growth factor-beta as a key feature of prostate cancer-derived stromal cells that has also been to stem cell phenotypes.					
15. SUBJECT TERMS prostate cancer stroma, stem cells					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	8	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7
Appendices.....	8

INTRODUCTION

The importance of tumor stroma in cancer development and progression has been recognized through its stimulating effects on cancer cell growth, migration and angiogenesis. The powers of tumor stroma have been further emphasized by its ability to convert nontumorigenic prostatic epithelial cells to tumorigenic ones. Tumor stroma shares many features with stroma in wound repair including key cell types and expressed molecules. Therefore, mechanisms underlying stromal reaction in wound repair may also play an important role in the development of tumor stroma. We hypothesize that mesenchymal stem cells, which serve as the source of tissue regeneration in wound repair, may also contribute to the development of the cancer-associated stroma. Our hypothesis is supported by observations in animal models suggesting that bone marrow mesenchymal stem cells contribute to tumor-associated stroma, and by the report that prostate cancer-derived stromal cells undergo anchorage-independent growth, a property of stem cells. Our objective in this project was to show that cancer-derived stromal cells capable of anchorage-independent growth have characteristics of stem cells and convert nontumorigenic prostatic epithelial cells to malignancy. This report details our project to date; we have requested a 6-month no-cost extension to finish our tasks due to unforeseen delays.

BODY

Our first designated task was to separate cells with or without anchorage-independent growth from cancer-derived stromal cells. Our belief that we would be able to identify such populations of cells was based on a previous publication by San Francisco et al. (2004), who found that 0.18% of cells cultured from normal prostatic stroma were capable of anchorage-independent growth whereas 1.2% of stromal cells from prostate cancers formed colonies in agar. We co-authored this publication and provided the cancer-derived stromal cells. However, the anchorage-independent growth assays were performed in our collaborator's lab. We were unable to replicate these results in our lab. We tested two different primary cultures derived from cancers, and two from normal tissues, in several different conditions. These included the medium in which we culture stromal cells [MCDB 105 supplemented with 5 µg/ml of fibroblast growth factor-2, 5 µg/ml of insulin and 5% fetal bovine serum (FBS)], the medium in the San Francisco et al. publication (DME with 10% FBS), soft agar, or ultra-low attachment dishes. In no circumstance did we observe anchorage-independent colony formation. The reasons for our inability to replicate the published results are unclear, but could be due to use of a different lot of serum or different primary cultures from those used in the previous study (the cell cultures used at that time are no longer available, since they have a finite lifespan).

Since we could not identify cells capable of anchorage-independent growth, we sought to identify other characteristics of our cancer-derived stromal cell cultures that we could use to isolate or identify putative mesenchymal stem cells. Using immunocytochemistry, we stained cancer-derived stromal cells with antibodies against proteins associated with mesenchymal stem cells. These included nanog, nestin, Oct 3/4, BCRPI, CD244, CD48, CD150, CD133, STRO-1, and Gli1. Of these, nestin was the only one for which the cells stained positively. Nestin is considered to be a general marker of stem/progenitor cells (Carriere et al., 2007). In future studies, we plan to stain stromal cells from normal tissues with anti-nestin. If they do not stain, we will be able to use nestin as a marker to further investigate the stem cell nature of cancer-

derived stromal cells. If normal cells stain as well, then nestin will not be useful as a stem cell marker of cancer-derived cells.

Another characteristic of stem cells is migratory ability. We thought that we might be able to use this ability to separate stem-like cells, so we grew normal and cancer-derived stromal cells to confluency, then wounded each monolayer. We observed the cultures each day microscopically to view cells migrating into the wounds. We concluded that neither normal nor cancer-derived cells were very migratory, with few cells migrating into the wound in either type of culture even after many days.

CD34 is a cell surface antigen that has been associated with mesenchymal stem cells (Tirode et al., 2007). We stained semi-confluent cultures of normal and cancer-derived stromal cells with antibody against CD34, and found that all of the cells were positive. CD34 did not distinguish normal from cancer-derived stromal cells. We also stained tissue sections of normal prostate and cancer to see if in fact CD34 was expressed differently in the cancer-associated stroma, but our ability to draw conclusions was hampered by the expression of CD34 on the vascular endothelium, making it difficult to discern staining specifically on stromal fibroblasts/myofibroblasts/smooth muscle.

CD90 is another cell surface antigen expressed by mesenchymal stem cells (Gindraux et al., 2007). Our genetic profiling study of normal versus cancer-derived stromal cells identified CD90 as more highly expressed in cancer-derived stromal cells compared to normal cells (Zhao et al., 2007). Our collaborator, Dr. Alvin Liu at the University of Washington, previously reported that CD90 was expressed more highly in the stroma of prostate cancer tissues than stroma of normal tissues (Liu et al., 2004). Dr. Liu measured CD90 mRNA expression in our cultured stromal cells by RT-PCR and confirmed that CD90 was higher in stromal cells from cancer. In the future, we plan to use CD90 to sort CD90-positive cells from CD90-negative cells in cancer-derived stromal cultures. We will then compare the stem cell characteristics of each of these populations (self-renewal, multilineage differentiation potential in Task 2) and their respective ability to convert nontumorigenic epithelial cells to tumor cells (Task 3).

The second task was to demonstrate self-renewal and multilineage differentiation potential uniquely of cells with anchorage-independent growth capability. Since we could not identify cells capable of anchorage-independent growth, we chose to carry out this experiment instead with cells derived from normal tissues versus cells derived from cancers.

Self-renewal: We have serially passaged more than five normal cultures and five from cancers to assess capability of infinite self-renewal, and none have this property. All became senescent after ~ 15 to 20 passages.

Multilineage differentiation: We first investigated the ability of normal versus cancer-derived stromal cells to undergo smooth muscle differentiation. Our lab previously reported that transforming growth factor (TGF)-beta caused normal prostatic stromal cells to differentiate into smooth muscle (Peehl and Sellers, 1997). Our previous experiments were performed with semi-confluent cultures. Here, we wanted to test cancer-derived clonal cell populations, because stem cells might make up only a small percentage of the total cancer stromal population. We allowed

normal or cancer-derived stromal cells to form small colonies, then changed them to smooth muscle-differentiating culture conditions (MCDB 105 with 1 ng/ml of TGF-beta). We then evaluated smooth muscle differentiation by labeling with antibody against smooth muscle alpha-actin. As we had previously reported, normal stromal cells differentiated into smooth muscle only in the presence of TGF-beta. In contrast, cancer-derived stromal cells expressed smooth muscle alpha-actin regardless of the presence or absence of TGF-beta. This was true of 100% of the cancer-derived stromal cell clones.

The smooth muscle differentiation of the cancer-derived stromal cells in the absence of exogenous TGF-beta possibly occurs because of elevated endogenous expression of TGF-beta in these cells compared to normal stromal cells. Elevated expression of TGF-beta was originally reported by us in collaboration with San Francisco et al. (2004), and was recently confirmed in a publication by Ao et al. (2007). Elevated expression of TGF-beta is characteristic of myofibroblasts, which is the typical phenotype of stromal cells in prostate and other tumors (Tuxhorn et al., 2001). The myofibroblastic phenotype is apparently retained during in vitro culture, as evidenced by expression of smooth muscle alpha-actin, a marker of myofibroblasts as well as smooth muscle cells. Ao et al. showed that expression of TGF-beta is required for cancer-derived stromal cells to convert nontumorigenic prostatic epithelial cells to tumors.

Other experiments to assess potential for multilineage differentiation are in progress. Normal and cancer-derived cultures have been placed in medium to promote osteoblastic differentiation, or placed in medium to promote chondrocyte differentiation.

Another characteristic of prostate cancer stroma is downregulation of proenkephalin (PENK), as reported by Goo et al. (2005). We again allowed normal or cancer-derived stromal cells to form small colonies, then changed the colonies to medium with or without TGF-beta. Immunocytochemistry with antibody against PENK showed that PENK was expressed by both types of cells, with or without TGF-beta, at apparently similar levels. Downregulation of PENK expression in cancer-derived stromal cells is apparently not retained in vitro, and is seemingly not an intrinsic feature of cancer-derived stroma. Our genetic profiling study of normal versus cancer-derived stromal cells also failed to identify PENK as a differentially expressed gene (Zhao et al., 2007), confirming our immunocytochemistry results.

The third task was to determine the in vivo tumor-promoting potential of stromal cells with anchorage-independent growth capability. This task has been delayed for several reasons. First, the technician who was trained to work with mice left the lab, and we only recently regained this expertise in the lab. Second, we could not identify anchorage-independent growth as a putative marker of stem cells. We searched for other markers to use, and CD90 appears to be appropriate since CD90 is expressed by mesenchymal stem cells and is overexpressed in cancer-derived stromal cells compared to stromal cells from normal tissues. In future studies, we will sort CD90-positive versus CD90-negative cells, combine with nontumorigenic prostatic epithelial cells, and implant under the renal capsule of immunocompromised mice. We hypothesize that CD90-positive cancer-derived stromal cells will preferentially convert epithelial cells to tumorigenicity. If so, this would support our hypothesis that mesenchymal stem cells are the key cells in cancer-derived stroma that promote cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

- Could not reproduce anchorage-independent growth as stem cell-like feature of cancer-derived stromal cells
- Determined that nestin, a general marker of stem/progenitor cells, is highly expressed in cancer-derived stromal cells
- Identified CD90, characteristically expressed by mesenchymal stem cells, as more highly expressed in stromal cells derived from cancer versus those derived from normal tissues
- Determined that smooth muscle alpha-actin, a marker of myofibroblasts/smooth muscle, is constitutively expressed in cancer-derived stromal cells, perhaps due to high levels of endogenous TGF-beta

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Although we could not reproduce previous findings of anchorage-independent growth of cancer-derived stromal cells, other characteristics of these cells are suggestive of a stem cell-like phenotype. The overexpression of TGF-beta remains the most reproducible and consistently reported characteristic of cultured prostate cancer-derived stromal cells. Whether this is indicative of a stem cell origin of cancer-associated stromal cells remains to be determined. Elevated expression of TGF-beta is characteristic of the “wound healing” gene activation pattern seen in cancer-associated stroma, and therefore high levels of TGF-beta may simply reflect this typical characteristic of the reactive stroma. On the other hand, high expression of TGF-beta is emerging as a feature of stem cells (McCarthy, 2007). TGF-beta appears to maintain stem cells in an undifferentiated, proliferative state. We and others also have found that CD90 is overexpressed in cancer-derived stromal cells. CD90 is expressed by mesenchymal stem cells, although not exclusively. During the no-cost extension of 6 months that we have requested for this project, we will take advantage of differential expression of CD90 to sort CD90-positive versus CD90-negative cells and determine whether CD90-positive cells have features of mesenchymal stem cells (multilineage differentiation potential) and are able to convert nontumorigenic prostatic epithelial cells to tumorigenicity.

REFERENCES

Ao, M., Franco, O.E., Park, D., Raman, D., Williams, K. and Hayward, S.W. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Res.* 67:4244-4253, 2007.

Carriere, C., Sealey, E.S., Goetze, T., Longnecker, D.S., and Korc, M. The nestin progenitor lineage is the compartment of origin for pancreatic intraepithelial neoplasia. *Proc. Natl. Acad. Sci. USA* 104:4437-4442, 2007.

Gindraux, F., Selmani, Z., Obert, L., Davasi, S., Tiberghien, P., Herve, P. and Deshaseeaux, F. Human and rodent bone marrow mesenchymal stem cells that express primitive stem cell markers can be directly enriched using the CD49a molecule. *Cell Tiss. Res.* 327:471-483, 2007.

Goo, Y.A., Goodlett, D.R., Pascal, L.E., Worthington, K.D., Vessella, R.L., True, L.D. and Liu, A.Y. Stromal mesenchyme cell genes of the human prostate and bladder. *BMC Urology* 5:17, 2005.

Liu, A.Y., Roudier, M.P. and True, L.D. Heterogeneity in primary and metastatic prostate cancer as defined by cell surface CD profile. *Am. J. Pathol.* 165:1543-1556, 2004.

McCarthy, N. Relative significance. *Nature Rev. Cancer* 7:320-321, 2007.

Opalenik, S.R. and Davidson, J.M. Fibroblast differentiation of bone marrow-derived cells during wound repair. *FASEB J.* 19:1561-1572, 2005.

Peehl, D.M. and Sellers, R.G. Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells. *Expt. Cell Res.* 232:208-215, 1997.

San Francisco, I.F., DeWolf, W.C., Peehl, D.M. and Olumi, A.F. Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. *Int. J. Cancer* 112:213-218, 2004.

Tirode, F., Laud-Duval, K., Prieur, A., Delorme, B., Charbord, P. and Delattre, O. Mesenchymal stem cell features of Ewing tumors. *Cancer Cell* 11:421-429, 2007.

Tuxhorn, J.A., Ayala, G.E. and Rowley, D.R. Reactive stroma in prostate cancer progression. *J. Urol.* 166:2472-2483, 2001.

Zhao, H., Ramos, C.F., Brooks, J.D. and Peehl, J.D. Distinctive gene expression of prostatic stromal cells cultured from diseased versus normal tissues. *J. Cell. Physiol.* 210:111-121, 2007.

APPENDICES

None.