AD_____

Award Number: W81XWH-06-1-0101

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

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

CONTRACTING ORGANIZATION: Stanford University
Stanford, CA 94305

REPORT DATE: December 2007

TYPE OF REPORT: Final Addendum

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-12-2007	Final Addendum	15 Jun 07 – 14 Nov 07

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

E-mail: 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)
US Army Medical Research and Materiel Command

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
The importance of tumor stroma in prostate cancer development and progression has been recognized through its stimulating effects on cancer cell growth, migration and angiogenesis. Our hypothesis was that the prostate cancer stroma is derived, at least in part, from mesenchymal stem cells (MSCs) that are proliferative, multipotent and self-renewing. Our objective was to demonstrate that cancer-derived stromal cells have characteristics of mesenchymal stem cells. Comparing primary cultures of stromal cells from cancer versus normal tissues, we observed (a) differential gene expression profiles, (b) increased expression of autotaxin, a motility factor, (c) increased expression of the MSC-associated antigen CD90, and (d) increased expression of transforming growth factor-beta, associated with tumor promotion, in CD90-positive cells. Many of these properties are consistent with a mesenchymal stem cell phenotype of prostate cancer-associated stroma, supporting our hypothesis.

15. SUBJECT TERMS
cancer stroma, stem cells, transforming growth factor-beta, CD 90, autotaxin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
unclassified	unclassified	unclassified	unlimited	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

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 is our final report and includes results obtained during a 6-month no-cost extension.

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). However, normal cells stained as well, so nestin is not useful as a specific stem cell marker of prostate cancer-derived stromal cells.

Another characteristic of stem cells is migratory ability. In a genetic profiling study of normal versus cancer-derived stromal cells, we identified autotaxin, a migration-stimulating factor, as more highly expressed in cancer-derived stromal cells than in normal cells (Zhao et al., 2007). We thought that we might be able to use migration 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.

To further characterize CD90 expression in cancer stromal cells (CAFs), we first stained CAFs with a biotin-conjugated monoclonal antibody against CD90 and an avidin-conjugated Alexa 488 fluor whose emission is similar as fluorescein (FITC). Under fluorescent microscopy, a small number of the CAFs showed much brighter green signal than others (Figure 1), suggesting that CD90 expression is heterogeneous among CAFs. The localization of the fluorescent signal was consistent with CD90 being a cell surface antigen.

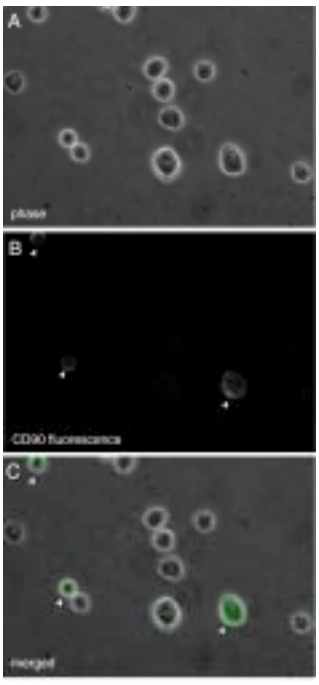

Figure 1. CD90 is differentially expressed among CAFs. (A) phase-contrast image of CAFs; (B) Fluorescence image of the same CAFs as in (A); (C) merged image of (A) and (B)

Therefore, we subsequently used antibody against CD90 to sort cells with high expression of CD90 from cells with low or no expression of CD90 in cancer-derived stromal cultures by fluorescence activated flow cytometry (FACS).

Cells were stained as described above. Dead cells were excluded from the analysis using DAPI staining. Doublets and cell clusters were excluded using forward scatter and side scatter plots. As shown in Figure 2, two distinct populations of cells expressing different levels of CD90 were detected by flow cytometry. The majority of cells expressed low level of CD90 whereas a small percentage of cells expressed much higher CD90. We collected cells within gate P6 as CD90-high cells and within P5 as CD90-low cells. The average CD90 intensity of CD90-high cells is at least 10-fold higher than that of CD90-low cells. The difference in CD90 levels between these two populations is not likely due to differences in cell size since they showed similar forward scatter and side scatter. These results demonstrated that CD90 is differentially expressed among CAFs and that CD90-high cells can be separated from CD90-low cells by FACS.

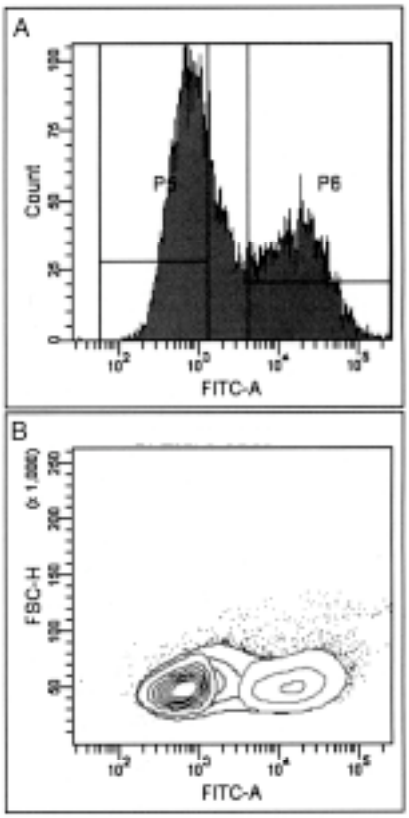

Figure 2. Isolation of CD90-high and CD90-low CAFs by FACS. (A) Histogram of CD90 expression in CAFs. (B) A probability contour graph showing CD90 expression in cells within P5 and P6.

Our next goal was to determine whether CD90-high cells (the putative stem cells of the CAFs) had properties associated with tumor promotion. CAFs express higher levels of transforming growth factor (TGF)-beta than normal stromal cells (San Francisco et al., 2004; Ao et al., 2007), and TGF-beta has been implicated as a key factor in the tumor-promoting ability of CAFs (Ao et al., 2007). We characterized TGF-beta1 expression in CD90-high and CD90-low cells using quantitative polymerase chain reaction (qPCR). In CAFs derived from two different donors, TGF-beta1 levels were 2- and 5-fold higher in CD90-high cells compared to CD90-low cells (Figure 3). Quantitative PCR analysis also confirmed that the CD90 level was more than 5-fold higher in CD90-high cells compared to CD90-low populations (data not shown). It has been shown that high levels of TGF-beta signaling

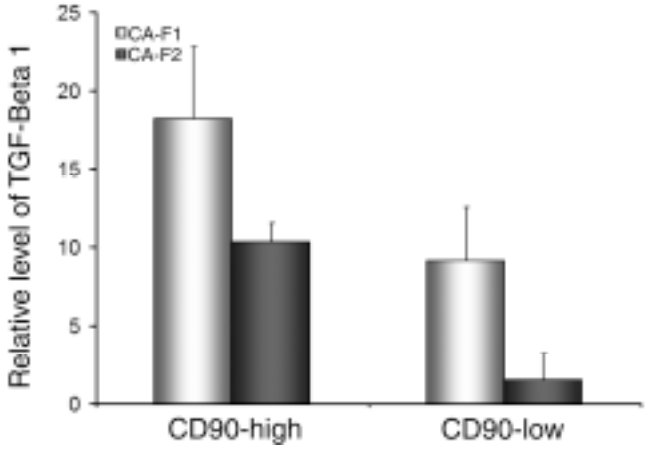

Figure 3. Quantitative polymerase chain reaction analysis of TGF-beta1 levels in CD90-high and CD90-low CAFs.

are present in the quiescent proximal region of ducts in the mouse prostate where putative stem cells reside. This high level of TGF-beta signaling is important in maintaining the dormancy of the stem cells. Our results raise the possibility that CD90-high cells may be enriched with stromal stem cells that employ high level of TGF-beta to maintain their stemness.

It has been shown that CAFs have the ability to transform initiated but nonmalignant human prostatic epithelial cells (BPH-1) into malignant tumor cells. The elevated expression of TGF-beta in CD90-high CAFs suggests that these cells may be responsible for the tumorigenic effects of CAFs through TGF-beta signaling. It has been shown that TGF-beta signaling is both necessary and sufficient to induce membrane-localized CXCR4, the stromal cell-derived factor-1 (SDF-1) receptor, in BPH-1 cells, and that suppressing the expression of CXCR4 abrogated the tumorigenic conversion of BPH-1 in response to CAFs.

Accordingly, we investigated the effects of CD90-high and CD90-low cells on CXCR4 expression in BPH-1 cells. We co-cultured BPH-1 cells with either CD90-high or CD90-low cells that had been separated by FACS. We then examined CXCR4 expression on BPH-1 cells by immunoflurescence staining using a monoclonal primary antibody against CXCR4 and an Alexa 488-conjugated secondary antibody. As expected, a subpopulation of BPH-1 cells treated with 5 ng/ml TGF-beta expressed CXCR4 clearly detectable on the cell surface (Figure 4B), whereas cells grown in control medium only showed background staining in the cytoplasm (Figure 4A). More importantly, BPH-1 cells co-cultured with CD90-high CAFs showed CXCR4 expression at the cell surface (Figure 4D), whereas cells co-cultured with CD90-low CAFs only displayed a low level of signal comparable to the background (Figure 4C). Our results demonstrated that CD90-high cells differ from CD90-low cells in that they have the ability to induce CXCR4 in BPH-1 cells, probably through secretion of TGF-beta. This finding supports the hypothesis that CD90-high cells (stem cells?) are responsible for the tumor-promoting activity of CAFs.

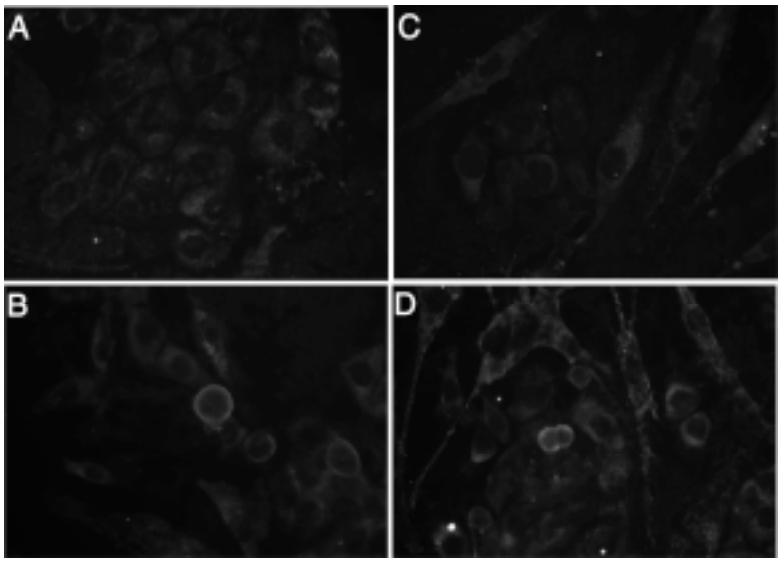

Figure 4 Immunoflurescence staining of CXCR4 in BPH-1 cells. BPH-1 cells were either cultured in serum-free RPMI-1640 without TGF- _ (A), with TGF- _ (5 ng/ml) (B), CD90-low CAFs (C), or CD90-high CAFs (D).

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.

Experiments to evaluate the potential for multilineage differentiation are still in progress. Normal and cancer-derived cultures were placed in medium to promote osteoblastic or chondrocyte differentiation. Clear evidence of differentiation was not obtained. However, in future studies,

we wish to evaluate CD90-positive (putative stem cells) versus CD90-negative cells for multilineage differentiation ability.

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 was delayed for several reasons and was one of the factors necessitating a no-cost extension. First, the technician who was trained to work with mice left the lab, and we had to regain this expertise in the lab. Second, we could not identify anchorage-independent growth as a putative marker of stem cells. However, as described above, we turned our focus to CD90-positive cells since CD90 is expressed by mesenchymal stem cells and is overexpressed in cancer-derived stromal cells compared to stromal cells from normal tissues.

The first necessity was to develop our animal assays. Accordingly, a member of the lab who is also working on epithelial stem cells took a course from Dr. Simon Hayward (an expert at implantation of prostate cells and tissues under the mouse renal capsule) at Vanderbilt University. Here, she mastered the techniques of making and implanting androgen pellets in SCID mice, making cell cultures for implants, placing cells and tissues under the renal capsule, and recovering the implants for immunohistological analysis. Recently, we sorted CD90-positive versus CD90-negative cancer-derived stromal cells, combined them with nontumorigenic prostatic epithelial cells (the BPH-1 cell line), and implanted the recombinants under the renal capsule of immunocompromised mice. After several months, we will sacrifice the mice and examine the implants. We hypothesize that CD90-positive cancer-derived stromal cells will preferentially convert BPH-1 epithelial cells to tumorigenicity. If so, this would support our hypothesis that CD90-positive/mesenchymal stem cells are the key cells in cancer-derived stroma that promote cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

- 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 CD90-high cells from cancer stroma express more TGF-beta than CD90-low cells

- Determined that CD90-high cells induce membrane-associated CXCR4 in epithelial BPH-1 cells, a property associated with tumorigenic potential, presumably through secreted TGF-beta
- 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

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

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 requested for this project, we took advantage of differential expression of CD90 to sort CD90-positive versus CD90-negative cells from cultured cancer-derived stroma. We determined that TGF-beta is expressed more highly by CD90-positive cells, suggesting that these cells (mesenchymal stem cells?) may indeed be the key players in tumor promotion. CD90-positive versus CD90-negative stromal cells have been combined with nontumorigenic prostatic epithelial BPH-1 cells and implanted under the renal capsule of SCID mice. If CD90-positive cells preferentially convert BPH-1 cells to tumors, this will support our hypothesis that mesenchymal stem-like cells are involved in the promotion of prostate cancer.

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.