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14. ABSTRACT Circulating Mesenchymal Stem Cells (MSCs) originating from the bone marrow have the ability to differentiate into cells of the mesoderm lineage and an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. MSCs have been detected in the perivascular space of many tumors, including those of the prostate, and have been shown to be a critical element in oncogenic progression. MSCs are inherently non-immunogenic, which prevents allogeneic MSCs from being rejected by normal host defense mechanisms. This immune-privileged status, together with their oncotropic properties, makes possible the infusion of allogeneic MSCs into patients for therapeutic purposes, such as the delivery of cytotoxic agents to sites of primary and metastatic prostate cancer. PRX302 is a PSA-activated aerolysin-based protoxin that forms membrane pores and leads to necrosis by a proliferation-independent mechanism at low picomolar concentrations. Importantly, PRX302 binds with low nanomolar affinity to GPI-anchor proteins, which are highly expressed on the surface of all mammalian cells. Therefore, MSCs can be genetically manipulated to express the PRX302 transgene endogenously from a 'safe harbor' locus. Based upon this rationale we hypothesize that human bone marrow-derived mesenchymal stem cells (hMSCs) can be used as a cell-based targeting vehicle to selectively deliver therapeutic agents, such as PRX302, to primary and metastatic sites of prostate cancer, and thus spare host toxicity.					
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Introduction

Circulating Mesenchymal Stem Cells (MSCs) originating from the bone marrow have the ability to differentiate into cells of the mesoderm lineage and an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. MSCs have been detected in the perivascular space of many tumors, including those of the prostate, and have been shown to be a critical element in oncogenic progression. MSCs are inherently non-immunogenic, which prevents allogeneic MSCs from being rejected by normal host defense mechanisms. This immune-privileged status, together with their oncotropic properties, makes possible the infusion of allogeneic MSCs into patients for therapeutic purposes, such as the delivery of cytotoxic agents to sites of primary and metastatic prostate cancer. PRX302 is a PSA-activated aerolysin-based protoxin that forms membrane pores and leads to necrosis by a proliferation-independent mechanism at low picomolar concentrations. Importantly, PRX302 binds with low nanomolar affinity to GPI-anchor proteins, which are highly expressed on the surface of all mammalian cells. Therefore, MSCs can be genetically manipulated to express the PRX302 transgene endogenously from a ‘safe harbor’ locus. Based upon this rationale we hypothesize that human bone marrow-derived mesenchymal stem cells (hMSCs) can be used as a cell-based targeting vehicle to selectively deliver therapeutic agents, such as PRX302, to primary and metastatic sites of prostate cancer, and thus spare host toxicity.

Body

Specific Aim 1: Optimize the recruitment efficiency of allogeneic human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to human prostate cancers in preclinical animal models to maximize their potential as a therapeutic delivery vehicle (**Months 1-12**).

Initially, we had proposed to quantify the efficiency of hBM-MSC homing to prostate cancer xenografted subcutaneously, orthotopically, and intratibially based on quantification using cells fluorescently-labeled with CM-DiI, i.e., counting fluorescent cells (numerator) per field of DAPI-labeled nuclei (denominator). While we were able to qualitatively demonstrate that systemically-infused hBM-MSCs (1×10^6) are able to traffic to prostate cancer xenografts (**Figure 1**), we were not able to accurately quantify these results using this system. This was due to our inability to resolve the number of cells per fluorescent signal due to inconsistent labeling,

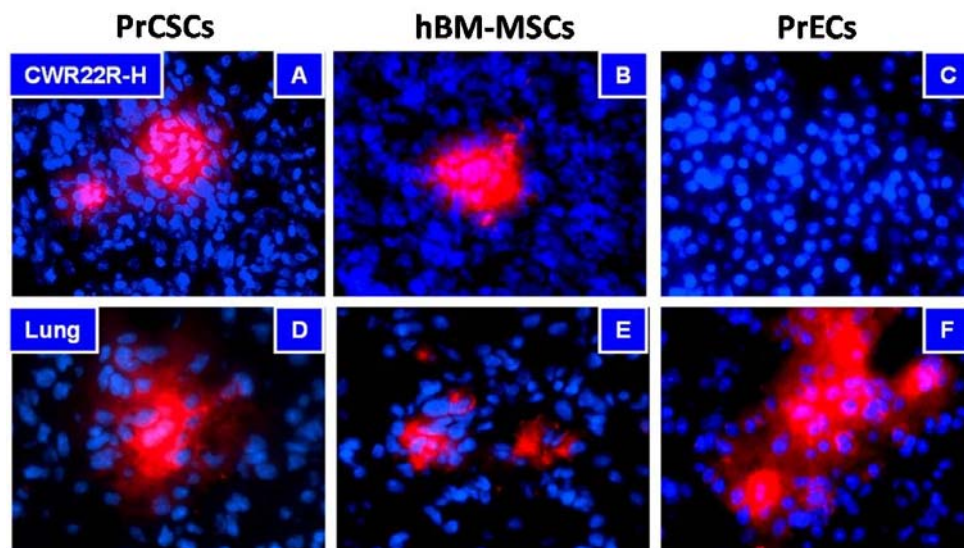


Figure 1: Tumor Trafficking of PrCSCs and hBM-MSCs to Human Cancer Xenografts in Mice. PrCSCs (**A**) and hBM-MSCs (**B**), but not PrECs (**C**), traffic to prostate cancer xenografts in vivo following systemic infusion. Fluorescently-labeled (CM-DiI, red) PrCSCs, hBM-MSCs, and PrECs (1×10^6) were infused intravenously (IV) into immunocompromised mice bearing subcutaneous CWR22RH xenografts (3/group). Four days post-infusion, lungs and tumors were harvested and analyzed by fluorescence microscopy for the presence of CM-DiI-labeled cells. In contrast to the xenografts, all three cell types were found entrapped in the lungs following infusion (**D-F**). Nuclei counterstained with DAPI (blue). At least three images analyzed per tissue per animal, representative images shown.

both in terms of the staining intensity per cell and the staining pattern, which made absolute quantification using this method difficult and inaccurate (**Figure 1**).

To overcome this challenge, we have decided to optimize two different technologies for this purpose within our lab. The first of these is designed to allow characterization of the kinetics of MSC trafficking within the murine xenograft models described. This will be achieved by utilizing a Luc2 expression vector; the Luc2 gene has been engineered to improve mammalian expression and overall sensitivity. The Luc2 gene has been amplified from the original pUAS-luc2 plasmid (#24343) obtained from Addgene and cloned into the pLenti-CMV-GFP-Puro expression vector (#658-5) also obtained from Addgene using standard molecular biology techniques to replace the GFP sequence with Luc2. By transducing hBM-MSCs with this lentiviral expression vector we were able to detect a bioluminescence signal from as few as 50 cells in vitro (**Figure 2**), which is a 4- to 12-fold improvement over the previous generation of luciferase expression constructs (1). This low limit of detection (i.e., enhanced sensitivity) is

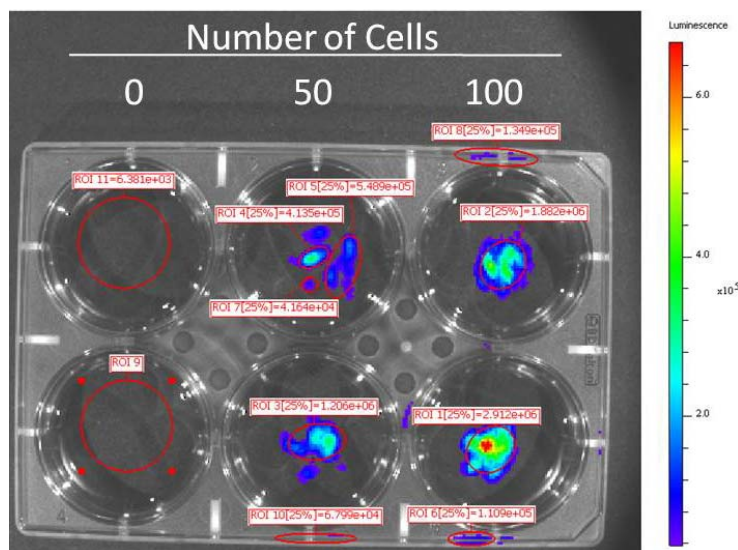


Figure 2: Enhanced sensitivity of bioluminescent signal from hBM-MSCs expressing Luc2. Cells were transduced with a lentiviral expression vector encoding the Luc2 gene. Cells were plated in a six-well plate at the indicated densities in duplicate and allowed to adhere overnight. Luciferin was added, and the bioluminescent signal was quantified using a Xenogen Bioluminescent Imaging System.

necessary to achieve accurate characterization of the trafficking kinetics of infused hBM-MSCs in vivo. An additional challenge has been the sensitivity of MSCs to polybrene (2), a reagent used to enhance the transduction efficiency of viral vectors; the use of which resulted in reduced proliferation and general toxicity that limited the use of these transduced hBM-MSCs for in vivo experiments. To circumvent this problem, polyamine sulfate was substituted for polybrene during the transduction, which has been shown to double transduction efficiencies without the toxicity typically observed with polybrene (3). hBM-MSCs have been successfully transduced with the Luc2 expression vector using an optimized polyamine sulfate transduction protocol for use in xenograft trafficking experiments that are currently in progress. This system will allow us to determine the kinetics of hBM-MSC trafficking to prostate cancer xenografts implanted at multiple locations throughout the murine host; thereby, allowing us to determine the optimal time to harvest the xenograft tissue for quantification of MSC homing.

Quantification of MSC trafficking will be achieved using the second of these optimized techniques, specifically, digital BEAMing PCR (4-5). This technology overcomes problems with amplification bias associated with traditional PCR to accurately quantify rare numbers of cells within a complex population at frequencies of less than 1 in 10,000 based upon the presence of mutations or polymorphisms in the DNA sequence. For these purposes we will use a panel of six SNPs from a set of 45 SNPs dispersed throughout the 22 human chromosomes that were previously shown to be highly variable in the population and have very little to no genetic linkage with each other (6), both of which will increase their potential to be informative (i.e., different) between the donor and recipient. For a panel of 6 SNPs, this equates to a probability of $2 \times 10^{-4}\%$ or a 1 in 4,049 chance that the donor would have an identical profile for all six SNPs analyzed as the recipient. Cell pellets from LNCaP, CWR22Rv1, and VCaP, in addition to, three primary bone marrow-derived MSC lines currently in use within the lab have been sent for SNP profiling. These experiments are being performed, in part, through an agreement with Inostics, Inc (Hamburg, Germany). Subsequent, xenograft experiments will validate this technology for the quantification of MSC trafficking to prostate cancer tissue for use in a clinical trial currently being planned to accurately quantify the number of allogeneic hBM-MSCs that home to cancerous prostates in men following systemic infusion.

These techniques are currently operational within our lab or our collaborators, and we are on track to successfully complete all proposed tasks within Specific Aim 1 over the next several months. In parallel with these experiments, we have optimized a protocol to a flow cytometry protocol to quantify the number of MSCs present in prostatectomy tissue within 3 hrs of surgery and prior to any selection resulting from expansion in tissue culture. In the 10 samples analyzed thus far, MSCs represent 0.01-1.1% of the total number of cells present in the digested prostatectomy tissue (7). The manuscript describing these results has recently been published and is attached as **Appendix 1**. Additional studies have been performed to determine optimal growth conditions with regards to FBS (10% vs. 20%) and oxygen concentrations (20%, 2%, and 1% O₂). Experiments demonstrating the relationship between these culture conditions, differentiation potential, and trafficking properties are currently in progress.

Specific Aim 2: Genetically modify hMSCs to endogenously express and secrete PRX302 using zinc-finger nuclease and integration-deficient lentiviral technologies to insert the transgene into a “safe-harbor” locus (HIV co-receptor CCR5) within the genome (**Months 1-12**).

This aim has been modified from the original proposal to incorporate a better understanding of the delivery platform and enhance the potential for success. The PRX302 protoxin (PSA-activated pro-aerolysin) was initially going to be inserted into the CCR5 locus for expression, a known safe-harbor. However, because the protoxin binds to GPI-anchored proteins on the surface of all mammalian cells, the most likely outcome in this system would be for the protoxin-expressing cells to self-sterilize as a result of the toxin binding to their own surface. Evidence for this is suggested by the fact that there is an approximately 40% reduction in the number of cells expressing PRX302 over several passages post-transduction and sorting as measured using flow cytometry (**Figure 3**). Importantly, this demonstrates that mammalian cells can synthesize and secrete the bacterial protoxin in a functional form, which is critical to the success of the proposed therapeutic strategy. This latter point is indicated by additional proof-of-principle experiments demonstrating that incubating cells in supernatant from PRX302-expressing cells results in significant cell lysis only in the presence of PSA (data not shown).

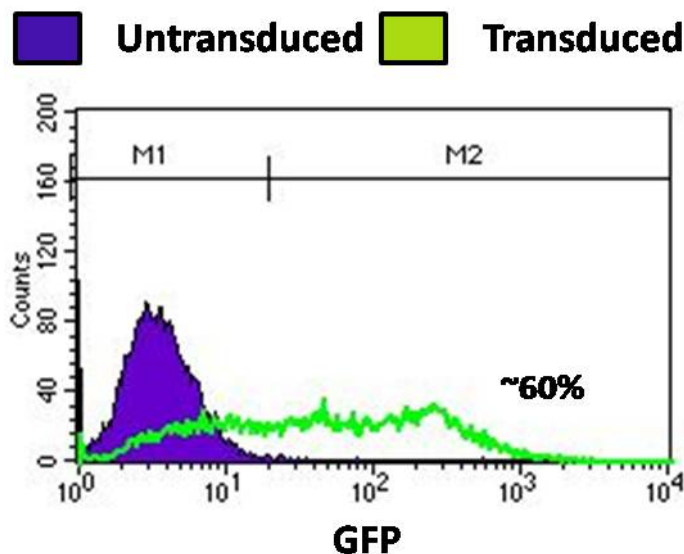


Figure 3: Mammalian cells transduced with a PRX302 expression vector including a GFP reporter. HEK293T cells were transduced with a lentiviral expression vector encoding the PRX302 transgene and a GFP expression cassette driven off a single CMV promoter. A T2A sequence separates the PRX302 and GFP sequences, which results in a 2A-mediated separation of the adjacent proteins via a translational skipping mechanism (8). This expression vector generates a nearly 1:1 ratio of the two proteins with much greater expression of the downstream gene than is typically achieved using more conventional IRES-containing vectors. Furthermore, stem cells readily silence expression of genes downstream of IRES sequences (9) making their use inconsistent for tagging purposes. This vector was generously provided by Dr. Cheng-Lai Fu and Dr. Hans Hammers, who generated the vector by cloning the T2A sequence into the pLVX-AcGFP1-N1 expression vector (PT3994-5) from Clontech.

Therefore, a more rational approach is to insert the protoxin transgene into the PIG-A locus, which is the first enzyme in the GPI-anchor biosynthesis pathway (10); and thereby, render the cells resistant to PRX302-mediated toxicity because they would lack the ability to make GPI-anchors that are required for aerolysin binding and activity. This resistance is clearly manifested in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients who are characterized by a

germline mutation in PIG-A, which makes them resistant to aerolysin-induced toxicity (11). In fact, PNH is currently diagnosed clinically using an assay that takes advantage of aerolysin binding, or the lack thereof in the case of PNH patients, to GPI-anchor proteins on the surface of peripheral blood mononuclear cells (PBMCs) (12). Zinc-finger nucleases (ZFNs) targeting the PIG-A locus have previously been generated with the help of our collaborator on this project, Dr. Linzhao Cheng (13). Vectors encoding these PIGA-targeted ZFNs have been obtained through a MTA with Dr. Keith Joung and Harvard Medical School and are currently present within the lab. Additionally, a integration-deficient lentiviral vector (IDLV) encoding the PIG-A homology arms required for homologous recombination-mediated insertion of the PRX302 transgene have been kindly provided by Dr. Linzhao Cheng as well (13). The PRX302 transgene is currently being sub-cloned from the pMMB66HE vector into this vector between the PIG-A homology arms. Upon successful generation of this vector, all three vectors (one with the PRX302 transgene flanked by the PIG-A homology arms and vectors for each of the ZFNs targeting the PIG-A locus) will be co-transfected into the target hBM-MSCs. Confirmation of PRX302 insertion into the target locus, and characterization of PRX302 transgene expression and secretion will then be performed as initially proposed along with a comparison of the trafficking efficiency of these genetically-modified hBM-MSCs to prostate cancer xenografts relative to their untransfected parental counterparts. Additional experiments will characterize any off-target, non-PIGA integration sites and their associated frequency in this system using both the Surveyor nuclease kit and ultra-deep sequencing.

Specific Aim 3: Evaluate the therapeutic efficacy and host toxicity of genetically modified hMSCs expressing the PRX302 transgene using this optimized homing protocol in preclinical proof-of-principal studies against a series of human prostate cancer xenografts growing in NOG mice (**Months 13-24**).

These experiments have not yet been initiated, but are set to commence over the next few months as results from the ongoing experiments in Specific Aims 1 and 2 are completed.

Key Research Accomplishments

- Tissue digestion and dissociation protocols for primary human prostatectomy tissue have been optimized
- Optimization of flow cytometry-based analyses to characterize co-incident expression of multiple MSC-related markers on a single cell
- Using these optimized protocols, we have demonstrated that MSCs are present in sites of human prostate cancer at a frequency of 0.01-1.1% of the total cells present.
- Optimal growth conditions for the in vitro expansion of MSCs have been delineated
- Stromal cells isolated from primary prostatectomy tissue have multi-lineage differentiation potential (i.e., adipocytes, osteoblasts, and chondrocytes) consistent with an MSC phenotype
- Both hBM-MSCs and MSCs isolated from prostate tissue have tumor-tropic properties following systemic infusion into nude mice bearing prostate cancer xenografts
- Viral transduction protocols have been optimized to both enhance transduction efficiency and reduce toxicity in MSCs
- hBM-MSCs have been transduced with a Luc2 expression vector, and bioluminescent detection has been documented in vitro with as few as 50 cells

- Mammalian cells have been successfully shown to synthesize and secrete the PRX302 protoxin in a functional form.

Reportable Outcomes

Publications:

- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Quantification of Mesenchymal Stem Cells (MSCs) at Sites of Human Prostate Cancer. *Oncotarget*. 2013 Jan; 4(1): 106-17.

Oral Presentations:

- **Brennen WN**. Characterization of Mesenchymal Stem Cells (MSCs) in the Human Prostate. 2012 Dec; Prostate Cancer Young Investigator's Forum, Baltimore, MD.

Poster Presentations:

- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Detection and Characterization of Mesenchymal Stem Cells (MSCs) in Human Prostate Cancer. 2012 Nov; Society for Basic Urologic Research (SBUR), Miami, FL.
*2012 Travel Award Recipient
- **Brennen WN**, Denmeade SR, and Isaacs JT. Mesenchymal Stem Cells (MSCs) as a Selective Delivery Vehicle for a PSA-activated Protoxin for Advanced Prostate Cancer. 2012 Mar; Multi-Institutional Prostate Cancer Program Retreat, Ft. Lauderdale, FL.

Conclusion

Studies arising from this fellowship have led to the quantification and characterization of MSCs present at sites of human prostate cancer, which has recently been published in the open-access journal *Oncotarget*. Optimal growth conditions for expansion of MSCs in culture have been determined, and the capacity of these cells to traffic to prostate cancer xenografts in vivo has been demonstrated. These experiments are essential prerequisites and have laid the foundation for all future work seeking to develop MSCs as drug delivery vehicles to sites of advanced prostate cancer. Challenges related to the accurate quantification of MSC tumor tropism in vivo and the efficient transduction of this cell type with lentiviral expression vectors have been overcome. Furthermore, proof of PRX302 expression and secretion by mammalian cells in a functional form has been demonstrated – critical components for the success of this therapeutic platform. The therapeutic strategy has been modified from its original incarnation to increase the probability of success by utilizing ZFNs targeting the PIG-A locus, rather than CCR5, and thereby, eliminating the potential for the protoxin-expressing cells to self-sterilize. Furthermore, all of the necessary reagents have been generated or obtained and are currently present within the laboratory to ensure successful completion of the proposed project in a timely manner.

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Quantification of Mesenchymal Stem Cells (MSCs) at Sites of Human Prostate Cancer

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ABSTRACT:

Circulating bone marrow-derived Mesenchymal Stem Cells (BM-MSCs) have an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. The prostate is bombarded by numerous infectious & inflammatory insults over a lifetime. Chronic inflammation is associated with CXCL12, CCL5, and CCL2, which are highly overexpressed in prostate cancer. Among other cell types, these chemoattractant stimuli recruit BM-MSCs to the tumor. MSCs are minimally defined as plastic-adhering cells characterized by the expression of CD90, CD73, and CD105 in the absence of hematopoietic markers, which can differentiate into osteoblasts, chondrocytes, and adipocytes. MSCs are immunoprivileged and have been implicated in tumorigenesis through multiple mechanisms, including promoting proliferation, angiogenesis, and metastasis, in addition to the generation of an immunosuppressive microenvironment. We have demonstrated that MSCs represent 0.01-1.1% of the total cells present in core biopsies from primary human prostatectomies. Importantly, these analyses were performed on samples prior to expansion in tissue culture. MSCs in these prostatectomy samples are FAP-, CD90-, CD73-, and CD105-positive, and CD14-, CD20-, CD34-, CD45-, and HLA-DR-negative. Additionally, like BM-MSCs, these prostate cancer-derived stromal cells (PrCSCs) were shown to differentiate into osteoblasts, adipocytes, & chondrocytes. In contrast to primary prostate cancer-derived epithelial cells, fluorescently-labeled PrCSCs & BM-MSCs were both shown to home to CWR22RH prostate cancer xenografts following IV injection. These studies demonstrate that not only are MSCs present in sites of prostate cancer where they may contribute to carcinogenesis, but these cells may also potentially be used to deliver cytotoxic or imaging agents for therapeutic and/or diagnostic purposes.

INTRODUCTION

The prostate is subjected to numerous infectious and inflammatory insults over the course of a man's lifetime, ranging from dietary carcinogens to physical trauma to viral and bacterial pathogens [1]. In fact, greater than 80% of men have evidence of inflammation in their prostate at biopsy [2]. Furthermore, prostatitis likely effects all men at some point during their life, at least acutely [1-2]. While many of these inflammatory lesions will be resolved naturally without intervention, a subset of these

will go on to develop clinical symptoms as a result of chronic inflammation. Chronic inflammation has been suggested as an initiating event in prostate carcinogenesis as evidence of a leukocytic infiltrate is frequently present at sites of prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA), prostate cancer precursor lesions [1].

Mesenchymal stem cells (MSCs) are adult stem cells that have recently gained attention as potent modulators of both the innate and adaptive immune responses [3-5]. MSCs have been minimally defined by

the International Society for Cell Therapy (ISCT) as adult stem cells of fibroblastoid morphology that can adhere to tissue culture plastic, express CD73, CD90, and CD105 in the absence of hematopoietic lineage markers, including CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR [4, 6-7]. Additionally, these cells have the ability to differentiate into cells of the mesoderm lineage, including adipocytes, chondrocytes, and osteoblasts [6], but may also include additional cell types such as pericytes [4, 8-9], myocytes [9-10], and neurons [11-13], though the latter is the subject of controversy [14-15]. Due to the lack of HLA-DR expression and the associated co-stimulatory molecules, MSCs are immunoprivileged and thus escape immune surveillance [3-4, 16]. Furthermore, MSCs have been shown to mediate immunosuppression through multiple mechanisms involving nearly every component of the immune system, both the innate and adaptive arms [3-5]. MSCs traffic to sites of inflammation through the action of soluble chemokines and cytokines emanating from these lesions [17-19]. MSCs have been shown to express a great number of the cognate receptors for these chemokines and cytokines, which have been shown to mediate their homing properties [17].

This latter point is particularly relevant, because the prostate has frequently been shown to contain sites of inflammation, and prostate cancer expresses high levels of pro-inflammatory stimuli, including CXCR4, CCL5, and CCL2 [18, 20-22]. In 2007, Lin et al. characterized stromal cells from benign prostatic hyperplasia (BPH) tissue that had multi-lineage differentiation potential consistent with MSCs [23]. However, because these stromal cells lacked the ability to differentiate into neurons, the authors concluded that these cells did not represent MSCs [23]. In 2010 and 2012, however, it was demonstrated that the ability of MSCs to differentiate into neuronal cells is highly dependent on the age of the donor [13, 24]. These studies documented that MSCs derived from older donors (>45) lose the ability to differentiate into neuronal cells [13, 24]. Therefore, since the Lin et al. study utilized BPH tissue from patients older than 45, this differentiation potential would be consistent with MSCs derived from older donors. In the data presented herein, we demonstrate that a population of cells can be isolated from primary prostate cancer specimens prior to expansion in tissue culture that is consistent with an MSC phenotype. These primary prostate cancer stromal cells or PrCSCs are

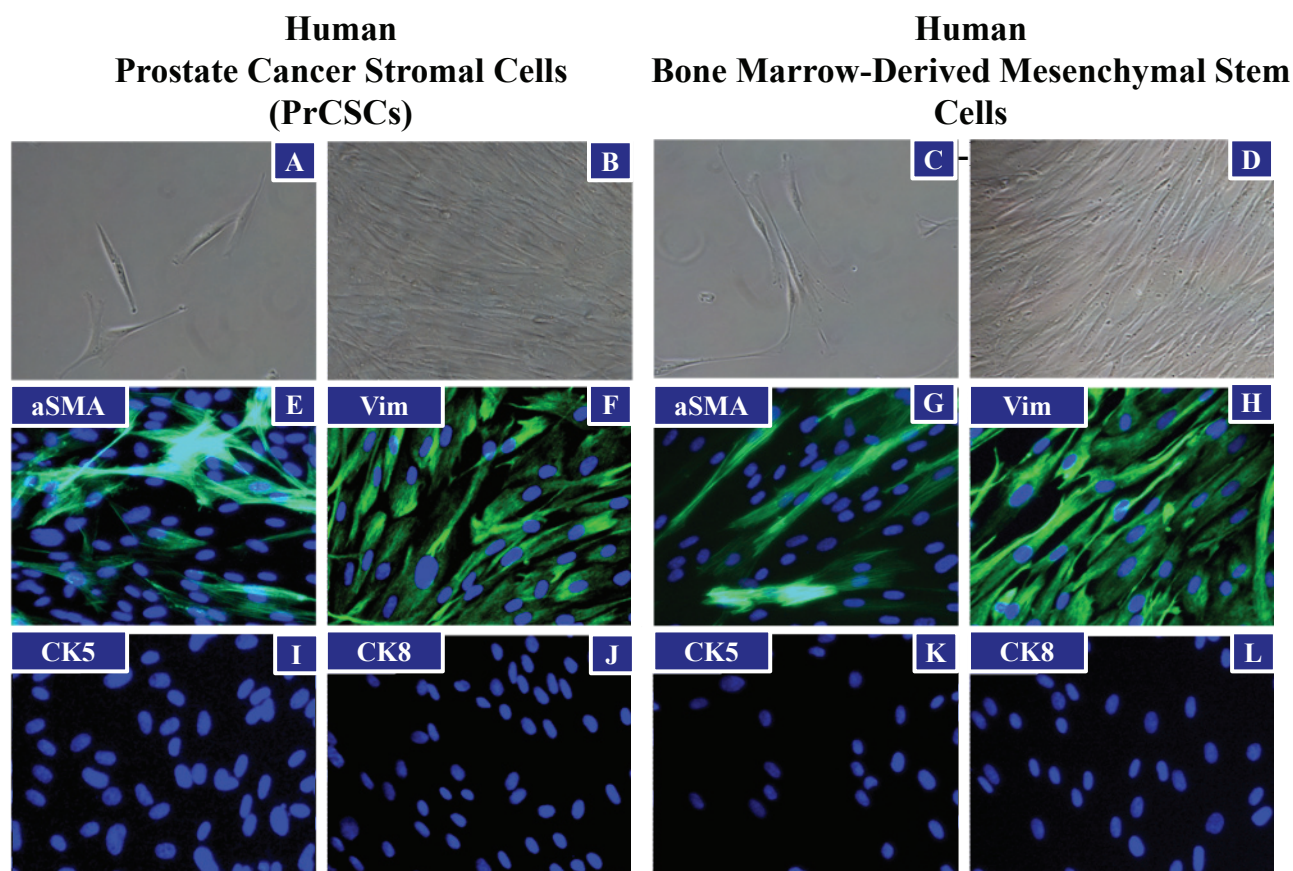


Figure 1: Morphological Similarities between PrCSCs and hBM-MSCs. Prostate cancer-derived stromal cells (PrCSCs) and human bone marrow-derived mesenchymal stem cells (hBM-MSCs) have similar morphologies at low (A and C) and high (B and D) densities (representative phase-contrast images). Both PrCSCs and hBM-MSCs stain positive for mesenchymal markers, alpha-smooth muscle actin (aSMA) (green, E and G) and vimentin (Vim) (green, F and H), but not epithelial markers, cytokeratin 5 (I and K) or cytokeratin 8 (J and L) by immunofluorescence. Nuclei counterstained with DAPI (blue, E-L).

FAP-, CD90-, CD105-, and CD73-positive in the absence of CD14, CD20, CD34, CD45, and HLA-DR expression. Furthermore, a subset of these cells is able to differentiate into osteoblasts, adipocytes, and chondrocytes; thereby, demonstrating their multipotent nature. Like bone marrow-derived MSCs (BM-MSCs), these PrCSCs can traffic to sites of prostate cancer in vivo.

RESULTS

Multi-lineage Differentiation Potential of Human Prostate Cancer-derived Stromal Cells

Tissue cores of human prostatectomy specimens were obtained immediately following surgery, dissociated into a single cell suspension, and placed in tissue culture (RPMI) media supplemented with 10% fetal bovine serum (FBS). From these explanted cells, outgrowth of fibroblast-like prostate cancer-derived stromal cells (PrCSCs) (Figure 1A, B) was observed that had a similar morphology to human bone marrow-derived MSCs (hBM-

MSCs) (Figure 1C, D). If a portion of the same cellular suspension was cultured in keratinocyte serum-free media (K-SFM), basal-like prostate-derived epithelial cells (PrECs) were obtained [25-28]. Both hBM-MSCs and PrCSCs stained positive for alpha-smooth muscle actin (aSMA) (Figure 1E, G) and vimentin (Vim) (Figure 1F, H), but not cytokeratins 5 (CK5) (Figure 1I, K) or 8 (CK8) (Figure 1J, L). These results are the absolute opposite of those obtained for PrECs, which are negative for aSMA and Vim, but positive for CK5 and CK8 [25-28]. Similar to hBM-MSCs (Figure 2Q, S, T), differentiation of PrCSCs into adipocytes (Oil Red O-positive) (Figure 2B, G, L), osteoblasts (Alizarin Red-positive) (Figure 2D, I, N), and chondrocytes (Safranin O-positive) (Figure 2E, J, O) was observed if the cells were cultured under the appropriate induction conditions, but not in the uninduced controls (Figure 2A, C, F, H, K, M, P, R). Furthermore, these cells were shown to be fibroblast activation protein (FAP)⁺, CD90⁺, CD105⁺, CD73⁺, and alpha-smooth muscle actin (aSMA)⁺ by flow cytometry in the absence of CD45, CD34, CD11b, CD19, and HLA-DR expression (Table 1). In contrast, PrECs do not differentiate into these cell types under the same conditions (data not shown). Importantly,

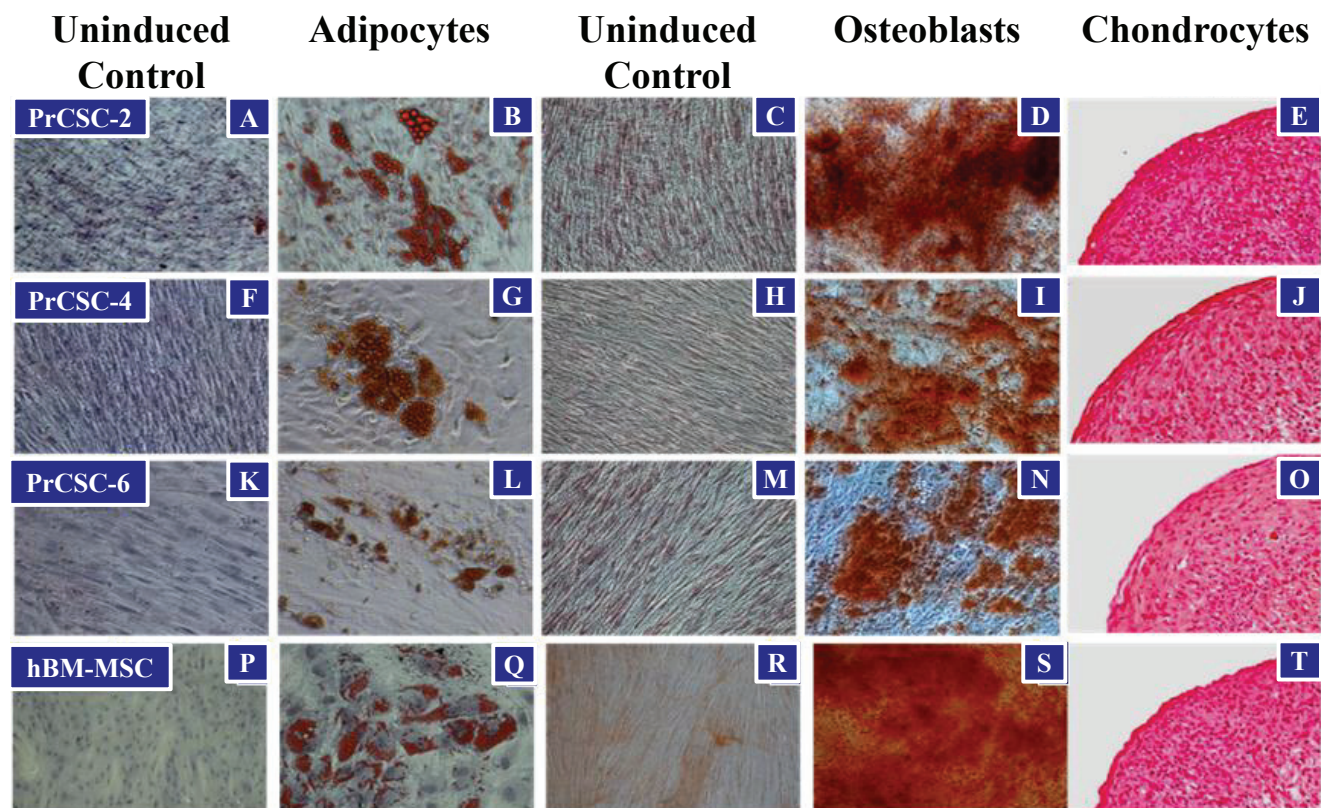


Figure 2: Multi-lineage Differentiation of PrCSCs and hBM-MSCs. PrCSCs derived from multiple patients (PrCSC-2, -4, -6) are able to differentiate into adipocytes (B, G, and L), osteoblasts (D, I, and N), and chondrocytes (E, J, and O) when placed in the appropriate induction media as defined by positive staining for lipid vacuoles (adipocytes, Oil Red O), calcium mineralization (osteoblasts, Alizarin Red S), and glycosaminoglycans (chondrocytes, Safranin-O), respectively. Differentiation indicated by red staining in each. In contrast, no differentiation is observed when these cells are not cultured in the presence of the various inducing factors (adipocytes: A, F, K, and P; osteoblasts: C, H, M, and R). Differentiation into these three lineages is one of the defining characteristics of mesenchymal stem cells as demonstrated by the hBM-MSC positive controls (Q, S, and T).

only a subset of cells within PrCSCs derived from a single donor possesses this tri-lineage differentiation potential (Figure 2A-O). In addition, not all PrCSCs derived from different patients were able to differentiate into all lineages (Table 1). Interestingly, the multi-lineage differentiation potential of the PrCSCs does not appear to correlate with Gleason Score (Table 1).

Quantification of Mesenchymal Stem Cells in Human Prostate Cancer

To eliminate potential artifacts resulting from selection events associated with tissue culture, we optimized a flow cytometry-based assay to directly quantify the number of MSCs present in human prostate cancer samples directly from the patient prior to expansion in culture. Again, tissue cores of prostatectomy specimens were obtained immediately following surgery and digested

into a single cell suspension using a combination of mechanical and enzymatic methods. Following labeling with either an MSC phenotyping cocktail (CD73, CD90, CD105, CD14, CD20, CD34, CD45, and HLA-DR) (Figure 3A) or an antibody isotype control cocktail (Figure 3B), these dissociated cells were analyzed by flow cytometry. MSCs within this population of cells were defined as being CD73, CD90, and CD105 triple-positive in the absence of CD14, CD20, CD34, CD45, and HLA-DR labeling (Figure 3). First, cells staining positive for the tested lineage markers were excluded from further analysis. Next, the CD73-positive cells within this lineage-negative population were selected. Finally, the number of MSCs present in the prostatectomy specimens were quantified by determining the number of CD73-positive, lineage-negative cells that were also double-positive for CD90 and CD105 (Figure 3). Of the 10 specimens analyzed in this study, MSCs represented between

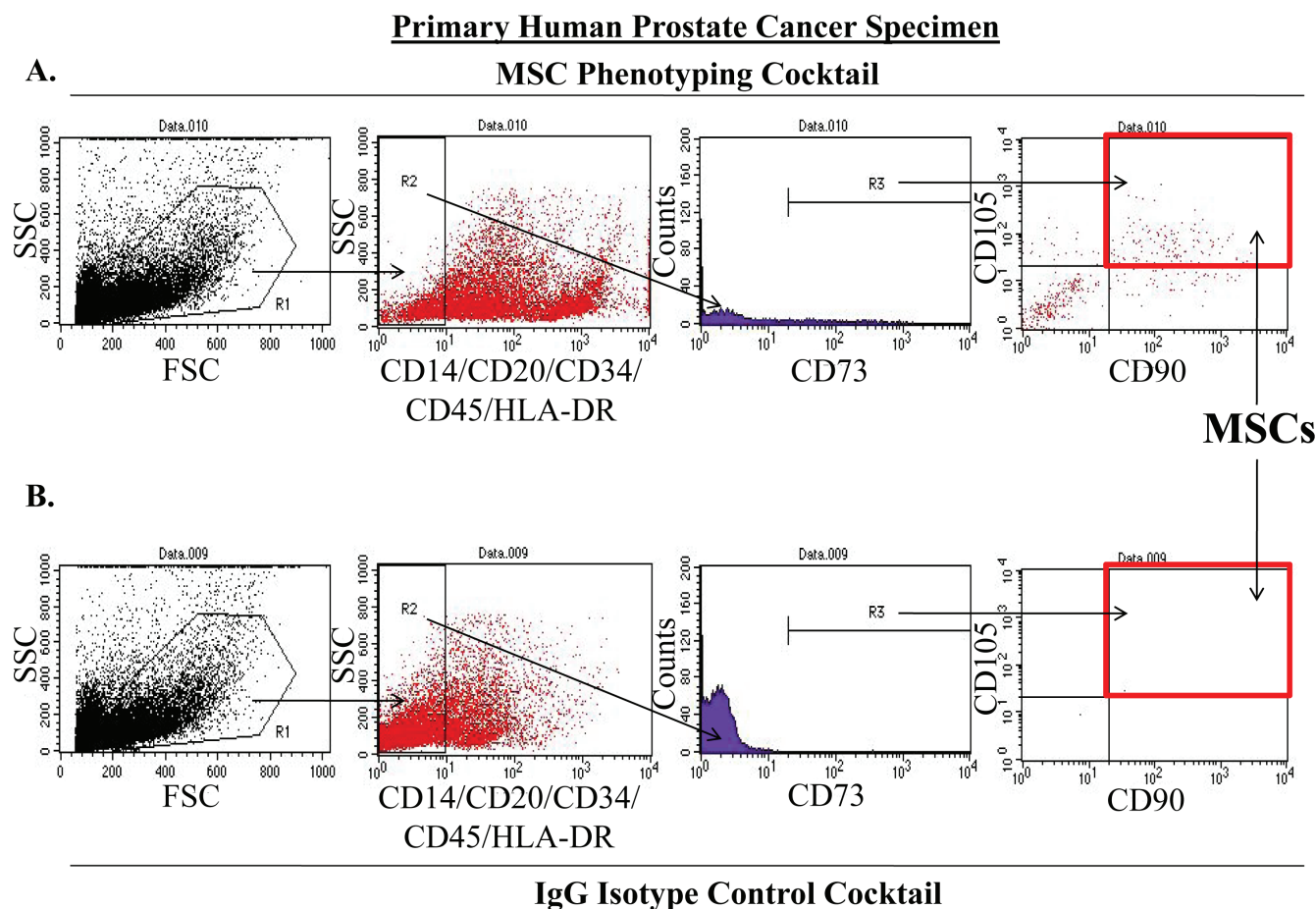


Figure 3: Method for Quantifying MSCs in Primary Human Prostatectomy Samples. MSCs were quantified from primary human prostatectomy specimens using an optimized flow cytometry assay (A-B). Prostatectomy samples were digested into a single cell suspension using a combination of enzymatic and mechanical methods. At least 10,000 cells were initially gated (R1) on the basis forward and side scatter (FSC and SSC, respectively). From this initial population, lineage-negative cells (CD14⁻, CD20⁻, CD34⁻, CD45⁻, HLA-DR⁻) were selected (R2) and analyzed for expression of CD73 (R3). These lineage-negative, CD73-positive cells were further analyzed for the co-expression of CD90 and CD105. MSCs were defined as being lineage-negative and triple-positive for CD73, CD90, and CD105 (red box). Final quantification was performed by subtracting the number of events meeting these criteria in the IgG isotype control cocktail analysis (red box, B) from the events detected in the sample stained with the MSC phenotyping cocktail (red box, A). Importantly, all samples were analyzed within 3 hrs post-surgery.

Table 1: Expression Profile and Differentiation Capacity of PrCSCs and hBM-MSCs.

	FAP	CD90	CD105	CD73	aSMA	CD45	CD34	CD11b	CD19	HLA-DR	Adipo-cytes	Osteo-blasts	Chondro-cytes	Gleason Score
hBM-MSC1	+	+	+	+	+	-	-	-	-	-	+	+	+	N/A
hBM-MSC2	+	+	+	+	+	-	-	-	-	-	+	+	+	N/A
PrCSC-1	+	+	+	+	+	-	-	-	-	-	-	+	-	3+3
PrCSC-2	+	+	+	+	+	-	-	-	-	-	+	+	+	3+3
PrCSC-3	+	+	+	+	+	-	-	-	-	-	+	-	-	4+3
PrCSC-4	+	+	+	+	+	-	-	-	-	-	+	+	+	4+3
PrCSC-5	+	+	+	+	+	-	-	-	-	-	-	-	-	4+3
PrCSC-6	+	+	+	+	+	-	-	-	-	-	+	+	+	4+4

approximately 0.01 and 1.1% of the overall population of cells within the digested prostatectomy tissue (Table 2). As with the multi-lineage differentiation potential of PrCSCs derived from comparable prostatectomy specimens, there does not appear to be a relationship between the quantity of MSCs present in a particular sample and Gleason grade; however, the small number of samples characterized in this analysis preclude any conclusive judgments (Table 2). For comparison, CD31+ endothelial cells represented 1.89% of the cell population in the one prostatectomy specimen analyzed.

Trafficking of Prostate Cancer-derived Stromal Cells & Mesenchymal Stem Cells to Prostate Cancer Xenografts

Additionally, hBM-MSCs are known to home to sites of cancer as a result of the inflammatory microenvironment present within these lesions. To determine whether PrCSCs also retained this ability, fluorescently-labeled cells (1×10^6) were administered intravenously (IV) to animals bearing CWR22RH xenografts (3 animals/group). While all cell types tested (PrCSCs, hBM-MSCs, and PrECs) were found entrapped in the lungs at 4 days post-infusion (Figure 4A, B, and C), only the hBM-MSCs and PrCSCs were able to traffic to the prostate cancer xenograft (Figure 4D, E, and F).

DISCUSSION

PrCSCs obtained directly from prostate cancer patients, prior to expansion in tissue culture, express CD90, CD73, and CD105 in the absence of CD14, CD20, CD34, CD45, and HLA-DR as demonstrated using an optimized flow cytometry assay (Figure 3, Table 2). Additionally, at least a subset of PrCSCs retains the ability to differentiate into osteoblasts, adipocytes, and chondrocytes (Figure 2). Therefore, PrCSCs fulfill all of the currently accepted criteria that are used to define MSCs. Importantly, both intra- and inter-patient

heterogeneity is apparent in the population of cells isolated according to the current methods. This is demonstrated by the fact that not all PrCSC cultures isolated from different patients retained their multi-lineage differentiation potential (Table 1), and not all cells within a single culture were able to differentiate into all lineages under the appropriate induction conditions (Figure 2). Additionally, each core from each patient was heterogeneous with respect to the amount of cancer present, the number of cancer foci, and the degree of inflammation, all of which likely effect the number of MSCs quantified in any given specimen. Of the 10 prostatectomy specimens analyzed, the number of MSCs ranged from 0.01-1.1% of the overall cell population (Table 2). In comparison, CD31+ endothelial cells, which are known to play absolutely critical roles in tumorigenesis [29-30], represented 1-2% of the cells within sites of prostate cancer. Despite MSCs representing a relatively minor population of cells within the tumors analyzed, their numbers can reach approximately 50% of the endothelial cell content, and therefore, they may potentially play a significant role in prostate tumorigenesis. Interestingly, MSCs are often found in close association with blood vessels where there reside in a perivascular niche [9].

MSCs have previously been shown to influence carcinogenesis in a variety of ways, including promoting proliferation, angiogenesis, and metastasis, in addition to the generation of an immunosuppressive microenvironment [5, 7, 31]. Several studies have also shown MSCs to have anti-tumorigenic properties mediated through immunostimulatory properties and suppression of Akt- and Wnt-mediated survival signals [31-34]. Thus far, only a few studies have examined the role of MSCs in prostate carcinogenesis in vivo, which have predominantly demonstrated no effect on tumor growth [33, 35-41]. Unfortunately, these studies have primarily relied upon the PC3 cell line; therefore, experiments extending these observations into a broader range of models are necessary prior to making any conclusive judgments on MSCs role in prostate carcinogenesis. To further complicate the situation, MSCs have also been shown to give rise to so-

Table 2: Quantification of MSCs in Primary Human Prostatectomy Samples.

Sample	Gleason Score	MSCs (%)
PCa-1	3+3	0.38
PCa-2	3+3	1.10
PCa-3	3+4	0.22
PCa-4	3+4	0.12
PCa-5	3+4	0.01
PCa-6	4+3	1.02
PCa-7	4+3	0.28
PCa-8	4+4	0.14
PCa-9	5+4	0.38
PCa-10	5+5	1.06

called carcinoma-associated fibroblasts or CAFs [42-44], which have been the subject of many investigations into cancer and its relationship with the supporting stroma [45-50]. Our own data suggests that it is relatively easy to expand MSCs from primary human tissue samples under standard culture conditions, and these cells together with their progeny can quickly become a dominant population in the culture. Furthermore, these PrCSCs/MSCs express both α SMA and vimentin (Figure 1), the co-expression of which is commonly used to define reactive fibroblasts or CAFs [50]. This would suggest that many previous studies investigating the role of stromal cells derived from primary human tissue were actually studying MSCs depending on the passage used during the analysis and the frequency of MSCs in the starting population. While the mechanisms underlying the effects of MSCs in carcinogenesis are not fully understood, they are likely related to the complex relationship that exists between MSCs and the immune system [3, 5] coupled with the heterogeneity of tumor microenvironments and the cytokine profile present [18].

Chronic inflammation potentially resulting from a variety of stimuli, including dietary products, infectious agents, corpora amylacea-induced physical trauma, hormonal changes, and urine reflux, is frequently associated with prostate cancer precursor lesions [1-2]. The presence of *Mycoplasma hominis* has also been suggested as a cause of prostate inflammation [51]; however, these results may have been derived from tissue collection artifacts associated with transrectal biopsies [52]. Regardless of the cause, chronic inflammation has been suggested as an initiating event for prostate cancer [1-2]. Additionally, prostate cancers typically express high levels of pro-inflammatory chemokines, including CXCL12 (SDF-1), CCL5 (RANTES), and CCL2 (MCP-1)

[20-22]. The expression of these cytokines has been shown to mobilize systemic reservoirs of inflammatory and immunomodulatory cells, including BM-MSCs, which are recruited to prostate cancer lesions [17-18]. MSCs express an extensive array of cytokine receptors, which have been shown to mediate their trafficking to sites of inflammation and cancer [17]. Furthermore, MSCs also secrete a large number of immunomodulatory, growth, and signaling molecules, including TGF- β , GM-CSF, RANTES, CCL2, VEGF, HGF, IL-6, and IL-10 [3-4, 19, 53], which may help to initiate a self-reinforcing loop that may lead to chronic inflammation under pathological conditions and contribute to carcinogenesis. Given the regenerative, immunomodulatory and immunotraficking properties of MSCs, it is not surprising to find these cells in the prostate during tissue regrowth [54], carcinogenesis (Figure 3, Table 2), and inflammation-associated pathologies, such as BPH [23]. Placencio et al. have previously demonstrated that bone marrow-derived MSCs contributed to prostate regrowth following testosterone supplementation in mice post-castration [54]. Previous work by Lin et al. has also demonstrated that stromal cells consistent with an MSC phenotype from older donors can be isolated from BPH tissue [23]. The authors concluded that these stromal cells did not represent MSCs due to their inability to generate neurons, a property that has been shown to decrease with the age of the donor [13, 24], and therefore, is likely explained by the prevalence of BPH in older men from which the tissue analyzed was obtained. An earlier study comparing CD90hi vs. CD90lo primary stromal cells isolated from prostate cancer patients also concluded that these cells did not represent MSCs [55]. However, it should be noted that the differentiation potential of these cells was not assayed. Furthermore, CD90hi cells

were compared to CD90^{lo} cells rather than CD90^{neg} cells, both of which may represent MSCs at different stages of differentiation and would explain the observed similarities in their expression profiles. The data presented herein clearly demonstrates that there is a rare population of CD90-positive MSCs present in tissue isolated from primary prostate cancer patients (PrCSCs) (Figures 2-3, Table 2).

As described above, there is extensive literature demonstrating that BM-MSCs can home to sites of prostate cancer based upon the inflammatory microenvironment present within these lesions [3, 17-19]. Due to the lack of HLA-DR expression and immunologic co-stimulatory molecules, these cells are non-immunogenic even in an allogeneic setting [3-4, 16]. This suggests that MSCs can be used to systemically deliver therapeutic or imaging agents to both primary and metastatic prostate cancer deposits throughout the body. Additionally, our data suggests that PrCSCs retain this tumor trafficking ability as well (Figure 4), which raises the possibility of using autologous cells derived from a patient's own prostatectomy specimen to target systemic disease; however, ethical concerns related to infusing patients with autologous cancer-associated stromal cells would be of significant concern with this latter approach. Much previous work has attempted to exploit the tumor-trafficking properties of MSCs derived from a variety

of non-malignant sources to deliver cytotoxic agents to various solid tumor types with mixed results [56-60]. Importantly, these studies failed to take into account that MSCs traffic to other sites throughout the body, including the lungs, bone marrow, and spleen, in addition to the tumor; therefore, dose-limiting toxicities can be manifested from the delivery of these compounds to peripheral non-target tissues. To circumvent this problem, a prodrug approach exploiting tumor- or tissue-selective activation of a therapeutic compound in which the MSCs were used as a vector to enhance drug accumulation within the tumor would potentially be of greater therapeutic benefit. Additionally, studies by Sarkar et al. have demonstrated that cell engineering approaches can be used to enhance the homing and engraftment efficiency of MSCs in target tissues by mimicking mechanisms of leukocyte extravasation [61].

In summary, primary human prostate cancer harbors a population of cells consistent with MSCs. Stromal cells derived from human prostatectomy specimens (PrCSCs) share an expression profile with MSCs derived from the bone marrow (BM-MSCs) for all cell surface markers analyzed. Like BM-MSCs, these PrCSCs have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes; thereby, demonstrating their multi-lineage differentiation potential. Both BM-MSCs and PrCSCs are able to traffic to prostate cancer xenografts in vivo,

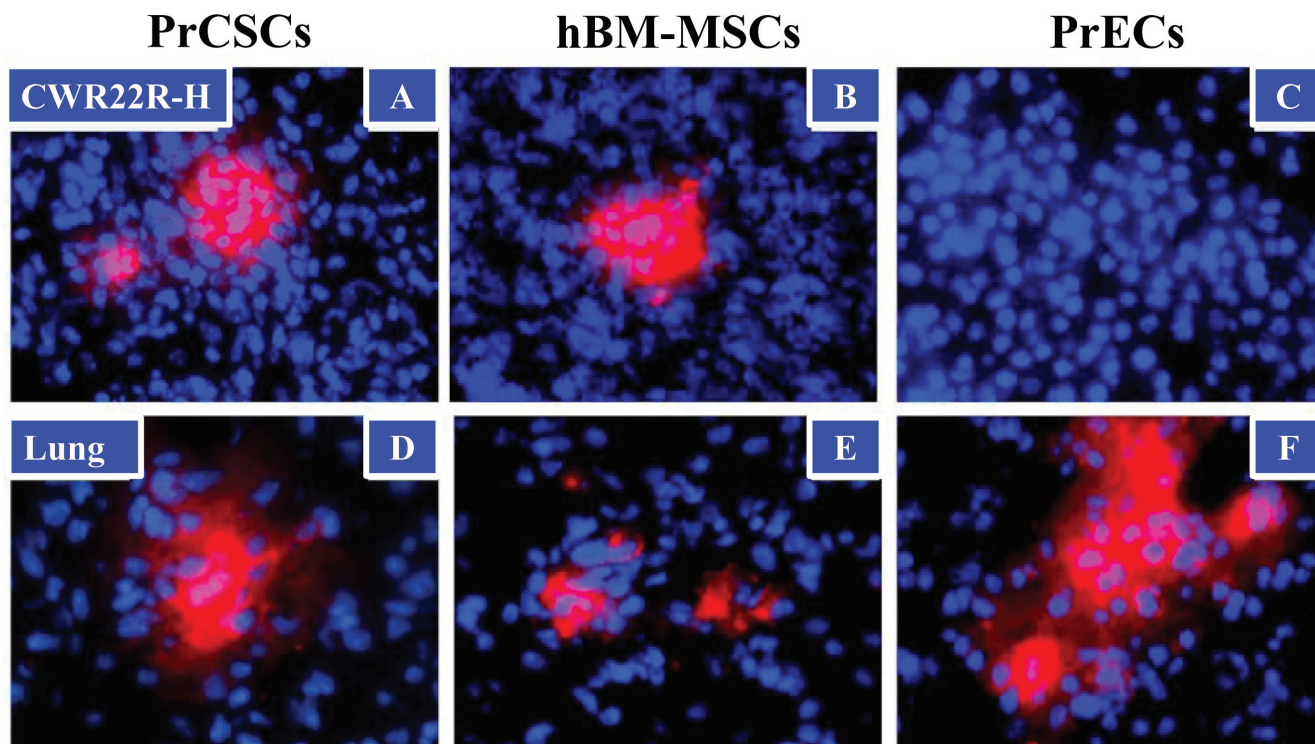


Figure 4: Tumor Trafficking of PrCSCs and hBM-MSCs to Human Cancer Xenografts in Mice. PrCSCs (A) and hBM-MSCs (B), but not PrECs (C), traffic to prostate cancer xenografts in vivo following systemic infusion. Fluorescently-labeled (CM-DiI, red) PrCSCs, hBM-MSCs, and PrECs (1×10^6) were infused intravenously (IV) into immunocompromised mice bearing subcutaneous CWR22RH xenografts (3/group). Four days post-infusion, lungs and tumors were harvested and analyzed by fluorescence microscopy for the presence of CM-DiI-labeled cells. In contrast to the xenografts, all three cell types were found entrapped in the lungs following infusion (D-F). Nuclei counterstained with DAPI (blue). At least three images analyzed per tissue per animal, representative images shown.

likely as a result of the pro-inflammatory cytokine and chemokine milieu present. Therefore, MSCs represent a potential drug delivery vector for future therapeutic approaches targeting both local and metastatic prostate cancer.

METHODS

Reagents

Rat anti-human CD11b-APC (clone M1/70.15.11.5), mouse anti-human CD19-PE (clone LT19), mouse anti-human CD34-PE (clone AC136), mouse anti-human CD45-APC (CLONE 5B1), mouse anti-human CD326(EpCAM)-FITC (clone HEA-125), mouse anti-human CD326(EpCAM)-PE (clone HEA-125), mouse anti-human CD326(EpCAM)-APC (clone HEA-125), and mouse anti-human HLA-DR-PerCP (clone AC122) antibodies were purchased from Miltenyi Biotec, Inc. (Bergisch Gladbach, Germany). Mouse anti-human HLA-DR-APC (clone LN3), mouse anti-human CD73-APC (clone AD2), mouse anti-human CD105-PE (clone SN6), mouse anti-human CD326(EpCAM)-biotin (clone 1B7) and mouse anti-human FAP (clone F11-24) were purchased from eBioscience (San Diego, CA). Mouse anti-human CD90-FITC (clone F15-42-1) was purchased from Millipore (Billerica, MA). Mouse anti-human aSMA-FITC (clone 1A4) was purchased from Abcam (Cambridge, MA). Mouse anti-human CK5 (clone XM26) was purchased from Vector Laboratories (Burlingame, CA). Mouse anti-human CK8 (clone LP3K) was purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-human vimentin (clone LN-6) was purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse Alexa Fluor 488, Roswell Park Memorial Institute (RPMI)-1640 medium, keratinocyte-serum free medium (K-SFM), Hank's Balanced Salt Solution (HBSS), L-glutamine, and penicillin-streptomycin were purchased from Life Technologies-Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bioproducts (West Sacramento, CA).

Primary Cell Isolation and Tissue Culture

hBM-MSCs were obtained from Lonza (Walkerville, MD). Primary prostate epithelial and stromal cells from patient radical prostatectomy specimens were isolated at our institution in accordance with an Institutional Review Board approved protocol according to previously published protocols [25-28, 62] for the cell cultures used in the differentiation assays, immunofluorescence staining, and cell surface expression studies. hBM-MSCs and PrCSCs were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1%

penicillin-streptomycin in a 5% CO₂, 95% air humidified incubator at 37°C. PrECs were grown in K-SFM with defined growth factors [25-28] in the same 5% CO₂, 95% air humidified incubator at 37°C.

Immunofluorescence

Immunofluorescent staining for aSMA, Vim, CK5, and CK8 were performed using the antibodies listed above according to previously published protocols [27-28]. Nuclei are counterstained with DAPI using ProLong Gold anti-fade with DAPI (Invitrogen). Images were captured using a Nikon (Melville, NY) Eclipse Ti Fluorescent scope equipped with a Nikon DS-Qi1Mc camera NIS-Elements AR3.0 imaging software.

Multilineage Differentiation

To assay adipogenic differentiation, 2 x 10⁵ cells were plated in a 6-well plate and allowed to reach 100% confluence (3 replicates/cell type) in an incubator with 5% CO₂ at 37°C. The media was then changed to hMSC adipogenic induction medium (Lonza) supplemented with h-insulin (recombinant), L-glutamine, MCGS, dexamethasone, indomethacin, IBMX (3-isobutyl-1-methyl-xanthine), GA-1000. According to the manufacturer's instructions, media was changed every three days alternating between induction and maintenance medium for three complete cycles. After the final cycle, cells remained in the maintenance medium for an additional 7 days prior to evaluation of adipogenic differentiation. Negative control cells were grown in maintenance media only. Adipogenic differentiation was assayed using the lipid stain Oil Red O (Sigma) to identify lipid vacuoles in differentiated cells.

To assay osteogenic differentiation, 3 x 10⁴ cells were plated in a 6-well plate and allowed to adhere overnight at 37°C in an incubator with 5% CO₂ (3 replicates/cell type). According to the manufacturer's instructions, the media was then changed to Osteogenic Induction media (Lonza) supplemented with dexamethasone, L-glutamine, ascorbate, MCGS, b-glycerophosphate. Media was changed every 3-4 days for 21 days. Negative control cells were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, penicillin-streptomycin. After 21 days, osteogenic differentiation was assayed by staining for calcium deposits with Alizarin Red S (Sigma).

To assay chondrogenic differentiation, 2.5 x 10⁵ cells were centrifuged at 150 x g for 5 min at room temperature and resuspended in 0.5 mL chondrogenic induction medium (Lonza) supplemented with dexamethasone, ascorbate, ITS, GA-1000, sodium pyruvate, proline, L-glutamine, and TGF-B3 in a 15 mL polypropylene conical tube according to the manufacturer's instructions (3 replicates/cell type). The caps were loosened a half-

turn and placed at 37°C in an incubator with 5% CO₂. The media was changed every 3 days for 21 days while being careful to avoid aspirating the pellet. After 21 days, cell pellets were fixed in formalin and paraffin-embedded for histological processing. Negative controls were cultured in the absence of TGF-B3. Chondrogenic differentiation was assayed by staining for glycosaminoglycans with Safranin-O (Sigma).

Analysis of Cell Surface Markers and MSC Quantification by Flow Cytometry

To analyze cell surface marker expression, prostatectomy cores were dissociated into a single cell suspension as described previously [25-28, 62]. Flow cytometry analyses were also performed as described previously [26-27]. Briefly, all antibody incubations, washes, and flow cytometric analyses were performed in MACS cell sorting buffer (Miltenyi). Antibody labeling was performed at 4°C for 20 min with a 1:10 dilution of the antibody in a volume of 100 µl per 1x10⁶ cells. The cells were washed in 1 mL cold cell sorting buffer, resuspended in 1.0 mL cell sorting buffer and passed through a 0.2 m filter into a flow analysis tube (BD Biosciences, Franklin Lakes, NJ). Analysis was performed on a BD FACSCalibur flow cytometer.

To obtain cell suspensions for quantification of MSCs by flow cytometry prior to expansion in tissue culture, the following protocol was optimized. Twenty-five 18-gauge biopsy needle cores (C. R. Bard, Inc., Tempe, AZ) were obtained and washed in HBSS. Five randomly selected cores were fixed, paraffin-embedded, and sectioned for H&E staining and pathological confirmation. The remaining cores were digested using a human tumor dissociation kit (Miltenyi) and a gentleMACS dissociator (Miltenyi) according to the manufacturer's instructions. The dissociated cell suspension was then passed through a 70 µm pre-separation filter (Miltenyi). The sample was centrifuged at 250 x g for 5 min and resuspended in RBC lysis buffer (Miltenyi) for 10 min at room temperature. The RBC-negative cell suspension was centrifuged at 250 x g for 5 min and resuspended in MACS cell sorting buffer (Miltenyi) to determine cell number and viability by trypan exclusion using a Cellometer Auto T4 (Nexcelcom Bioscience, Lawrence, MA) prior to downstream flow cytometry applications.

All antibody incubations, washes, and flow cytometric analyses were performed in MACS cell sorting buffer (Miltenyi). Antibody labeling was performed at 4°C for 10 min with a 1:10 dilution of with a MSC Phenotyping Cocktail (anti-CD14-PerCP, anti-CD20-PerCP, anti-CD34- anti-PerCP, anti-CD45-PerCP, anti-CD73-APC, anti-CD90-FITC, and anti-CD105-PE) or Isotype Control Cocktail (Mouse IgG1-FITC, Mouse IgG1-PE, Mouse IgG1-APC, Mouse IgG1-PerCP, and Mouse IgG2a-PerCP)

provided in the human MSC phenotyping kit (Miltenyi) in a volume of 100 µl per 1x10⁶ cells according to the manufacturer's instructions. Additionally, anti-HLA-DR-PerCP (Miltenyi) was added to the MSC Phenotyping Cocktail. The cells were washed in 1 mL cold cell sorting buffer, resuspended in 0.5 mL cell sorting buffer and passed through a 0.2 µm filter into a flow analysis tube. Analysis was performed on a BD FACSCalibur flow cytometer. All compensation controls were performed using anti-EpCAM antibodies directly conjugated to FITC, PE, APC, or Biotin followed by anti-Biotin-PerCP on aliquots of the same cell suspension to ensure proper gating and instrument settings prior to sample analysis. For sample analysis, cell suspensions labeled with either the Isotype Control or MSC Phenotyping Cocktails were gated (R1) on the basis of forward and side scatter (FSC & SSC, respectively) (Figure 3). Cells gated in R1 were then selected based on being lineage-negative (R2), i.e., negative for CD14, CD20, CD34, CD45, and HLA-DR expression. Next, CD73-positive cells (R3) within these lineage-negative cells were further analyzed for co-expression of CD90 and CD105. MSCs were defined as cells that were triple-positive for CD90, CD73, and CD105 in the absence of the tested lineage markers and quantified by subtracting the number of events, if any, that met these criteria in the isotype control sample. This corrected number was used as the numerator to determine the percentage of MSCs present in the sample. At least 10,000 events were collected in R1, which defined the number of total cellular events and was used as the denominator in the above calculation. Importantly, all samples were processed and analyzed within 3 hrs post-surgery.

Cell Trafficking to Prostate Cancer Xenografts in vivo

Animal studies were performed according to protocols approved by and performed in accordance with the guidelines of the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. For CWR22RH xenografts, 50 mg of minced tumor tissue that had passed through a sterile tissue strainer and washed with HBSS was implanted subcutaneously in 100 µl of 80% Matrigel (BD Biosciences, Sparks, MD) in the flanks of NOG-SCID mice.

To assay tumor trafficking, human PrCSCs, hBM-MSCs (Lonza), or PreCs were fluorescently-labeled ex vivo with CM-DiI (Invitrogen) and washed according to the manufacturer's instructions. Subsequently, 1x10⁶ cells were injected intravenously into NOG-SCID mice bearing subcutaneous CWR22RH tumors (3 mice/group). Animals were euthanized by CO₂ asphyxiation at 4 days post-infusion. The lungs and tumors were harvested from each mouse, flash frozen in VWR Clear Frozen Section

Compound (Radnor, PA), and 4 μ m sections were cut on a Shandon Cryotome E (Thermo Scientific, Waltham, MA). Nuclei are counterstained with DAPI using ProLong Gold anti-fade with DAPI (Invitrogen). Images were captured using a Nikon (Melville, NY) Eclipse Ti Fluorescent scope equipped with a Nikon DS-Qi1Mc camera NIS-Elements AR3.0 imaging software.

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