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Rapid Selection of Mesenchymal Stem and Progenitor Cells in Primary Prostate Stromal Cultures

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Abstract

BACKGROUND—Carcinoma-associated fibroblasts (CAFs) are a dominant component of the tumor microenvironment with pro-tumorigenic properties. Despite this knowledge, their physiologic origins remain poorly understood. Mesenchymal stem cells (MSCs) can be recruited from the bone marrow to areas of tissue damage and inflammation, including prostate cancer. MSCs can generate and have many overlapping properties with CAFs in preclinical models.

METHODS—Multiparameter flow cytometry and multipotent differentiation assays used to define MSCs in primary prostate stromal cultures derived from young (>25 yrs) organ donors and prostate cancer patients compared with bone marrow-derived stromal cultures. Population doubling times, population doublings, cell size, and differentiation potential determined under multiple culture conditions, including normoxia, hypoxia, and a variety of media. TGF- β measured by ELISA.

RESULTS—MSCs and stromal progenitors are not only present in normal and malignant prostate tissue, but are quickly selected for in primary stromal cultures derived from these tissues; becoming the dominant population within just a few passages. Growth potential inversely associated with TGF- β concentrations. All conditions generated populations with an average cell diameter >15 μ m. All cultures tested had the ability to undergo osteogenic and chondrogenic differentiation, but unlike bone marrow-derived MSCs, primary stromal cultures derived from normal prostate tissue lack adipogenic differentiation potential. In contrast, a subset of stromal cultures derived from prostate cancer patients retain the ability to differentiate into adipocytes; a property that is significantly suppressed under hypoxic conditions in both bone marrow- and prostate-derived MSCs.

CONCLUSIONS—Primary prostate stromal cultures are highly enriched in cells with an MSC or stromal progenitor phenotype. The use of primary cultures such as these to study CAFs raises interesting implications when considering their overlapping properties. The lack of adipogenesis in stromal cultures derived from normal prostates suggests they have a lineage-restricted progenitor phenotype. The retention of adipogenic differentiation in cultures from a subset of prostate cancer patients suggests the active recruitment of less committed progenitors or MSCs from the bone

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marrow as a function of disease progression. This recruitment can potentially be exploited for prognostic purposes or a cell-based platform for the systemic delivery of cytotoxic agents to sites of prostate cancer.

Keywords

mesenchymal stem cell; MSC; carcinoma-associated fibroblast (CAF); stromal progenitor; normal prostate

INTRODUCTION

Cancer progression is complex process involving dynamic and reciprocal interactions between cancer cells and their surrounding microenvironment, including fibroblasts, endothelial cells, myeloid-derived suppressor cells (MDSCs), macrophages, and other inflammatory cells, in addition to mesenchymal stem or stromal cells (MSCs, [1,2]). Together with the extracellular matrix (ECM), this collective milieu referred to as the tumor microenvironment has been implicated in all stages of tumor development and progression [1–4]. Cancer cells are dependent on this supporting stroma not only for their nutrient supply via the circulation, but for growth and survival signals produced by the stromal cells themselves. Paracrine factors secreted by the cancer not only pervert the surrounding non-malignant cells of the parenchyma but recruit circulating cells from distant sources such as the bone marrow in order to establish a microenvironment permissive to tumor growth, survival, invasion, and metastasis [5].

This tumor-associated stroma can represent a significant proportion of the tumor mass, as much as 90% or more in cancers characterized by a desmoplastic reaction [3]. Carcinoma-associated fibroblasts (CAFs) in particular are among the most abundant stromal cell populations within the tumor and have been implicated in progression to a metastatic lethal state in multiple studies [3–10]. CAFs secrete growth factors that stimulate tumor growth, angiogenesis, and other tumorigenic properties, in addition to remodeling the extracellular matrix, a reservoir of extracellular growth, and survival factors. Pioneering work by Dr. Cunha and colleagues demonstrated that immortalized, but non-tumorigenic prostate epithelial cells generated tumors 500-times greater in size when mixed with CAFs relative to those co-inoculated with their normal counterparts (i.e., NAFs, [8]). Though there is some ambiguity regarding an exact definition of CAFs, they are commonly defined by the co-expression of alpha-smooth muscle actin (α SMA) and vimentin. Over the years, much work has gone into characterizing CAFs and defining their origin. Proposed sources include tissue resident fibroblasts, local, and regional progenitors, in addition to infiltrating bone marrow-derived MSCs [6,11–17].

MSCs are recruited from the bone marrow to sites of tissue damage and inflammation, including those present at sites of prostate cancer [1]. MSCs are minimally defined as cells with fibroblastic morphology that can adhere to tissue culture plastic and co-express CD73, CD90, and CD105 in the absence of hematopoietic lineage markers including CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR [1,6,18,19]. Additionally, MSCs are multipotent cells capable of differentiating into a variety of mesenchymal lineages, including

osteoblasts, chondrocytes, and adipocytes, among others [1,6,18,19]. Following recruitment of MSCs to injured tissue, they respond to signals in the tissue microenvironment to aid in the repair process through immunomodulatory, trophic and regenerative processes [1,20]. These same physiological pathways can be subverted in pathophysiologic processes, including cancer.

MSCs have been associated with many pro-tumorigenic properties including promoting proliferation, angiogenesis, metastasis, and the generation of an immunosuppressive microenvironment [1,21]. This latter property may be particularly important given the association between chronic inflammation and prostate cancer progression that eventually leads to an anergic immune microenvironment [22–25]. MSCs suppress multiple components of the innate and adaptive arms of the immune system, including effects on T-cells, B-cells, NK cells, dendritic cells, macrophages, and others [1,26–29]. Interestingly, a subset of prostate cancer patients have an elevated number of MSCs in their tumors [6], suggesting MSCs have the potential to contribute to prostate cancer progression through many of the mechanisms described above including the generation of CAFs. In fact, preclinical models have demonstrated that the recruitment of MSCs promotes prostate cancer metastasis [30].

Herein, we demonstrate that primary prostate stromal cultures from normal and prostate cancer tissue donors are enriched in MSCs. Furthermore, this progenitor cell population is quickly selected for in tissue culture and becomes the dominant population within just a few passages. Similar enrichment is observed whether cultures are initiated from a single cell suspension following mechanical and enzymatic dissociation or from traditional explant cultures. This raises important implications for the in vitro study of CAFs, which are frequently performed using such cultures. Additionally, growth of bone marrow-derived MSCs (BM-MSCs) in a variety of media were examined and compared to those for primary prostate stromal cultures. Of note, a proprietary defined media low in TGF- β available from Rooster Biotech consistently promoted the most robust growth of both bone marrow- and prostate-derived stromal cultures; however, none of the conditions tested produced cultures with an average cell diameter <15 μ m. In contrast to BM-MSCs, primary stromal cultures initiated from normal prostate tissue are unable to undergo adipogenesis; whereas, a subset of those from prostate cancer patients retain canonical MSC tri-lineage differentiation potential. These observations indicate that primary stromal cultures from normal prostate tissue from young (<25 yrs) donors are enriched in mesenchymal progenitor cells that have undergone lineage restriction. This further suggests that less committed progenitors or MSCs are actively recruited from the bone marrow as a function of prostate cancer pathogenesis or the host response to it.

METHODS

Reagents

Roswell Park Memorial Institute (RPMI)-1640 medium, Minimum essential medium—alpha media (α MEM), Hank's Balanced Salt Solution (HBSS), L-glutamine, and penicillin-streptomycin were purchased from Life Technologies-Invitrogen (Carlsbad, CA). MSC growth medium (MSCGM), in addition to osteogenic, adipogenic, and chondrogenic

induction media were purchased from Lonza (Basel, Switzerland). StemPro MSC SFM CTS (StemPro) and CELLstart were purchased from ThermoFisher Scientific (Waltham, MA). hMSC High Performance Media (Rooster) was purchased from RoosterBio, Inc. (Frederick, MD). Fetal bovine serum (FBS) was purchased from Gemini Bioproducts (West Sacramento, CA).

Primary Tissue Sources

All tissue was collected in accordance with Johns Hopkins Institutional Review Board (IRB)-approved protocols. Bone marrow-derived MSCs (BM-MSCs) were either purchased from RoosterBio (n = 2) or obtained from healthy bone marrow donors fulfilling Foundation for the Accreditation of Hematopoietic Cell Therapy (FACT) criteria through the Biospecimen Repository Core at Johns Hopkins (n = 3). Prostate tissue from young men (<25 yrs old, n = 2) was obtained through a rapid organ donor program organized by the National Disease Research Interchange (NDRI). Tissue was perfused, surgically harvested, and delivered within 24 hr of the time of death consistent with standard organ donor protocols to maximized viability. Prostate cancer tissue was obtained from patients undergoing radical prostatectomy (n = 4) at the Brady Urological Institute at Johns Hopkins through the Tissue Services core according to previously described protocols [6].

Briefly, fifty 18-gauge biopsy needle cores (Angiotech, Vancouver, BC, Canada) were obtained from the peripheral zone of prostates from young donors or radical prostatectomy tissue and washed in HBSS. Five randomly selected cores were fixed, paraffin-embedded, and sectioned for H&E staining and pathological examination. The remaining cores were mechanically minced and then enzymatically digested using a human tumor dissociation kit (Miltenyi Biotec, Inc. Bergisch Gladbach, Germany) and a gentleMACS dissociator (Miltenyi) according to the manufacturer's instructions. The dissociated cell suspension was then passed through a 70 μ m pre-separation filter (Miltenyi) and centrifuged at 2,500 rpm for 5 min. The single cell suspension was 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). Importantly, this dissociation protocol is completed within 1 hr; thereby, minimizing artifacts associated with long exposure to digestive enzymes and prolonged ex vivo incubation. Cells were then analyzed by flow cytometry or plated for expansion in tissue culture.

Cell Culture

Following digestion into a single cell suspension, cultures were initiated by plating the cells in the indicated media. For cultures grown in StemPro, flasks were first coated with; CELLstart for 1 hr at 37°C according to the manufacturer's instructions. RPMI, α MEM, and StemPro were also supplemented with 1% L-glutamine and 1% penicillin. StemPro basal medium CTS plus Supplement CTS were mixed at the indicated ratios and stored according to the manufacturer's instructions. MSCGM was supplemented with mesenchymal cell growth supplement (MCGS), L-glutamine, and GA-1000 according to the manufacturer's instructions. Rooster High Performance Media was supplemented with the GTX Booster according to manufacturer's instructions. Cultures were incubated under normoxic (20% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂) atmospheric conditions as specified at 37°C in a

humidified incubator with regular media changes every 3–4 days. Cultures were subcultured upon reaching ~80% confluency with 0.25% trypsin/1 mM EDTA, and viability determined by trypan exclusion using a Cellometer Auto T4 (Nexcelcom Bioscience). Cells were re-plated at a density of 1,000 viable cells/cm². Population Doubling Time (PDT) was calculated over the initial exponential expansion phase for 3 passages using the following equation: $((\text{culture time} \times \log [2]) / (\log (\text{final cell yield}) - \log (\text{starting cell number}))) \times 24$. Cell diameter was determined at each passage throughout the culture period using the Cellometer Auto T4.

Quantification of MSCs by Flow Cytometry

MSCs in primary tissue or culture were quantified as previously described [6]. Briefly, cells were labeled with a MSC Phenotyping Cocktail (anti-CD14-PerCP, anti-CD20-PerCP, anti-CD34-PerCP, anti-CD45-PerCP, anti-CD73-APC, anti-CD90-FITC, and anti-CD105-PE) or an Isotype Control Cocktail (Mouse IgG1-FITC, Mouse IgG1-PE, Mouse IgG1-APC, Mouse IgG1-PerCP, and Mouse IgG2a-PerCP) included in the human MSC Phenotyping kit (Miltenyi) in a volume of 100 µl per 1×10^6 cells for 10 min at 4°C according to the manufacturer's instructions. Anti-HLA-DR-PerCP (Miltenyi) was also added to the MSC Phenotyping Cocktail. The cells were washed, resuspended, and passed through a 0.2 µm filter into a flow analysis tube. All incubations, washes, and analyses were performed in MACS cell sorting buffer. Analysis was performed using a BD FACSCalibur flow cytometer. For analysis of primary tissue samples, 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. For analysis of the stromal cultures, compensation controls were performed using anti-CD90-FITC, anti-CD105-PE, anti-CD73-APC, and anti-CD73-biotin followed by anti-biotin-PerCP individually.

To identify MSCs, cell suspensions labeled with either the Isotype Control or MSC Phenotyping cocktails were gated using forward and side scatter (FSC and SSC, respectively) to identify the single cell population (R1, Fig. 1A). Next, the lineage-negative population (i.e., CD14[−], CD20[−], CD34[−], CD45[−], and HLA-DR[−]) within R1 were selected (R2). The CD73⁺ cells from this lineage-negative population were then selected (R3) followed by gating on the CD90 and CD105 double-positive population (MSCs). Therefore, MSCs were defined on the basis of being CD73, CD90, CD105 triple-positive in the absence of hematopoietic lineage markers (CD14, CD20, CD34, CD45, and HLA-DR); a definition consistent with the minimal criteria outlined by the International Society for Cellular Therapy (ISCT) [19]. The percentage of MSCs within the tissue or culture was calculated by dividing the number of MSCs identified by the total number of single cell events analyzed (R1) and multiplying by 100. At least 10,000 events were collected for all samples.

Differentiation Assays

Differentiation assays were performed as previously described [6]. Briefly, adipocyte differentiation was assayed by plating 2×10^5 cells/well in triplicate in a six-well plate and allowing them to reach 100% confluence while incubating at 37°C in a standard tissue culture incubator. Media was changed to adipogenic induction medium (Lonza)

supplemented with h-insulin (recombinant), L-glutamine, MCGS, dexamethasone, indomethacin, IBMX (3-isobutyl-1-methyl-xanthine), and GA-1000. According to the manufacturer's instructions, cells were cultured for three cycles consisting of 3 days on induction media followed by 4 days of maintenance media supplemented with h-insulin, L-glutamine, MCGS, and GA-1000. After the final cycle, cells were cultured for an additional 7 days in maintenance media prior to evaluation of adipogenic differentiation using the lipid stain Oil Red O (Sigma). Negative controls were grown in RPMI + 10% FBS. For quantification of adipogenic differentiation potential, 0.5×10^5 cells/well were plated from a representative BM-MSC and PrCSC culture in duplicate in a 24-well plate with the differentiation protocol was performed as described above. Plates were incubated for the duration of the induction protocol under either normoxic (20% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂) atmospheric conditions as indicated. Following staining with Oil Red O at the end of the assay, wells were washed 3× with 60% isopropanol for 5 min/wash and then the dye was solubilized in 100% isopropanol for 15 min. The absorbance was read at 492 nm with fold change in absorbance calculated over the uninduced negative control.

Osteogenic differentiation was assayed by plating 3×10^4 cells/well in triplicate in a six-well plate and allowing them to adhere overnight at 37°C in a standard tissue culture incubator. The media was changed to osteogenic induction media (Lonza) supplemented with dexamethasone, L-glutamine, ascorbate, MCGS, and b-glycerophosphate according to the manufacturer's instructions. Media was changed every 3–4 days for 21 days prior to evaluation for osteogenic differentiation by staining for calcium deposits using Alizarin Red S (Sigma). Negative controls were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin.

Chondrogenic differentiation was assayed by centrifuging 2.5×10^5 cells at 150g in a 15 ml polypropylene conical at room temperature and resuspending them in 0.5 ml chondrogenic induction medium (Lonza) supplemented with dexamethasone, ascorbate, ITS, GA-1000, sodium pyruvate, proline, L-glutamine, and TGF-β3 according to the manufacturer's instructions. The caps were loosened a half-turn and placed at 37°C in a standard tissue culture incubator. The media was changed every 3–4 days for 21 days without aspirating the pellet. Pellets were fixed in formalin and paraffin-embedded for histology. Chondrogenic differentiation was evaluated by staining for glycosaminoglycans with Safranin-O (Sigma). Negative controls were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin.

TGF-β Quantification: ELISA

TGF-β1 Levels in media were determined using a Human TGF-beta 1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) following acidification of the sample to activate latent TGF-β according to the manufacturer's instructions.

RESULTS

Validation of Analytical and Functional MSC Assays Using Canonical Human Bone Marrow-Derived MSCs

The rapid expansion of stromal cells in primary cultures suggests the presence of a stem or progenitor cell population (i.e., MSCs). To test this hypothesis, analytical and functional assays for the identification of human MSCs were validated using primary stromal cultures initiated from human bone marrow aspirates, the prototypical source of MSCs (i.e., BM-MSCs). A multi-parameter flow cytometry assay based on the co-expression of CD73, CD90, and CD105 in the absence of CD14, CD20, CD34, CD45, and HLA-DR expression was optimized as previously described (Fig. 1A, [6]). The multi-lineage differentiation potential of these cultures was confirmed by assaying osteoblast, adipocyte, and chondrocyte differentiation when incubated in the presence of the appropriate induction media (Fig. 1B). Thus, both analytical and functional MSC assays were validated using canonical human bone marrow-derived MSC cultures.

Growth of Human Bone Marrow-Derived MSCs in Tissue Culture

BM-MSCs have traditionally been cultured at low density in a variety of base media including α MEM and RPMI-1640 supplemented with 10–20% FBS, though multiple more specialized media have since been developed [31]. Therefore, population doublings were monitored over time in stromal cultures derived from independent bone marrow donors ($n = 3$ –5 donors/condition) that were grown in either RPMI + 20% FBS, α MEM + 10% FBS, MSCGM, StemPro, or Rooster media under standard tissue culture atmospheric conditions [i.e., 20% O₂, 5% CO₂]. Under these conditions, BM-MSCs have an average population doubling time (PDT) of ~89 hr (SE: ± 21 hr); however, substantial heterogeneity is observed between the different media. Notably, the fastest growth under the conditions tested occurs using Rooster media (PDT $\pm$ SE: $\sim 31 \pm 2$ hr, [Fig. 2A]). Furthermore, the greatest number of population doublings (PDs) observed was also consistently achieved using Rooster media [PD $\pm$ SE: 22.1 ± 3.4 , (Fig. 2B)]. Cell diameter (mean $\pm$ SE: 19.6 ± 0.7 μ m) was comparable in the different media analyzed and tended to get larger with serial passaging of the culture over time (Fig. 2C); though it should be noted that cells cultured in StemPro were smaller on average (mean $\pm$ SE: 17.1 ± 0.3 μ m) than those observed in the other conditions tested. Primary stromal cultures grown in each of these media were highly enriched (>75%) in cells that were analytically and functionally consistent with an MSC phenotype based on the previously described flow cytometry and differentiation assays (Table I).

Growth of Prostate Stromal Cells From Normal and Malignant Donors in Tissue Culture

We have previously demonstrated that MSCs can be identified in radical prostatectomy tissue from patients with prostate cancer [6]. To determine if primary stromal cultures from prostate cancer patients (i.e., PrCSCs) have in vitro growth requirements similar to BM-MSCs, growth of these cultures was examined under the same conditions described above. Cultures were initiated by dissociating the primary tissue into a single cell suspension prior to plating, though similar results were obtained using cultures established using tissue explants. In contrast to BM-MSCs in which the PDT varied over a nearly fourfold range

(~31–122 hr), the growth of PrCSCs was more consistent with an average PDT of ~44 hr (SE: ± 11 hr, [Fig. 3A]) under the conditions tested. This variation is predominantly driven by the cultures grown in MSCGM, which exhibited a significantly slower PDT than the other conditions analyzed (PDT $\pm$ SE: $\sim 81 \pm 25.5$ vs. 35.2 ± 2.8 hr). Again, the greatest number of PDs was achieved using Rooster media (PD $\pm$ SE: 41.2 ± 10.0 vs. 20.4 ± 4.8 , [Fig. 3B]). Notably, the fastest PDTs and greatest overall number of PDs in both BM-MSCs and PrCSCs was achieved using media with the lowest concentrations of TGF- β 1 (Fig. 4), which has previously been implicated in MSC growth and differentiation [32–37]. Cell diameters in the different media (mean $\pm$ SE: 19.2 ± 0.6 μ m) were comparable to BM-MSCs with the smallest cells observed using StemPro (Fig. 3C). Interestingly, PrCSC cultures were typically smaller in size than BM-MSCs at the earliest passages, though both increased significantly in size with serial passaging.

Normal prostate tissue was obtained from young men (<25 yrs) through a rapid organ donor program. Similar growth kinetics were observed using primary stromal cultures derived from these young organ donors (i.e., nPrSC). However, a greater number of overall PDs was typically achieved relative to the PrCSC cultures, which is consistent with a younger donor age.

Primary Stromal Cultures From Human Prostate Tissue are Enriched in MSC Properties

Next, primary stromal cultures derived from human prostate tissue were analyzed at early passage (p1-6) for phenotypic MSC markers using the multiparameter flow cytometry assay described above. All primary stromal cultures analyzed were highly enriched (>50%) in cells consistent with an MSC phenotype independent of the media used and whether the tissue donor had prostate cancer or not (i.e., PrCSCs or nPrSCs, respectively; [Table I]). Similar results have also been obtained using explant cultures.

Furthermore, these primary prostate stromal cultures consistently underwent osteogenic and chondrogenic differentiation when placed in the appropriate induction media. (Table I). However, in contrast to BM-MSCs, stromal cultures derived from normal prostate tissue did not undergo robust adipogenesis (Table I). The inability of nPrSCs to differentiate into adipocytes, suggests they have undergone lineage restriction indicative of a more progenitor-like state. In contrast and consistent with our previous observations [6], a subset of stromal cultures initiated from radical prostatectomy tissue do exhibit adipogenic differentiation potential (Table I). Collectively, these data demonstrate that stromal progenitors quickly become the dominant population within primary stromal cultures, and suggests that less committed progenitors or MSCs are actively recruited from the bone marrow to the prostate in men with prostate cancer.

Selective Outgrowth of MSCs and Stromal Progenitors in Primary Human Prostate Stromal Cultures

The rapid enrichment of MSCs and stromal progenitors in primary cultures could be the result of either selection or adaptation in tissue culture. Therefore, a primary stromal culture from one of these young donors was analyzed longitudinally. This analysis demonstrated a progressive accumulation of MSCs within the culture from 0.03% of all cells in the primary

tissue to >80% of the population by passage 3 (Fig. 5). Similar results were obtained using other cultures and are consistent with the highly clonogenic nature of MSCs, which were initially defined as colony forming unit-fibroblasts (CFU-Fs, [1,20]).

To further ascertain whether these observations are a function of selection or adaptation, a primary culture was initiated with 100,000 cells from the prostate of a young organ donor that initially contained 0.75% MSCs (i.e., 750). After 7 days, the culture contained ~75,000 cells of which 33.0% were determined to be MSCs according to the analytical flow cytometry assay (i.e., ~25,000). These observations are consistent with a selective outgrowth of the initial MSC population based on a PDT of ~31 hr under the conditions used.

Effect of Oxygen Tension on the Growth and Differentiation of Bone Marrow- and Prostate Cancer-Derived Stromal Cells

Previous studies have demonstrated that reduced oxygen tension mimicking the bone marrow microenvironment promotes maintenance of a more “stem-like” state via induction of telomerase expression and inhibition of differentiation [38–43]. Though a trend toward faster PDTs was observed in BM-MSCs cultures grown under hypoxic conditions (i.e., 1% O₂, 5% CO₂, [Fig. 2A]), this did not necessarily translate into significant increases in overall PDs (Fig. 2B). Low oxygen concentrations also did not have a significant impact on PDT and PDs in PrCSC cultures (Fig. 3).

As adipogenesis is the most informative assay differentiating bone marrow and prostate-derived stromal progenitors, the effect of hypoxia on this functional capacity was investigated in representative cultures from each tissue source. Consistent with prior reports [41–43], hypoxia substantially suppresses adipogenic differentiation of BM-MSCs as demonstrated by a significant reduction in Oil Red O staining to baseline levels detected in uninduced cultures (Fig. 6). Similarly, hypoxia significantly inhibits adipogenesis in PrCSCs (Fig. 6). Though it should be noted that PrCSCs typically undergo adipogenesis less efficiently than BM-MSCs even under normoxic conditions (Fig. 6), suggestive of a more heterogeneous population of cells within these cultures.

DISCUSSION

Carcinoma-associated fibroblasts (CAFs) are associated with a robust literature regarding their protumorigenic properties [3–10]. Despite this history, the physiologic origins of this cell type remain unclear. CAFs have been suggested to arise from normal tissue-resident fibroblasts as a result of continuous exposure to paracrine factors in the tumor microenvironment [15]. CAFs are frequently defined by the co-expression of α SMA and vimentin, which is also a defining feature of myofibroblasts found in the reactive stroma present in areas of wound repair [4]. The expression of some but not all smooth muscle markers has led some to speculate that CAFs are a product of smooth muscle de-differentiation [17,44,45]. Of note, the ratio of smooth muscle present in the stroma decreases during prostate cancer progression relative to the normal gland, which is inversely associated with the increased prevalence of CAFs [4,7,44]. A third reported source of CAFs are mesenchymal stem cells (MSCs) recruited from local and distant sources, such as regional adipose tissue and the bone marrow [1,13,14,16].

MSCs are estimated to represent from 0.01 to 0.001% of the cells present in the bone marrow where they contribute to the hematopoietic stem cell (HSC) niche [18,46,47]. In addition to this supporting role in HSC maintenance, they can also be mobilized from the bone marrow into systemic circulation where they traffic to sites of inflammation and tissue damage [48–50]. At these sites of damage, MSCs contribute to tissue repair through regenerative processes and the secretion of trophic and immunomodulatory factors [1,20]. Indeed, MSCs have been identified in tissues throughout the body, including the prostate [6,32,51,52]. However, it is likely that many of these MSCs are tissue-resident progenitors serving in a “first responder” capacity with the bone marrow representing a reserve reservoir that can be mobilized in response to chemokines and cytokines such as G-CSF produced during periods of inflammation and tissue damage [1,48–50].

Bone marrow transplant studies using transgenic mice have demonstrated that as many as 20–40% of CAFs are of bone marrow origin [7,10,53,54]. However, the presence of MSCs in peripheral tissues in addition to the bone marrow suggests that a larger percentage of CAFs may in fact be derived from MSCs than was previously thought. MSCs and CAFs demonstrate many similarities and overlapping properties, including pro-tumorigenic effects within certain contexts [1,13,14,21]. As shown here, primary prostate stromal cultures from prostate cancer patients are highly enriched in cells consistent with an MSC phenotype, which has interesting implications considering CAFs are often studied using such cultures. Consistent with this idea, we also previously demonstrated these primary prostate stromal cultures are enriched in α SMA- and vimentin-positive cells [6]. The enrichment of MSCs within these cultures is consistent with the selective outgrowth of a pre-existing population of stem or progenitor cells with greater proliferative potential relative to other more terminally differentiated cell types present in the initial culture. This observation is consistent with prior studies showing a progressive loss of differentiated smooth muscle cells in primary stromal cultures over time [13,17,44,45,55].

MSCs recruited into peripheral tissues react to local cues in the microenvironment that regulate context-specific responses. One consequence of this local programming is a tissue-specific restriction of differentiation potential as exemplified by the lack of adipogenesis observed in primary stromal cultures from normal prostate tissue. The ability of these same cultures to differentiate into osteoblasts and chondrocytes suggests they have undergone lineage restriction by factors in the local microenvironment that suppress differentiation down certain lineages while maintaining others. This lineage restriction is indicative of a more progenitor-like phenotype being dominant in primary prostate stromal cultures derived from normal tissue as opposed to canonical MSCs, which are typically associated with a broader differentiation potential. Preliminary evidence suggests this lineage restriction is a functional consequence of TGF- β and AR signaling in the prostate, which drives MSCs down the smooth muscle lineage; a concept with strong support in the literature [35–37,56–59]. Of note, the greatest number of overall PDs and fastest PDTs were observed in cultures grown in media containing low TGF- β concentrations. Importantly, TGF- β is a critical mediator of MSC growth and differentiation, in addition to being thought of as the primary driver of the CAF phenotype within the tumor microenvironment [32–37].

In contrast to normal tissue, primary stromal cultures derived from a subset of prostate cancer patients maintain the ability to undergo adipogenesis, suggesting the active recruitment of MSCs or less committed progenitors from the bone marrow as a function of disease progression, or the host response to it. The recruitment of undifferentiated stromal progenitors from the bone marrow is consistent with observations regarding a general loss of smooth muscle in prostate cancer [17,44]. Of note, hypoxia has previously been shown to inhibit MSC differentiation [41–43], which was confirmed herein via suppression of adipogenesis in primary stromal cultures derived from bone marrow and prostate tissue grown under low (i.e., 1%) oxygen concentrations. Hypoxia-induced suppression of adipogenesis is mediated through HIF-1 α dependent inhibition of PPAR γ 2 transcription, a critical factor in adipocyte differentiation [60]. Notably, HIF-1 α is stabilized in BM-MSCs even under normoxic conditions, which is mediated at least partially via TGF- β signaling and suggests that HIF-1 α is an important regulator of MSC fate [61–63]. Transitioning MSCs from hypoxic to normoxic conditions decreases HIF-1 α levels and restores adipogenic differentiation potential [39–41,61]. Upregulation of HIF-1 α is an early event in prostate carcinogenesis and hypoxic areas are commonly present in clinical prostate cancer [64,65]. Provocatively, perhaps the increasingly hypoxic nature of tumors as they grow maintains recruited MSCs in a more “stem-like” state, which further contributes to the altered microenvironment that fuels progression to a lethal phenotype.

The recruitment of MSCs to sites of prostate cancer also suggests they can be used as cell-based vectors to deliver cytotoxic molecules or other agents of interest [1]. Indeed, the baseline homing efficiency of systemically-infused BM-MSCs to sites of primary prostate cancer is currently being evaluated by our group in a phase 0 pre-prostatectomy clinical trial (NCT01983709). Further development of such vectors requires that optimal conditions for the ex vivo expansion of MSCs are determined. Toward this goal, MSCs cultured in High Performance Media from Rooster Biotech were consistently shown to have faster PDTs and undergo a greater number of PDs overall compared to the other media analyzed in this study. However, MSCs expanded under all of the conditions tested were 15.0 μ m in diameter, a property associated with increased mechanical entrapment, and poor systemic circulation following intravenous (IV) infusion [66–68]. Indeed, preliminary experiments in animal models have demonstrated that MSCs grown under these conditions are largely trapped in the lung very quickly following IV administration. Though systemically infused MSCs can be detected in prostate cancer xenografts post-injection [6], targeting occurs with relatively poor efficiency under the conditions tested. These observations suggest that alternative strategies for the ex vivo expansion of MSCs will need to be evaluated for efficient systemic targeting using cell-based delivery platforms. One possibility is culturing MSCs at extremely low density (<50 cells/cm²) or in suspension culture, which has been shown to produce cultures with an average cell diameter as small as 12 μ m [68–70]. Interestingly, MSCs with a smaller diameter have also been associated with more stem-like properties, including enhanced self-renewal, in addition to greater expansion, clonogenic, and differentiation potential [69–72].

In conclusion, mesenchymal stem and progenitor cells quickly become the dominant population within primary prostate stromal cultures due to selection of these pre-existing populations within the culture. The use of primary cultures such as these to study CAFs

raises interesting implications, particularly when considered in light of their overlapping properties. Though robust growth of MSCs was consistently observed using a proprietary media available from Rooster Biotech, this may not necessarily correlate with optimal properties in the expanded culture depending on the desired application. Additionally, the presence of MSCs with broader differentiation potential in primary stromal cultures from a subset of prostate cancer patients compared to those from normal prostate tissue suggests the recruitment of less committed progenitors from the bone marrow as a function of disease progression. This recruitment can potentially be exploited for prognostic purposes or as a cell-based platform for the selective delivery of therapeutic, diagnostic, and imaging agents to sites of prostate cancer.

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Fig. 1. Validation of analytical and functional Mesenchymal Stem Cell (MSC) assays. (A) Identification of MSCs by multiparameter flow cytometry. Following labeling with the MSC phenotyping cocktail, the viable single cell population based on forward (FSC), and side (SSC) scatter is gated (R1). From this population, the lineage-negative fraction (CD14⁻, CD20⁻, CD34⁻, CD45⁻, and HLA-DR⁻) is selected (R2) followed by gating on the CD73⁺ population (R3). Next, the CD90⁺ and CD105⁺ double-positive population is quantified (upper-right quadrant). Therefore, MSCs are defined as the CD73⁺, CD90⁺, and CD105⁺

triple-positive fraction in the absence of the indicated hematopoietic lineage markers. The percentage of MSCs in the tissue is defined by dividing the number of MSCs identified by the total number of events analyzed (R1). **(B)** Multipotent differentiation potential of bone marrow-derived mesenchymal stem cells (MSCs) confirmed following culture in the appropriate induction media. Examples of adipogenic, osteogenic, and chondrogenic differentiation as demonstrated by positive Oil Red O staining of lipid droplets, positive Alizarin Red S staining for calcium mineralization, and positive Alcian Blue staining of glycosaminoglycans, respectively. In contrast, no differentiation is observed when cells are cultured in the absence of the respective induction factors. Representative images at 20 $\times$.

Fig. 2.

Growth of bone marrow-derived mesenchymal stem cells (BM-MSCs) in tissue culture using different media. (A) Population doubling times (PDT) were calculated over the initial expansion phase (3 passages) for BM-MSCs grown in the respective media in either normoxic (20% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂) atmospheric conditions. (B) Overall population doublings (PD) were determined for these same cultures under the indicated culture conditions. Error bars represent standard error. (C) Average cell diameter

was measured at each passage over the duration of culture period. Mean values reported $\pm$ standard error (SE) with minimum and maximum diameters observed listed in parentheses.

Fig. 3.

Growth of prostate cancer-derived stromal cells (PrCSCs) in tissue culture using different media. (A) Population doubling times (PDT) were calculated over the initial expansion phase (3 passages) for BM-MSCs grown in the respective media in either normoxic (20% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂) atmospheric conditions. (B) Overall population doublings (PD) were determined for these same cultures under the indicated culture conditions. Error bars represent standard error. (C) Average cell diameter was measured at

each passage over the duration of culture period. Mean values reported $\pm$ standard error (SE) with minimum and maximum diameters observed listed in parentheses.

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Fig. 4.

TGF-β1 concentrations in different media as determined by ELISA. TGF-β concentrations in RPMI and αMEM are derived from FBS supplementation (20% and 10%, respectively). StemPro and Rooster are proprietary media with supplements included from the manufacturer, though it should be noted that StemPro is a serum-free medium.

Fig. 5.

MSCs and stromal progenitors are selected for in primary prostate stromal cultures. Primary culture was initiated from a single cell suspension derived from the prostate of a young (<25 yrs) organ donor. Culture was expanded in RPMI under normoxic conditions. MSCs initially represented 0.03% of all cells in the donor tissue (p0) as determined by flow cytometry. During serial passaging, this population quickly became the dominant fraction of the culture (>80% of all cells by p3) due to a selective outgrowth of the initial MSC population. Similar results were attained with other cultures whether initiated from a single cell suspension or from traditional explant cultures.

Fig. 6.

Hypoxia suppresses adipogenic differentiation in bone marrow- and prostate cancer-derived stromal cells (BM-MS-C and PrCSC, respectively). Matched cultures from individual tissue donors were expanded in Rooster media under normoxic (20% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂). Upon reaching confluence, the adipogenic induction protocol was initiated with cultures maintained in their respective atmospheric conditions for the duration of the induction period. At the end of the induction protocol, cultures were stained with Oil Red O as described. Dye was solubilized in 100% isopropanol, absorbance read at 492 nm, and fold change in absorbance over matched uninduced controls calculated. Assay was performed in duplicate for each culture. Representative cultures shown for each tissue source. Error bars represent standard error.

TABLE I
Primary Stromal Cultures Derived From Bone Marrow or Prostate Tissue are Enriched
in Analytical and Functional MSC Properties

Tissue	Sample	% MSCs	Adipocytes	Osteoblasts	Chondrocytes
Bone marrow	BM-MSC-1	89.1	+	+	+
	BM-MSC-2	77.4	+	+	+
	BM-MSC-3	80.0	+	+	+
Normal prostate	nPrSC-1	77.3	-	+	+
	nPrSC-2	72.5	-	+	+
Prostate cancer	PrCSC-1	79.7	-	+	+
	PrCSC-2	53.0	-	+	+
	PrCSC-3	94.9	+/-	+	+
	PrCSC-4	63.3	+	+	+

All primary stromal cultures analyzed were highly enriched (> 50%) in cells consistent with an MSC phenotype by multiparameter flow cytometry at an early passage (< 3). Unlike canonical bone marrow-derived MSCs with tri-lineage differentiation potential (adipocytes, osteoblasts, and chondrocytes), stromal cultures initiated from normal prostate tissue are unable to undergo adipogenesis indicative of a more lineage-restricted progenitor cell phenotype. In contrast, primary stromal cultures from a subset of prostate cancer patients retain their adipogenic potential, suggesting less committed progenitors or MSCs are actively recruited from the bone marrow to the prostate in men with prostate cancer.