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Original Article

Stromal modulation of bladder cancer-initiating cells in a subcutaneous tumor model

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Abstract: The development of new cancer therapeutics would benefit from incorporating efficient tumor models that mimic human disease. We have developed a subcutaneous bladder tumor regeneration system that recapitulates primary human bladder tumor architecture by recombining benign human fetal bladder stromal cells with SW780 bladder carcinoma cells. As a first step, SW780 cells were seeded in ultra low attachment cultures in order to select for sphere-forming cells, the putative cancer stem cell (CSC) phenotype. Spheroids were combined with primary human fetal stromal cells or vehicle control and injected subcutaneously with Matrigel into NSG mice. SW780 bladder tumors that formed in the presence of stroma showed accelerated growth, muscle invasion, epithelial to mesenchymal transition (EMT), decreased differentiation, and greater activation of growth pathways compared to tumors formed in the absence of fetal stroma. Tumors grown with stroma also demonstrated a greater similarity to typical malignant bladder architecture, including the formation of papillary structures. In an effort to determine if cancer cells from primary tumors could form similar structures *in vivo* using this recombinatorial approach, putative CSCs, sorted based on the $CD44^{+}CD49f^{+}$ antigenic profile, were collected and recombined with fetal bladder stromal cells and Matrigel prior to subcutaneous implantation. Retrieved grafts contained tumors that exhibited the same structure as the original primary human tumor. Primary bladder tumor regeneration using human fetal bladder stroma may help elucidate the influences of stroma on tumor growth and development, as well as provide an efficient and accessible system for therapeutic testing.

Keywords: Bladder cancer, cancer stem cell (CSC), subcutaneous tumor model, stroma, sphere

Introduction

Bladder cancer is the second most common genitourinary malignancy, with transitional cell carcinoma representing 90% of the cases [1]. Although BCG immunotherapy is effective in up to 75% of patients [2], treatments for bladder cancer have not significantly advanced in the last 30 years. Advances in therapeutics heavily depend on disease models. Subcutaneous models involving the direct injection of bladder tumor cells suffer from the inability of the tumor to form biologically relevant architecture [3]. Conversely, transgenic models [4-6] lack flexibility and require a longer incubation period. Here, we present a subcutaneous bladder tumor model with the ability to create papillary architecture, a model that may uniquely support the development of therapeutics.

The last few decades of cancer research have focused on the identification of oncogenes and tumor suppressors involved in the emergence of tumors. The effects of the surrounding stromal tissue have been largely ignored until recently. While the quantity of stroma does not appear to correspond with malignancy [7], signaling between tumor and stromal tissue is important for the formation of a complex tumor microenvironment and can influence the phenotype of the tumor [8].

Tumor cells are known to directly influence their surrounding stroma through invasion and angio-

genesis [9]. Growth factors such as VEGF, EGF, FGF, and TGF-β, as well as other cytokines, modulate the tumor microenvironment to promote a more permissive stroma and facilitate tumor growth [10, 11]. We postulated that tumor-adjacent stroma influences both the growth and differentiation of malignant cells.

Recent strides have been made in the identification and characterization of cancer stem cells (CSCs), the cancer-initiating cells within the tumor. By targeting CSCs specifically with new therapies, the risk of tumor recurrence is expected to be greatly reduced [12], justifying the inclusion of and focus on CSCs in any new tumor model. Breast cancer stem cells have been demonstrated to engage in stromal remodeling [13], but a more focused view of the interaction between CSCs and the surrounding stroma is necessary.

Stem cells have been shown to grow in three-dimensional spheroids in culture [14, 15]. This culture system has proven effective at growing cancer stem cells as well [16-18] and allowed for the culture of immortalized cell lines with cancer stem cell properties. For this study, we utilized the SW780 transitional bladder carcinoma cell line, which we found form spheres when grown in serum-free media. These cells exhibit the cancer-initiating cell phenotype, defined as $CD44^{+}CD49f^{+}$. When combined with fetal bladder mesenchymal cells in a novel subcutaneous model, the SW780 spheroids develop tumors that recapitulate primary tumor architecture. Therefore, the addition of stroma to a tumor model may promote both larger and less differentiated tumors.

Materials and methods

Cell lines and tumor samples

The SW780 immortalized human urinary bladder cell line (ATCC #CRL-2169) and the HT-1376 immortalized human urinary bladder cell line (ATCC #CRL-1472) were used in our *in vitro* and *in vivo* models. SW780 monolayer cells were maintained in RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Sphere cultures for staining and implantation were maintained in floating culture as previously described [19, 20] on uncoated plates in RPMI 1640 with L-glutamine supplemented with 2% B-27 serum-free, 1% Pen/Strep, 2 μg/ml heparin, 20 ng/ml FGF, and 20 ng/ml EGF. When passaged, spheres were gravity-separated for 15-20 minutes before fresh media was added. Fresh bladder tumor samples were surgically resected, immediately suspended in PBS or DMEM, and maintained at 4°C until processed. Tumors were digested in 0.25% collagenase IV-DMEM for 4 hours and plated as above. Experiments were performed under IRB approved protocol #11-001363.

Acquisition, isolation, and culture of fetal bladder stroma

Human fetal bladder tissue was acquired from 16-17 week gestation specimens in accordance with federal and state guidelines. Fetal bladder, prostate, and urethra were removed *en bloc*. A portion of the specimen was fixed in formalin and paraffin-embedded to confirm correct anatomic localization. The remainder of the tissue was mechanically and enzymatically digested as previously described [21]. Dissociated bladder cell suspensions were sequentially filtered through 100-micron and 40-micron filters, and then passed through a 23-gauge needle. Cells were counted with a hemocytometer and resuspended in RPMI supplemented with 10% FBS and 1% Pen/Strep, and Methyltrienolone R1881 (Sigma) for culture in vitro. After 3 passages, cells were cryopreserved and thawed/expanded as needed for use in recombination assays.

Sphere formation efficiency assay

To quantify the percentage of cells that produce spheres, we adapted a previously described MatriGel culture system [22]. SW780 monolayer cells using 0.05% Trypsin-EDTA incubated at 37°C for 5-6 minutes before quenching with RPMI 1640 with L-glutamine supplemented with 10% FBS. Media was replaced with sphere media and cells were resuspended at 2.5×10^4 cells/mL. 40 μL of cell mixture was then mixed with 60 μL ice-cold MatriGel, and well-mixed. 100 μL of MatriGel mixture was then pipetted around the rim of a chilled 12-well plate. Plate was then swirled to evenly distribute the mixture around the edges and incubated at 37°C for 30 minutes to allow the MatriGel to set. 1 mL of sphere media was then gently added to the center of each well. 500 μL of media was aspirated and replaced with fresh

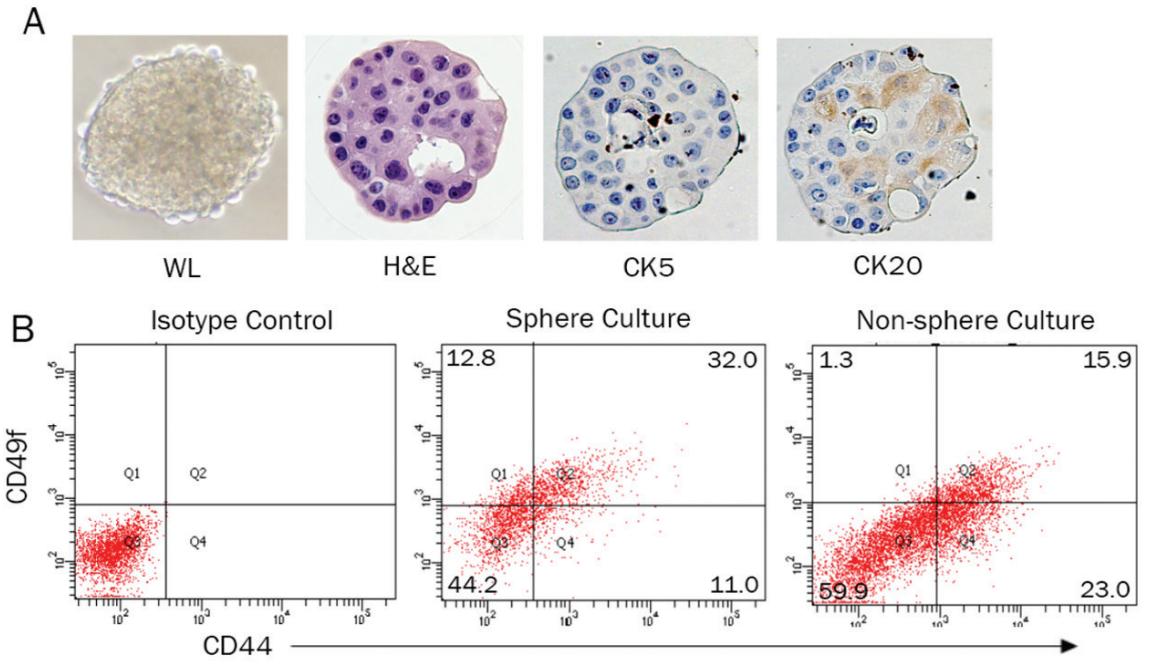

Figure 1. Spherical cultures express both luminal and basal markers. SW780 spheres from serum-free culture were fixed and bisected for IHC (A). Sections were stained with basal (CK5) and luminal (CK20) cell markers, as well as H&E. Representative views are shown, and images were captured at 400X magnification. SW780 spheres were dissociated and compared to SW780 cells grown in monolayer by flow cytometry (B). Cells were stained with anti-CD44-PE and anti-CD49f-FITC antibodies or isotype controls.

sphere media every three days. Spheres were counted at 7 days and 14 days.

Immunohistochemistry

Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological analysis. Immunohistochemistry was performed using antibodies against CK5/6 (Invitrogen), CK20 (DAKO), CD44 (eBioscience), EGFR (BIOCARE), pS6 (Cell Signaling), E-cadherin (BD Biosciences), and N-cadherin (ZYMED). Slides were probed with biotinylated goat anti-rabbit or goat anti-mouse secondary antibodies and with streptavidin conjugated to HRP. Photographs were taken using an Axio Imager 2 (Zeiss).

Flow cytometry

Cell surface marker analysis was performed by flow cytometry using the LSR II (BD Biosciences). Cells were incubated with antibodies against CD44 conjugated to PE (BD Biosciences) and against CD49f conjugated to FITC (BD Biosciences). Mouse IgG antibodies conjugated with each fluorochrome were used as isotype controls.

Subcutaneous tumor model

Non-obese diabetic severe combined immunodeficient gamma (NSG) mice between 6 and 8 weeks old were used for *in vivo* subcutaneous tumor growth experiments. Approximately 10^5 monolayer cells were suspended in 0.1 mL of media for inoculation. Cultured SW780 spheres and patient-derived spheres were prepared for xenograft implantation by first gravity-separating spheres for 15-20 minutes. Approximately 500 spheres were suspended in 0.1 mL of media for inoculation, and an equal quantity of Matrigel (BD Biosciences) was added. All mice were inoculated subcutaneously in the lower flank. Mice were monitored daily, and tumor growth was observed. Mice were sacrificed when tumor size reached 1 cm.

Results

Spherical cultures exhibit a cancer stem cell phenotype

To establish the validity of spheres as a cancer stem cell culture method, SW780 cells were grown in a Matrigel suspension and allowed to form spheres. The efficiency of sphere forma-

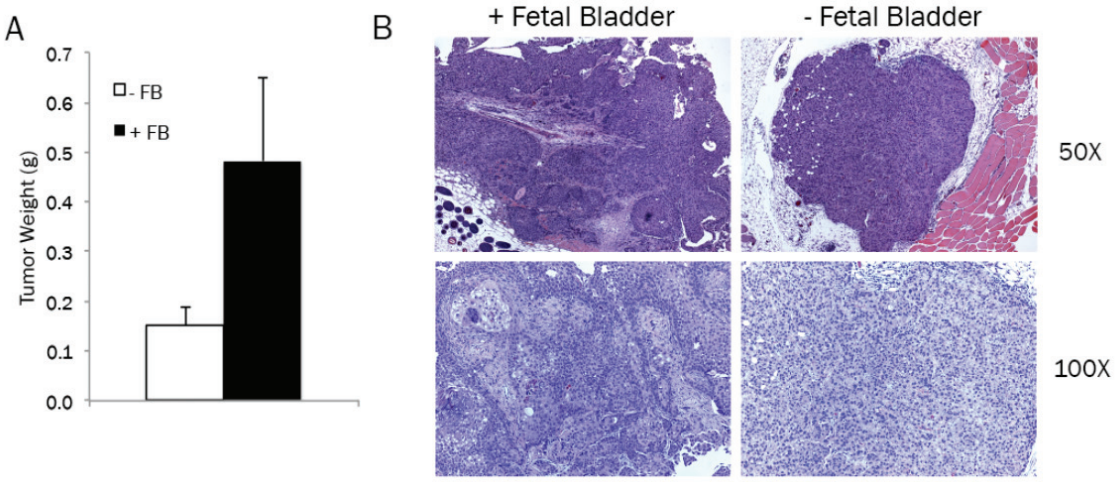

Figure 2. Stromal influence promotes increased tumor growth and invasiveness. NSG mice were implanted subcutaneously with SW780 spheres with and without the presence of fetal bladder stroma. After 4 weeks, tumors were harvested and weighed (A) (n=7 mice/group, $P < 0.001$). Nearby satellite tumors were also included in this weight. Error bars indicate standard deviation from the mean. Tumor sections were stained with H&E for histology. Tissue architecture (B) was compared in representative images captured at 50-100X magnification. Evidence of muscle invasion (C) was noted in images captured at 400X magnification.

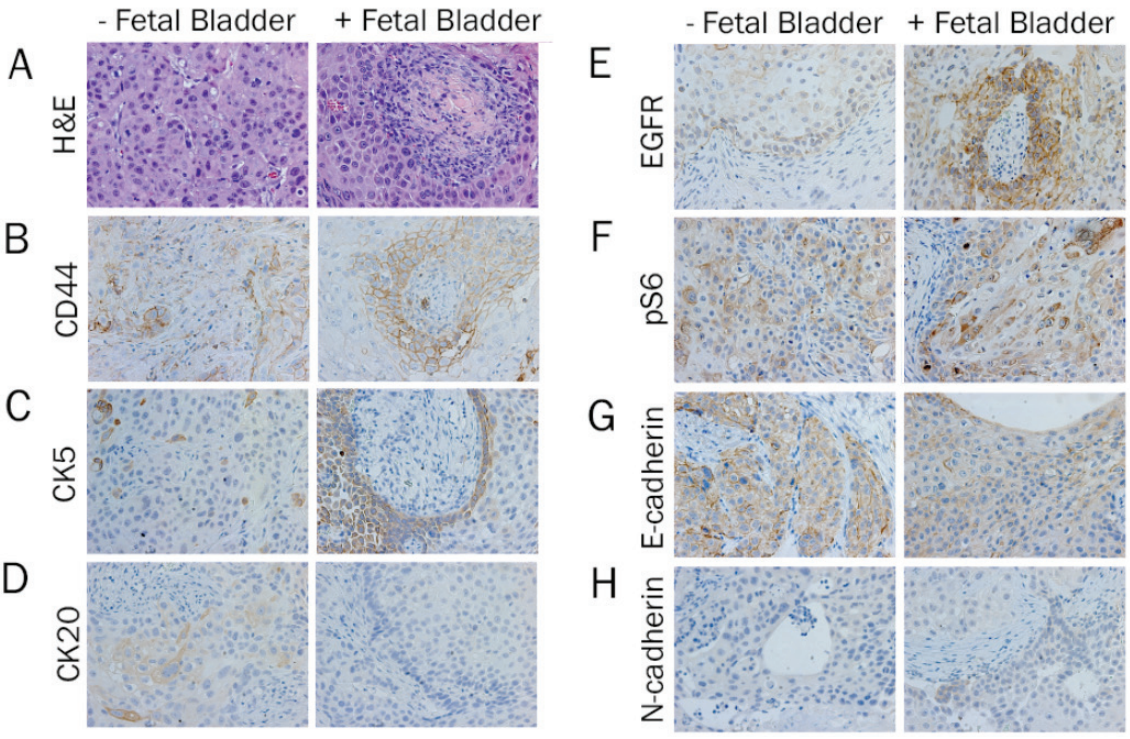

Figure 3. Tumors formed in the presence of stroma are less differentiated and functionally distinct. NSG mice were implanted subcutaneously with SW780 spheres with and without the presence of fetal bladder. After 4 weeks, tumors were harvested and embedded in paraffin for IHC. Slides were stained with H&E (A) for histology, as well as differentiation markers CD44, CK5, and CK20 (B-D). Tumor sections were also stained for functional markers: EGFR, pS6, E-cadherin, and N-cadherin (E-H). Representative views are shown, and images were captured at 400X magnification.

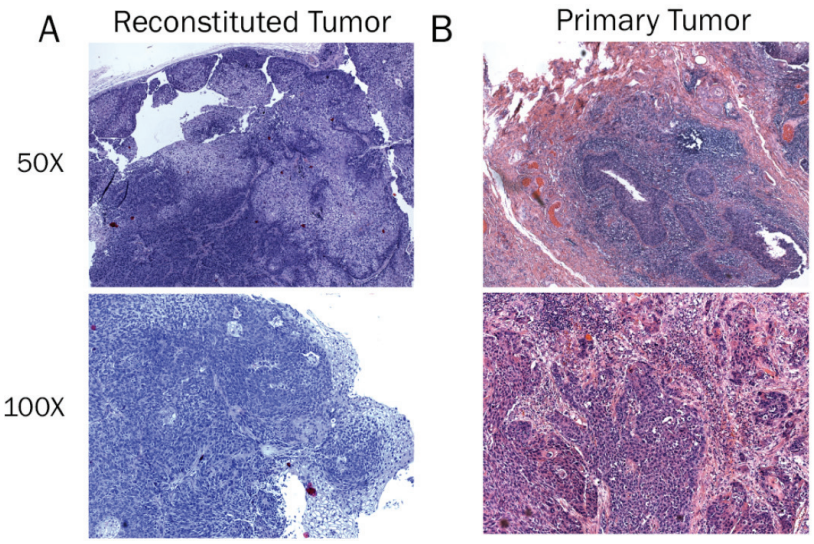

Figure 4. Subcutaneous model using human cells recapitulates both murine and primary tumor architecture. Primary human bladder tumor was sorted to isolate the $CD44^{+}CD49f^{+}$ population. Cells were combined with human fetal bladder and injected subcutaneously into NSG mice. Tumor sections were stained with H&E for histology (A). Pathology slides from the original human bladder tumor were stained with H&E for histology (B). Representative views are shown, captured at 50X and 100X magnification.

tion was measured at 2-4% (data not shown). Spheres from floating culture were fixed and sectioned for staining with basal and luminal markers to determine the cell phenotype (**Figure 1A**). The spheres showed expression of CK20, a luminal marker, towards the center and expression of the basal marker CK5 in the periphery. SW780 spheres were also shown to have a more undifferentiated phenotype than monolayer cells by measuring cell surface levels of the stem cell markers CD44 and CD49f (**Figure 1B**).

Stromal influence on tumor growth and function

To test the influence of stromal cells on tumors in an *in vivo* model, NSG mice were challenged subcutaneously with spheres with or without human fetal bladder stroma. Tumors were harvested, weighed, and stained. Supporting the assertion that the influence of stroma creates a more aggressive tumor, mice challenged with spheres and fetal bladder mesenchyme developed larger tumors than mice challenged with spheres alone (**Figure 2A**). The tumors with stroma present also showed evidence of invasion into the muscle (**Figure 2C**) and surrounding satellite tumors (data not shown), which were not observed in tumors formed without the influence of stroma. Histological analysis (**Figure 2B**, **3A**) showed that tumors formed in the presence of stroma grew papillary architecture, creating a malignant bladder-like structure in the subcutaneous compartment.

The tumors that included fetal bladder also showed higher expression of basal markers CD44 (**Figure 3B**) and CK5 (**Figure 3C**), while conversely, the expression of the luminal marker CK20 is decreased (**Figure 3D**). This indicates the presence of less differentiated malignant cells, which are associated with more advanced tumors [23].

The stromal-influenced tumors expressed more EGFR (**Figure 3E**) and pS6 ribosomal protein (**Figure 3F**), indicating an increase in growth factor signaling. A decrease in E-cadherin (**Figure 3G**), suggested an early stage of epithelial-to-mesenchymal transition (EMT) although we did not observe a corresponding increase in N-cadherin (**Figure 3H**). Both early EMT and activation of growth pathways would be expected in a more advanced tumor.

Primary human bladder tumor was also used to validate this model. A primary tumor was sorted to isolate $CD44^{+}CD49f^{+}$ cells previously described as the cancer stem cell population [24]. NSG mice were challenged with sorted cells combined with fetal bladder. The resulting tumor (**Figure 4A**) not only formed papillary architecture comparative to the murine model (**Figure 2B**), but the architecture recapitulated the structure of the original primary tumor (**Figure 4B**).

Discussion

With this work, we suggest a model for bladder cancer that creates a subcutaneous tumor that

exhibits typical human bladder tumor architecture. It maintains the benefits of subcutaneous implantation while gaining more physiological relevance, allowing comparisons to orthotopic or induced models.

The use of spheres to seed the tumor is validated by their demonstrated expression of the cancer stem cell (CSC) phenotype and their ability to express markers of both basal and luminal cells (**Figure 1**). As CSCs have the ability to differentiate into the heterogeneous malignant cell types found in a tumor [25], they are ideal for implantation. Their use isolates the model from extraneous factors and increases tumor-forming efficiency, as seen in breast cancer with $CD44^{+}CD49^{hi}$ cells [24].

Within the framework of this model, we can also explore the stromal contribution to tumor growth and progression. Fetal bladder induced significantly accelerated tumor growth (**Figure 2A**). The tumors were also less differentiated, showed signs of EMT, and began invading the surrounding muscle (**Figure 2C**, **3**), indicating a more advanced disease. The effects appear to be mediated, at least partially, by stromal activation of growth pathways (**Figure 3E**, **3F**), as has been previously observed [26, 27].

For this tumor model to be physiologically relevant, it should mimic typical bladder tissue architecture. With the addition of the stromal component, the tumors effectively recreated malignant bladder tissue (**Figure 2B**). Similar effects were seen when primary human CSCs were used in place of a cell line (**Figure 4A**). Most significantly, the model recapitulated the architecture seen in the primary tumor (**Figure 4B**).

This novel model of subcutaneous bladder cancer presents a unique opportunity to analyze the influence of the surrounding stroma to tumor growth. The elucidation of the role of stroma represents a significant area of research interest, and a subcutaneous model is particularly well suited to disentangle the contribution of malignant cells and stroma to the tumor microenvironment.

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