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14. ABSTRACT

To study circulating prostate cancer cell adhesion behavior and the contribution to distance metastasis of prostate cancer, we applied a dynamic flow-based E-selectin+SDF-1 coated microchannel system to isolate and characterize a subpopulation of adhering prostate cancer cells. We found a positive correlation of cells' aggressiveness with their adhesion/rolling ability. By using this device we then sorted prostate cancer cell lines (PC3 and DU-145) into rolling and floating populations, and tested for their behavior *in vitro* and *in vivo*. Results from our *in vitro* studies found that rolling cancer cells are more aggressive in nature with higher growth rate and invasion and form bigger and more colonies in soft agar and 3D sphere formation assays. To test further *in vivo*, an orthotopic implantation mouse model is underway. In summary, this flow based device provides a platform for isolating the most dangerous aggressive form of cancer cells which can be used for human patients' peripheral blood for isolating and characterizing and to form a process for customized cancer treatment.

15. SUBJECT TERMS

prostate cancer, metastasis, selectin, circulating, Micro-vascular

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INTRODUCTION

Prostate cancer is the second most common cause of male cancer death in the USA. The major cause of cancer patient mortality is metastasis. Recent studies found that epithelial origin cancer cells can undergo epithelial-to-mesenchymal transition (EMT), which plays an important role in metastasis, where the cancer cells gain the genes that can transform them to behave as 'leukocyte like' cells to be transported through the blood stream to their metastatic destinations (1). Like leukocytes, Prostate Cancer (PRCA) cells preferentially adhere and roll on bone marrow endothelial cells (BMECs) (2, 3) where abundant E-selectin is expressed (4), subsequently initiating a cascade of activation events that eventually leads to the invasion, migration, and ultimately development of metastatic tumors (5-7). All of the observations support the notion that prostate cancer adhesion to the BMECs is a key step in the metastasis cascade. Selectin plays a very important role for capturing cancer cells from the blood stream and migrating them to the site of metastasis. (3). Activated prostate cancer cells become firmly adhered through integrin mediated interactions, and finally, the cells migrate across the endothelium under the direction of SDF-1 gradient (3). To study the mechanisms by which prostate cancer cells adhere to vessel endothelial cells, we applied a dynamic flow-based E-selectin coated microchannel system, cylindrical flow chamber. This selectin-mediated system mimics physiological events involved in normal cell trafficking such as recruitment of leukocytes during the inflammatory response and homing of hematopoietic stem cells to the bone marrow (8-10). We successfully established parameters' for the dynamic flow-based E-selectin+SDF-1 coated microtubes, which can capture PRCA cells and allow us to study the circulating tumor cell behavior and its contribution to tumor metastasis. It was concluded that, a tube coated with 40 μg/ml of E-selectin and 10μg/ml of SDF-1β with shear stress 1 dyne/cm^2 is the best possible combination to collect the smallest more potent PRCA cells from the tube based circulation model (preliminary data). We used this selectin and SDF-1β coated micro-renathane tubing system, which mimics pro-metastatic vascular endothelial cell surface, for the capturing of 'rolling/adhesion/attached' group of PRCA cells. We found that the 'rolling/adhesive/attached' group of cells posses more aggressive and stem cell type characteristics compared to the 'floating' in our *in vitro* assay. In summary, our experimental *in vitro* data confirmed that cell rolling is a critical step in controlling cancer metastasis, and this novel flow-based device provides a system to capture and enrich metastatic cancer cells from the circulation. So further evaluation is necessary not only to identify the pathways/genes that are responsible for cancer metastasis, but also to provide a pure population for preclinical drug screening. It opens up a process for customized cancer treatment which can have more potent action and also reduce the disadvantage of multiple drug side effects.

BODY

We worked on Task 1 and Task 2 in this period of time and accomplished the following areas.

We submitted task 1 in our work statement as:

Task 1: Correlation of cancers' aggressiveness with their adhesion /rolling capacity in static and dynamic flow-based status (timeframe: months 1-12).

1a: Determination of cell rolling capacity in a dynamic state (timeframe: months 1-12).

1b: Determination of cell adhesion ability in a static condition (timeframe: months 1-8).

1c: Determination of cell metastatic behavior *in vitro* (timeframe: months 1-12).

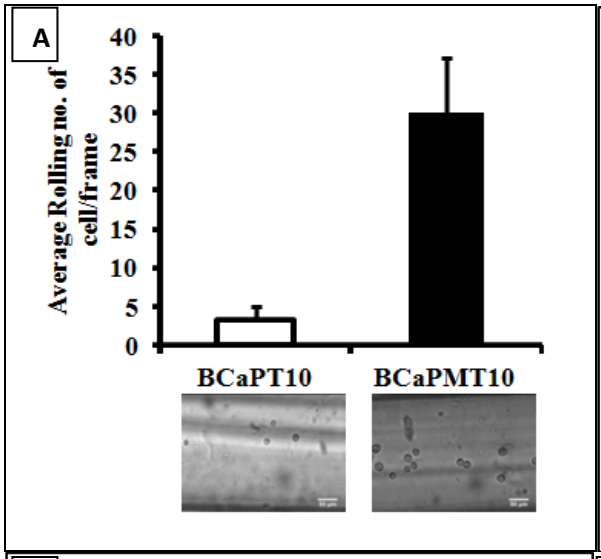
Accomplishment of Task 1:

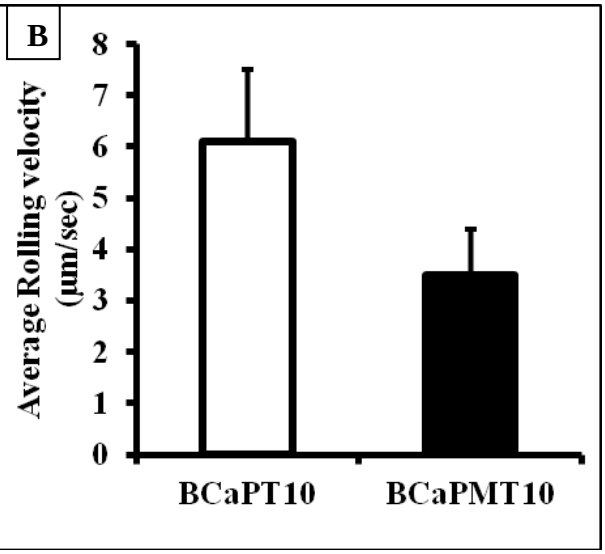
Using a dynamic flow-based system, we analyzed the adhesion/rolling/attached subgroup of LNCaP, CWR22R, and BPH-1 series, and correlate with their aggressiveness. We have shown the rolling capacity of LNCaP and CWR22R series in our preliminary data, and now we continue to analyze the third cell type- BPH-1 series, from nontumorigenic, low grade tumorigenic (BCaPT10) to metastastic (BCaPMT10) cell lines. Cells which roll on the E-selectin and SDF-1β (R and D Co.) coated micro-renathane tube (Braintree Scientific, inc.) were considered as 'rolling/adherent/attached' cells. To perform rolling capacity, determined by rolling cell number and rolling cell velocity, we perfused BCaP10 and BCaPM10 cell lines through 2 separate coated (E-selectin + SDF-1β) tubes under microscopic guidance. Cells' rolling behavior was video recorded for analysis. In our body, metastatic prone circulating cancer cells tether, roll and ultimately adhere on the endothelial cell surface of the capillary to where the shear stress is low. They then exert from the capillary for distant metastasis (1). *In vitro*, under shear stress the metastatic/aggressive type of prostate cancer cells should roll on the inner surface of the blood vessel mimicking E-selectin/SDF-1 coated tube and eventually adhere. So the rolling/adherent/attached cell number should be higher in more aggressive prostate cancer cells. On the other hand, prostate cancer cells which roll faster would not adhere on the surface; they would roll a few times and ultimately flush away under shear flow. The cells which do not roll at all would be given '0'in rolling velocity.

We defined rolling cells as cells that translated at an average velocity <50% of the calculated free stream velocity for more than 2 seconds while remaining in the field of view [FOV: 432 μm × 324 μm, using a 20x Plan Fluorite objective, NA 0.40 (Olympus America Inc., Center Valley, PA)]. Rolling cells that interacted with other cells were not included (11). We used shear stress 1 dyne/cm^2, which is the lowest shear stress in the capillary (12), to analyze BPH-1 series and we found that metastatic subtype BCaPM10 displayed higher rolling cell number as compared to the tumorigenic, low grade BCaPT10. As expected, rolling velocity of the BCaPT10 was higher as compared to the BCaPM10 cells. Next, we compare their invasiveness *in vitro* using BD matrigel coated (BD Bioscience) invasion chamber. We found

that metastatic BCaPM10 cells show higher invasiveness as compared to tumorigenic BCaPT10 cells (Figure 1).

Figure 1





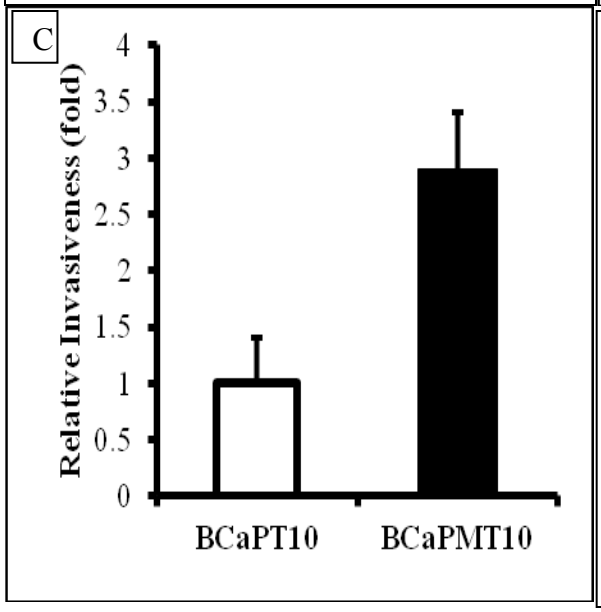


Figure 1. Rolling Cell Number, Velocity and Invasiveness of BPH-1 Series. Under shear stress 1 dyne/cm^2 metastatic BCaPM10 subtype demonstrated higher number of cells rolled/adhere on the E-Selectin/SDF-1β coated inner surface of micro-renathane tube compared to their contemporary subtype tumorigenic BCaPT10 cell line (**A**). Under shear stress 1 dyne/cm^2 BCaPT10 cell line demonstrated higher rolling velocity compared to BCaPM10 subtype on the E-Selectin/SDF-1β coated micro-renathane tube (**B**). Invasion Assay shows BCaPMT10 cells have higher invasiveness compared to BCaPT10 cells using BD matrigel coated invasion chamber (**C**).

We submitted task 2 in our work statement as:
Task 2: Sorting prostate cancer cells into adhesion (attached) and non-adhesion (floating) subpopulations and comparison of these two populations' metastatic behaviors *in vitro* and *in vivo* (timeframe: months 5-24).

2a: Fractionation of prostate cancer (PRCA) cells based on rolling capacity (timeframe: months 5-20).

2b: Characterization of sorted cells metastatic behavior *in vitro* (timeframe: months 7-18).

2c: Confirmation of metastatic potential on those rolling PRCA cells *in vivo* by the orthotopic injection mice model (timeframe: months 13-24).

Accomplishment of Task 2:

We performed part of the Task 2a & 2b for sorting and analyzing of prostate cancer cell lines. We found that the rolling/adhesive/attached cells are more aggressive and posseses more cancer stem cell characteristics compared to the floating population in our *in vitro* assays.

For sorting prostate cancer cells, PC3 and DU-145 cell lines were used. Each type of cells was infused through the immobilized E-selectin and SDF-1β coated micro-renathane tube at 1dyne/cm^2 shear stress. Rolling/adherent/attached cells were collected separately from non-rolling (floating) cells. All the parameters were tested for the sorted PC3 cells, the same as we did with DU-145 cell line (1dyne/cm^2 shear stress, and the same concentration of immobilized E-selectin and SDF-1β). We noticed that PC3 cells have higher adhesiveness to selectin/SDF-1 coated tube as compared to DU-145 cells. The results are described in the following.

Viability testing of Sorted Cells:

To maintain the characteristics of sorted PC3 cells (floating *vs.* rolling), we collected sorted cells and characterized their behavior *in vitro* and *in vivo* for no more than two passages after sorting. First, cell viability of sorted floating and adhesion cells were determined by trypan blue staining (using Vi-CELL-XR Cell Viability Analyzer, Beckman Coulter). As shown in Table 1, the viability of all three populations is similar, ranging from 96-86%. Interestingly, the adhesion cells exhibit the lowest viability among all three, but the difference is not significant.

Table 1:

	Parental: Before sorting	Floating: After sorting	Rolling: After sorting
Viable cells (%)	96.8	94.4	86.7

Aggressiveness of Sorted cells:

Collected 'rolling' and 'floating' cells were then compared for their aggressiveness *in vitro*, freshly sorted cells were used to perform growth assay (MTT) and invasion assay using BD matrigel invasion chambers (BD Biosciences)) and static adhesion assay on HMEC-1 cells. As shown in Figure 2, rolling/adhesion/attached prostate cancer cells showed higher aggressive behavior than floating cells, where rolling cells have higher proliferation rate by the MTT assay, more invasiveness by invasive chamber assay, and possess more adhesiveness to endothelial HMEC-1 cells by a static adhesion assay.

In addition, we also tested their anchorage independent growth ability by the colony formation assay. As expected, the rolling cells formed more and bigger colonies than floating cells (figure 3). The stem cell characteristics were also compared between floating and rolling cells, due to their potential contribution to metastasis. As shown in Figure 4, the rolling PC3 cells exhibit bigger in size and higher number of 3D-sphere, the standard assay to characterize stem cells.

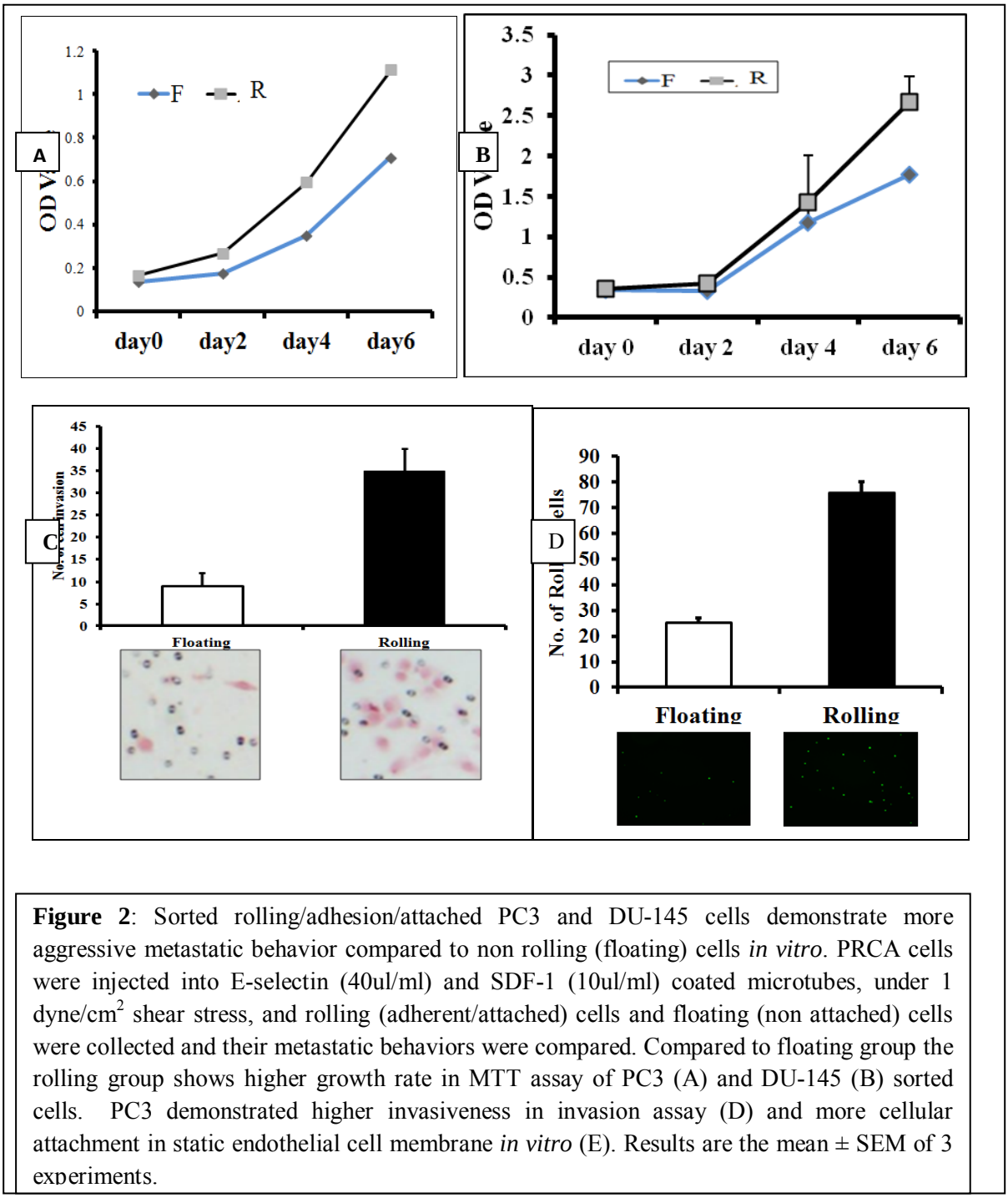
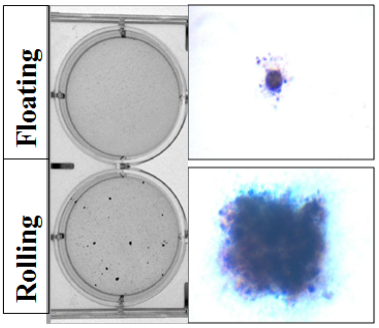


Figure 2: Sorted rolling/adhesion/attached PC3 and DU-145 cells demonstrate more aggressive metastatic behavior compared to non rolling (floating) cells *in vitro*. PRCA cells were injected into E-selectin (40ul/ml) and SDF-1 (10ul/ml) coated microtubes, under 1 dyne/cm^2 shear stress, and rolling (adherent/attached) cells and floating (non attached) cells were collected and their metastatic behaviors were compared. Compared to floating group the rolling group shows higher growth rate in MTT assay of PC3 (A) and DU-145 (B) sorted cells. PC3 demonstrated higher invasiveness in invasion assay (D) and more cellular attachment in static endothelial cell membrane *in vitro* (E). Results are the mean ± SEM of 3 experiments.

Figure 3



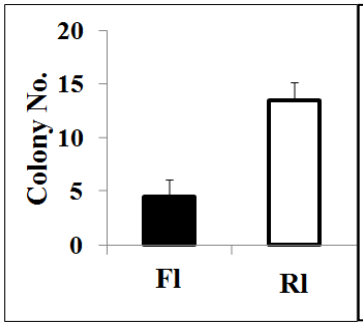
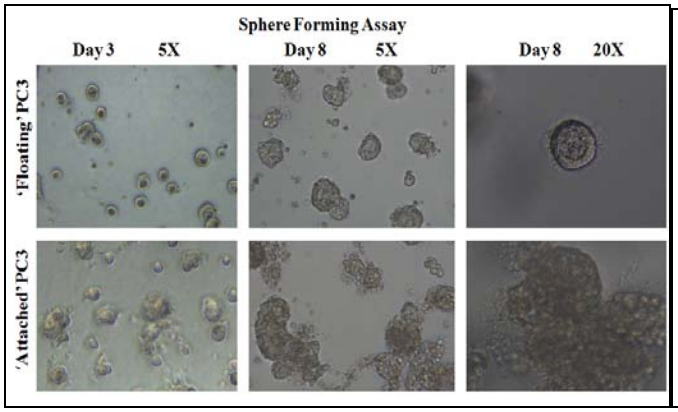


Figure 3. In Anchorage-independent colony-forming assay, PC3-rolling cells demonstrated higher number and bigger sized colony compared to PC3-floating group when they were grown in 4% noble agar for 3 weeks.

Figure 4



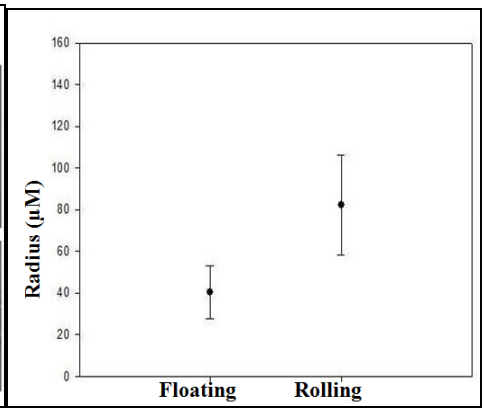


Figure 4. In Sphere Forming Assay. When freshly sorted PC3 floating and rolling cells were 4D cultured using matrigel (BD bioscience), both floating and rolling cells developed sphere form day 8. But rolling cells demonstrated higher number and bigger sized spheres compare to the rolling subtype.

After seeing these encouraging results *in vitro*, we are now sorting cells and confirm their aggressiveness/metastasis behavior *in vivo* as proposed in Task 2c.

In summary, we have provided evidence showing this flow based E-selectin/SDF coated tube can allow us to sort a small percentage of prostate cancer cells that represent the most dangerous populations in circulation.

After seeing these encouraging results *in vitro*, we concluded that this flow-based device can capture a small population of most aggressive prostate cancer cells in the circulation. We now continue our efforts to confirm if this remains the same *in vivo* orthtopic xenograft mouse model as proposed in Aim 2/ Task 2c.

Key Research Accomplishments

- Establishment of an *in vitro* system to analyze the rolling behavior of prostate cancer cell lines.
- Establishment of a sorting system of prostate cancer cells based on their rolling behavior: rolling/adhesion cells (<5%) and floating cells (>95%).
- Correlation of prostate cancer cells' aggressiveness with their rolling behavior.
- In vitro characterization of sorted prostate cancer cells (floating vs. rolling) behavior and aggressiveness.

Reportable Outcomes

- Participated in a poster session of prostate cancer basic research in the American Urological Association (AUA) annual meeting in Atlanta, Georgia May 19 – May 23, 2012 (please see the appendix for documents).

CONCLUSION:

We used this selectin and SDF-1 coated micro-renathane tubing system, to capture the 'rolling/adhesion/attached' subpopulation of prostate cancer cells, such as PC-3 and DU145 cells under constant flow (shear stress 1 dyne/cm^2). Based on their adhesion ability, prostate cancer cells were sorted into: rolling/adhesion/attached fraction which contains potentially a more aggressive cells population, and non-adhesion/floating fraction which were less aggressive sub-population. We found that the 'rolling/adhesive' cells are more aggressive in nature, as compared to the 'floating' cancer cells by *in vitro* assays including cell proliferation assay (MTT assay), invasion Assay, and static adhesion assay on HMEC-1 cells and anchorage-independent assay (sulf agar colony formation assay). They also demonstrated higher stemcell characteristics where bigger and more spheres were formed in rolling population. In summary, our experimental data have confirmed that cell rolling is a critical step in controlling cancer metastasis, and this novel flow-based device provides a way to capture and enrich metastatic cancer cells in the circulation. This not only leads to identifying the pathways/genes that are responsible for cancer metastasis, but also provides a pure population for preclinical drug screening. It opens up a process for customized cancer treatment which can have more potent action and also reduce the disadvantage of multiple drug side effects.

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AUA ANNUAL MEETING

May 19–23, 2012
Atlanta, GA USA

MEETING PROGRAM

American Urological Association
Education and Research, Inc.

Sunday, May 20, 2012 **1:00 pm - 3:00 pm**

Moderated Poster Session 19

PROSTATE CANCER: BASIC RESEARCH III

B 308 @ Georgia World Congress Center

Moderators: *Ralph Buttyan and Alexander Parker*

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Koji Hatano, Yasutomo Nakai,* Suita, Japan, *Yasuhide Miyamoto,* Osaka, Japan, *Yasuhi Kaneda, Norio Nonomura,* Suita, Japan

Certificate of Attendance

This certifies that

Sayeda Yasmin-Karim

has participated in the 2012 Annual Meeting of the
American Urological Association
in Atlanta, GA
May 19-23, 2012

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