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Soft agar-based selection of spontaneously transformed rat prostate epithelial cells with highly tumorigenic characteristics

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Abstract

The critical molecular and cellular mechanisms involved in the development and progression of prostate cancer remain elusive. In this report, we demonstrate that normal rat prostate epithelial cells (PEC) undergo spontaneous transformation at high passage ($p > 85$) evidenced by the acquisition of anchorage independent growth when plated on soft agar and tumorigenicity when injected into immunodeficient mice. In addition, we also report the discovery of a minor subpopulation of spontaneously transformed PEC derived from high passage PEC with the ability to migrate through a layer of 1% agar and form expanding colonies on the underlying plastic substratum. Comparison of these soft agar invasive (SAI) cells with low ($p < 35$), mid ($p 36-84$) and high passage ($p > 85$) PEC identified marked differences in cell morphology, proliferation and motility. The SAI subpopulation was more tumorigenic than the high passage anchorage independent cultures from which they were isolated, as manifested by a decreased latency period and an increase in the size of tumors arising in immunodeficient mice. In contrast, low and mid passage cells were unable to grow on soft agar and failed to form tumors when injected into immunodeficient mice. Screening with antibody-based signaling arrays identified several differences in the altered expression levels of signaling proteins between SAI derived cells and low

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

or high passage PEC, including the up-regulation of EGFR and MAPK-related signaling pathways in SAI-selected cells. In summary, these studies suggest that the SAI assay selects for a novel, highly tumorigenic subpopulation of transformed cells that may represent an early step in the progression of slow growing prostatic carcinomas into more rapidly growing and aggressive tumors.

Keywords

prostate epithelial cells; neoplastic conversion; tumorigenicity; cancer stem cell

Introduction

Prostate cancer is the most commonly diagnosed cancer among males excluding skin cancers. The American Cancer Society estimates that in the United States 164,690 men will be diagnosed with and 29,430 men will die of prostate cancer (American Cancer Society, 2018). Moreover, the incidence is currently on the rise. The development of more effective treatment modalities will require a better understanding of the critical molecular and cellular pathways involved in the genesis and progression of prostate cancer. There is general agreement that solid tumors are composed of a heterogeneous population of cells that differ in their proliferative patterns, ability to self-renew, differentiation status and malignant potential. More recently, accumulating evidence supports the notion that solid tumors, including prostate cancer, may result from subpopulations of proliferative cells with a high capacity for self-renewal and the ability to grow in immunodeficient mice (Reya et al., 2001; Clarke and Fuller, 2006). This evidence includes results from research showing that a CD34⁺ subpopulation of human acute myeloid leukemic (AML) cells was the source of tumors in immunodeficient mice that recapitulated the phenotypic heterogeneity of the original tumor (Bonnet and Dick, 1997). Additional evidence comes from studies describing subpopulations of tumor cells with a high self-renewal capacity and tumorigenicity in carcinomas isolated from the prostate (Collins, 2005), liver (Ma, 2007), breast (Al-Hajj et al., 2003), lung (Eramo et al., 2008), brain (Singh et al., 2003), pancreas (Li et al., 2007), endometrium (Rutella et al., 2009), colon (Ricci-Vitiani et al., 2007) and head and neck (Prince et al., 2007).

In previous work, we developed a spontaneous transformation model for rat bile duct epithelial cells (BDEC) that culminated at high passage (p>85) in anchorage independent growth when plated on soft agar and tumorigenicity when injected into immunodeficient mice (Rozich et al., 2010). In the present report, we show that a rat prostate epithelial cell (PEC) line established without prior carcinogen treatment or immortalization also undergoes neoplastic conversion *in vitro*. Similar to our previous studies with BDEC, we found that with increasing passage PEC acquired several of the classical hallmarks of neoplastic cells (Hanahan and Weinberg, 2000), including anchorage independent growth when plated in soft agar and tumorigenicity when injected into immunodeficient mice. In addition, we identified a subpopulation of high passage anchorage independent cells with the ability to migrate through a layer of soft agar and expand into well-defined colonies on the underlying plastic substratum. Further analysis revealed several differences between this soft agar

invasive (SAI) cell population and the high passage anchorage independent cells from which they were selected. These included distinct differences in morphology, proliferation, motility and surface phenotype. SAI cells were also distinguished by their expression of several signaling proteins in pathways known to modulate cell growth and survival, findings consistent with the differences in tumorigenicity between the unselected high passage and SAI-selected PEC. These findings demonstrated that soft agar selection offers a simple means for isolating highly tumorigenic subpopulations present in established lines of neoplastic prostate epithelial cells.

Materials and methods

Cell culture

The origin and isolation of the rat prostate epithelial cells was previously described (Britt et al., 2004; Mills et al., 2012). To provide a basis for discussion, low, medium and high passage cells were operationally defined as passages (p) 1–35, 36–85 and greater than 85, respectively. For selection of anchorage independent soft agar colonies and soft agar invasive cells, complete culture medium containing 1% agarose was added to tissue culture dishes and high passage rat PEC (1×10^6 cells/well) suspended in complete culture medium containing 0.35% agar were seeded on top of the 1% agarose substratum. Plates were incubated at 37°C for approximately 2–3 weeks and examined microscopically to identify anchorage independent soft agar colonies and/or soft agar invasive cells proliferating on the underlying plastic substratum. To harvest colonies of SAI cells, the agarose was carefully removed and the colonies of adherent cells were cultured in complete medium and passaged as described for adherent PEC cultures (Mills et al., 2012).

Cell proliferation assays were performed as previously described (Rozich et al., 2010) using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI). Briefly, PEC cultures were seeded in 24-well plates at a concentration of 5×10^4 cells/well. Formazan, the end product of the MTS assay, was read at 490 nm on a Bio-Kinetics EL312 automated plate reader (Bio-Tek, Winooski, VT).

Indirect immunofluorescence microscopy

Rat PEC subcultures were seeded in two-well permanox chamber slides (Nalge Nunc International, Rochester, NY, USA) at a density of 1×10^5 cells/ml and incubated for 48–72 hours in a 5% CO₂ humidified chamber as described (Mills et al., 2012). Following three washes in PBS, cells were fixed and permeabilized for 15 min in ice-cold acetone. To minimize non-specific binding of antibodies, acetone-fixed cells were blocked for 10 min in 1% bovine serum albumin (BSA)/PBS with 10% normal goat serum (Sigma-Aldrich, Inc.). Primary and secondary antibodies were diluted in 1% BSA/PBS with 10% normal goat serum and incubated sequentially with cells for 45 min at room temperature. Cells were examined by fluorescence microscopy using a Nikon Eclipse E800 microscope (Nikon Instruments, Inc., Melville, NY) fitted with a Spot digital color camera (SPOT Imaging Solutions, Sterling heights, MI). Antibody dilutions were as follows: MAb OC.2 (Hixson and Allison, 1985), 1:1000; and MAb OC.5 (Hixson et al., 2000), 1:1000; Alexa Fluor 488

goat anti-mouse IgG and Alexa Fluor 594 goat anti-mouse IgM secondary antibodies (Molecular Probes, Eugene, OR), 1:400.

Cell motility assays

Cell motility of low passage, mid passage, high passage and soft agar invasive PEC was measured using the Cellomics Cell Motility Kit (Pierce Biotechnology, Rockford, IL) as described in the manufacturer's instructions. Briefly, blue fluorescent microspheres were coated onto collagen-covered 96-well plates and overlaid with 500 cells per well in a total volume of 100 µl complete PEC culture media. Cells were incubated at 37°C for 24 hours in a 5% CO₂ humidified chamber and photographed using an inverted microscope with a Carl Zeiss digital camera (SPOT). A minimum of 50 cells in 10 random views for each cell line were quantified for the distance that they had migrated. ImageJ software was used for the quantitative evaluation of cell motility. Briefly, tracks created by a single cell were circled using the free hand tool and analyzed using the ImageJ software (<http://rsbweb.nih.gov/ij/>), as described (Nogalski et al., 2012).

Cell lysates and western blotting

For screening of the Kinexus Kinex Antibody Arrays, low passage, high passage and SAI PEC lysates were prepared according to the manufacturer's instructions (Kinexus Vancouver, BC, Canada). For immunoblot analysis, cells were lysed in situ with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and one Complete Protease Inhibitor tablet [Roche Diagnostics Co.]) or with the M-PER Mammalian Extraction Reagent (Pierce Chemical Co.) as described in the manufacturer's instructions. Detergent insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C. Supernatant aliquots were mixed with four volumes of 5X non-reducing SDS sample buffer (BioRad Laboratories, Inc., Hercules, CA) and proteins separated by 7.5% SDS-PAGE were transferred onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad Laboratories) and immunoblotted as described (Mills et al., 2012). Reactivity was visualized by chemiluminescence with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Antibody dilutions were as follows: polyclonal anti-EGFR (IgG; Santa Cruz Biotechnology, Dallas, TX), 1:400; polyclonal anti-raf-1 (IgG; Abcam, Inc., Cambridge, MA), 1:500; polyclonal anti-Stat1 (IgG; Santa Cruz Biotechnology), 1:2000; polyclonal anti-Stat1 (phospho Y701 (IgG; Abcam, Inc.), 1:1000; monoclonal anti-β-actin (IgG; Sigma-Aldrich, Inc., St. Louis, MO), 1:30,000; anti goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Biosource, Camarillo, CA), 1:25,000.

Tumorigenicity assay

All animal protocols carried out in these studies used protocols approved by the Rhode Island Hospital Institutional Animal Care and Use Committee. Tumorigenicity was assayed as previously described (Rozich et al., 2010). Briefly, cells were grown to approximately 80% confluence before being harvested with trypsin/EGTA. 10×10^6 cells suspended in HBSS were injected into the left front flank of 4–6 week old female beige/nude/xid triple deficient mice (Harlan Laboratories). Mice were closely monitored and kept for up to one

month. At the time of sacrifice, tumors were measured, excised, weighed and frozen in a hexane/acetone bath and stored at -80°C .

Statistical analysis

The values in the figures are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis of the data was performed by a Student's *t* test using Statistica for Windows v. 8.0. Values of $P < 0.05$ were considered as significant.

Results

Rat prostate epithelial cells show morphologic change with increasing passage number

In previous studies, we found that with continued passage, rat bile duct epithelial cells accumulated neoplastic characteristics, some or all of which were required for the induction of anchorage independent growth in vitro and the ability to form tumors in vivo. Briefly, by midpassage (p31–84), BDEC showed alterations in morphology, onset of aneuploidy, increased growth rate with growth factor independence, decreased cell adhesion and loss of cholangiocyte markers expressed at low passage ($p < 30$). At high passage ($p > 85$), an increasing number of BDEC expressed activated ErbB2/Neu, exhibited anchorage independent growth on soft agar and had acquired the ability to form tumors when injected into immunodeficient mice (Rozich et al., 2010). Here, we describe a similar in vitro model of spontaneous transformation using a continuous line of rat prostate epithelial cells (PEC) established from the dorsal-lateral prostate without immortalization or prior carcinogen treatment. As shown in Figure 1, with continuous passage under normal culture conditions, PEC at high passage (p102) were less tightly packed, more randomly organized, and more frequently spindle shaped than their low passage (p29) counterparts. In addition, high passage cells retained a higher level of refractivity after attachment and spreading as compared to low passage cells (Fig. 1 a, b).

High passage PEC are anchorage independent and contain a soft agar invasive subpopulation

We have previously reported that high passage ErbB2/Neu positive BDEC displayed anchorage independent growth when plated on top of a soft agar substratum (Rozich et al., 2010), a property associated with cell transformation and highly correlated with *in vivo* tumorigenicity (Shin, 1975 et al.; Colburn et al., 1978). In the present study, we tested low, mid and high passage PEC for anchorage independent growth on soft agar. Consistent with our previous findings with BDEC, we found that low and mid passage PEC did not form detectable colonies when cultured on soft agar (data not shown). In contrast, high passage PEC ($p > 85$) had become anchorage independent and formed large spheroid aggregates when plated on or in soft agar. Of considerable interest, high passage anchorage independent PEC contained a subpopulation of cells that migrated through the soft agar and adhered to the underlying plastic substratum where they proliferated and formed well-defined colonies. When examined by phase microscopy, the morphology of these soft agar invasive (SAI) cells was distinct from both low and high passage cells. As shown in Figure 1c, the SAI cells had lost the characteristic spindle shape of the high passage cells (Fig. 1b) and exhibited a tightly packed arrangement more similar in appearance to low and mid passage cells. The SAI cell

subpopulation was only present in the high passage anchorage independent cell population, and was not detected when lower passage ($p < 85$), anchorage dependent cells were cultured on soft agar.

Proliferative changes during spontaneous transformation and following soft agar invasion

In previous work, we observed that high passage ($p > 85$) rat BDEC proliferated more rapidly than low passage cells ($p < 30$) (Rozich et al., 2010). This increase in proliferation at high passage also occurred with PEC cells. As shown in Figure 2, at the 24-hour time point, high passage PEC (p99) showed an approximate 2-fold higher rate of growth as compared to low passage cells (p31). In contrast, SAI cells selected from p99 high passage cells showed a statistically significant decreased rate of proliferation relative to unselected high passage cells, a rate that was only slightly higher than that displayed by low passage cells. A similar trend was observed at the 48-hour time point but the mid passage cells exhibited higher rates of proliferation that more closely resembled the unselected high passage cell population.

Rat PEC show an increase in motility with increasing passage

Cell motility assays were performed to determine if high passage or SAI-derived PEC cells displayed an increase in cell motility, an *in vitro* characteristic frequently correlated with invasive and metastatic growth. Relative motility was determined from the length of the trail of fluorescent beads displaced by the movement of single cells with cell migration expressed as arbitrary units of motility. As shown in Figure 3, paths formed by low (p31; Panel A) and mid (p54; Panel B) passage cells showed little motility. In contrast, the high passage (p137; Panel C) and SAI-derived (Panel D) cells cleared paths significantly larger than low and mid passage cells.

Rat PEC show differences in the expression of rat bile duct epithelial markers following transformation and soft agar invasion

In earlier work, we have shown that the cultured rat PEC used in the present study exhibited strong reactivity with common epithelial markers such as E-cadherin and desmoplakin (Halpert et al., 1996). More recently we reported that BD.1/eIF3a, a previously defined cholangiocyte marker (Yang et al., 1993), was also expressed by rat PEC. Expression was found to vary with passage number with low ($p < 30$) and high ($p > 85$) passage PEC expressing high levels of BD.1/eIF3a and mid-passage PEC (p31–84) displaying a marked decrease in expression (Mills et al., 2012). Here, we have extended these findings and show that SAI PEC display differential reactivity with several additional MAbs previously demonstrated to react with liver epithelial cells. As shown in Figure 4, a small percentage of low (p26) and high (p99) passage PEC were positive for OC2 (Hixson and Allison, 1985), a marker expressed by 100% of the freshly selected SAI cell population (Fig. 4). Conversely, marker OC.5 (Hixson et al., 2000) was expressed by a high percentage of low and high passage PEC but were barely detected in freshly selected SAI cells (Fig. 4).

SAI PEC are more tumorigenic than high passage PEC

A defining hallmark of cellular transformation is the ability to form tumors when injected into immunodeficient mice (Lai et al., 2005). Consistent with a previous report by Rozich et

al. (2010) describing changes in tumorigenicity of BDEC as a function of passage number, we found that high passage, anchorage independent PEC (p91) formed large subcutaneous tumors when injected into immunodeficient mice (Fig. 5a) (Rozich et al., 2010). Tumors excised at day 28 had an average weight of 0.22 ± 0.12 grams ($n=3$). In contrast, after 35 days (the longest time point examined) no tumors were found in immunodeficient mice injected with low and mid passage anchorage dependent PEC (data not shown). PEC SAI cells, on the other hand, were more tumorigenic than high passage PEC as evidenced by a decreased latency period and a marked increase in tumor size (Fig. 5b) with an average weight of 0.77 ± 0.32 grams when excised at day 21 ($n=5$). Due to the large tumor burden in mice injected with SAI-derived cells, it was necessary to sacrifice these animals prior to the 28-day end point used for the high passage PEC tumorigenicity studies. For the purposes of this study, each excised tumor was considered as a single growth and the total weight of excised tissue per animal was reported as shown in Figure 5. Excised tumors were limited to the injection site, as additional growths were not identified in any of the sacrificed animals following a survey of removed organs or the peritoneal cavity.

Changes in signaling proteins associated with spontaneous transformation and SAI-selection

To determine if there were changes in signal transduction pathways associated with spontaneous transformation (i.e., low to high passage) and/or following the selection of the SAI-derived cell subpopulation from high passage anchorage independent cells, we characterized the reactivity of low passage, high passage and SAI-derived PEC lysates against Kinexus antibody arrays that targeted known signaling proteins. The results are given in Tables 1 and 2. Z-ratios represent the difference between the averages of the observed protein Z-scores divided by the SD of all of the differences in the comparison. The average intensity of all spots within a sample subtracted from the raw intensity for each spot, and then divided by the standard deviations (SD) of all of the measured intensities within each sample renders the Z-score (Cheadle et al., 2003)

Pairwise comparisons between the low passage and spontaneously transformed high passage PEC lysates identified several signaling proteins that were differentially expressed in pairwise comparisons. As shown in Table 1, as cells progressed from low to high passage, the expression levels of several members of the epidermal growth factor (EGF) family, (phosphorylated EGFR (Y1197) and ErbB2), and proteins associated with the MAPK signaling pathway (p38/Erk6, Mek2, and Ask1/MAP3K5) were found to be up-regulated in high passage as compared to the low passage cells. Also found to be upregulated in high passage cells were proteins involved in cell cycle regulation (cyclin D1), cyclin-dependent kinases (CDK6, CDK10), and several kinases previously associated with cancer progression (PDK1, PKC α and RSK1/3).

Several differences were also observed in the expression levels of signaling proteins in SAI-derived cell lysates as compared to high passage PEC lysates (Table 2), differences that further support our contention that SAI selects for a unique subpopulation of neoplastic anchorage independent cells. When compared to high passage PEC, the SAI-selected cell lysates showed higher levels of phosphorylated EGFR (T693) and phosphorylated MAP

kinases Erk1/2 and JNK 1/2/3. Other proteins found to be upregulated in SAI-derived lysates included Fas and HSP90a/b, a molecular chaperone required for the stability and function of conditionally activated signaling proteins and is implicated in cancer cell growth and survival (Zuo et al., 2012). SAI cells also showed a down-regulation of a number of proteins as compared to high passage-derived lysates including Akt2 and Raf1, PDK1, Ask1 and several proteins associated with cell cycle regulation (cyclin D1, CDK6, CDK8 and CDK10).

To validate the Kinexus antibody array analysis, expression levels of EGFR and several downstream components of the mitogen-activated protein kinase (MAPK) signaling pathway were determined by immunoblot analysis. Results shown in Figure 6A and 6B confirmed differences between low passage, high passage and SAI-selected cells in the expression levels of EGFR and raf-1. Specifically, validation by immunoblot analysis showed that low and high passage cells expressed low levels of EGFR and raf-1 protein in comparison to SAI-derived cell lysates that expressed increased levels of both proteins. Also, since EGFR has been shown to up-regulate expression of STAT1 (Han et al., 2013), we examined the expression levels of STAT1 and phosphorylated STAT1 in low passage, high passage and SAI-derived PEC cell lysates. As shown in Figures 6C and 6D, low levels of STAT1 and pSTAT1 (pY701) expression were detected in low passage cells and increased dramatically in both high passage and SAIselected lysates. The immunoblot data for raf-1 (Fig. 6B) was at odds with the Kinexus data that showed down-regulated expression of raf-1 in the SAI lysate as compared to high passage lysate, a finding that highlights the importance of further validating the reported Kinexus antibody array data. In spite of this discrepancy between the Kinexus and immunoblot results, both methods of analysis show that SAI and high passage cells display distinct differences in signaling profiles that support the uniqueness of SAI-derived cells.

Discussion

The present study describes the characterization of a continuous long-term line of rat PEC that acquire anchorage independent growth and tumorigenicity at high passage ($p>85$). The PEC line developed in this study was initially isolated from the dorso-lateral lobes of the rat prostate and established as a continuous cell line without prior carcinogenic treatment or immortalization using recombinant DNA (Britt et al., 2004; Mills et al., 2012). The origin of the cell line described here is in contrast to many previously described primary rat prostate epithelial cell lines that have focused on ventral prostate, a preference owing in part to the small size and nondescript appearance of the rat dorsal and lateral prostate lobes. Many protocols describing the long-term maintenance of rat prostate epithelial cultures require the pre-treatment of rats with carcinogens or testosterone prior to cell isolation or cell immortalization in primary culture using recombinant DNA methodologies. As an animal model for studying human prostatic cancer, the rat dorso-lateral region also more closely resembles human prostate than does the rat ventral lobe (Dunning, 1963; Makarovskiy et al., 1999). Furthermore, in terms of studying human prostatic cancer, the rat dorso-lateral region is prone to spontaneous and induced neoplasms and most closely resembles the outer or peripheral zone of the human prostate gland, a region that is most often associated with intraepithelial neoplasms (Makarovskiy et al., 1999; Valkenburg and Williams, 2011).

The ability of transformed cells to proliferate under anchorage independent conditions is highly correlated with in vivo tumorigenicity, suggesting that the molecular mechanisms mediating anchorage dependent growth are related to those that underlie the aggressive growth properties of naturally occurring tumors (Yang and Kraus, 1997). In the present study, we found that only high passage cells had acquired anchorage independence as evidenced by their ability to form colonies in soft agar, a well-accepted hallmark of transformation. As the cultured rat PEC progressed towards the spontaneous transformation that occurs at high passage, we found several significant differences between low and high passage cells, operationally defined as $p < 35$, and $p > 85$, respectively. These included distinctions in cell morphology, rate of proliferation and relative motility.

High passage cells also contained a minor subpopulation not detected in low or mid passage cultures with the ability to migrate through the soft agar, attach to the underlying plastic and form well-defined colonies of proliferating cells. To the best of our knowledge, this SAI subpopulation has not been previously reported. Subsequent examination of the SAI-selected subpopulation revealed a number of significant differences between the unselected anchorage independent high passage cells and the SAI subpopulation that included differences in cell morphology, proliferation, expression of defined cell surface antigens, tumorigenicity, and expression levels of several proteins involved in functionally defined signal transduction pathways often associated with cancer progression.

Phenotypic differences were defined using several unique monoclonal antibodies recognizing cell surface epitopes expressed by normal and carcinogen treated rat liver epithelial cells of ductal origin (Hixson et al., 2000; Simper-Ronan et al., 2006; Rozich et al., 2010; Mills et al., 2012) and at high levels by rat prostate epithelial cells. This cross reactivity is perhaps not surprising as the prostate, like the liver, is endodermally derived and like the biliary tree, is composed of a complex ductal network (Berman et al., 2004). Previous work describing a spontaneous transformation model for rat BDEC showed changes in cell surface antigen profiles as the cells progressed from low ($p < 35$) to high ($p > 85$) passage (Rozich et al., 2010). Similar distinctions in the reactivity of low passage, high passage and SAI-selected rat PEC were found with MAbs specific for the cholangiocyte markers OC2 and OC5, differences that identified a novel and distinct antigenic phenotype (OC.2⁺/OC.5⁻) for rat PEC SAI-selected cells. At present, the identity of these reactive antigens (i.e., OC.2 and OC.5) remains unknown. However, work is in progress to identify their corresponding antigens as their identity may prove relevant to human prostate cell differentiation, carcinogenesis and, owing to their localization at the cell surface, may contribute to efforts aimed at isolating antigen-defined cell subpopulations for subsequent investigation.

Multiple studies have previously demonstrated alterations in the regulation or expression of cell signaling proteins associated with tumor progression and cancer stem cells (Karamboulas and Ailles, 2013; Guille et al., 2013). While the molecular mechanisms driving prostate tumor progression are not fully understood, overexpression of several growth factors and their receptors, such as epidermal growth factor receptor (EGFR), have been previously described (Marks et al., 2008). When PEC lysates were screened against Kinexus antibody arrays targeting signaling proteins, SAI cell lysates showed increased

expression of EGFR (ErbB-1) and several proteins belonging to signaling cascades that act downstream of EGFR, including raf-1, pERK-1 and phosphorylated STAT1 and STAT3. EGFR is a well-described cell surface receptor tyrosine kinase that belongs to the ErbB family of receptors that includes ErbB2 (Her2/neu). Activation of EGFR following ligand binding has been shown to elicit subsequent activation of several downstream signaling cascades, most notably, the MAPK, Akt and JNK pathways that regulate cellular proliferation, motility and adhesion. Furthermore, overexpression or dysregulated activation of EGFR-related pathways has been associated with tumor cell growth, therapeutic resistance and the capacity for self-renewal of cancer stem cells (Mimeault, 2012; Dasgupta et al., 2012). Enrichment of the EGFR expressing subpopulation by SAI selection will allow us to further examine the role of this protein and associated signaling pathways during spontaneous transformation, invasion and tumorigenicity.

Selected SAI cells are not unique to transformed cultures of rat prostate epithelial cells. We have also isolated this unique cell population from cultures of human DU145, RWPE-2 and PC-3 prostatic carcinoma cells, human Hep G2 hepatoma cells, and the spontaneously transformed rat BDE1.1 bile duct epithelial cell line (Yang et al., 1993). Further characterization and tumorigenicity testing of these SAI-selected cell subpopulations are currently in progress. Taken together, these studies suggest that SAI-selection represents a novel cell-based selection strategy for isolating and characterizing a novel tumorigenic subpopulation of cells from transformed, anchorage independent epithelial cell cultures. The identified changes described here suggest that the SAI cell population may help drive tumor formation and represents an attractive target for the development or application of anti-tumor therapeutic strategies.

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Highlights:

- Model of a spontaneous transformation of prostate cancer cells (PEC) was developed
- A novel soft agar invasive subpopulation (SAI) of transformed PEC was isolated and described
- SAI cells show differences in morphology, proliferation, surface phenotype and tumorigenicity
- Differential expression of signaling proteins between low, high and SAI-derived cell populations

Figure 1. PEC undergo morphological changes with increasing passage and following SAI selection.

Phase contrast micrographs demonstrate changes in cell morphology between low passage (p29), high passage (p102) and following selection by SAI. Low passage cultures form tightly packed, well-organized colonies (A). In contrast, cells of high passage cultures are more randomly organized and spindle-shaped (B). SAI-selected cells lose the characteristic spindle shape of the high passage cells and form more tightly packed colonies that more closely resemble the organizational pattern of low passage cultures (C). Scale bar represents 50 μm .

Figure 2. Cell proliferation assay.

High passage PEC (p99) show an approximate 2-fold higher rate of growth, as compared to low passage cells (p31), at the 24-hour time point. Rate of proliferation of SAI cells selected from p99 high passage cells was decreased compared to high passage (p99) cells and was only slightly higher than the one displayed by low passage (p31) cells. A similar proliferative trend was observed at the 48-hour time point. * $P < 0.05$ versus p99 high passage cells. All experiments were done in triplicate and data are presented as mean $\pm$ SD.

Figure 3. High passage and SAI-selected PEC exhibit increased motility as compared to low passage PEC.

Low passage (p31; Panel A), mid passage (p54; Panel B), high passage (p137; Panel C) and SAI-selected PEC (Panel D) were seeded into a 96-well plate coated with blue fluorescent microbeads, incubated for 24 hours, fixed and photographed using a SPOT digital camera as described in the Materials and methods section. As individual cells move they push aside the beads and leave visual tracks that are proportional to the magnitude of cell movement. Panel E, quantification of phagokinetic cell tracks using ImageJ software. Data are presented as mean $\pm$ SD values of triplicate experiments with $n = 50$ for each cell type. Average track area made by high passage (Panel C) and SAI-selected PEC (Panel D) were significantly larger as compared to those made by low (Panel A) and mid passage (Panel B) cells (Panel E; * $P < 0.05$).

Figure 4. The antigenic cell surface profile of rat PEC changes with increasing passage number and SAI selection.

Indirect immunofluorescence showed loss of OC.5 and gain of OC.2 expression in SAI cells as compared to low (p26) and high passage (p99) cells. OC.5 was expressed by a large number of low passage cells (A) but was absent in SAI-selected cells (E). Low (B) and high (D) passage cells expressed OC.2 in a low number of cells but 100% of SAI cells were positive for OC.2 (F). The scale bar represents 100 μ m.

Figure 5. Rat PEC SAI-derived cells form larger more aggressive tumors than unselected high passage PEC.

Rat high passage (p93) and SAI-selected prostate epithelial cells (PEC) were tumorigenic when injected into immunodeficient beige/nude/xid mice. Panel A. The high passage-derived tumor shown is representative of tumorigenicity at four weeks (28 days) postinjection of high passage (p93) rat PEC. Panel B. Shown is a representative SAI-derived PEC tumor at three weeks (21 days) post-injection. The SAI-derived tumors showed a shorter latency period than high passage derived tumors. Panel C. The average weight of the excised tumors at the time of sacrifice was 0.22 ± 0.12 grams ($n = 3$, excised at 28 days post-injection) and 0.76 ± 0.32 grams ($n = 5$, excised at 21 day post-injection), for high pass and SAI-derived injected cells, respectively (* $P < 0.05$). In contrast, low passage (p17) PEC did not form tumors by five weeks (35 days) when injected into immunodeficient mice (not shown).

Figure 6. Western blot analysis of signaling proteins.

Whole cell lysates from low passage (p18), high passage (p97) and soft agar invasive cells (SAI) were prepared for the analysis and immunoblotted with anti-EGFR, anti-raf-1, anti-STAT1, anti-pSTAT1 and anti-actin antibodies as reported. Western blot analysis shows that SAI cells express higher levels of EGFR (Panel A) and raf-1 (Panel B) as compared to low and high passage cells. Levels of STAT1 (Panel C) and pSTAT1 (pY701) (Panel D) expression were enhanced in SAI cells. Shown are representative immunoblots for each antibody. All immunoblot experiments were repeated at least three times with similar results.

Table 1.

Differently expressed signaling proteins in high passage PEC when compared to low passage cells identified with Kinexus microarray analysis.

Target protein name	Phospho Site	Full Target Protein Name	Z-ratio (PEC-H, PEC-L)
PDK1	S241	3-Phosphoinositide-dependent protein-serine kinase 1	7,15
PKCz/ι	T410/T412	Protein-serine kinase C zeta/lambda	3,86
EGFR	Y1197	Epidermal growth factor receptor-tyrosine kinase	3,30
p38g MAPK (Erk6)	Pan-spec	Mitogen-activated protein-serine kinase p38 gamma (MAPK12)	3,01
PKA R2a	Pan-spec	cAMP-dependent protein-serine kinase regulatory type 2 subunit alpha	2,62
ASK1 (MAP3K5)	Pan-spec	Apoptosis signal regulating protein-serine kinase	2,58
IGF1R	Pan-spec	Insulin-like growth factor receptor protein-tyrosine kinase	2,55
ErbB2 (HER2)	Pan-spec	ErbB2 (Neu) receptor-tyrosine kinase	2,24
Cyclin D1	Pan-spec	Cyclin D1 (PRAD1)	2,22
MEK2 (MAP2K2)	Pan-spec	MAPK/ERK protein-serine kinase 2 (MKK2)	2,20
PI4KCB	Pan-spec	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	2,06
MEK2 (MAP2K2)	Pan-spec	MAPK/ERK protein-serine kinase 2 (MKK2)	1,97
CDK10	Pan-spec	Cyclin-dependent protein-serine kinase 10 [PISSLRE]	1,84
p21 CDK11	Pan-spec	cyclin-dependent kinase inhibitor 1 (MDA6)	1,67
RSK1/3	T359+S363/ T356+S360	Ribosomal S6 protein-serine kinase 1/3	1,67
p38a MAPK	T180+Y182	Mitogen-activated protein-serine kinase p38 alpha	1,65
PKCz	Pan-spec	Protein-serine kinase C zeta	1,59
CDK6	Pan-spec	Cyclin-dependent protein-serine kinase 6	1,57
Hsp90a/b	Pan-spec	Heat shock 90 kDa protein alpha/beta	-1,54
ROKb (ROCK1)	Pan-spec	RhoA protein-serine kinase beta	-1,57

Table 2.

Differently expressed signaling proteins in soft agar invasive cells when compared to high passage cells identified with Kinexus microarray analysis.

Target protein name	Phospho Site	Full Target Protein Name	Z-ratio (SAI, PEC-H)
FAS	Pan-spec	Tumor necrosis factor superfamily member 6 (Apo1, CD95)	2,26
EGFR	T693	Epidermal growth factor receptor-tyrosine kinase	2,09
pp32	Pan-spec	Acidic leucine-rich nuclear phosphoprotein 32 family member A	1,86
PKCz/1	T410/T412	Protein-serine kinase C zeta/lambda	1,58
CD45	Pan-spec	Leukocyte common antigen CD45 receptor-tyrosine phosphatase (LCA, T200)	1,56
CDK1/2	T161	Cyclin-dependent protein-serine kinase 1/2	1,46
Rb	Pan-spec	Retinoblastoma-associated protein 1	1,40
Erk1 + Erk2	[T202+Y204] + [T185+Y187]	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase) + Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)	1,37
JNK1/2/3	T183 + Y185	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) 1/2/3	1,34
Hsp90a/b	Pan-spec	Heat shock 90 kDa protein alpha/beta	1,29
RIPK1	Pan-spec	Receptor-interacting protein-serine kinase 1	1,18
CDK1/2	Y15	Cyclin-dependent protein-serine kinase 1/2	1,09
Msk1	S376	Mitogen & stress-activated protein-serine kinase 1	1,04
Raf1	Pan-spec	Raf1 proto-oncogene-encoded protein-serine kinase	-1,03
PKBb (Akt2)	Pan-spec	Protein-serine kinase B beta	-1,07
CDK5	Pan-spec	Cyclin-dependent protein-serine kinase 5	-1,18
MEK5 (MAP2K5)	Pan-spec	MAPK/ERK protein-serine kinase 5 (MKK5)	-1,25
PAK1/2/3	T423/402/436	p21-activated kinase 1/2/3 (serine/threonine-protein kinase PAK 1/2/3)	-1,27
ASK1 (MAP3K5)	Pan-spec	Apoptosis signal regulating protein-serine kinase	-1,66
JNK1/2/3	Pan-spec	Jun N-terminus protein-serine kinases (stress-activated protein kinase (SAPK)) 1/2/3	-1,66
CDK10	Pan-spec	Cyclin-dependent protein-serine kinase 10 [PISSLRE]	-1,77
PAK1	Pan-spec	p21-activated kinase 1 (alpha) (serine/threonine-protein kinase PAK 1)	-1,78
PDK1	S241	3-Phosphoinositide-dependent protein-serine kinase 1	-1,80
PI4KCB	Pan-spec	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	-1,89
Cyclin D1	Pan-spec	Cyclin D1 (PRAD1)	-1,95
CDK6	Pan-spec	Cyclin-dependent protein-serine kinase 6	-2,72
CDK8	Pan-spec	Cyclin-dependent protein-serine kinase 8	-3,70