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# Deletion of *Ptp4a3* reduces clonogenicity and tumor-initiation ability of colitis-associated cancer cells in mice

**Julie M. Cramer**[#a,b], **Mark W. Zimmerman**[#c,2], **Tim Thompson**[d], **Gregg E. Homanics**[c,e], **John S. Lazo**[c,3], and **Eric Lagasse**[a,b]

[a]Department of Pathology, 200 Lothrop Street, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15261

[b]McGowan Institute for Regenerative Medicine, 450 Technology Drive, Suite 300, University of Pittsburgh, Pittsburgh, PA, 15219

[c]Department of Pharmacology and Chemical Biology, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, PA, 15260

[d]Department of Bioengineering, 3700 O'Hara St, University of Pittsburgh, Pittsburgh, PA, 15261

[e]Department of Anesthesiology, University of Pittsburgh, 3550 Terrace Street, Pittsburgh, PA, 15261

[#] These authors contributed equally to this work.

## Abstract

The PTP4A3 gene is highly expressed in human colon cancer and often associates with enhanced metastatic potential. Genetic disruption of the mouse *Ptp4a3* gene reduces the frequency of colon tumor formation in mice treated in a colitis-associated cancer model. In the current study, we have examined the role of *Ptp4a3* in the tumor-initiating cell population of mouse colon tumors using an *in vitro* culture system. Tumors generated *in vivo* following AOM/DSS treatment were isolated, dissociated, and expanded on a feeder layer resulting in a $CD133^+$ cell population, which expressed high levels of *Ptp4a3*. Tumor cells deficient for *Ptp4a3* exhibited reduced clonogenicity



**To whom correspondence should be addressed:** Eric Lagasse, PharmD, PhD McGowan Institute for Regenerative Medicine University of Pittsburgh 450 Technology Drive Suite 300 Pittsburgh, PA 15219 Phone: 412-624-5285 Fax: 412-624-5363 lagasse@pitt.edu.
[2]Present address: Department of Pediatric Oncology, 450 Brookline Avenue, Dana-Farber Cancer Institute, Boston, MA, 02215
[3]Present address: Department of Pharmacology, University of Virginia, P.O. Box 800793, Charlottesville, VA, 22908
Julie M. Cramer – juc24@pitt.edu
Mark W. Zimmerman - markw_zimmerman@dfci.harvard.edu
Tim Thompson - tnt15pitt@gmail.com
Gregg E. Homanics - homanicsge@anes.upmc.edu
John S. Lazo - lazo@virginia.edu



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**Authors Contributions** JC and MZ conceived and designed the study, carried out the experiments, interpreted the data, and wrote the manuscript. TT assisted with experiments and data analysis for *in vitro* cell culture. GH, JL and EL assisted with experimental design, data analysis, and writing of the manuscript. All authors read and approved the final manuscript.

and growth potential relative to WT cells as determined by limiting dilution analysis. Importantly, expanded tumor cells from WT mice readily formed secondary tumors when transplanted into nude mice, while tumor cells without *Ptp4a3* expression failed to form secondary tumors and thus were not tumorigenic. These results demonstrate that *Ptp4a3* contributes to the malignant phenotype of tumor-initiating cells and supports its role as a potential therapeutic target to inhibit tumor self-renewal and metastasis.

### Keywords

Phosphatase of Regenerating Liver 3 (PRL-3); *Ptp4a3*; azoxymethane (AOM); colon cancer; tumor-initiating cells; clonogenicity; limiting dilution analysis

## Introduction

Colon cancer is a complex disease in which normal epithelial cells abandon normal proliferative control and become malignant. These tumors comprise many different cell types that feature functional heterogeneity and unique roles in malignancy. The pathogenesis and recurrence of colon cancer following treatment likely depends on a specific subpopulation of tumor-initiating cells present within the tissue capable of self-renewal, multipotency and higher clonogenicity than differentiated cells (1–3). The process of metastasis in particular may depend on the ability of colon tumor-initiating cells to extravasate and clonally expand in secondary locations. This result is the consequence of a combination of genetic aberrations and environmental signals, which are often enhanced by inflammatory conditions such as ulcerative colitis. Although the 5-year survival rate for colorectal cancer is 90% when diagnosed at the local stage, the survival decreases to only 12% if metastases are found (4). Therefore, a better understanding of the genes that contribute to the metastatic process should elucidate opportunities for therapeutic intervention to reduce the lethality of this disease.

Protein tyrosine phosphatase 4A3 (PTP4A3) is highly expressed in human colon tumors and a potential driver of the metastatic cascade (5). We have recently reported that disruption of the mouse *Ptp4a3* gene reduces colon carcinogenesis in a colitis-associated cancer model (6), although the manner in which PTP4A3 facilitates the malignant phenotype is not well understood. Several lines of evidence have emerged suggesting that PTP4A3 may have a role in the biology of tumor-initiating cells. We previously reported upregulation of *Ptp4a3* expression in pre-neoplastic colon tissue after acute exposure to azoxymethane (AOM). PTP4A3 has been reported to mediate both p53 and TGFβ signaling which are well described mediators of cell fate and tumorigenesis (7, 8). Additionally, a recent report observed that a PTP4A3 small molecule inhibitor prevents the tumorigenesis of human lung cancer stem cells and sensitizes them to combination chemotherapy (9). Therefore, it is possible that PTP4A3 has an important role in the tumorigenicity of tumor-initiating cells of colon cancer.

In the current study, we investigated the effects of *Ptp4a3* loss on the tumor-initiating cell population of colon tumors from wildtype (WT) or *Ptp4a3*-knockout (KO) mice using an *in vitro* culture system previously used to study human metastatic colon cancer stem cells (10).

The expanded tumor cells were ubiquitously $CD133^+$ and exhibited enhanced clonogenicity at higher passages. Interestingly, expanded cells derived from *Ptp4a3*-KO tumors demonstrated reduced clonogenicity as well as the inability to form secondary tumors compared to WT tumor cells expressing *Ptp4a3*. These findings provide the first strong evidence for the involvement of *Ptp4a3* in mediating the clonogenicity of colon tumor-initiating cells and suggest that this phosphatase could potentially be a target for cancer therapy directed at self-renewal as well as metastasis.

## Methods

### Genetically engineered mice and colitis-associated cancer model

Experimental *Ptp4a3* mutant mice were produced by mating heterozygous breeding pairs and offspring were genotyped by Southern blot analysis. Colon tumors from the colitis-associated cancer model were generated following treatment with AOM/DSS (Dextran Sodium Sulfate) as previously described (6). Briefly, mice were administered a single IP injection of AOM (12.5 mg/kg) followed by a 1 week treatment with DSS (2.5%) in drinking water and 2 weeks of normal water. The DSS cycle was repeated two additional times and mice were sacrificed at 16 weeks after the beginning of treatment. All animal experiments were performed in accordance with the guidelines of the University of Pittsburgh Animal Care and Use Committee.

### Tumor cell culture and expansion

Single tumor cells were isolated from primary tissue and expanded as previously described (10). Briefly, colon tumor tissue (n=4 tumors/genotype) from AOM/DSS treated *Ptp4a3* WT (Floxed/Floxed) and KO (−/−) mice (n=2 mice/genotype) was isolated, pooled and stored in Hank's Balanced Salt Solution (HBSS) on ice containing 10% FBS (Invitrogen). Tumors were transferred to EBSS/10mM EGTA/1% HEPES (Life Technologies, NY/Sigma-Aldrich, MO/Mediatech, VA) and minced into small (<2 mm) pieces. Tissue was then transferred to a tube and incubated for 5 min at room temperature. After an EBSS wash, the tissue was treated three times with a cocktail containing 1 mg/mL collagenase II (Life Technologies, NY) and 20 mg/ml DNase I (Roche, IN) in HBSS/1% HEPES for 20 min. Tissue/cell suspensions were passed through a 100 μm cell strainer (Fisher, PA) to isolate single cells from undigested tissue. Trypsinization for 20 min was then performed to achieve complete dissociation of the tumor tissue. Cells were plated onto a confluent layer of previously irradiated LA7 (ATCC: CRL-2283) cells (feeder layer) at ~80,000 cells/$cm^2$ in DMEM/F12 supplemented with 0.5% FBS, 25 mg/mL gentamicin (Sigma-Aldrich, MO) and 1% Insulin-Transferrin Selenium (ITS) (Mediatech, VA). Initial cultures (P0) were passaged at 2–3 weeks post-plating (70% confluence) by incubating with EBSS/10mM EGTA/1% HEPES followed by 0.25% trypsin/0.1% EDTA. Following initial expansion, cultures were maintained by passaging at ~70% confluence onto new feeder cells. For immunohistochemistry of expanded tumor cells, cells were plated onto 4-well chamber slides (Thermo Scientific, NY). The isolation procedure was performed three separate times for each genotype to eliminate variability associated with individual experiments and tumor samples.

### Fluorescence Activated Cell Sorting and Cell Cycle Analysis

Flow cytometry and Fluorescence Activated Cell Sorting (FACS) were used to assess cell surface marker expression and colony-forming unit (CFU) frequency via Limiting Dilution Analyses (LDA). Single cell suspensions were immunolabeled with Epithelial Cell Adhesion Molecule (EpCAM), CD29, CD104 (Biolegend, CA), CD49f, CD13, CD44, CD81, CD24, CD9, CD54 (BD Bioscience, MA), and/or CD133 (eBiosciences, CA) specific antibodies (200,000 cells/tube) and analyzed on the MACSQuant™ (Miltenyi Biotec, CA) or BD FACSAriaII™ (BD Biosciences, MA). Dead cells were detected with Sytox Blue at 10uM (Life Technologies, NY). Post-acquisition analysis was carried out in FlowJo (http://www.treestar.com). LDAs were performed by sorting 1–1000 $EpCAM^+$ cells/well into respective rows of 96-well plates (Corning, NY) previously seeded with irradiated LA7 feeder cells. The number of wells responding with the formation of one or more colonies was counted at four weeks and input into L-Calc (Stem Cell Technologies, Vancouver, BC, Canada) to determine CFU frequency. The CFU frequency (i.e. 1/935, one cell out of 935) is the prevalence of cells capable of forming colonies in a particular cell population, in this case, $EpCAM^+$ cells of each cell type. To assess their nuclear content, prior to preparing the expanded WT and *Ptp4a3*-KO tumor cells for flow cytometry analysis as outlined above, the cells were incubated with Hoechst 33342 (Life Technologies, NY) for 60 min.

### Growth curve

Two sets of WT and *Ptp4a3*-KO cells were independently compared using the same number of starting cells ($1.2\times10^6$) on day 0 in a 24-well plate seeded with feeder cells. Wells were analyzed by flow cytometry in triplicate on days 1, 4, 8 and 11 to determine the absolute number of $EpCAM^+$ cells in a defined volume using the MACSQuant™ cell analyzer. Triplicates were averaged and plotted against time on a line graph to reflect growth rate.

### Quantitative RT-PCR

Gene expression levels were assayed by quantitative RT-PCR. Total RNA was isolated from tumor cells and tissues with Trizol reagent (Invitrogen) and resuspended in nuclease-free water. First strand synthesis was performed with the iScript cDNA synthesis kit (Bio-rad) using 1 μg of total RNA. Gene expression was assayed with 2X SYBR Green Mastermix (Bio-rad) with real-time detection performed with the iCycler thermocycler (Bio-Rad). PCR was performed by incubating at 95°C for 5 min, followed by 39 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min. The Ct values for determining relative gene expression were normalized to β-*actin* as an endogenous control. The following primer sets were used for each target gene: *Ptp4a3* (F-CGGGATGAAGTACGAGGACG, R-GCGTGTGTGGGTCTTTGAAC), *Muc2* (F-AGTCTGCTCTGTGAAGTGCC, R-GGCAAACACAGTCCTTGCAG), β-*actin* (F-TCATGAAGTGTGACGTTGACATCCGT, R-CCTAGAAGCATTTGCGGTGCACGATG).

### Immunohistochemistry

Tissues were first isolated and rinsed in ice-cold phosphate buffered saline (PBS). Samples were immediately submerged in 10% neutral buffered formalin, incubated at room temperature overnight, and dehydrated through an ethanol gradient. Fixed tissues were then

embedded in paraffin and sectioned onto glass slides. Sections were deparaffinized and rehydrated prior to staining. For chamber slides, slides were rinsed in PBS, fixed in 4% paraformaldehyde, rinsed again and frozen prior to staining. For both tissue and chamber slides, samples were permeabilized with 0.1% Triton X100 (Sigma-Aldrich, MO) for 10 min, blocked with 5% Bovine Serum Albumin (BSA) for 30 min, and stained with hematoxylin & eosin or primary antibody against Mucin2 (MUC2) (Santa Cruz, TX) in PBS containing 0.5% BSA (Fisher, PA) for one hour followed by anti-rabbit Alexa Fluor secondary (Life Technologies, NY) for 30 minutes followed by counterstain with Hoechst 33342. Images were taken using an IX71 inverted microscope (Olympus, PA).

### Subcutaneous tumor growth

Subcutaneous injection of tumor cells allowed assessment of tumorigenic potential after *in vitro* expansion. Expanded tumor cells (passage seven) were washed, trypsinized, resuspended in cold HBSS/Matrigel (BD Biosciences, CA) 1:1 and stored on ice. Nude mice were injected with either $5\times10^5$ (n=3) or $1\times10^6$ (n=3) cultured tumor cells subcutaneously. Mice were injected in both flanks with WT (left) and *Ptp4a3*-KO (right) tumor cells and monitored for tumor formation. Mice were sacrificed at 18 weeks post-injection of cells or the point when tumors reached 20 mm in diameter.

### Statistics

Assays for quantitative RT-PCR and cell cycle analysis were quantified and analyzed statistically by two-tailed T-test, and the growth curve was analyzed by repeated measures ANOVA. In both cases, significance was defined as $p<0.05$.

## Results and Discussion

### In vitro expansion of mouse colon tumor cells

Homozygous loss of *Ptp4a3* resulted in decreased colon tumor frequency when mice were treated in a colitis-associated cancer model (6). Despite decreased tumor frequency, mice without functional PTP4A3 developed tumors that were histologically similar to WT mice (Supplemental Fig. 1a). This observation led to our hypothesis that loss of PTP4A3 likely impairs the malignant phenotype of tumor-initiating cells in the colon. To examine this hypothesis, we applied a primary culture system previously used to study human metastatic colon cancer (10), a novel approach to studying the widely used mouse AOM/DSS colon cancer model. This methodology allowed us to analyze the tumor-initiating cell population of these tissues which are required for tumorigenesis and *in vivo* tumor self-renewal (11). The culture system enriched the population of epithelial tumor cells expressing Epithelial Cell Adhesion Molecule (EpCAM) from ~60% in primary cells to ~94% in expanded cells for both genotypes (Fig. 1a). When plated on the feeder layer, cells from tumors of both genotypes were capable of forming distinct epithelial cell colonies among the feeder layer (Supplemental Fig. 1b, outlined). Adaptation of this culture system for use in a mouse model is an inherently powerful technique owing to the potential for genetic engineering in mice, making it an ideal system for studying the oncogenetic factors that contribute to the properties of colon tumor-initiating cells.

## Expanded WT and Ptp4a3-KO tumor cells have the characteristics of tumor-initiating cells

We then assayed for the presence of cell surface markers on primary and expanded tumor cells. While $EpCAM^+$ primary tumor cells initially exhibited restricted expression of CD133, there was considerable enrichment for $CD133^+$ cells following expansion (Fig. 1b). Previous studies indicate that CD133 is a murine stem cell and cancer stem cell marker (12), which suggests that this culture system enriches for tumor-initiating cells, although some reports suggest it is possible that CD133 marks other cells of the intestine (13). Looking at cells double positive for both EpCAM and CD133, we assayed for the presence of other cell surface markers including CD24 and CD54 (cell adhesion molecules), CD104, CD49f, and CD29 (integrins), CD9, CD81 and CD151 (tetraspanins), as well as CD13 and CD44 (cancer stem cell-associated) (Fig. 1c). No significant differences were observed in the expression of these markers on expanded WT and *Ptp4a3*-KO cells. We observed strong expression of tetraspanins and integrins on all expanded cells. These markers have known functions in proliferation, cell motility, cell death and tumor formation and metastasis. The expression of CD49f and CD29, specifically, demonstrated the presence of additional stem-like markers (14, 15). CD49f has been observed to not only enhance multipotency, but is thought to be involved in metastasis and represents a poor prognostic factor for patients with advanced CRC (15, 16). Interestingly, CD29 has been reported to directly interact with PTP4A3 to promote the motility, invasion and metastasis of colon cancer (17). Additionally, colon tumor-initiating cells that co-express CD133 and CD29 have been shown to exhibit self-renewal potential (18). The mechanism by which PTP4A3 might enhance the self-renewal of tumor cells through CD29 is not clear, but it is known that CD29 together with E-cadherin are involved in cell membrane interactions that affect tumor self-renewal and differentiation in colon cancer, although the mechanism of this activity is not well understood (19). Taken together, the expression pattern of the expanded WT and *Ptp4a3*-KO tumor cells is indicative of tumor-initiating ability.

Furthermore, *Ptp4a3* expression was elevated in both primary WT tumor tissue (6.7-fold) and expanded WT tumor cells (8.7-fold) relative to normal colon cells (Fig. 1d). The expression of *Mucin2* (*Muc2*), a marker of mature goblet cells of the intestine, was decreased in primary tumors relative to normal colon, and below detectable levels in expanded cells (Fig. 1d). Together, these findings suggest that expanded cells exhibited stronger tumor-initiating cell properties compared to the more differentiated cell types often found in primary tumor tissue.

## Ptp4a3-KO tumor-initiating cells have reduced clonogenicity and growth potential but normal cell cycle progression

Limiting dilution analysis (LDA) was used to assess the clonogenic potential of $EpCAM^+$ WT and *Ptp4a3*-KO tumor cell populations. LDA is a quantitative measurement used to determine the colony-forming unit (CFU) frequency of a specific cell population, which is associated with stem cell activity and may influence tumor formation (20) and/or metastatic potential (21, 22). When primary $EpCAM^+$ tumor cells were sorted in limiting dilution (P0), WT tumor cells exhibited a higher CFU frequency than *Ptp4a3*-KO tumor cells, indicating a self-renewal advantage associated with expression of PTP4A3 (Table 1). Subsequently, expanded tumor cells deficient for PTP4A3 maintained reduced colony-forming ability

relative to expanded WT tumor cells at each passage. As a result, when tumor cells were visualized after plating, there were more colonies present in WT relative to *Ptp4a3*-KO tumor cell cultures (Fig. 2a). Higher magnification and staining for $EpCAM^+$ tumor cells confirmed the ability to distinguish tumor cell colonies from the $EpCAM^-$ feeder layer background (Fig. 2b). We also observed an increase in CFU frequency in both genotypes at subsequent passages, indicating that the culture conditions enriched for cells exhibiting the tumor-initiating cell characteristic of self-renewal while also maintaining reduced self-renewal in cells deficient for *Ptp4a3* (Table 1). The standard error (SE) CFU Frequency Range gives a slight indication that the difference in CFU frequency between WT and *Ptp4a3*-KO begins to diminish at 6 passages. Although a much higher number of experiments would need to be performed to confirm or deny this, it could be the result of consistent enrichment in both genotypes, and would suggest that other factors, in addition to *Ptp4a3*, play a role in the ability to form colonies. This phenomenon could also suggest that *in vivo* extrinsic factors, in combination with intrinsic factors, result in the low frequency of primary tumors observed in *Ptp4a3*-KO mice compared to WT mice.

Next, growth curve analysis was performed to quantitatively measure the growth of WT and *Ptp4a3*-KO tumor-initiating cells. After similar determination of the total number of cells at day 1, significantly higher numbers of $EpCAM^+$ cells were observed in WT relative to *Ptp4a3*-KO samples at all subsequent time points (Fig. 2c). Since PTP4A3 has previously been implicated in cell cycle progression (23), we examined the cell cycle distribution of the expanded tumor-initiating cells. Interestingly, we did not observe a significant difference in the percentage of cells in G1, S and G2 phases when comparing WT and *Ptp4a3*-KO tumor-initiating cells (Fig. 2d). Additionally, no difference in apoptosis was observed between WT and *Ptp4a3*-KO tumor-initiating cells (data not shown). Unimpaired cell cycle progression in this model suggests that while overall cell numbers are decreased in the *Ptp4a3*-KO population, this is likely the result of reduced clonogenic and self-renewal potential rather than a direct effect on tumor cell proliferation. This finding also supports our previous observations that although *Ptp4a3*-KO mice exhibit fewer colon tumors, they are similar in size to WT tumors, suggesting that the *in vivo* role of PTP4A3 is in mediating tumor-initiation properties rather than tumor cell proliferation.

## WT tumor-initiating cells retain multipotentiality and tumorigenicity while Ptp4a3-KO tumor-initiating cells are not tumorigenic

We further examined the multipotency of expanded tumor-initiating cells by immunofluorescent staining for MUC2. As expected, a reduction in goblet cell differentiation was observed when comparing normal colon epithelium to primary tumor tissue demonstrating the presence of limited differentiation normally associated with colon tumors (Fig. 3a). Furthermore, MUC2 staining was undetectable in expanded tumor-initiating cell cultures, suggesting that the culture system maintains expanded cells in a progenitor state, limiting differentiation (Fig. 3a). To examine the tumorigenic potential of tumor-initiating cells from WT and *Ptp4a3*-KO tumors, cells were injected subcutaneously into the flank of nude mice (Fig. 3b). Following transplant of expanded WT tumor cells, 5 out of 6 mice formed secondary tumors (Supplemental Table 1). The same mice were injected in the opposite flank with expanded *Ptp4a3*-KO tumor cells, resulting in no

detectable tumors at those sites (Supplemental Table 1). Pathological examination of WT secondary tumors showed highly similar tissue structure when comparing them to primary AOM-derived colon tumors in mice (Fig. 3c). Furthermore, upon immunofluorescent staining, MUC2 expression in WT secondary tumors resembled that of primary tumors suggesting that expanded WT tumor cells retained the multipotency inherent to tumor-initiating cells of the colon (Fig. 3c). This indicates that while both WT and *Ptp4a3*-KO cells share characteristics of tumor-initiating cells *in vitro*, a diminished self-renewal phenotype in cells deficient for *Ptp4a3* resulted in a loss of ability to form secondary tumors *in vivo*.

### Conclusions

In this report we have adapted a powerful technique used to study human solid tumor-initiating cells for use in a mouse model system. Using a clonogenic assay, we showed that the colony forming activity of both primary and expanded *Ptp4a3*-KO tumor cells is lower than their WT tumor cell counterparts. Colonies are formed by cells with self-renewal potential, therefore, the decrease in frequency of colony-forming tumor cells from *Ptp4a3*-KO mice is likely due to a defect in self-renewal potential. Furthermore, previous reports have demonstrated that increased CFU frequency is associated with tumorigenicity and metastatic potential (20, 21). While we observed that both expanded WT and *Ptp4a3*-KO tumor cells shared the characteristics of tumor-initiating cells, with nearly identical expression of cell surface markers assayed, *Ptp4a3*-KO tumor cells were significantly deficient in clonogenicity and tumorigenesis.

Our data strongly and logically suggest a decreased self-renewal potential in *Ptp4a3*-KO tumor-initiating cells and, in consequence, an inability to form secondary tumors *in vivo*. The process of tumorigenesis after carcinogen treatment is exceedingly complex and undoubtedly includes the role of stromal cells, including endothelial cells, which are known to express *Ptp4a3*. However, after identical treatment *in vitro*, only WT expanded tumor cells and not *Ptp4a3*-KO expanded tumor cells, were tumorigenic after secondary transplant, suggesting that *Ptp4a3* has an important intrinsic function in tumor cells that is necessary for optimal tumorigenicity. This indicates that loss of *Ptp4a3* may result in a defect related to the inherent self-renewal potential of tumor-initiating cells. This finding may further explain why *Ptp4a3* deficient mice exhibit less colon tumors in the AOM/DSS model of colon cancer and how PTP4A3 may be involved in the metastasis of human colon tumors. The metastatic cascade is dependent on many biological processes and the findings presented here indicate for the first time that PTP4A3 may contribute to metastasis by enhancing the clonogenicity and self-renewal properties of tumor-initiating cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **(PTP4A3)** | Protein tyrosine phosphatase 4a3 |
| **(AOM)** | Azoxymethane |
| **(DSS)** | Dextran Sodium Sulfate |
| **(CFU)** | Colony Forming Unit |
| **(LDA)** | Limiting Dilution Analysis |
| **(FACS)** | Fluorescence Activated Cell Sorting |
| **(MUC2)** | Mucin2 |
| **(WT)** | Wildtype |
| **(KO)** | Knockout |
| **(EpCAM)** | Epithelial Cell Adhesion Molecule |

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## Highlights

- Primary mouse colon tumor epithelial cells from the AOM model can be studied by *in vitro* expansion
- Expanded cells express surface markers associated with stemness, proliferation and cancer
- *Ptp4a3* deficient tumor cells exhibit reduced clonogenicity and self-renewal relative to WT cells
- Loss of *Ptp4a3* completely ablates the potential of expanded cells to form secondary tumors *in vivo*
- We report a novel link between *Ptp4a3* and the self-renewal potential of tumor-initiating cells

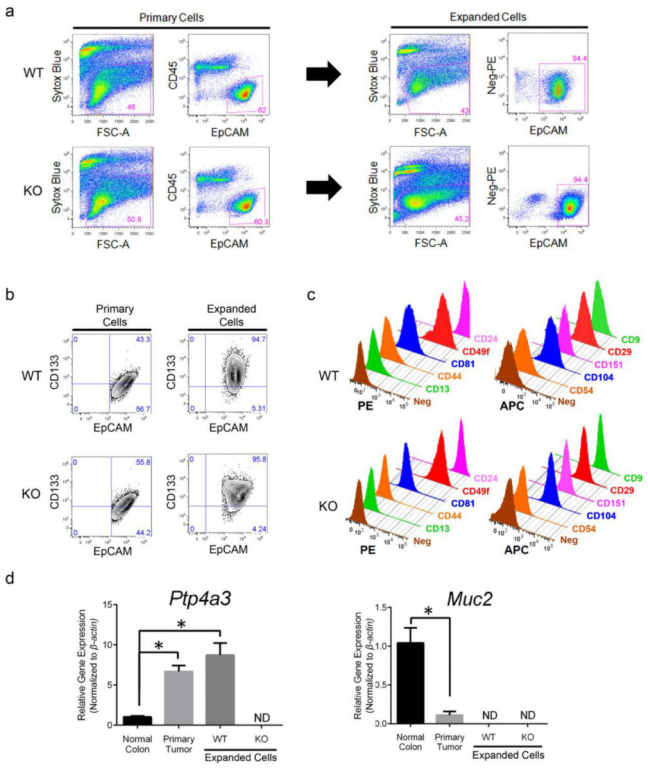


**Figure 1. Isolation and expansion of primary mouse epithelial cells from AOM/DSS-induced tumors**

**(a)** Flow cytometric analysis of primary colon tumor cells freshly isolated from WT and *Ptp4a3*-KO mice (left four plots). Gating strategy used to identify live cells (Sytox Blue$^{-}$) from total cells. Gating of EpCAM$^{+}$ cell population from live cells, excluding blood cells (CD45$^{+}$). Bulk primary WT and *Ptp4a3*-KO tumor cells were expanded in the feeder layer system and analyzed for expression of EpCAM (right four plots). Gating strategy used to identify live cells (Sytox Blue$^{-}$) from total cells. Gating of EpCAM$^{+}$ cell population from live cells demonstrates a similar expansion of epithelial cells from 62% (WT) and 60.3% (*Ptp4a3*-KO) of live cells in primary to 94.4% (WT) and 94.4% (*Ptp4a3*-KO) in expanded tumor cells (EpCAM$^{+}$), excluding feeder layer (EpCAM$^{-}$). **(b)** Representative contour plots (5% probability) of primary and expanded tumor cells immunolabeled with CD133 and EpCAM, stained with Sytox Blue and analyzed by flow cytometry. Plots display percent of live cells in quadrants set to unstained control. **(c)** Representative histograms of expanded WT (top) and *Ptp4a3*-KO (bottom) tumor cells stained for various surface markers and analyzed by flow cytometry. Brown curves represent unstained cells in all four plots and serves as a negative control. Analyses were performed on live, EpCAM$^{+}$, CD133$^{+}$ cells stained with additional PE and APC antibodies presented in two plots for WT and *Ptp4a3*-KO. Within each cell type, the same color curves denote antibodies that were co-stained (ex: pink curves, CD24-PE and CD151-APC). **(d)** Quantitative RT-PCR analysis of *Ptp4a3* and *Muc2* gene expression in primary WT tumors and expanded WT and *Ptp4a3*-KO tumor cells relative to normal colon tissue (*p<0.05).

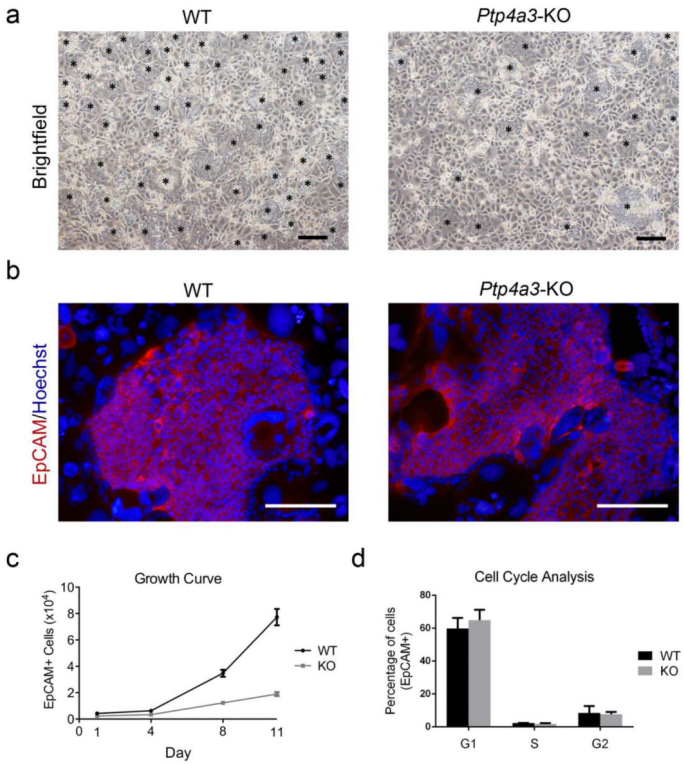


**Figure 2. PTP4A3 knockout impairs growth without significantly effecting cell cycle progression**

**(a)** Representative images of expanded WT and *Ptp4a3*-KO tumor cells at seven days after plating, with tumor cell colonies indicated with asterisks (scale bar = 200μm). **(b)** $EpCAM^+$ (red) colonies of expanded tumor cells on feeder layer confirm identification of tumor cell colonies versus $EpCAM^-$ feeder cells in both WT and *Ptp4a3*-KO cultures. Counterstain Hoechst 33342 (bar = 100μm). **(c)** Growth curve analyses were performed by plating $1.2\times10^6$ cells and measuring the number of $EpCAM^+$ cells by flow cytometry on days 1, 4, 8 and 11. Significantly higher cell counts were observed in WT cultures relative to *Ptp4a3*-KO cells at each time point assayed ($p<0.0001$). **(d)** Cell cycle distribution was determined by Hoechst 33342 staining and flow cytometry for each of n=3 sets of WT and *Ptp4a3*-KO cells (passages 1, 3 and 4) following expansion. The percentage of G1, S and G2 phase cells was not statistically different between genotypes ($p>0.05$).

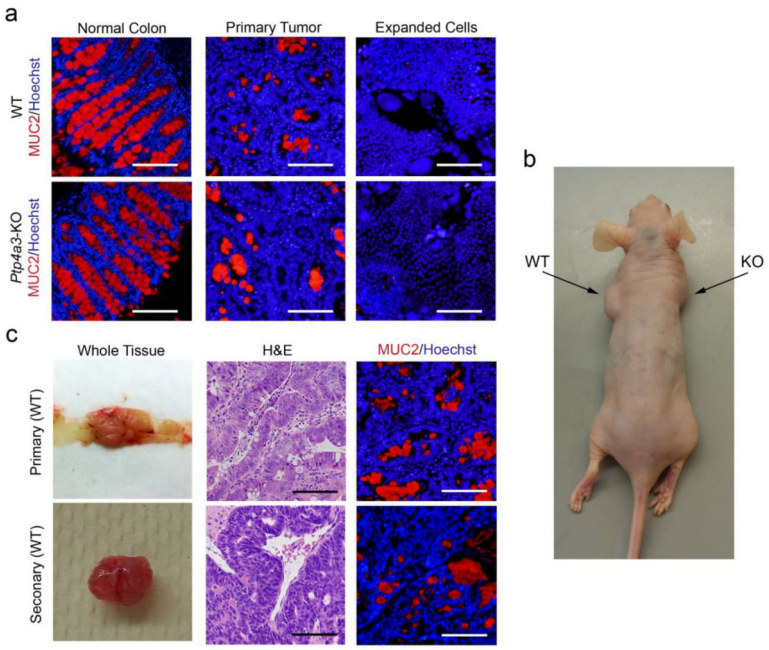


**Figure 3. Culture conditions limited differentiation of expanded tumor-initiating cells while maintaining tumorigenicity and multipotency of WT tumor cells**

**(a)** Immunofluorescent staining of normal colon, primary colon tumors and expanded tumor cells assayed for MUC2 (red) expression and counterstained with Hoechst 33342 (bar = 100μm). **(b)** Representative image of a secondary tumor-bearing nude mouse 16 weeks after injection of tumor cells after seven passages *in vitro*. WT expanded cells were transplanted into the left flank and *Ptp4a3*-KO cells were transplanted to the right flank. **(c)** Comparison of primary and secondary WT tumor tissue revealed similar tissue structure and composition with sections stained with H&E and immunostained for MUC2 (red) (bar = 100μm).

## Table 1

**Ptp4a3-KO tumor cells exhibit decreased CFU compared to WT tumor cells**

Limiting dilution analysis was performed on $EpCAM^+$ cells of both genotypes at the indicated passages. The colony forming unit (CFU) frequency is the prevalence of cells capable of forming colonies in a particular cell population, represented as a ratio. Standard Error (SE) CFU Frequency Range represents the upper and lower range predicted by standard error determination.

| Genotype | Passage | Sorted Population | CFU | SE CFU Frequency Range |
|---|---|---|---|---|
| WT | 0 | $EpCAM^+$ | 1/935 | 1/1153 – 1/758 |
| KO | 0 | $EpCAM^+$ | 1/4729 | 1/6003 – 1/3726 |
| WT | 3 | $EpCAM^+$ | 1/86 | 1/111 – 1/67 |
| KO | 3 | $EpCAM^+$ | 1/447 | 1/550 – 1/363 |
| WT | 6 | $EpCAM^+$ | 1/9 | 1/12 – 1/6 |
| KO | 6 | $EpCAM^+$ | 1/31 | 1/39 – 1/25 |