University of Nevada, Reno

Stem-Cell Like PDGFRα Cells are Induced in Chronic Intestinal Partial Obstruction

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by

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Abstract

The gastrointestinal tract harbors a wide array of smooth muscle cells, motor neurons, and epithelial and stem cells. This makes it an attractive model system for the study of regeneration following mechanical injury. This project investigated the regenerative capacity of the gut by injuring the murine gastrointestinal tract and characterizing the changes that occur in several cell populations critical to gastroenterological function. To accomplish this, a chronic partial intestinal obstruction surgery was used to stimulate intestinal tissue, and changes to cellular phenotype and behavior were characterized using histology and immunofluorescence. We found that the injury induced smooth muscle hypertrophy, which involved the proliferation of $CD34^{+}/CD29^{-}/CD45^{+}$ cells. Moreover, mature smooth muscle cells dedifferentiated into a proliferative, $PDGFR\alpha^{+}$ phenotype under hypertrophic and cultured conditions. Future directions will evaluate the potential of these cells for use in cell therapy, and will translate these findings to analagous aganglionic human disorders.

Acknowledgements

The completion of my thesis would not have been possible without the passionate mentorship of my supervisor, Seungil Ro, PhD. In addition to phenomenal technical support, he has provided me with many life lessons I will take as I progress into the next phase of my career.

I would also like to thank my various postdoctoral mentors. Paul Park, MD, PhD undertook the gallant chandelle of introducing me to laboratory work when I was new to science; his sense of patience and desire to see students improve are both unparalleled. I am also indebted to Moon Young Lee, MD, PhD, who helped me improve my troubleshooting skills and taught me the importance of carefully managing time.

Additionally, thanks are due to the other full-time members of the Ro laboratory: Chanjae Park, who supported me while troubleshooting antibodies, as well as Robyn Berent, Lisa Wei, Brian Jorgensen, David O'kane, and George Madders, who provided logistical support and comic relief. Finally, I would like to thank the other undergraduates in the Ro laboratory for their collegiate attitudes.

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Introduction

In humans and mice, the gastrointestinal (GI) tract is a smooth muscle-lined organ responsible for breaking down and absorbing nutrients (Sanders et al. 2012). Rhythmic contraction of GI smooth muscle cells (SMC) is necessary for successful elimination of waste, and is coordinated by motor neurons named interstitial cells of Cajal (ICC). In addition to ICC-based electrical signals, GI peristalsis is coordinated by a series of ion channels and SMC mechanosensors. The GI tract also contains a population of platelet derived growth factor receptor alpha (PDGFRα)-expressing cells, which are implicated in anchoring epithelial tissues to their surrounding organs and serve a heretofore-unknown function (Betsholtz et al. 2001).

Due to the intricate relationship between SMC, ICC, and PDGFRα cells, it is unsurprising that many human GI disorders result from dysfunction in these cell types (Grover et al. 2012). One disorder of interest is the chronic partial intestinal obstruction, which results from physical blockage of the gastrointestinal tract and causes hernia, severe pain, and can be fatal if untreated for prolonged periods of time (Agresta et al. 1999). One mouse model of this disorder has shown that GI injury drives ICC to dedifferentiate or transdifferentiate into a SMC-like cell population (Chang et al. 2001). This report suggests that the partial obstruction (PO) prevents maintenance of adult ICC phenotype in a way that interferes with the proper functioning of GI tissue.

To better understand the impact of this ICC-deficient disorder on GI functioning, our group sought to expand the partial obstruction model used by

Chang et al. (2001) with a focus on the behavior of SMC and PDGFRα cells. Using histology and immunofluorescence experiments, we showed that PO reduces the numbers of SMC and ICC and results in a large population of proliferative stem cell-like cells, and that this change appeared to be driven by a hypertrophic or inflammatory mechanism. Fate mapping revealed that some of these proliferative cells were derived from SMC and had been induced to express low levels of PDGFRα. Cell culture demonstrated that SMC could be induced to dedifferentiate into proliferative PDGFRα cells *in vitro*. We screened these proliferative cells for other markers and found that they express a high number of mesenchymal stem cell antigens. We are continuing these immunofluorescence experiments to detect changes in other major GI antigens following injury, to acquire a more comprehensive picture of which cell types are implicated in the injury-response. Taken together, this project has expanded our understanding of SMC biology by uncovering a specific pathway through which mature GI populations respond to mechanical injury.

Moving forwards, we will take data from our laboratory's smooth muscle transcriptome browser (not yet published) to determine which proteins are most likely upregulated during injury. Our preliminary data suggests that proteins altered during injury will be related to ICC functionality, such as thrombospondin 4 (THBS4), cKIT, ANO1, PGP9.5. Since injury results in an increase in the number of proliferative cells, we also predict upregulation of stem cell antigens such as CD29, CD34, and CD45. Finally, we intend to investigate expression of transcription factors

known to induce phenotypic switching, such as MyoD. Changes in the expression or localization of these proteins will allow us to hypothesize a mechanism through which the partial obstruction injury alters intestinal cell phenotype.

Methods

Generation of transgenic mouse lines.

To track the behavior of SMC following dedifferentiation, we aimed to generate a Cre-Stop-Lox mouse line. To this end, we purchased smMHC$^{iCre\text{-}ERT2/+}$ and Rosa26$^{lacZ/lacZ}$ mice from Jackson Laboratories and mated them. At 3 weeks of age, tamoxifen injections were given to induce iCre activity, which permitted lacZ expression in mice that were expressing the smMHC promoter at that time. Tamoxifen injections were performed again at 4 and 6 weeks of age. This enabled generation of mice that permanently expressed lacZ in cells that had previously expressed the smooth muscle myosin heavy chain (smMHC) marker. We are currently optimizing the age at which mice receive tamoxifen injection to ensure that the largest number of smMHC-expressing cells are induced to express lacZ.

Partial obstruction surgery model

Two weeks after receiving their first tamoxifen injection, mice were subject to our partial obstruction surgery, which was adapted from Chang et al. (2001). This protocol currently has IRB approval. Mice were anesthetized using isoflurane, and a synthetic ring was inserted around the distal ileum of the animal's GI tract in a

recovery surgery. Age-matched control mice were given a sham surgery in which the intestines were manipulated using forceps, but no synthetic ring was applied. Mice were sacrificed after two weeks.

Tissue preparation

For tissue extraction, mice were anesthetized using isoflurane and the animal was killed by displacement of the cervical spine. Intestines were fixed in 4% paraformaldehyde/PBS for 80 minutes at 4°C and briefly washed in TBS. To dehydrate the intestines for freezing, the fixed GI was then incubated overnight in 20% sucrose in PBS at room temperature. The intestines were then flash-frozen in a cryosectioning reagent (33% Optimal Cutting Temperature Compound in 20% sucrose in PBS). Flash-freezing was conducted by immersing the tissue in 95% ethanol that had been chilled by liquid nitrogen. Molds were then cryosectioned at negative 20°C to generate slides.

Immunohistochemistry

Cryosectioned slides were air dried for 15 minutes before being washed twice in TBS for 5 minutes. Slides were then immersed in 0.1% Tween-20/TBS before a 15-minute incubation with egg whites to block endogenous avidin. To block nonspecific binding, tissues were incubated with 4% dry milk and 0.01% Tween-20 in TBS. Tissues were then incubated overnight at 4°C with the associated antibody at a concentration specified by the manufacturer of the antibody in 4% dry milk and

0.01% Tween-20 in TBS. The next day, slides were washed three times for one minute each in TBS before being incubated for one hour with secondary and biotin-conjugated antibody at a concentration specified by the manufacturer in 4% dry milk and 0.01% Tween-20 in TBS. Slides were then washed three more times for one minute each in TBS before being incubated with 594-conjugated streptavidin for one hour at a concentration of 1:450 in 4% dry milk and 0.01% Tween-20 in TBS. Slides were then washed five times for one minute each in TBS. After drying for one hour, slides were coverslipped using Invitrogen Prolong DAPI Coverslipping Reagent (Invitrogen). Slides were cured overnight at room temperature and sealed using nail polish purchased at Walmart.

Hematoxylin and Eosin Staining

Slides were washed in deionized water for 1 minute before being treated with hematoxylin for 90 seconds. Slides were then washed a second time in deionized water before being treated with eosin for 30 seconds. Slides were then put through a dehydration gradient of absolute ethanol, 90% ethanol in water, 80% ethanol in water, 70% ethanol in water, and xylene for 10 minutes each. Samples were the air-dried for 10 minutes and coverslipped using Permount (Fisher).

X-gal Staining

Cryosectioned samples were treated with 5-bromo-4-chloro-3-indolyl β-d-galactosidase using the β-gal Staining Kit (Life Technologies). The protocol was

modified so that samples were incubated overnight at 37°C in a humidified chamber before removal of the β-galactosidase reagent. This resulted in stronger LacZ labeling.

Results

Partial Obstruction Surgery Results in Severe Morphological Changes

The partial obstruction surgery resulted in gross distension of the intestines from the duodenum through the cecum (Fig 1a-b). H&E imaging revealed that hypertrophy occurred throughout all layers of the submucosa, mucosa, and muscularis, but was most significant in the circular muscle and serosa (Fig 1c-f).

Hypertrophic Tissue Has Non-Native Origin

X-gal labeling marked both muscularis layers, and light staining was observed in the serosa (Figure 2a-b). Immunofluorescence labeling of LacZ revealed that numerous $LacZ^{+}$ cells were present in the intestinal serosa (Fig 2c). High magnification images of the serosa confirmed that virtually none of the LacZ-expressing cells maintained Myh11 expression (Fig 2d-e). While these LacZ-expressing cells were largely $Ki67^{+}$, there were also a large number of serosal cells determined to be $LacZ^{-}$; $Ki67^{+}$ (Fig 3a).

Partial Obstruction-Induced Cells are Proliferative and PDGFRα$^+$

To establish the current identity of the LacZ$^-$; Ki67$^+$ cells, we generated a PDGFRα-GFP expressing mouse line and subject the animals to partial obstruction surgeries. We found that the majority of the cells in the serosa were PDGFRα$^+$ as indicated by GFP expression; virtually all of these co-expressed Ki67 and CD45 (Fig 3a). We screened these cells for common stem cell markers and found that they are negative for the mesenchymal stem cell marker CD29, but express high levels of the hematopoietic stem cell marker CD34 (Fig 3b-c).

PDGFRα-Expressing Cells can be Converted to Smooth Muscle Cells in Vitro

When PDGFRα-GFP cells were cultured, they proliferated over several days and quickly reached confluence (Fig 4b). While these cells were initially GFP$^-$, they gained expression of this reporter and their morphology slowly became elongated. Conversely, culture of SMC-GFP cells lost GFP expression over the course of time (Fig 4a). The increase in GFP expression and change in shape both indicate the adoption of a differentiated state.

Discussion

Our gross partial obstruction data are consistent with previous findings, and our H&E stains revealed that SMCs are significantly impacted by the injury (Fig 1b). Our immunofluorescence experiments demonstrated that while the cells populating the injured area associated with SMCs, they do not express SMC-specific markers

and have strong proliferative character (Fig 2). These findings indicate that SMCs cannot independently account for the injury-response phenotype.

We performed further immunofluorescence experiments to establish an identity for the injury-associated cells, and found that they express low levels of the protein Platet Derived Growth Factor Receptor Alpha (PDGFRα). The Platelet Derived Growth Factor (PDGF) ligand has long been associated with induction of target cells following mechanical injury, so it is not surprising to see PDGFRα-expressing in the partial obstruction injury (Jawien et al. 1992). Their Ki67 expression strongly suggest that they behave in this context as stem cells which drive a response to the injury (Fig 3).

To further investigate the idea that PDGFRα cells act as stem cells, we isolated them from PDGFRα-GFP mice and cultured them. Their initial expression of Ki67 and high proliferation rate (determined by number of nuclei) when cocultured with smooth muscle growth factors strongly suggests a stem cell identity (Fig 4b). Further, they were able to convert into Myh11 expressing cells over time, indicating a role consistent with that of a smooth muscle stem cell (Fig 4a). The reduction of both Srf and Myh11 expression indicates the loss of mature SMC phenotype, suggesting that the cells have adopted a primitive and proliferative lineage (Fig 4c). This idea is recapitulated by the loss of Myocd and Elk1 transcription, which are both well-known vascular smooth muscle markers.

Taken together, we have expanded on previous literature by identifying a specific class of cell that could drive an intestinal injury-response mechanism.

Further experiments should investigate whether this phenomenon occurs in human intestinal injury.

Figures

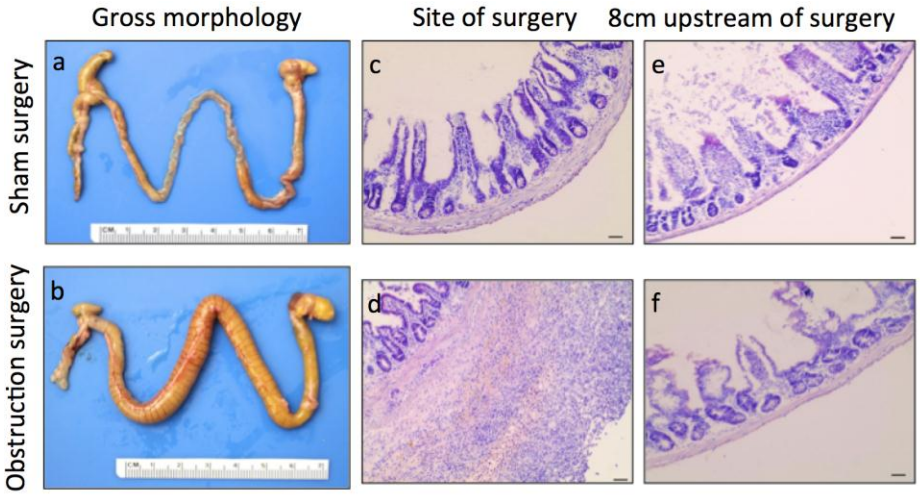

Figure 1. *a-b*, Gross morphology of the small intestines from the stomach to the colon. *c-f*, H&E bright field imaging at 40x; scale bar = 10µm.

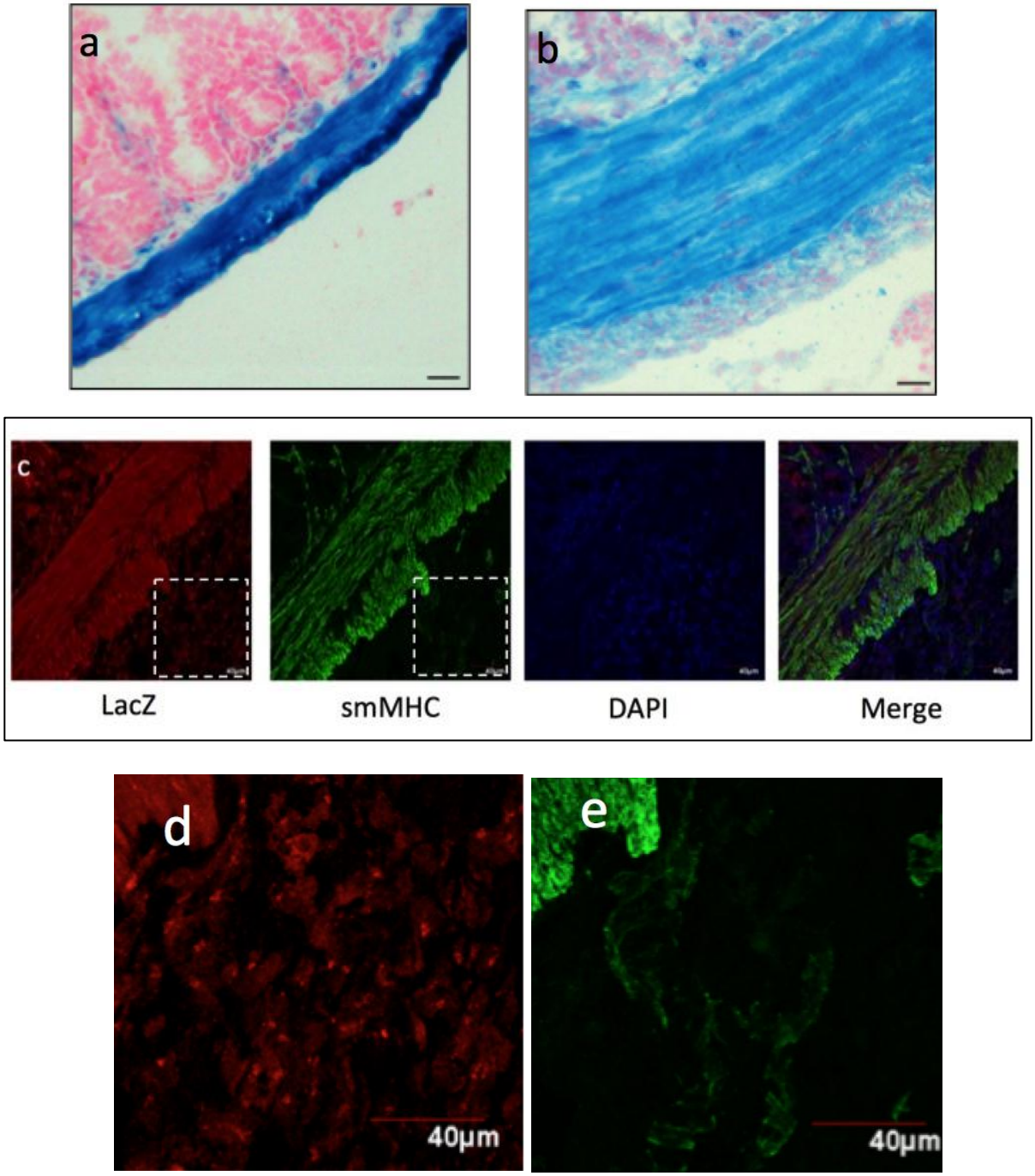

Figure 2. *a-b,* X-gal imaging of wild type (*a*) and partial obstruction (*b*) tissue at 40x; scale bar = 10μm. *c*, Confocal microscopy of partial obstruction tissue at 40x with DAPI and antibodies against LacZ (red) and smMHC (green); scale bar = 40μm. *d-e*, Close-up of serosal tissue for LacZ and smMHC shown in *c*; scale bar = 40μm.

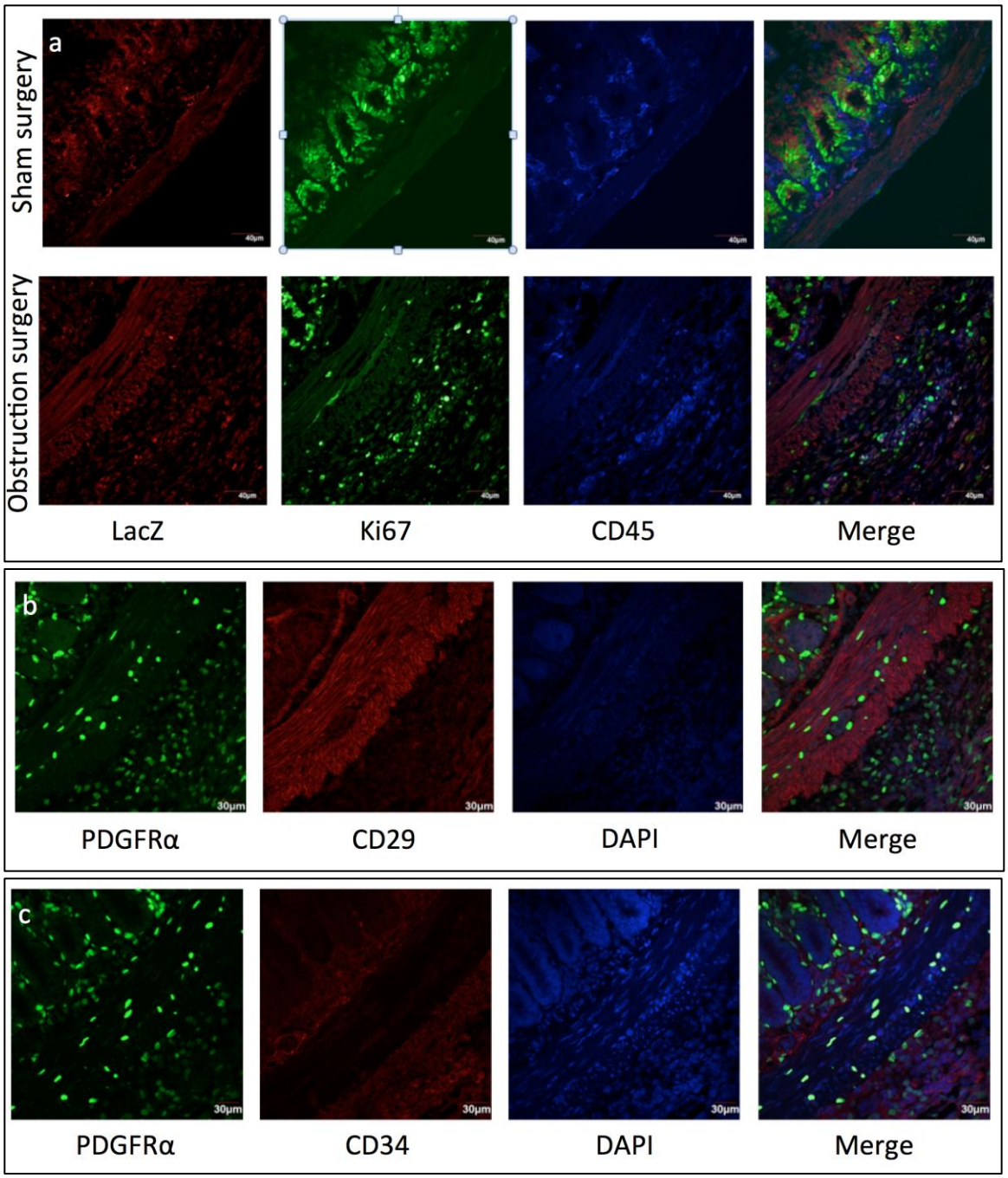

Figure 3. *a*, Confocal microscopy of sham and partial obstruction surgeries at 40x reveals differential LacZ and PDGFRα expression; scale bar = 30μm. *b*, Confocal microscopy at 40x reveals low levels of CD29 expression in partially obstructed serosa; scale bar = 30μm. *c*, Confocal microscopy at 40x reveals elevated levels of CD34 expression in partially obstructed serosa; scale bar = 30μm.

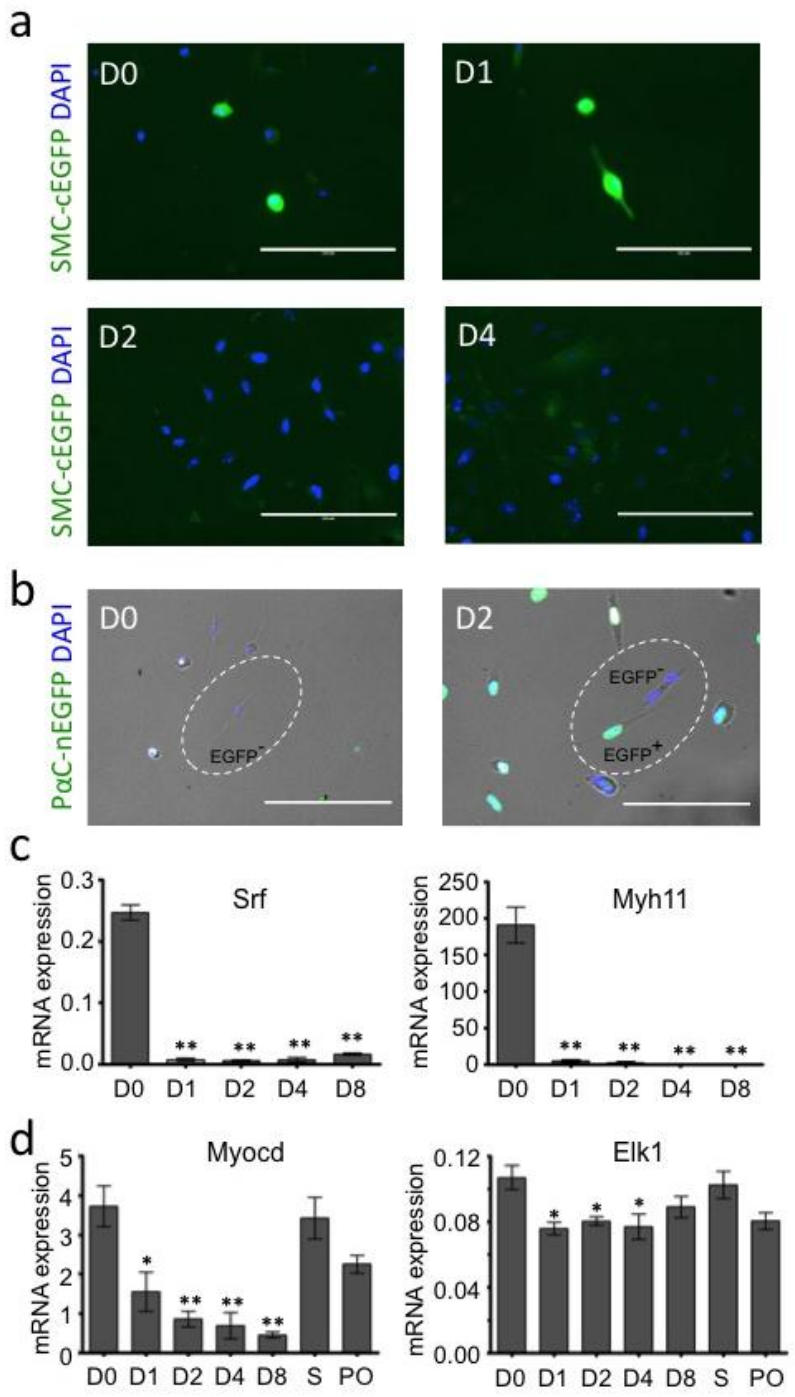

Figure 4. Cultured SMCs adopt stem-cell like, PDGFR-expressing character. *a*, culture of MYH11-GFP cells in smooth muscle growth medium. *b*, culture of PDGFRα-GFP cells in smooth muscle growth medium. *c*, QPCR analysis of Srf and Myh11 transcription levels over different days of SMC culture. *d*, QPCR analysis Myocd and Elk1 transcription levels over different days of culture. S = sham, PO = partial obstruction.

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