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Differential expression of genes related to purinergic signaling in smooth muscle cells, PDGFRα-positive cells and interstitial cells of Cajal in the murine colon

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Abstract

Background—Purinergic signaling provides regulation of colonic motility. Smooth muscle cells (**S**MC), interstitial cells of Cajal (**I**CC) and platelet-derived growth factor receptor α–positive (**P**DGFRα$^+$) cells are electrically coupled and form a functional (SIP) syncytium that constitutes the receptive field for motor neurotransmitters in the *tunica muscularis*. Each cell type in the SIP syncytium has specialized functions in mediating motor neurotransmission. We compared gene transcripts for purinergic receptors and membrane-bound enzymes for purine degradation expressed by each cell type of the SIP syncytium.

Methods—Fluorescence-activated cell sorting (FACS) was used to purify SMC, ICC and PDGFRα$^+$ cells from mixed cell populations of colonic muscles dispersed from reporter strains of mice with constitutive expression of green fluorescent proteins. Differential expression of functional groups of genes related to purinergic signaling was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Key Results—We detected marked phenotypic differences between SMC, ICC and PDGFRα$^+$ cells. Substantial numbers of genes of importance in purinergic neurotransmission were enriched in PDGFRα$^+$ cells in relation to SMC and ICC. Notably, genes related to mediating effects and extracellular biotransformation of enteric purinergic inhibitory neurotransmitters were strongly expressed by PDGFRα$^+$ cells.

Conclusions & Inferences—Our results demonstrate differential expression of genes for proteins involved in purinergic signaling in the SIP syncytium. These results may further clarify the specific functions of each cell type, identify novel biomarkers for postjunctional cells and provide hypotheses for further studies to understand the physiological roles of cells of the SIP syncytium.

Keywords

purine genes; interstitial cells; PDGFRα; colon; ATP; NAD

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DISCLOSURE
No competing interests declared.

AUTHOR CONTRIBUTION
LP performed the qRT-PCR analysis, analyzed results and participated in drafting the paper. KMS and VMY designed the experiments, interpreted results and wrote the paper. All work was performed in the Department of Physiology and Cell Biology of the University of Nevada School of Medicine.

INTRODUCTION

Extracellular purine, pyridine and pyrimidine substances (also referred to as extracellular nucleotides and nucleosides) are known to elicit cell surface receptor-mediated signals in mammalian cells and to function as neurotransmitters, neuromodulators and autocrine and paracrine mediators[1–4]. In the distal gastrointestinal (GI) tract, including the colon, purinergic signaling regulates excitability and motility, and adenosine 5′-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+) and adenosine 5′-diphosphate ribose (ADPR) have been proposed to be neurotransmitters[5–9]. Extracellular nucleotides are rapidly metabolized by cell surface enzymes to biologically active and inactive metabolites[10]. In some cases a substance can be produced from multiple extracellular substrates. For example, adenosine 5′-monophosphate (AMP) and adenosine can be formed by multiple-step enzymatic degradation of either ATP, NAD^+ or ADPR[8,10]. The biological effects of extracellular nucleotides and their metabolites are mediated by P1 and P2 purine receptors on effector cells, that are selectively activated by adenosine (P1 receptors) or adenine and pyrimidine nucleotides (P2 receptors). Human diseases of the GI tract display distinct purine gene dysregulation profiles[11], and such changes can result in functional effects but also provide novel pathways for diagnostics and possible therapeutics.

Smooth muscle cells (SMC) are traditionally considered to be the primary effectors in neuromuscular transmission in visceral smooth muscles[12]. In the GI tract however, SMC do not appear to be exclusive or even suitable targets for neurogenic purines, because these compounds elicit predominantly small inward currents in GI SMC[7]. This response contrasts with the $P2Y_1$ receptor-mediated activation of robust outward K^+ currents and hyperpolarization in intact colonic muscles in response to purine neurotransmitter(s). The outward current is generated by activation of small-conductance Ca^{2+}-activated K^+ channels (SK3) and possibly additional K^+ conductances[5,7,13]. Recently, a new class of interstitial cells, immunoreactive for platelet-derived growth factor receptor-α (PDGFRα$^+$ cells), have been shown to respond to extracellular purines by binding of $P2Y_1$ receptors and activation of SK3 currents[14].

Morphological studies show that varicose enteric nerve fibers are closely associated with PDGFRα$^+$ cells and interstitial cells of Cajal (ICC), and both classes of interstitial cells form gap-junctions with SMC[15,16]. Myogenic regulation of motility and post-junctional neurogenic responses result from the integrated activity of the SMC/ICC/PDGFRα$^+$ cell (SIP) syncytium[17]. However, a detailed description of the role of each cell type of the SIP syncytium in extracellular purinergic signaling is not yet available. The topology of the various purinergic components at the cell surface not only organizes the signal transduction machinery, but also controls the final cellular response. One way to discover the potential cellular targets of extracellular nucleotides is to compare cell-specific expression profiles of genes or proteins involved in signaling and metabolism. In the present study we have isolated cells from strains of mice with cell-specific expression of fluorescent reporters in SMC, ICC, and PDGFRα$^+$ cells. The cells were purified by fluorescence activated cell sorting (FACS), and comparisons of the purine effectorsomes (i.e. P1 and P2 purinergic receptors and membrane-bound (ecto-) enzymes that degrade extracellular nucleotides) were made using quantitative PCR.

MATERIALS AND METHODS

Mice and tissue preparation

Transgenic mice with green fluorescence proteins (eGFP or copGFP) tagged to cell-specific promoters (e.g. smMHC for SMC, Kit for ICC and PDGFRα for PDGFRα$^+$ cells) were used to isolate each cell type and determine purine gene expression. C57BL/6 mice and

Pdgfra$^{tm11(EGFP)Sor}$/J heterozygote mice, 3–6 weeks of age (both from Jackson Laboratory, Bar Harbor, ME, USA), smMHC/Cre/eGFP mice, 3–6 weeks of age (donated by Dr. Michael Kotlikoff, Cornell University), and Kit$^{+/copGFP}$, 3–6 weeks of age[18] were anaesthetized with isoflurane (AErrane; Baxter, Deerfield, IL, USA) and killed by cervical dislocation. After longitudinal laparotomy the colons were removed and opened along the mesentery and rinsed free of content with ice-cold Krebs-Ringer solution (mM): NaCl 118.5; KCl 4.5; $MgCl_2$ 1.2; $NaHCO_3$ 23.8; KH_2PO_4 1.2; dextrose 11.0; $CaCl_2$ 2.4. The mucosa and submucosa were peeled away and cells were isolated from *tunica muscularis* of the entire colon. Mice were maintained and experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* All protocols were approved by the Institutional Animal Use and Care Committee (IUCUC) at the University of Nevada.

Tissue dispersion and cell purification

Colonic muscles (25±5 mg tissue weight) were equilibrated in Ca^{2+}-free Hanks' solution and cells were dispersed as described previously[19,18]. eGFP-PDGFRα cells, eGFP-SMC, and CopGFP-ICC were purified by fluorescence-activated cell sorting (FACS) (Becton Dickinson FACSAriaII) using the blue laser (488 nm) and the GFP emission detector (530/30 nm). Expression of genes in each sorted cell type was compared against expression in the total cell population (TCP) of colonic *tunica muscularis*. TCP represents all cells dispersed from *tunica muscularis*. Each experiment with sorted cells was performed with colonic muscles from three mice of each reporter strain. TCP were prepared from 6 mice (2 for each cell specific reporter strain).

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from PDGFRα$^+$ cells, SMC, and ICC using illustra RNAspin Mini RNA Isolation kit (GE Healthcare, Little Chalfont, UK). Concentration and purity of RNA was measured using a ND-1000 Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE), comparative amounts of RNA were used for first-strand cDNA synthesized using SuperScript III (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. PCR was performed with specific primers (Table 1) using Go-Taq Green Master Mix (Promega Corp., Madison, WI, USA). PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. qRT-PCR was performed with the same primers as PCR using Fast Sybr green chemistry (Applied Biosystems, Foster City, CA, USA) on the 7900HT Real Time PCR System (Applied Biosystems).

Statistical analyses

Gene expression was compared between eGFP-SMC, eGFP-PDGFRα$^+$and CopGFP-ICC, and each cell type was also compared with TCP of colonic *tunica muscularis* of corresponding control animals. Regression analysis of the mean values of three multiplex qPCRs for the log10 diluted cDNA was used to generate standard curves. Unknown amounts of messenger RNA (mRNA) were plotted relative to the standard curve for each set of primers and graphically plotted using Microsoft Excel. Primer efficiencies of 90–110% were only accepted for analysis. This gave transcriptional quantification of each gene relative to the endogenous *Gapdh* standard after log transformation of the corresponding raw data. In pilot studies *Gapdh* was tested on all three cell types used in the present study and represents an appropriate control for qPCR analyses. All data were expressed as means ± S.E.M. Student's *t*-test was used where appropriate to evaluate differences in the data, and $P < 0.05$ taken to indicate statistically significant differences.

RESULTS

1. Cell markers in sorted SMC, PDGFRα$^+$ cells and ICC

The qRT-PCR analyses demonstrated that the FACS-sorted cells were highly enriched with cell specific markers: *Kit*, *Pdgfra* and *Myh11* were enriched in sorted CopGFP-ICC, eGFP-PDGFRα$^+$ cells, and eGFP-SMC, respectively. The *Kit*-enriched cells had negligible expression of either *Pdgfra* or *Myh11* whereas the *Pdgfra*-enriched cells had negligible expression of either *Kit* or *Myh11*. Likewise, the *Myh11*-enriched cells showed negligible expression of either *Pdgfra* or *Kit*. Each purified cell population had negligible expression of *Uchl1*, the gene encoding PGP9.5, indicating that the cell populations examined were relatively free of neurons. These data demonstrate the relative purity of the cell populations analyzed by qRT-PCR (Fig. 1).

2. Purinergic Receptors

2.1. P1 Receptors—Among genes encoding the four types of adenosine receptors (P1 receptors) PDGFRα$^+$ cells were enriched with *Adora1* and *Adora3* and transcripts for *Adora2a* and *Adora2b* were not resolved (Fig. 2). Expression of *Adora1* and *Adora3* was stronger in PDGFRα$^+$ cells than in SMC, ICC or TCP. In contrast, *Adora2b* was expressed more in SMC than in ICC or in TCP. *Adora2a* was modestly expressed in SMC and ICC but less than in TCP, suggesting that this receptor is mainly expressed on neurons or other non-SIP cells.

2.2. P2 Purinergic Receptors—All P2X receptor genes were expressed more highly in the PDGFRα cells than SMC. PDGFRα$^+$ cells were enriched (i.e. showed greater expression than in the TCP) with *P2rx1, P2rx3, P2rx4, P2rx5, P2rx6,* and particularly with *P2rx7* (Fig. 3A, Table 2). Therefore, PDGFRα$^+$ cells might be a target for extracellular ATP acting on ionotropic P2X receptors. ICC expressed *P2rx4* and *P2rx6* more than the TCP. ICC also expressed a low level of *P2rx7* that was significantly less than in PDGFRα$^+$ cells or the TCP. Among the genes for P2Y receptors SMC were enriched with *P2yr14* (Fig. 3B, Table 2). The gene for *P2yr14* was also enriched in PDGFRα$^+$ cells, but the expression was lower than in SMC. PDGFRα cells showed higher expression of *P2ry1, P2ry2, P2ry12, and P2ry13,* than TCP or SMC. ICC were enriched with *P2ry2*, *P2ry4*, and *P2ry6* suggesting that these cells might be targeted by extracellular pyrimidine substances rather than purines.

3. Cell surface nucleotide-metabolizing enzymes

Degradation of extracellular ATP and NAD$^+$ is accomplished in multiple steps by several enzymes (Fig. 4). Therefore, we next sought to determine the relative expression in SIP syncytium of key enzymes involved in extracellular purine biotransformation.

3.1. CD39/Ecto-nucleoside triphosphate diphosphohydrolase 1 (*Entpd1*) and CD73/ecto-5′-nucleotidase (*Nt5e*)—*Entpd1*, the gene that encodes CD39, was expressed more in PDGFRα cells than in SMC or ICC (Fig. 5A). In fact, *Entpd1* expression in PDGFRα cells appeared higher than in TCP, but this did not reach statistical significance. The expression of *Entpd1* in SMC was less than in the TCP. ICC showed negligible expression of *Entpd1*. Therefore, PDGFRα$^+$ cells appear to possess the machinery necessary for catabolism of extracellular ATP. We also found that SMC are enriched with *Nt5e*, the gene that encodes the ecto-5′ nucleotidase CD73, whereas PDGFRα$^+$ cells and ICC expressed much less *Nt5e* (Fig. 5A). Therefore, adenosine generated by extracellular nucleotides that are autocrine and paracrine mediators in the vicinity of the SIP syncytium may have more rapid and possibly greater effects on SMC than on other cells.

3.2. NAD glycohydrolases CD38 and CD157/Bst1—Colonic *tunica muscularis* express *Cd38* and *Cd157/Bst1* since both genes were found expressed in TCP (Fig. 5A). None of the cells comprising the SIP syncytium expressed levels of Cd157*/Bst1*. However, *Cd38* was expressed in all three cell types: interestingly, *Cd38* showed stronger expression in PDGFRα$^+$ cells than in the TCP, whereas *Cd38* expression in SMC or ICC was less than in TCP. This might be an important observation considering the neurotransmitter role of NAD^+, reported recently, and the observation that PDGFRα$^+$ cells express the proper machinery required for mediating purinergic motor neurotransmission in the colon[5,7,9]. SMC or ICC, on the other hand, do not appear to express significant amounts of *Cd38*, suggesting that CD38-mediated catabolism of NAD^+ does not primarily occur at the SMC or ICC cell membranes.

3.3. Ecto-nucleotide pyrophosphatase/phosphodiasterases (*Enpp*)—PDGFRα$^+$ cells highly expressed *Ennp1* and *Enpp3* whereas *Enpp2* was not resolved in these cells (Fig. 5B). In contrast, SMC showed strong expression of *Enpp2* and modest expression (less than TCP) of *Enpp1* and *Enpp3* (Fig. 5B). ICC were somewhat enriched with *Enpp1* (but much less than the PDGFRα$^+$ cells), expressed negligible amounts of *Enpp3*, and expressed unresolvable levels of *Enpp2* (Fig. 5B).

3.4. Mono-ADP ribosyl transferases (ARTs, *Art1-5*)—Here we demonstrate that the TCP of the muscularis expresses no *Art1, Art2.1* and *Art2.2,* but *Art3, Art4,* and *Art5* transcripts were resolved (Fig. 5C). PDGFRα$^+$ cells showed strong expression of *Art4,* low expression of *Art3,* and unresolvable expression of other *Art* genes. SMC and ICC showed stronger expression of *Art5* than the TCP. *Art3* expression was greater in SMC than in PDGFRα$^+$ cells and ICC. Therefore, *Art4* appears to be characteristic for PDGFRα$^+$ cells, whereas *Art5* is characteristic for SMC and ICC.

4. Small-conductance Ca^{2+}-activated K^+ channels (SK channels, *Kcnn1-3*)

Consistent with previous reports[14] we confirmed that *Kcnn3* is strongly enriched in PDGFRα$^+$ cells vs. the TCP and, importantly, the expression of *Kcnn3* in these cells far exceeded the expression of *Kcnn3* in either SMC or ICC. Other members of the *Kcnn* family of genes were expressed at very low levels in cells of the SIP syncytium. Therefore, PDGFRα$^+$ cells are distinguished as the cells in the SIP syncytium that have an enriched transcriptional and protein expression of *Kcnn3*[14, 20]. *Kcnn2* and *Kcnn4* showed relatively low expression in all SIP cells (Fig. 6), and *Kcnn1* (which encodes SK1 channels that are less sensitive to apamin than SK2 and SK3) was more highly expressed in SMC than in other SIP cells.

DISCUSSION

In this study we compared relative levels of gene transcripts that encode proteins involved in extracellular purinergic signaling in the 3 cell types of the SIP syncytium in murine colonic *tunica muscularis*. Each cell type displayed specific profiles of gene transcripts, suggesting cell-specific functions within the SIP syncytium in terms of mediating the effects of purinergic neurotransmitters and paracrine mediators and in achieving deactivation of these signals.

Adora1,2a,2b,3

In the GI tract, adenosine is involved in the regulation of gut sensory and secretomotor functions[21], in suppression of enteric nervous system excitability[22,23], and in immune and inflammatory responses[11,22–24]. Thus, knowing the preferential distribution of adenosine receptor genes in the functional SIP syncytium might facilitate identification of novel

therapeutic targets for adenosine receptor agonists and antagonists. SMC were enriched with *Adora2b*, whereas PDGFRα$^+$ cells were enriched with *Adora1* and *Adora3*, but lacked *Adora2a* or *Adora2b. Adora2a* was expressed in colon muscularis, but apparently in cell types different from those of the SIP syncytium. Therefore, we demonstrate here the molecular basis for differential effects of adenosine on cells in the SIP syncytium, and these will need further characterization by physiological experiments.

P2rx1-7 and P2ry1,2,4,6,12-14

$P2X_{1-7}$ receptors are ligand-gated ion channels that are activated by ATP25, whereas P2Y receptors are G-protein coupled receptors that are activated by adenine, pyridine and pyrimidine nucleotides[26]. NAD$^+$ can activate $P2X_7$ receptor through mono-ADP-ribosylation on the cell membrane[27]. NAD+5,7 and ADPR[8,28,29] are also ligands for the $P2Y_1$ receptor. The latter findings may be particularly important for the functions of the GI tract because the effects of the enteric purinergic inhibitory neurotransmitter(s) appear to be mediated exclusively by $P2Y_1$ receptors (coupled to inhibitory responses mainly through SK3 channels) and NAD$^+$ and ADPR, but not ATP, mimic the enteric inhibitory purinergic neurotransmitter[5,7,8,13,29]. Therefore, the observation that *P2ry1* was far more highly expressed by PDGFRα$^+$ cells than by SMC, ICC or TCP is of importance for the integrated response of the SIP syncytium to purinergic neural regulation. The present study supports the notion that SMC is not the primary target for the purinergic inhibitory motor neurotransmitter in the colon. In fact, our data confirm previous findings[14,30,31] that PDGFRα$^+$ cells have the molecular machinery (i.e., $P2Y_1$ receptors and SK3 channels) to receive and transduce neurotransmitters released from enteric inhibitory nerve terminals. ICC may mediate effects of extracellular UTP and UDP and derivatives since ICC demonstrated expression of *P2ry2*, *P2ry4* and *P2ry6*. PDGFRα$^+$ cells also expressed more *P2ry2* and *P2ry4* than SMC. SMC and PDGFRα$^+$ cells might be targets for UDP-sugars[26] as the two cell types expressed *P2ry14*.

Entpd1 and Nt5e

Extracellular nucleotide metabolism can regulate ligand concentrations at receptor sites, limit membrane receptor desensitization, terminate receptor activation by agonists, or generate intermediate products with similar or different signaling properties than the released nucleotide[32]. ENTPDase1 (CD39) is the primary ecto-nucleotidase involved in the degradation of extracellular ATP. It is anchored to cell membranes[32] and this restricts the effectiveness of this enzyme to cell-surface localized catalysis. Genetic polymorphisms of CD39 have been linked to Crohn's disease[33] and CD39 gene deletion in mice exacerbates dextran sodium sulphate-induced colitis[34]. In the present study we found that *Entpd1* was expressed more in PDGFRα $^+$cells than in SMC or ICC or TCP, suggesting that ATP added to or released in muscles can result in production of ADP and AMP at the surface of PDGFRα $^+$cells.

Ecto-5′-nucleotidase (CD73/*Nt5e*) is a membrane-bound glycoprotein that catalyzes the extracellular dephosphorylation of AMP to adenosine[35,36]. CD73-mediated hydrolysis of AMP to adenosine is a step in the degradation not only of ATP (via formation of ADP and AMP), but also of NAD$^+$ (via formation of ADPR and AMP)[32]. Human CD73 is also an orthologue of a bacterial enzyme that recognizes NAD$^+$ and nicotinamide mononucleotide (NMN) as substrates[37]. The physiological relevance of these catalytic activities of CD73 in the GI tract needs further investigation. The acute phase response in Crohn's disease and ulcerative colitis is associated with *Nt5e* gene dysregulation in mucosal biopsies[11] but the expression of this gene in muscle layers has not been determined. *Nt5e* was more highly expressed by SMC than by ICC or PDGFRα$^+$ cells, and therefore, adenosine would be formed primarily near SMC receptors.

Cd38 and Bst1/Cd157

CD38 is a type II transmembrane protein that degrades NAD^+ to cyclic ADP-ribose via its ADP-ribosyl cyclase activity and NAD^+ to ADPR and nicotinamide via its NAD^+ hydrolase activity[38]. Its role in the colon has not been determined. In colonic muscles isolated from $CD38^{-/-}$ mice we found that the effects of the endogenous purine neurotransmitter and the degradation of NAD^+, were intact[8], suggesting that either other enzymes that degrade NAD^+ were overexpressed in $CD38^{-/-}$mice, or other enzymes are normally responsible for degradation of NAD^+. CD157 is the only other enzyme in mammals that shares homology with CD38 and NAD-glycohydrolase and cyclase activities. In the present study, expression of *Bst1/Cd157* was not resolved in any of the cells in the SIP syncytium, although expression of this gene was noted in the TCP. However, *Cd38* was expressed in all three cell types. It is important to note that $PDGFR\alpha^+$ cells showed the strongest expression of *Cd38*, suggesting that the first steps in the degradation of NAD^+ that is released from motor nerve terminals may occur at $PDGFR\alpha^+$ cells.

Enpp1-3

Three of the seven members of the ecto-nucleotide pyrophosphatase/phosphodiasterase (E-NPP) family -ENPP1, ENPP2, and ENPP3 – are known to hydrolyze nucleotides[39]. NPPs hydrolize ATP to ADP and AMP as well as ADP to AMP[39]. NPPs also degrade NAD^+ to AMP and NMN as well as ADPR to AMP[32]. All substrates for NPPs are present within the interstitium of colonic muscles[5,7,8], but the role of NPPs in degradation of these nucleotides in the GI tract is unknown. We found that $PDGFR\alpha^+$ cells highly expressed Enpp1 and Enpp3 whereas SMC highly expressed Enpp2. This is an interesting distinction, because NPP1 and NPP3 are type II membrane proteins with a large extracellular domain, whereas NPP2 is a secreted protein and exists only in soluble form and is synthesized as a pre-pro-enzyme[32]. Further studies are warranted to understand the functional relevance of such distinction in the SIP syncytium in the colon.

Art1-5

Mammalian ARTs constitute a family of structurally-related proteins that transfer ADP-ribose from NAD^+ to target proteins. Therefore, ART activity in the extracellular compartment provides sophisticated regulatory mechanisms for cell communication[40]. In mice, ADP-ribosylation by ART2.1 and ART2.2 causes activation of the $P2X_7$ receptor on cell surface[41]. ART3 and ART4 show deviations from the consensus motif of arginine-specific ARTs, and enzyme activity has not been demonstrated for either of these proteins[42]. ART3 and ART4 may have acquired novel substrate specificity, or they may have lost enzyme activity and acquired new function(s). ART5 is predicted to be a secretory enzyme[43]. We demonstrate here that expression of *Art1*, *Art2.1* and *Art2.2* could not be resolved in murine colon muscularis. $PDGFR\alpha^+$ cells highly expressed *Art4*, whereas *Art5* was abundant in SMC and ICC. The functional relevance of *Art* expression in the SIP syncytium remains to be determined.

Kcnn1-3

Responses to purinergic inhibitory neurotransmitter(s) in the large intestine are mediated specifically by $P2Y_1$ receptors[5,7,13,29] and activation of K+ conductances that are sensitive to apamin5,7,44. Four closely related genes, *Kcnn1-4*, encode SK and IK channels. SK2 and SK3 channels are very sensitive to apamin[45] and may, therefore, mediate part of the inhibitory response to the enteric purinergic neurotransmitter. Previous studies reported SK3 channel immunoreactivity in Kit-negative fibroblast-like cells[30] and *Kcnn3* expression in $PDGFR\alpha^+$ cells[14]. $PDGFR\alpha^+$ cells were shown to have the ultrastructure of cells referred to as 'fibroblast-like'[31]. In our studies $PDGFR\alpha^+$ cells were highly enriched with transcripts of

Kcnn3, whereas *Kcnn1* was most highly expressed in SMC. The expression of *Kcnn3* far exceeded the expression of any other *Kcnn* family genes in the SIP syncytium. Therefore, these observations support the notion that PDGFRα$^+$ cells are a primary effector in purinergic neurotransmission.

While this study provides the most comprehensive investigation of the purinergic 'effectorsome' in colonic muscles, it is incomplete in fully describing the molecular apparatus responsible for activation and inactivation of purine motor neurotransmission. This is because we have not yet quantified cell-specific protein expression resulting from gene translation, and tests have not been performed to determine whether the proteins encoded by expressed genes are functional in the SIP syncytium. We also know little about the proteins expressed by motor nerve terminals, glia, or immune cells that might also participate in purinergic signaling, generation of active metabolites, or deactivation of signals. Even if motor neurons could be purified, we have no definitive method for determining the extent to which genes expressed encode proteins trafficked to nerve terminals. Immunohistochemistry can be a useful method for determining cellular localization of proteins, but it is notoriously inadequate as a means of quantifying protein expression and absence of labeling can signify poor immunoreactivity as well as lack of expression. Full understanding of the molecular and functional milieu of the SIP syncytium must await more definitive assays of functional proteins in situ.

Summary

The three cell types composing the SIP syncytium in the murine colon (i.e. SMC, ICC and PDGFRα$^+$ cells) display different expression profiles of key genes involved in purine signaling. Therefore, each cell type may have very specific roles in mediating and terminating purinergic signals. SMC demonstrate strong expression of *Adora2b, Art5, Enpp2, Nt5e and P2ry14,* modest expression of *P2rx1*, *Art3, Art4,* and lack *Adora1, Adora3, Art1, Art2.1, Art2.2,* and *Cd157/Bst1*. PDGFRα$^+$ cells demonstrate strong expression of *Adora1, Adora3, Art4, Enpp1, Enpp3, P2rx7, P2rx1, P2rx4, P2rx5, P2ry1, P2ry2, P2ry12, P2ry13,* and *P2ry14*, modest expression of *P2rx3, P2rx6, Cd38* and *Entpd1*and lack *Adora2a, Adora2b, Art1, Art2.1, Art2.2, Art5, Enpp2,* and *Cd157/Bst1.* ICC show strong expression of *P2ry2*, *P2ry4*, *P2ry6, Art5* and modest expression of *Adora2b* and *P2rx4.* These data can be used to examine targets of specific purine effects, to examine (patho)physiological consequences of extracellular purines on cells of the SIP syncytium, and identify cells of the SIP syncytium.

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Abbreviations

ADP	adenosine 5′-diphosphate
ADPR	adenosine 5′-diphosphate ribose
AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate

GI	gastrointestinal
GFP	green fluorescence protein
ICC	interstitial cells of Cajal
NAD^+	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAADP	nicotinic acid adenine dinucleotide phosphate
PDGFR	platelet-derived growth factor receptor
SK	small-conductance K^+ channel
SMC	smooth muscle cells
qRT-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
TCP	total cell population
UDP	uridine 5′-diphosphate
UTP	uridine 5′-diphosphate

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Key Messages

- The present study demonstrates that smooth muscle cells (SMC), interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor α positive (PDGFRα$^+$) cells (SIP syncytium) in the *tunica muscularis* demonstrate differential expression of genes related to purinergic signaling, suggesting cell-specific functions within the SIP syncytium in mediating enteric purinergic signals.
- Motility of the colon is regulated by purinergic neurotransmitters and paracrine mediators that may target specific cell types. We compared gene transcripts for purinergic receptors and membrane-bound enzymes for purine degradation expressed by each cell type of the SIP syncytium.
- SMC, ICC and PDGFRα$^+$ cells were isolated from colonic muscles of reporter strains of mice with cell-specific expression of green florescent proteins and were purified by fluorescence-activated cell sorting. Expression of genes was evaluated and compared by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).
- Our results demonstrate marked phenotypic differences between SMC, ICC and PDGFRα$^+$ cells. Notably, genes related to mediating effects and biotransformation of enteric purinergic inhibitory neurotransmitters were highly expressed by PDGFRα$^+$ cells.

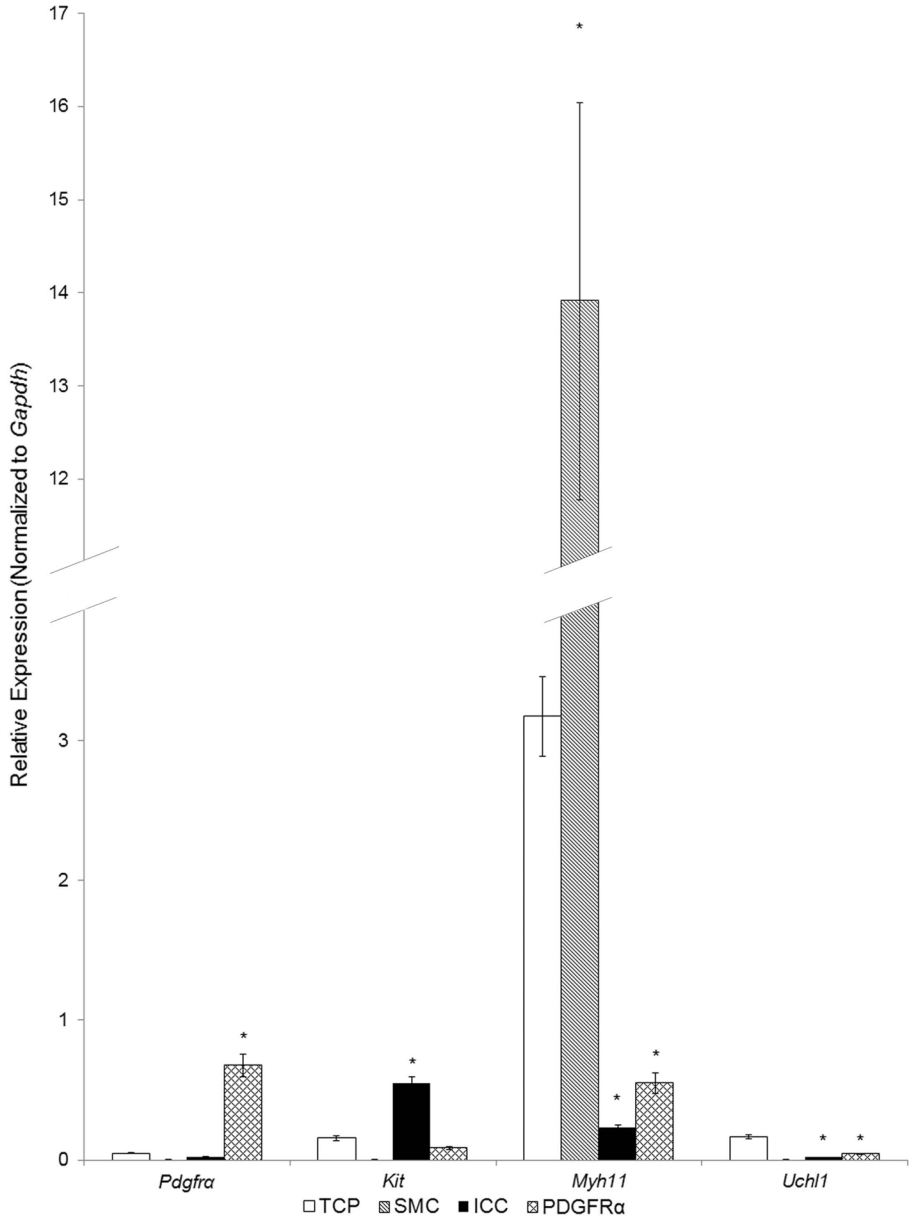

Fig. 1. Expression of cell type markers in SMC, ICC, and PDGFRα$^+$ cells of the murine colon by qRT-PCR analysis

The expression of cell type markers *Pdgfra* (PDGFRα$^+$ cells), *Myh11* (SMC), *Kit* (ICC) were compared with the expression of these genes in total cell population (TCP) of dispersed *tunica muscularis*, in which isolation of mRNA was performed without cell sorting (n=6). Sorted SMC (n=3) were minimally positive for *Pdgfra* and *Kit*, but demonstrated strong enrichment in transcripts of *Myh11*. Sorted PDGFRα$^+$ cells (n=3) were minimally positive for *Myh11* and *Kit*, but were enriched with *Pdgfra.* Sorted ICC (n=3) were minimally positive for *Myh11* and *Pdgfra,* but enriched with transcripts of *Kit.* After sorting, all three populations of purified cells displayed negligible transcripts of *UChl1*, the gene encoding the neural protein PGP9.5. The results were normalized to expression of the housekeeping gene *Gapdh.* Asterisks indicate P<0.05 when compared to the transcript isolated from TCP.

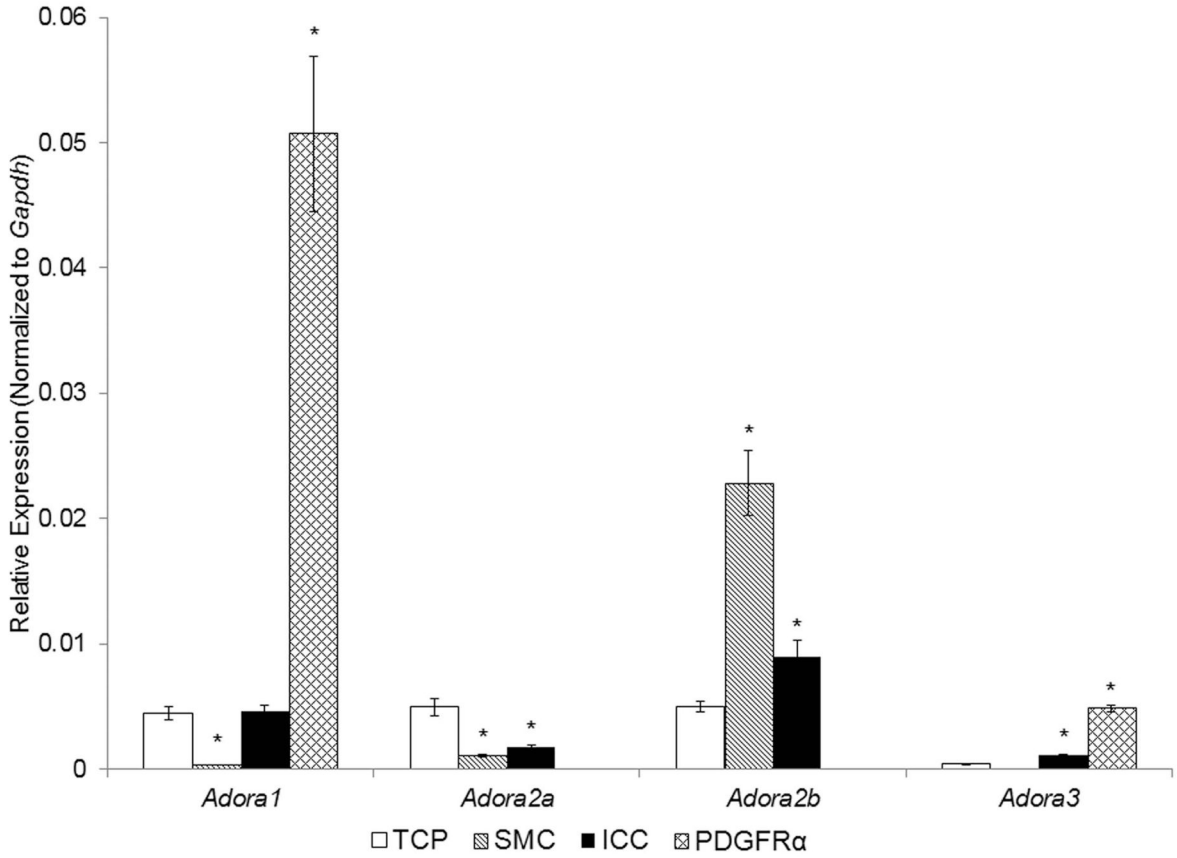

Fig. 2. Expression of genes for P1 receptors in SMC, ICC, and PDGFRα$^+$ cells of the murine colon by qRT-PCR analysis

Note that SMC and ICC were enriched with *Adora2b*, whereas PDGFRα$^+$ cells were enriched with *Adora1* and *Adora3*. TCP, total cell population (n=6), Sorted SMC, ICC and PDGFRα$^+$ cells (each n=3). Asterisks indicate P<0.05 when compared to the transcript isolated from TCP.

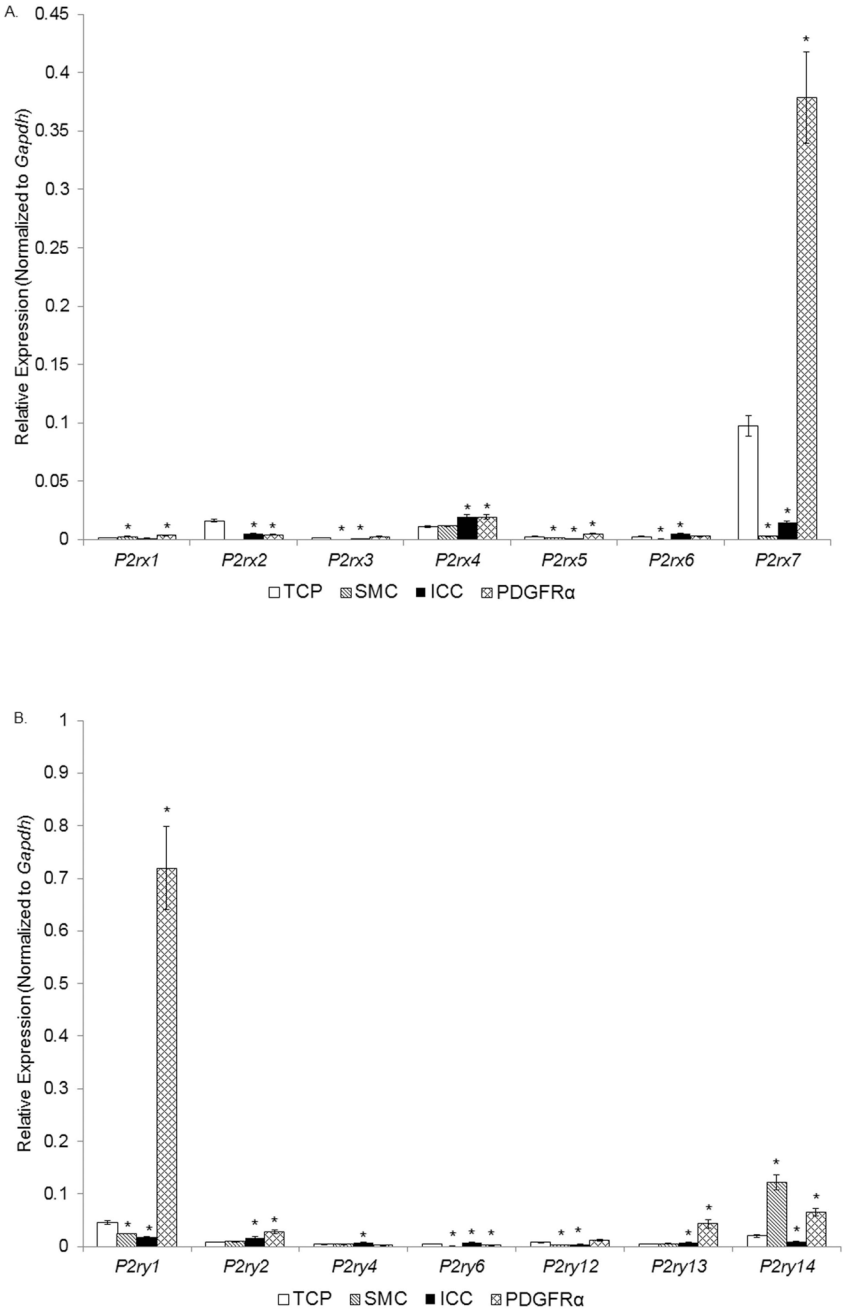

Fig. 3. Expression of genes for P2X receptors (panel A) and P2Y receptors (panel B) in SMC, ICC, and PDGFRα$^+$ cells of the murine colon by qRT-PCR analysis

Note that PDGFRα$^+$ cells are enriched with *P2rx7* and *P2rx4* as well as *P2ry1*, *P2ry2*, *P2ry12*, *P2ry14*, and *P2ry14*, whereas SMC were enriched with *P2ry14*. ICC showed strong expression of *P2rx4*, *P2ry2*, *P2ry4*, and *P2ry6*. TCP, total cell population (n=6), Sorted SMC, ICC and PDGFRα$^+$ cells (each n=3). Asterisks indicate $P<0.05$ when compared to the transcript isolated from TCP.

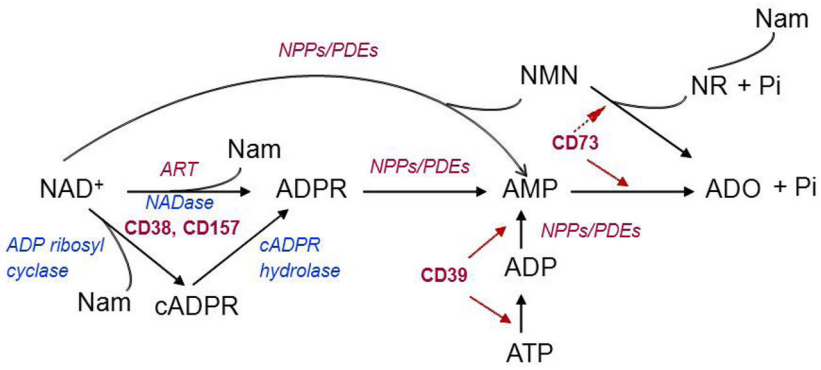

Fig. 4.
Biotransformation pathways for extracellular purines.

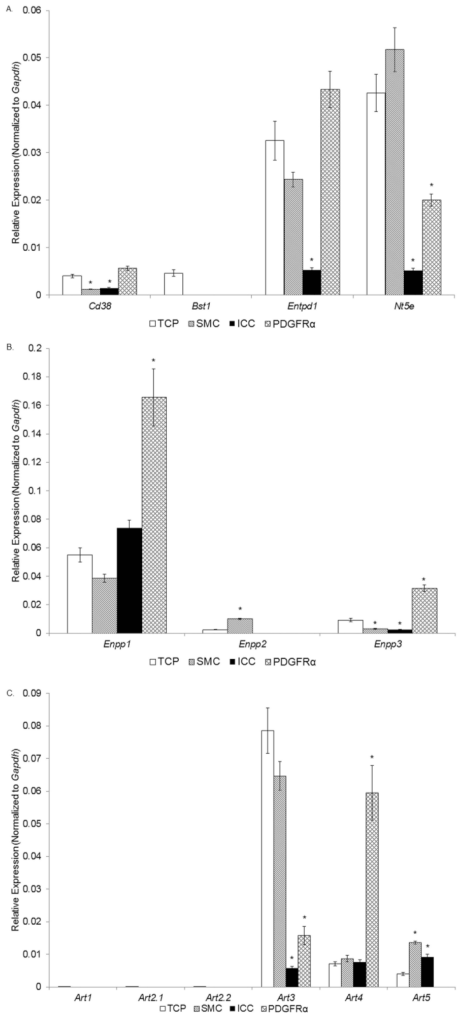

Fig. 5. Expression of genes for ecto-nucleotidases (panel A), ecto-nucleotide pyrophosphatases/ phosphodiasterases (panel B), and mono-ADP ribosyl transferases (panle C) in SMC, ICC, and PDGFRα$^+$ cells of the murine colon by qRT-PCR analysis

Note that PDGFRα$^+$ cells show relatively higher expression of *Cd38* and *Entpd1* and low expression of *Nt5e*. SMC showed relatively strong expression of *Entpd1. Bst1* was resolved in neither cell type. TCP, total cell population (n=6), Sorted SMC, ICC and PDGFRα$^+$ cells (each n=3). Asterisks indicate $P<0.05$ when compared to the transcript isolated from TCP.

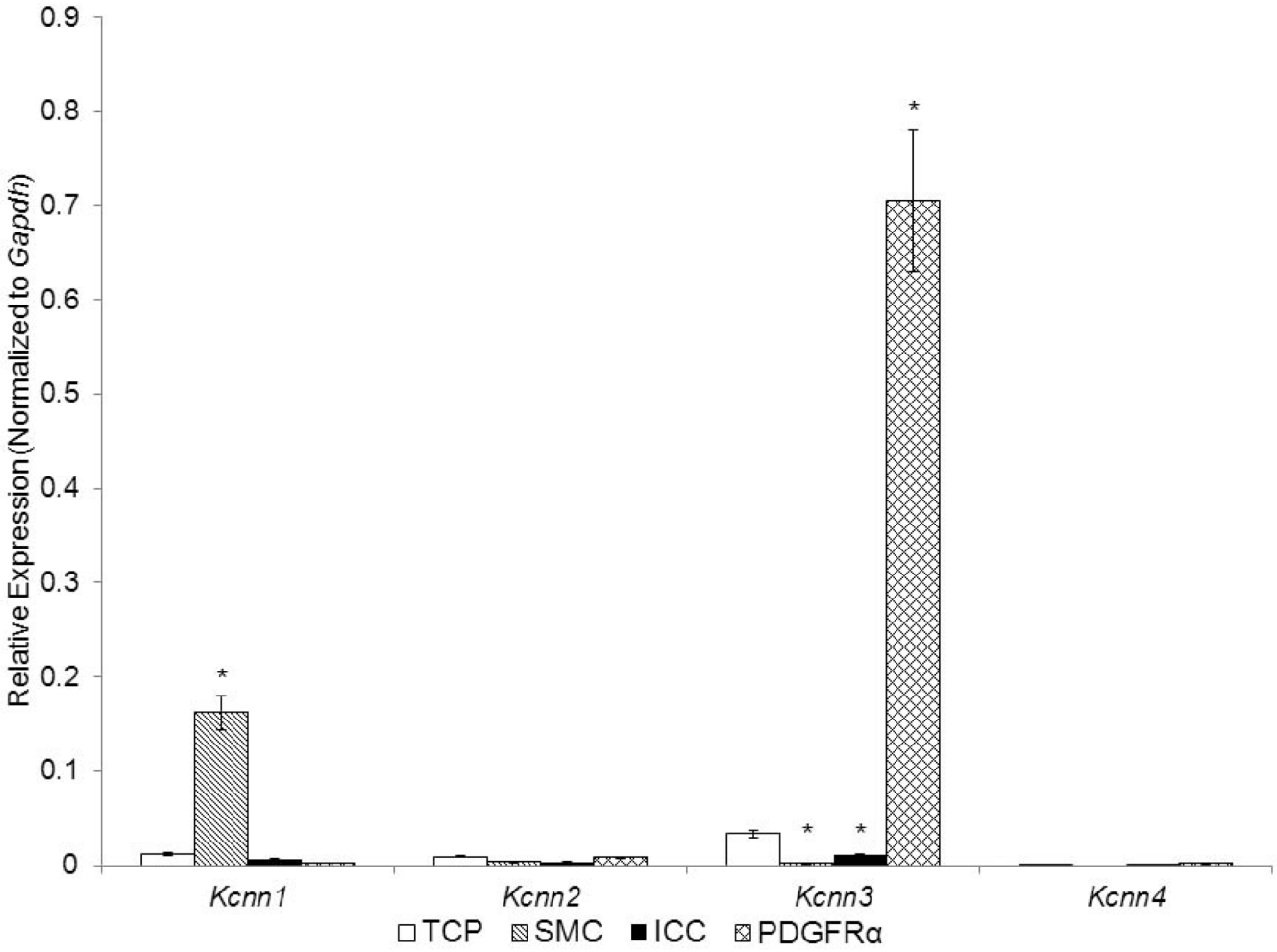

Fig. 6. Expression of genes for small-conductance Ca^{2+}-activated K^+ channels in SMC, ICC, and PDGFRα^+ cells of the murine colon by qRT-PCR analysis

Note that PDGFRα^+ cells demonstrate strong expression of *Kcnn3*, whereas SMC show strong expression of *Kcnn1*. TCP, total cell population (n=6), Sorted SMC, ICC and PDGFRα^+ cells (each n=3). Asterisks indicate $P<0.05$ when compared to the transcript isolated from TCP.

Table 1

Primer sequences used for qRT-PCR [Gene Name, Primer Sequences, Product length (bp), Accession Number]

Primer Name	Primer Sequence	Expected Size	Accession #
Gapdh-F	GCCGATGCCCCCATGTTTGTGA	178 bp	NM_008084
Gapdh-R	GGGTGGCAGTGATGGCATGGAC		
Pdgfra-F	ATGACAGCAGGCAGGGCTTCAACG	195 bp	NM_011058
Pdgfra-R	CGGCACAGGTCACCACGATCGTTT		
Kit-F	CGCCTGCCGAAATGTATGACG	162 bp	NM_021099
Kit-R	GGTTCTCTGGGTTGGGGTTGC		
Myh11-F	CAGCTGGAAGAGGCAGAGGAGG	198 bp	NM_013607
Myh11-R	AACAAATGAAGCCTCGTTTCCTCTC		
Uchl1-F	CGATGGAGATTAACCCCGAGATG	169 bp	NM_011670
Uchl1-R	TTTTCATGCTGGGCCGTGAG		
Adora1-F	AACATTGGGCCACAGACCTA	144 bp	NM_001008533
Adora1-R	TGTCTTGTACCGGAGAGGGA		
Adora2a-F	CCATTCGCCATCACCATCAG	165 bp	NM_009630
Adora2a-R	CAAGCCATTGTACCGGAGTG		
Adora2b-F	CCCTTTGCCATCACCATCAG	159 bp	NM_007413
Adora2b-R	TTTATACCTGAGCGGGACGC		
Adora3-F	GATGCACTTCTATGCCTGCC	139 bp	NM_009631
Adora3-R	AGTGGTAACCGTTCTATATCTGACT		
P2ry1-F	ACCGAGGTGCCTTGGTCGGT	140 bp	NM_008772
P2ry1-R	CCGGTCTTGGTCAGGGCACA		
P2ry2-F	AGCACCATCAATGGCACCTGGGA	132 bp	NM_008773
P2ry2-R	CACGACGTTCAGGCACAACCC		
P2ry4-F	GGCCCTCAATGCCCCAACCC	169 bp	NM_020621
P2ry4-R	GCCAGTGCCAAAGGGCCAGT		
P2ry6-F	ACCGCACTGTGTGCTACGAC	136 bp	NM_183168
P2ry6-R	CGGCGGGCCATGCGACAATA		
P2ry12-F	GGCTGACGTCACTGAACGCCTG	162 bp	NM_027571
P2ry12-R	TCTCTTCGCTTGGTTCGCCACC		
P2ry13-F	CAACACCACTGGGATGCAGGGCT	157 bp	NM_028808
P2ry13-R	GCTGGGGATGTGGACGAACACC		
P2ry14-F	ACCCTCCAAACCAGCCCTGC	197 bp	NM_133200
P2ry14-R	AGGCCCATGAGAAAGTCAGCCA		
P2rx1-F	CCACGCTTCAAGGTCAACAGGC	180 bp	NM_008771
P2rx1-R	GATACCAACCACCCCGCCCTTC		
P2rx2-F	TTGTGCATGGACAGGCAGGGAAA	185 bp	NM_153400
P2rx2-R	ACTTGAGGGGTGCCTTGGGGT		

Primer Name	Primer Sequence	Expected Size	Accession #
P2rx3-F	CTTCCGTGGGAGTGGGGACTGTT	186 bp	NM_145526
P2rx3-R	GCCCCCGAGTCTGTGGACTGC		
P2rx4-F	TGACGCTGGTGTGCCAACGC	176 bp	NM_011026
P2rx4-R	GGCAGAAGGGATCCGTCCGC		
P2rx5-F	TCCGTCTCAGGGGGAGAACGT	138 bp	NM_033321
P2rx5-R	TCCCCAGCGTGACAGTCGGT		
P2rx6-F	GAGAGTGGTGCTGTGCCCAGGAAG	188 bp	NM_011028
P2rx6-R	CGGAACACTGGGCAGTACGGA		
P2rx7-F	CAGACTACACCTTCCCTTTGCAG	179 bp	NM_011027
P2rx7-R	GCCAGTCTGGATTCCTTTGCTC		
Cd38-F	CAAGAACCCTTGCAACATCACAAGAG	147 bp	NM_007646
Cd38-R	GTGAACATCTTTCCCTGGATCCAAG		
Bst1-F	GAGATCTGGGTTATGCATGACGTTG	127 bp	NM_009763
Bst1-R	GTCGGTAGTCATTGATACAGCTGTG		
Entpd1-F	GTACCTGAGTGAGTACTGCTTCTC	168 bp	NM_009848
Entpd1-R	GCTGGGATCATGTTGGTCAAGTTC		
Nt5e-F	AACGGTGGAGATGGATTCCAGATG	151 bp	NM_011851
Nt5e-R	GACTTGCTGCAGAGAACTTGATCC		
Enpp1-F	AAATCTTTCTTGCCGGGAGC	110 bp	NM_008813
Enpp1-R	AGAAGGTCAGGGGCTCAATC		
Enpp2-F	TTTGGTCTGTGAGCATCCCT	179 bp	NM_015744
Enpp2-R	CGGAGTAAGAGGTGAGCCAT		
Enpp3-F	GCACTACAGAATACGCCTGG	183 bp	NM_134005
Enpp3-R	CATAGCTTTGCCGTACCCAC		
Art1-F	AGGGATGGAGTTCAGGTTCAA	181 bp	NM_009710
Art1-R	TCCGCGTATACTTTGTTGGC		
Art2.1-F	ATACTCATGAAGAGGAGGTGTT	188 bp	NM_007490
Art2.1-R	ACAGCTCTCTCTAGATCCTAAGC		
Art2.2-F	CCTCGTGAAGAGGAGGTGTT	189 bp	NM_019915
Art2.2-R	GCTCTCTCTGGATCCTGATATACTG		
Art3-F	ATTCCTCGGGGGTCTGAAAA	142 bp	NM_181728
Art3-R	TTCTGGCATTCTGTCAGGGT		
Art4-F	CACTGAGGCTCCTCTTAAGGT	184 bp	NM_026639
Art4-R	CTGGTTCAGCCAGGTTAGGT		
Art5-F	TTTTGGGGCTCCTATCCAGG	180bp	NM_007491
Art5-R	TTCTCCCCACCCAAATAGGC		
Kcnn1-F	GTTGTTGGTCTTCAGCGTCTCCTC	150 bp	NM_032397
Kcnn1-R	GTCTCCATAGCCAATGGACAAGAAG		
Kcnn2-F	ACTATCTGCCCAGGAACTGTGCTC	128 bp	NM_080465

Primer Name	Primer Sequence	Expected Size	Accession #
Kcnn2-R	CATTGCTCCAAGGAAGTTGCTAGTG		
Kcnn3-F	CTGCTGGTGTTCACCATCTCTCTG	138 bp	NM_080466
Kcnn3-R	GTCCCCATAGCCAATGGAAAGGAAC		
Kcnn4-F	AAGATGCTGGCCGCCATCCACA	157 bp	NM_008433
Kcnn4-R	TCTTCTCCAGGGCACGGTGCGA		

Table 2

Genes for P2 purinergic receptors: fold differences for the comparison of expression in smooth muscle cells (SMC, n=3), interstitial cells of Cajal (ICC, n=3) and PDGFRα$^+$ cells (n=3) vs. total cell population (TCP, n=6).

Gene Symbol	Gene Name	Fold Difference SMC vs TCP	P-Value	Fold Difference ICC vs TCP	P-Value	Fold Difference PDGFRα$^+$ vs TCP	P-Value
P2rx1	purinergic receptor P2X, ligand-gated ion channel, 1	1.45	0.0059	−1.16	0.5143	2	0.0316
P2rx2	purinergic receptor P2X, ligand-gated ion channel, 2	0	0.0000	−3.25	0.0012	−3.94	0.0003
P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3	−55.8	0.0055	−3.66	0.0158	1.53	0.0844
P2rx4	purinergic receptor P2X, ligand-gated ion channel, 4	1.05	0.7840	1.79	0.0035	1.77	0.0342
P2rx5	purinergic receptor P2X, ligand-gated ion channel, 5	−1.91	0.0258	−5.07	0.0038	1.85	0.0249
P2rx6	purinergic receptor P2X, ligand-gated ion channel, 6	−5.09	0.0008	2.07	0.0009	1.1	0.3311
P2rx7	purinergic receptor P2X, ligand-gated ion channel, 7	−33.61	0.0079	−6.78	0.0124	3.88	0.0008
P2ry1	purinergic receptor P2Y, G-protein coupled 1	−1.87	0.0087	−2.51	0.0035	15.98	0.0000
P2ry2 2	purinergic receptor P2Y, G-protein coupled	1.08	0.5929	1.94	0.0110	3.3	0.0033
P2ry4	purinergic receptor P2Y, G-protein coupled 4	1.02	0.9396	1.99	0.0167	−1.67	0.1645
P2ry6	purinergic receptor P2Y, G-protein coupled 6	−4.02	0.0000	1.53	0.0159	−2.22	0.0002
P2ry12	purinergic receptor P2Y, G-protein coupled 12	−2.48	0.0015	−1.62	0.0076	1.53	0.1043
P2ry13	purinergic receptor P2Y, G-protein coupled 13	1.07	0.5779	1.5	0.0083	8.78	0.0004
P2ry14	purinergic receptor P2Y, G-protein coupled 14	6.01	0.0009	−2.24	0.0107	3.19	0.0004