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Transcriptome analysis of mesenchymal stromal cells of the large and small intestinal smooth muscle layers reveals a unique gastrontestinal stromal signature

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ABSTRACT

Mesenchymal stromal cells in the muscle layer of the large intestine are essential for the regulation of intestinal motility. They form electrogenic syncytia with the smooth muscle and interstitial cells of Cajal (ICCs) to regulate smooth muscle contraction. Mesenchymal stromal cells are present in the muscle layer throughout the gastrointestinal tract. However, their area-specific characteristics remain ambiguous.

In this study, we compared mesenchymal stromal cells from the large and small intestinal muscle layers. Histological analysis using immunostaining showed that the cells in the large and small intestines were morphologically distinct. We established a method to isolate mesenchymal stromal cells from wild-type mice with platelet-derived growth factor receptor-alpha (PDGFRα) as a marker on the cell surface and performed RNAseq. Transcriptome analysis revealed that PDGFRα^{+} cells in the large intestine exhibited increased expression levels of collagen-related genes, whereas PDGFRα^{+} cells in the small intestine exhibited increased expression levels of channel/transporter genes, including *Kcn* genes. These results suggest that mesenchymal stromal cells differ morphologically and functionally depending on gastrointestinal tract. Further investigations of the cellular properties of mesenchymal stromal cells in the gastrointestinal tract will aid in optimizing methods for the prevention and treatment of gastrointestinal diseases.

1. Introduction

The intestinal tract undergoes complex peristaltic movements during the release of its contents via the coordinated action of a group of cells in the muscular layer. In the large intestine, there are three distinct cell types involved in the control of gastrointestinal motility: interstitial cells of Cajal (ICCs), mesenchymal stromal cells, and smooth muscle cells. ICCs are pacemaker cells that generate electrical slow waves and cause phasic contractions in the smooth muscle layer of the intestine. Mesenchymal stromal cells form a gap junction-mediated syncytium with smooth muscle cells, receive neurotransmitters from neurons, and regulate smooth muscle contractions [1]. In particular, mesenchymal stromal cells in the large intestinal muscular layer mediate inhibitory neurotransmission by opening purine receptor-mediated SK3 channels to regulate the digestive tract [2]. Mesenchymal stromal cells have platelet-derived growth factor receptor-alpha (PDGFRα) on their cell surface and are present in the muscle layer throughout the gastrointestinal tract; however, their area-specific functions were ambiguous.

PDGFRα signaling plays essential roles in cell growth and differentiation and contributes to tissue fibrosis. The cells are the origin of fibrotic scarring and lead to loss of tissue integrity during fibrotic pathologies. In the gastrointestinal tract, constant activation of PDGFRα signaling results in marked fibrosis of the submucosal connective tissue [3]. PDGFRα mutation-induced constant activation is observed in

Abbreviations: BSA, bovine serum albumin; ICC, interstitial cell of Cajal; PBS, phosphate-buffered saline; PDGFRα, platelet-derived growth factor receptor-alpha; TPM, transcripts per million.

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gastrointestinal stromal tumors [4]. In response to intestinal injury, PDGFRα$^+$ cells produce proinflammatory cytokines, leukocyte chemoattractants, and matrix proteins such as *Col1a1*, *Postn*, *Sparc*, and *Has1* [5], indicating that PDGFRα$^+$ cells are the main target involved in fibrotic pathogenesis in the gastrointestinal tract. As observed in Crohn's disease, intestinal fibrosis is prevalent in the lower gastrointestinal tract, suggesting the differences in the cellular characteristics of PDGFRα$^+$ cells in the large and small intestines.

In various tissues, mesenchymal stromal cells have an essential role in maintaining organ integrity. Mesenchymal stromal cells in the intestinal mucosal layer are essential for maintaining intestinal mucosal epithelial cells. For example, autophagy in PDGFRα$^+$ cells contributes to the homeostasis of intestinal mucosal epithelial cells [6]. Various ligands secreted by PDGFRα$^+$ mesenchymal stromal cells are necessary for maintaining the stem cell niche. Wnts, R-spondin 3 [7], and bone morphogenetic proteins [8,9] maintain the intestinal environment's homeostasis. PDGFRα$^+$ cells also promote the migration of CD103$^+$ CD11b$^+$ dendritic cells into intestinal lymph nodes and are essential for postnatal intestinal maturation [5]. There are several reports of transcriptome analysis on mesenchymal stromal cells of the gastrointestinal tract. However, previous studies have not informed the characteristics of the cell population in the muscle layer [8], or only performed transcriptomic analyses of the intestinal mucosal layer [9,10]. Many researchers used PDGFRαH2BeGFP mice [11] to analyze PDGFRα$^+$ cells, but the mice exhibit reduced expression of PDGFRα, resulting in inadequate PDGF signaling and impaired progression of fibrosis in the liver [12]. Based on these observations, it is difficult to define the PDGFRα$^+$ cells isolated from the transgenic mice as cells in their normal state.

In this study, we have developed a method to isolate PDGFRα$^+$ mesenchymal stromal cells from wild-type mice with high purity and compared the cells in the muscle layer of the large and small intestines. Bulk RNA-sequencing (RNA-seq) revealed the differences in the cell properties depending on gastrointestinal tract areas. Interpreting the molecular mechanisms of mesenchymal stromal cells will facilitate future research and development of strategies to prevent and treat stromal cell-associated gastrointestinal diseases.

2. Materials and methods

2.1. Animal preparation

C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). Eight to nine weeks of mice were used for experiments. All mice were supplied with food and water ad libitum and maintained under constant temperature and humidity in a 12-h dark/light cycle. All animal experiments were approved by the Experimental Animal Care and Use Committee of the University of Tokyo and performed in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines.

2.2. Immunohistochemical analysis

Small and large intestines of mice were sampled, avoiding the sphincter, and their lamina propria were peeled off using forceps and discarded. For immunofluorescence staining, only the muscular layers were pinned onto a silicon-based dish and fixed with ice-cold acetone for 10 min. After washing with Tris-buffered saline, the samples were mixed with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for blocking and incubated with goat polyclonal anti-mouse PDGFRα (1:400; R&D Systems, Minneapolis, MN, USA; Cat# AF1062) and rabbit anti-mouse-c-kit (GeneTex, Alton Pkwy Irvine, CA, USA; Cat# GTX10410) antibodies at 4 °C overnight. After washing with PBS, samples were incubated with Alexa Fluor 594-conjugated donkey anti-goat IgG (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. The samples were counterstained with 4′,6-diamidino-2-phenylindole (1:5000; DOJINDO, Kumamoto, Japan). Fluorescent images were captured using a confocal laser scanning microscope (ECLIPSE Ti; Nikon, Tokyo, Japan).

2.3. Flow cytometry and PDGFRα$^+$ cell isolation

Mice were euthanized via cervical dislocation following the Experimental Animal Care and Use guidelines of the University of Tokyo. Large and small intestines were removed and washed by flushing with Ca^{2+}-free Hanks' medium using a 10-mL syringe. Fat and mesenteric blood vessels were carefully removed, and the intestinal tissues were opened longitudinally. Muscle layers were isolated from the intestinal mucosal layers using forceps and used in this experiment. The muscular layers of the intestines were digested using Ca^{2+}-free Hanks' medium containing 2 mg/mL collagenase type II, 2 mg/mL BSA, 2 mg/mL trypsin inhibitor, and 0.3 mg/mL ATP for 30 min at 37 °C. After passing through the 18- and 21-gauge needles each 3–5 times consecutively, the samples were incubated with 0.1 mg DNase and dispersed (1 U/mL) for 10 min at 37 °C, followed by filtration to generate single-cell suspensions. Mononuclear cells were stained with the Zombie Violet Fixable Viability Kit (Cat# 423113; BioLegend, San Diego, CA, USA) for 15 min at 15–25 °C, followed by incubation with CD31-Alexa 488 conjugated (Clone 390; BioLegend), CD45-Alexa 488 conjugated (Clone 30-F11; BioLegend), and PDGFRα-PE-conjugated (Cat#FAB1062P; R&D Systems) antibodies for 30 min at 4 °C. Zombie Violet$^-$CD31$^-$CD45$^-$PDGFRα$^+$ cells were collected as a PDGFRα$^+$ live cell population. We collected 1×10^5 cells from each gastrointestinal tract region of a mouse and used them for RNAseq. This experiment was repeated three times. Flow cytometry data were analyzed using the FlowJo software version 10.7.1 (BD Biosciences, San Jose, CA, USA).

2.4. Bulk RNA-seq library construction

Total RNA was extracted from small and large intestinal PDGFRα$^+$ live cells isolated using flow cytometry. RNA-seq libraries were constructed using the Clontech Smart-seq v4 Ultra Low Kit (Cat# 634888; TaKaRa) and the NEBNext Ultra II RNA Library Prep Kit for Illumina (Cat# 7770; Illumina). Libraries were sequenced using Illumina NovaSeq 6000 (Illumina), according to the manufacturer's instructions.

2.5. Bulk RNA-seq and data processing

Data analysis was performed using the R package (version 4.1.0). The paired-end sequencing reads were aligned against the mouse reference genome (GRCm38) using STAR (version 2.7.10a) [13] and counted using featureCounts (version 1.6.4) [14] to obtain the read count. Quantified read counts from each sample were combined into a count matrix, with each row representing a unique gene ID and each column representing the raw counts for each unique sample. Bulk RNA-seq data are available in the DDBJ Sequenced Read Archive under the accession numbers DRR419065-DRR419070.

2.6. Differential gene expression analysis

To normalize the raw counts and calculate the p-values, R package "DESeq package" (version 1.32.0) [15] was used. The fold-change was log2 scaled, and the Benjamini–Hochberg method was used to correct the p-values for multiple testing. Differentially expressed genes (DEGs) between two different conditions (i.e., PDGFRα$^+$ cells of the large intestine vs. PDGFRα$^+$ cells of the small intestine) were identified using transcripts with a baseMean >100, a 2-fold change (log2 fold-change, >1 or < -1), and a 5% false-discovery rate cutoff for the thresholds. DeSeq results are available in the Genomic Expression Archive under the accession numbers E-GEAD-568.

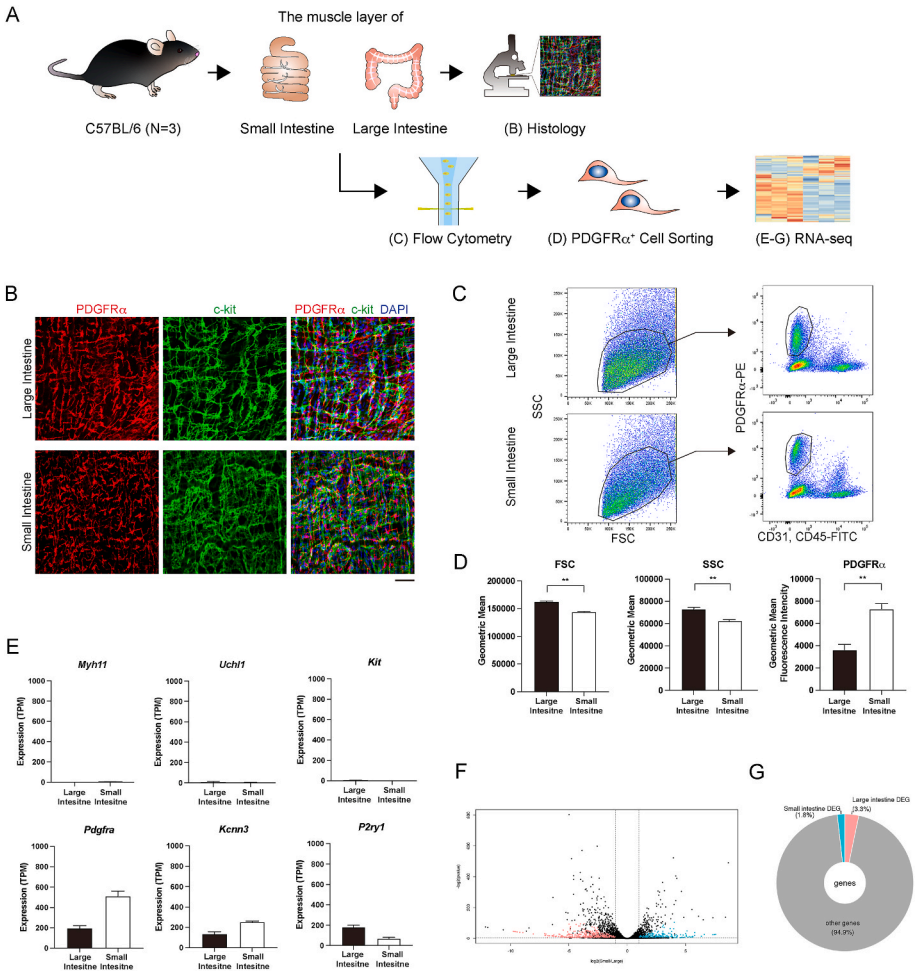

Fig. 1. Mesenchymal stromal cells differ morphologically and transcriptionally between the muscle layer of the large and small intestines. (A) Schematic diagram of the experimental design used for immunostaining, isolating mesenchymal stromal cell from the small and large intestines, and performing bulk RNA-sequencing (RNA-seq). (B) Immunohistochemical analysis of PDGFRα (red) and c-kit (green) in the small and large intestines. (C and D) PDGFRα$^+$ cells were identified via flow cytometry. (E) Transcripts per million (TPM) values of smooth muscle cell marker (*Myh11*), nerve cell marker (*Uchl1*), interstitial cells of Cajal marker (*Kit*), and mesenchymal stromal cell markers (*Pdgfra, Kcnn3, and P2ry1*). (F) Volcano plot and (G) percentage of small and large intestinal specific DEGs. Scale bar, 50 mm (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.7. Gene Ontology (GO) analysis

To deduce the potential functions of the signature genes in the large and small intestinal PDGFRα$^+$ cell populations, we mapped DEGs to their respective biological GO using the R package "clusterProfiler" (version 4.0.5) [16].

2.8. Channel/transporter and heatmap analyses

To normalize the expression levels of raw counts in the small and large intestinal PDGFRα-positive cells, transcripts per million (TPM) reads were used. When searching for functional channels and transporters in PDGFRα$^+$ cells of the large and small intestines, only genes with a TPM > 5 were employed. The definitions of potassium, cation, chloride, and sodium channel subunits expressed in PDGFRα$^+$ cells of the larger and small intestines are cited from the data used in S3 Fig. of a previous study [17]. To determine the expression levels of functional channels/transporters in PDGFRα$^+$ cells of the large and small intestines, we conducted bidirectional hierarchical clustering analysis to cluster Z-scores and samples based on the Euclidean distance with the R package "Pheatmap package" (version 1.0.12) [18].

2.9. Statistical analysis

Histograms are expressed as the mean ± standard deviation, and *t*-tests were performed for two-group comparisons. The sequencing data were graphed and analyzed using GraphPad Prism software (version 9; GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P < 0.05$ ($^{**}P < 0.01$).

3. Results

3.1. Comparison of PDGFRα+ mesenchymal stromal cells in the muscle layers of the small and large intestines

To investigate the differences in mesenchymal stromal cells of the muscle layers of the small and large intestines, we performed immunostaining and isolated PDGFRα$^+$ cells using flow cytometry from C57BL/6 mice (Fig. 1A). PDGFRα$^+$ cells in the large and small intestines were morphologically distinct (Fig. 1B). Flow cytometry also confirmed that PDGFRα$^+$ cells from the large intestine showed higher forward scatter and side scatter than those in the small intestine (Fig. 1C and D). Bulk RNA-seq was performed using isolated PDGFRα$^+$ cells to analyze their gene expression profiles (Fig. 1A). We obtained 13.8–17.1 million reads, of which 80.8–82.2% were mapped onto the genome. The transcriptomes included 26608 known genes. Expression profiles of PDGFRα$^+$ cells were similar between the small and large intestines (Spearman's rank correlation coefficient = 0.95–0.97). We determined the TPM values of markers specific to each cell type in the isolated cell population [17]. The isolated cell population rarely contained smooth muscle cells and ICCs, confirming the presence of genes specific to mesenchymal stromal cells (Fig. 1E). PDGFRα$^+$ cells in the small and large intestines expressed almost all the common genes. However, several small and large intestinal-specific DEGs were identified (1.8 and 3.3%, respectively) (Fig. 1F and G).

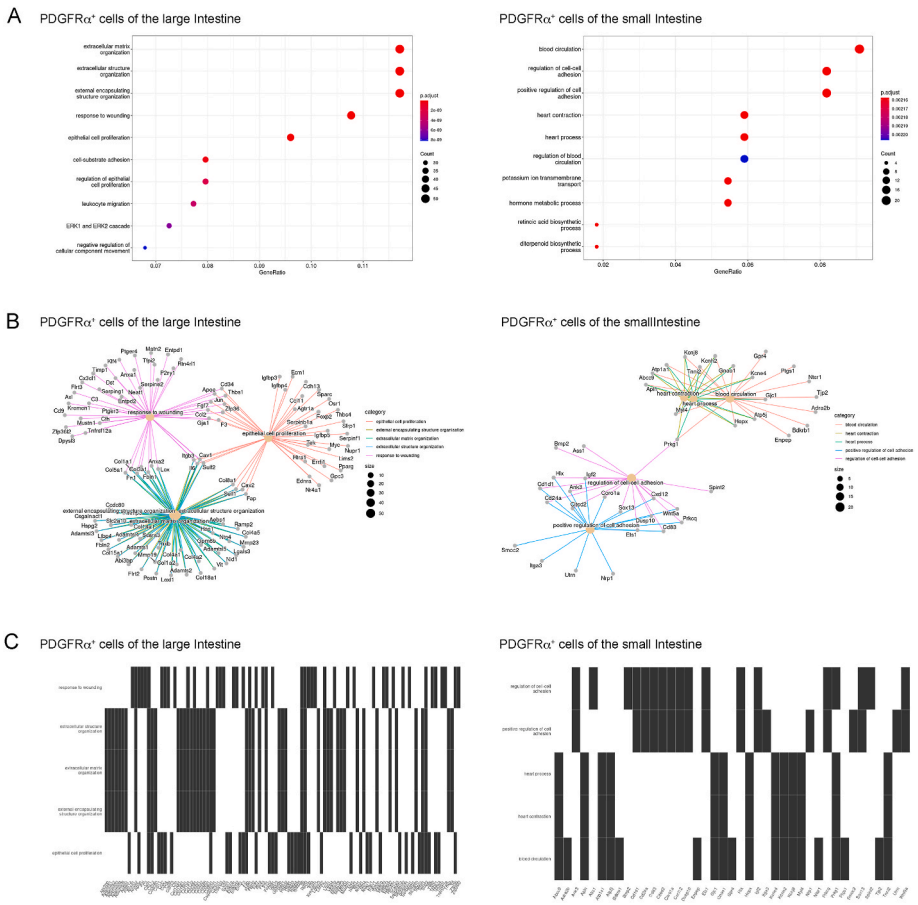

Fig. 2. Gene Ontology (GO) analysis of PDGFRα$^+$ cells in the muscle layer of the small and large intestines. (A) Representative GO terms enriched in the differentially expressed genes (DEGs) of PDGFRα$^+$ cells in the small and large intestines. (B) cnetplot and (C) heatplot of top five GO terms in small and large intestinal PDGFRα$^+$ cells.

3.2. GO analysis of the specific DEGs of PDGFRα$^+$ cells in the large and small intestines

We performed GO analysis of the specific DEGs to estimate the functional differences in PDGFRα$^+$ cells between the small and large intestines. DEGs in the large intestine were significantly enriched in the extracellular organization (extracellular matrix, extracellular structure, and external encapsulating structure organization), response to wounding, and epithelial cell proliferation. In contrast, DEGs in the small intestine were significantly enriched in blood circulation, regulation of cell–cell adhesion, positive regulation of cell adhesion, heart contraction, and heart processes, suggesting that the functions of PDGFRα$^+$ cells in the small and large intestines may be different (Fig. 2A and B). Focusing on different genes, we found that the levels of *Adamts* and collagen family genes of the extracellular matrix were upregulated in the large intestine, whereas *Kcn* genes were upregulated in the small intestine (Fig. 2C).

3.3. Comparative analysis of ion channels and transporters expressed in PDGFRα$^+$ cells

To further estimate the functions of PDGFRα$^+$ cells in the muscle layer of the small intestine, we analyzed the expression levels of ion channels/transporters. We adopted a list of previously reported channel/transporter genes and their functions in PDGFRα$^+$ cells [17]. Interestingly, 39% of the genes highly expressed in PDGFRα$^+$ cells in the muscle layer of the small intestine were associated with channels/transporters, whereas only 8% of the genes in the large intestine were associated with channels/transporters (Fig. 3A). We found 37 and 98 genes involved in channels/transporters in the DEGs of large and small intestinal PDGFRα$^+$ cells, respectively (Fig. 3B). Examining the channels/transporters individually (Fig. 3C). In terms of genes, the expression levels of *Atp* genes, including *Atp5b* and *Atp5j* in the hydrogen transporters, and *Kcn* genes, including *Kcne4* and *Kcnj8* in the potassium channels, were higher in the small intestinal PDGFRα$^+$ cells than the large intestinal PDGFRα$^+$ cells (Fig. 3D and E). Consistent with previous studies, the calcium regulator Gas6 was highly expressed in both small and large intestinal PDGFRα$^+$ cells [17]. However, for calcium channels such as Cacna1g, Cacna1h, and Cacna1d, no tissue-specific expression differences were found. (Fig. 3F).

4. Discussion

In the body, various organs contain stromal cells that fill the gaps between parenchymal cells. Stromal cells are composed of multiple cell types, including vascular component, hematopoietic, and fibroblast-like mesenchymal stromal cells; however, it is difficult to analyze them due to the lack of cell surface markers defining mesenchymal stromal cells. Although fibroblast-like mesenchymal stromal cells are known to be the cells of origin of fibrosis under pathological conditions [19], functional analysis of these cells has been limited. During 2009–2011, PDGFRα$^+$ was identified as a marker of fibroblast-mesenchymal stromal cells in various tissues [20–22]. Single-cell analysis revealed that fibroblast-mesenchymal stromal cells have different gene expression patterns in different organs [23]. PDGFRα$^+$ stromal cells are required for organ maintenance in some tissues [24]. The characteristics of PDGFRα$^+$ cells in the colonic muscle layer were investigated from anatomical and electrophysiological perspectives. PDGFRα$^+$ mesenchymal stromal cells

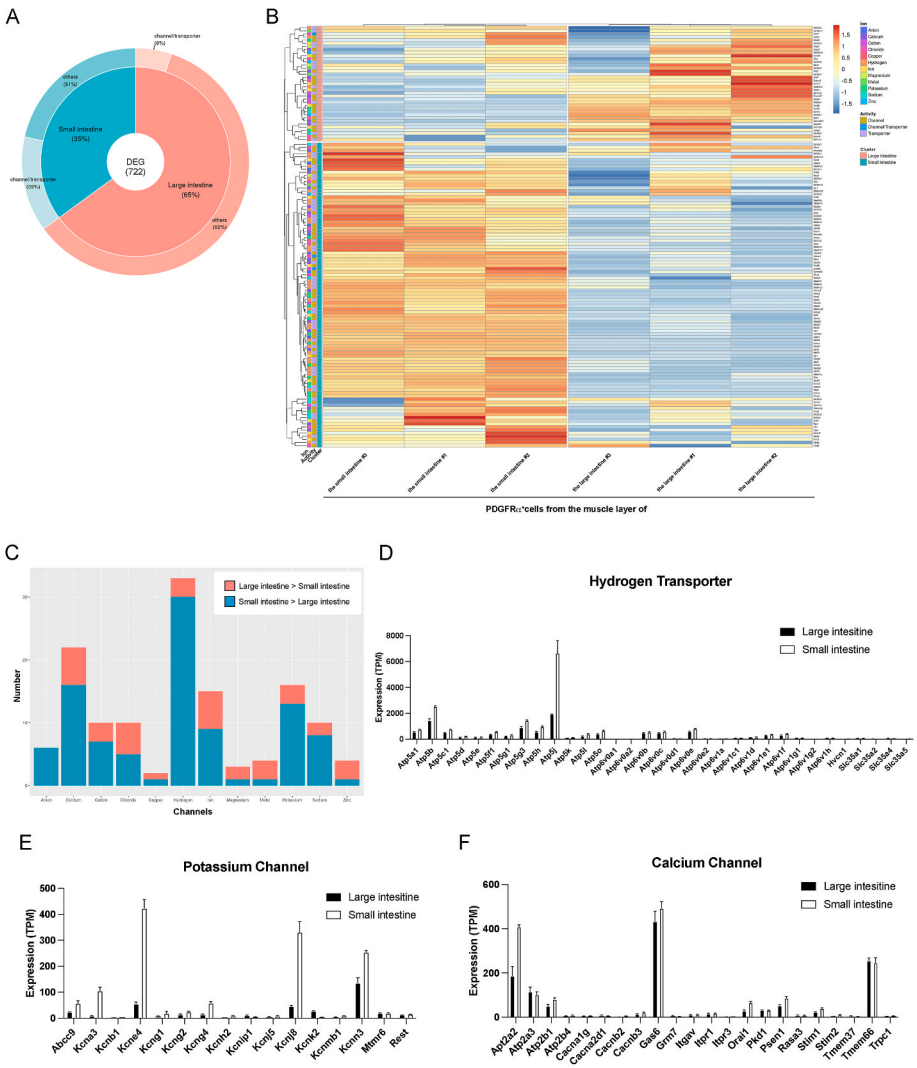

Fig. 3. Differential expression levels of ion channels and transporters in PDGFRα$^+$ cells of the small and large intestine. (A) Percentage of channel/transporter-related DEGs among total DEGs in small and large intestinal PDGFRα$^+$ cells. (B) Pheatmap based on the TPM values to visualize the similarities and dissimilarities among channel/transporter-related DEGs. Color scale value is based on Z-score. (C) Barplot of pheatmap result for each ion channel/transporter. (D–F) Barplot of hydrogen transporter, potassium channel, and calcium channel-related genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

are present in the muscle layer of throughout the gastrointestinal tract areas; however, their area-specific characteristics remain ambiguous.

Here, we show that PDGFRα$^+$ cells in the large and small intestinal muscle layers from wild-type mice have different characteristics in their cell morphology and transcriptome profiles. While PDGFRα$^+$ cells in both tissues showed similar transcriptome profiles, we found differences in the expression levels of channel/transporter genes and those associated with fibrosis, suggesting that mesenchymal stromal cells may have specific functions at different gastrointestinal sites. PDGFRα$^+$ cells in the large intestine muscle layer exhibited increased expression levels of *Adamts* and collagen family genes, suggesting that they may contribute to the structural maintenance and fibrosis of the organ. In contrast, PDGFRα$^+$ cells in the small intestine muscle layer exhibited high expression levels of channel/transporter genes, suggesting that they may play an essential role in regulating gastrointestinal motility, as reported for PDGFRα$^+$ cells in the large intestine [1]. We confirmed that adrenoceptor alpha (*Adra*)-*1a*, *Adra1b*, and potassium calcium-activated channel subfamily N member 3 (*Kcnn3*), known to be expressed in mouse colonic PDGFRα$^+$ cells forming smooth muscle cell–ICC–PDGFRα$^+$ cell (SIP) syncytium [1], were not only expressed in the colon, but also in the small intestine, suggesting the possibility of SIP syncytium formulation in the small intestine.

We found that PDGFRα$^+$ cells in the small intestine highly expressed hydrogen and potassium transporters. Potassium channels play important roles in the development and progression of several human diseases, including Crohn's disease, inflammatory bowel disease, and ulcerative colitis [25]. In the large intestine, purinergic motor neurotransmission occurs via P2Y1 receptors (P2Y1Rs) expressed on PDGFRα$^+$ cells, inhibiting intestinal motility [26]. High expression of P2Y1R in the colon, as shown in Figs. 1E and 2B, suggests the possibility of developing therapeutic therapies to target the colon during treatment effectively. We hope that this study will facilitate future research into the prevention and treatment of gastrointestinal diseases involving stromal cells.

There is one transcriptome analysis report on small intestinal PDGFRα$^+$ cells [17]. However, due to difficulties in cell fractionation, they used PDGFRαH2BeGFP mice and pooled the cells from 30 mice for analysis, resulting in the detection of the smooth muscle cell marker *Myh11*, which is not usually detectable in the mesenchymal stromal cell population. PDGFRα$^+$ cells isolated using our method comprised a high-purity cell population. We showed in Fig. 1E that our transcriptome analysis detected almost no other cell marker genes, such as *Myh11*, *Uchl1*, or *Kit*. Our transcriptome data based on PDGFRα$^+$ cells from wild-type mice may contribute to understanding the functions of PDGFRα$^+$ cells in the intestinal muscle layers.

Ethics approval and consent to participate

Experiments using mice were approved by the Experimental Animal Care and Use Committee of the University of Tokyo and performed in accordance with the Animal Research: Reporting of In Vivo Experiments

(ARRIVE) guidelines.

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Author Contributions

T.C., T.K., N.K., and M.H. designed the study and interpreted the data. T.C., T.K., K.K., M.I-U., and A.U. performed the experiments and analyzed the data. T.C., T.K., K.K., N.K., and M.H. wrote, reviewed, and revised the manuscript. All the authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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