Japanese Journal of Gastroenterology and Hepatology

Research Article

ISSN 2435-121 | Volume 6

PAR2 Involved in Colonic Platelet-Derived Growth Factor Receptor-A-Positive Cell Proliferation in Diabetic Mice

Yu-Jia L[1,2], Jun-Ping A[3], Huang X[2], Hong-Li L[2], Han-Yue F[2], Ni-Na S[2], Wen-Xie X[1,2*] and Chen J[1*]

[1]Department of Pediatric Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

[2]Department of Anatomy and Physiology, Shanghai Jiao Tong University College of Basic Medical Science, Shanghai, 200025, China

[3]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China

[*]Corresponding author:

Jie Chen,
Department of Pediatric Surgery, XinHua Hospital, Affiliated to Shanghai Jiao Tong University School of Medicine, 1665 Kong Jiang Road, 200092, Shanghai, China, E-mail: jiechen1974@163.com

Wen-Xie Xu,
Department of Anatomy and Physiology, Shanghai Jiao Tong University College of Basic Medical Science, Shanghai, 200025, China,
Tel: +86-21-34205639,
E-mail: wenxiexu@sjtu.edu.cn

[&]Author Contribution:

Yu-Jia L, Jun-Ping A, and these authors are contributed equally to this work.

Received: 12 Feb 2021
Accepted: 01 Mar 2021
Published: 06 Mar 2021

Citation:

Chen J, Wen-Xie X. PAR2 Involved in Colonic Platelet-Derived Growth Factor Receptor-A-Positive Cell Proliferation in Diabetic Mice. Japanese J Gstro Hepato. 2021; V6(2): 1-10

Keywords:

Diabetic mice; Colon; PAR2; PDGFRα+ cells; Proliferation

1. Abstract

1.1. Background: Our previous study indicated that STZ-induced diabetes led to colonic PDGFR α+ cell proliferation accompanied by slow colonic transit in mice, however, the mechanism is unclear.

1.2. Aims: This study was designed to investigate whether protein as e-activated receptor 2 (PAR2) mediates PDGFR α+ cell proliferation.

1.3. Methods: Western blotting, immunohistochemistry and quantitative PCR were applied to this study.

1.4. Results: The present study showed that PDGFRα, PAR2 and Ki-67coexpression was increased in the diabetic colonic muscle layer. The expressions mRNA and protein of PDGFRα and PAR2 were also observably enhanced in the diabetic colonic muscle layer. Mice treated with 2-furoyl-LIGRLO-amide (2-F-L-a), a PAR2 agonist, exhibited significant colon elongation and increased smooth muscle weight. In the 2-F-L-a-treated mice, PDGFRα, PAR2 and Ki-67 coexpression was increased, and PDGFRα and PAR2 mRNA and protein expression was significantly enhanced in the colonic smooth muscle layer. 2-F-L-a also increased proliferation and PDGFRα expression in NIH/3T3 cells cultured in high glucose, while LY294002, a PAR2 antagonist, decreased cell proliferation and PDGFRα expression. The expressions of protein and mRNA in PI3K and Akt and the protein expression of p-Aktin diabetic and 2-F-L-a-treated mice were markedly reduced in colonic smooth muscle. 2-F-L-a also reducedPI3K, Akt and p-Akt protein expressions in NIH/3T3 cells, while LY294002, a PAR2 antagonist, increased this expression.

1.5. Conclusions: The results indicate that PAR2 involve the proliferation of PDGFRα+ cells by the PI3K/Akt signaling approach in the colon of STZ-induced diabetic mice, which contribute to the slow transit and constipation that are associated with diabetes.

2. Introduction

Diabetes is a universal metabolic sickness that seriously affects human health and can cause serious complications that involve multiple organ systems; the incidence of diabetes worldwide has significantly increased in recent decades [1]. Diabetes is a systemic disease that affects the nervous system, urinary system, cardiovascular system and digestive system. The dysmotility is a familiar chronic accompanying symptom of gastrointestinal tract in diabetes mellitus [2-4]. The main manifestations of this disorder are low esophageal pressure, insuffi-

cient gastric motility, gastric emptying disorders, delayed gastrointestinal transit (gastroparesis), abdominal distension, nausea, vomiting, constipation, and diarrhea and so on, and these symptoms seriously influence the survival quality of patients, as well as the absorption of hypoglycemic drugs and the control of blood glucose. To date, the mechanisms underlying chronic constipation in DM patients remain unclear. Our previous study demonstrated that STZ-induced type 1 diabetes mellitus resulted in slow transit constipation in a mouse model. Our research further found that diabetes upregulated expressions of purinergic neurotransmitter P2Y1 receptor and SK3 channel in colonic smooth muscle tissue and led to slow transit constipation in diabetic mice [5]. Therefore, it is necessary to explore the reason of high expression of SK3 channels in colon smooth muscle tissue caused by diabetes.

Recently, studies have found that in the alimentary tract, in addition to interstitial cells of Cajal (ICCs), there is also a interstitial cell called platelet-derived growth factor receptor α positive cells (PDGFRα+ cell) and exhibit similar morphology and properties as ICCs. In the gastrointestinal tract ICCs and PDGFRα+ cells form gap junctions with the smooth muscle cell to constitute SIP (SMCs, ICCs and PDGFRα+ cells) syncytium6-8. The SIP syncytium is an important structure of the enteric nerve that regulates smooth muscle motility. The ICCs and PDGFRα+ cells, are distributed around the varicosity of nerve endings, and the neurotransmitters that are released by the enteric nerve regulate smooth muscle motility through these two kinds of interstitial cells; for example, the excitatory and inhibitory effects of Ach and ON occur via ICCs, and the inhibitory effects of purinergic neurotransmitters occur via PDGFRα+ cells9-11. Our previous study showed that in mice with STZ-induced type I diabetes, colon lengthening significantly decreased colon motility and reduced colon transit and that colonic PDGFRα+ cells were significantly hyper plastic and hyper functional; however, the mechanism of diabetes-induced PDGFRα cell proliferation was unclear [5, 12].

The protease-activated receptor (PARs) family has seven transmembrane fragments with PAR1, PAR2, PAR3 and PAR4 subunits, belonging to G protein-coupled receptors. PARs are widely distributed in epithelial cells, intestinal neurons, and immune cells, such as lymphocytes, macrophages and mast cells; these receptors are involved in the regulation of digestive tract function under physiological and pathological conditions [13–17]. For example, PARs participate in gastrointestinal motility, secretion of digestive mucosa, changes in epithelial permeability, regulation of visceral sensory function and pathogenesis of colitis. The colonic SIP syncytium mainly expresses PAR1 and PAR2, whereas PAR3 is expressed at significantly lower levels than PAR1 and PAR2, and the distribution of PAR1 and PAR2 in PDGFRα+ cell is much higher than that in ICCs and smooth muscle cells [16]. Recent research have indicated that positive PAR2 in blood samples of atherosclerotic patients and in vascular smooth muscle cells of human treated with oxidized LDL after transfection-mediated PAR2 over expression promotes cell proliferation and migration and disrupts the quiescent condition of VSMCs [18]. Whether PARs are involved in the cell proliferation processes caused by various pathogenic factors, especially the oxidative stress of diabetes is unknown, and the answer to this question is of great significance for explaining the pathogenesis of many diabetic complications. Based on these considerations, this study aimed to investigate whether PAR2 is participated in proliferation of colonic PDGFRα+ cell in STZ induced diabetic mice.

3. Materials and Methods

3.1. Ethics Statement

All the animals were approved by the local ethic committee from Shanghai; China. This study was conducted according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of the P.R.C. (STCC Publication No. 2, revised 1988). The protocol was approved by the Committee on the Ethics of Animals at Our University and got the permit number of 686-2009 (Hu).

3.2. STZ-treated Diabetic mouse model

Adult male ICR mice (5wk old) were maintained at 22°C under a 12-h light/dark cycle and given free access to water and food, In total 90 mice were included in this research. Mice was injected with STZ (Sigma Aldrich, St. Louis, MO,200 mg/kg; USA), which dissolved in ice-cold citrate buffer (0.1 mol/L). The normal mice were injected with the same quantity of citrate buffer.1 week and 2 months later, we measured the blood glucose levels of STZ-treated mice; if the blood glucose of the mice were both beyond 16.7 mmol/L, we confirmed the establishment of STZ-induced diabetic mouse.

3.3. PAR2 Agonist Treatment

Adult male ICR mice (3wk old) were maintained at 22°C under a 12-h light/dark cycle and given free access to water and food, in total 30 mice were included in this research. The mice in the 2-F-L-atreatment group were intraperitoneally injected with 2-F-L-a (1 μM/L; 0.2 ml/10 g; Tocris Bioscience, Ellisville, MO, USA) dissolved in ddH2O, and the mice in the control group were injected with the same volume of saline.

3.4. NIH/3T3 Fibroblast Cell Culture

NIH/3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 25 mmol/L glucose and supplemented with 100 mg/mL streptomycin, 10% heat-inactivated bovine serum (BS) and 100 U/mL penicillin. The cells were incubated at 37 °C and 5% CO2. The NIH/3T3 cells were seeded in plates with 50 mmol/L glucose (high glucose), the expression of PI3K, PDGFRα, Akt and p-Akt was measured12 h and 24 h later.

3.5. Preparation of Colonic Smooth Muscle Tissue

The mice were sacrificed by cervical dislocation, and the entire colon

was removed from the diabetic which was placed in Krebs solution with a carbonated mixture (95% O2and 5% CO2). The control mice had the same processing pattern. The Krebs solution contained the following components: glucose, 11.5; NaCl, 121.9; KCl, 5.9; MgSO4, 1.2; NaHCO3, 15.5; CaCl2, 2.4 and KH2PO4, 1.2, all concentrations in mmol/L. The colon was cut along the mesentery, and the colon pellets were flushed out using Krebs solution. The colon tissues were pinned to a rubber plate, and the submucosa and mucosa were carefully shifted under a microscope. The tissue samples were stored at -80 °C.

3.6. Immunostaining

Tissue samples or NIH/3T3 cells were embedded as frozen tissue blocks after dehydrated in 20% sucrose and fixed with ice-cold 4% paraformaldehyde for 6~8 h, then were cut into frozen sections with thicknesses of 8~10 μm at -20 °C. The sections were incubated in 0.1 M PBS (phosphate-buffered saline) with 10% normal goat serum for 2 h to block nonspecific binding and then cultured with a goat anti-PDGFRα antibody (AF1062,1:200; R&D Systems, USA) and a rabbit anti-Ki-67 antibody (GB13030, 1:50; Wuhan Good Biotech Co., China) or a rabbit anti-PDGFRα antibody (1:1000; #3174; Cell Signaling Technology, Danvers, MA, USA) and 24 h later a PAR2 (SAM11) antibody (1:50; sc-13504; Santa Cruz Biotechnology, Inc.) in Triton X-100 (Sigma Aldrich, St. Louis, MO, 0.5%; USA) at 4 °C. The samples were washed with 0.1 M PBS for 30 min and then incubated at room temperature with a Cy3-conjugated anti-goat IgG (1:300; GB21404, Wuhan Good Biotech Co., China), an Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson Immuno Research, 1:100, USA), an Alexa Fluor 488-conjugated goat anti-mouse IgG (A0428, 1:200; Beyotime Institute of Biotechnology, China) and DAPI (2 h). Images were got using a fluorescence microscope (ZEISS Axiovert 200, Japan).

3.7. RNA Isolation and Reverse-Transcription Quantitative PCR

According to the manufacturer's procedure, we extracted the total RNA from the smooth muscle layers of colon via an (Tiangen, Beijing, China) RNA simple Total RNA kit, then synthesized first-strand cDNA which used a Prime Script RT Reagent Kit (Takara, Dalian, China) with gDNA Eraser. Reverse-transcription quantitative PCR which was used the Fast Start Universal SYBR Green Master Mix (Roche, Mannheim, Germany) on a 7500 Real-Time PCR System (Applied Biosystems Waltham, MA, USA) performed with ad hoc primers. The ΔCT method determined the expression of the target genes relative to that of the endogenous control. The primer sequences what we used it is down below:

PDGFRα: F-CTGGTGGTCATTTGGAAGC and R-GGAGTCGTAAGGCAACTG;

PAR2: F-CACCTGGCAAGAAGGCTAA and R- CAACTGGACTGAAGCTCTAC;

PI3K: F-AGATGAGACAGCCAGACT and R-TCTTCAAGCCTGAGGTTTCCTA;

GAPDH: F-TGCGACTTCAACAGCAACTC and R-ATGTAGGCAATGAGGTCCAC.

3.8. Western Blot Analysis

Protein samples were obtained from the colon muscle layers lysed in RIPA (radioimmunoprecipitation assay) buffer (P0013, 1:10; Beyotime Chemical Co., Jiangsu, China) and 1:100PMSF solution. The suspensible material was centrifuged for 15 min at 12000 rpm and 4 °C, combined with 4 × loading buffer, and stewed for 5 min in a boiling water bath. Equal amounts of protein (30 μg/lane) were separated on 10% or 7.5% SDS-PAGE gels and consequently transferred to PVDF(polyvinylidenedifluoride) membranes. We incubated the PVDF membranes which were blocked with 5% nonfat milk in 0.1% TBST (Tris-buffered saline/Tween 20) overnight with primary antibodies at 4 °C, then with secondary antibodies for 2h at indoor temperature. Which the antibody dilutions and antibodies we used were as follows: PAR2 (SAM11) antibody (1:500; sc-13504; Santa Cruz Biotechnology, Inc.), a PDGFRα rabbit antibody, PI3 kinase p110α (C73F8) rabbit antibody, protein kinase B(Akt) rabbit antibody, phospho-Akt (ser473) rabbit antibody, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit antibody, anti-rabbit IgG, HRP-linked antibody, anti-mouse IgG, HRP-linked antibody (1:1000; #3174; #4249; #4691; #4060; #2118;#7074;#7076; Cell Signaling Technology, Danvers, MA, USA), we got the anti-tubulin antibody(1:1000; AT819)from Beyotime Chemical Co.

3.9. Cell Survival Assay

Living cells was counted using a Cell counting kit-8 (Beyotime, Jiangsu, China), which combining monosodium salt, 1-methoxy PMS [5-methylphenazinium, methyl sulfate]and WST-8 [2- (2–methoxy-4–nitrophenyl)-3-(4–nitrophenyl)–5-(2,4disulfophenyl)-2H-tetrazolium]. Briefly, the cells were joined in 96-well tissue culture plates for 24 h, the cells were incubated with control medium or drug. Next, 20 μl of kit reagent was modified and incubated with the cells for 1 h in 5% CO2at 37 °C. Then, the formazan dye was quantified by detecting the optical density at 480 nm using an ELISA plate reader. The absorbance was correlated with the amount of metabolically active cells.

3.10. Statistical Analysis

The results were calculated statistically by using the computer program Gragh Pad Prism6 (La Jolla, CA, USA) or Image J software. The data were expressed as the means ± standard error of the mean (SEM). Among the multiple groups, quantitative data were examined using one-way ANOVA by Bonferroni's post hoc test. Unpaired Student's t-test were used to determine statistical significance for two group comparisons. The data were analyzed at a P-values<0.05 level of significance.

4. Results

4.1. PAR2 and Pdgfrα Coexpression in Colonic Muscle Tissue of Diabetic Mice

PDGFRα+ cells play anvital function in the regulation of colonic motility through handling potassium channel, for example, SK3 channels. In addition, the PAR2 ligand binds to PAR2 to activate SK3 in PDGFRα+ cells and to hyperpolarize the cell membrane potential, which induces SIP-mediated smooth muscle relaxation. However, whether PAR2 contributes to colonic motility disorder induced by diabetes is currently unclear, so the coexpressions of PDGFRα and PAR2 in colonic muscle tissues were investigated in STZ-induced diabetic mice. The data indicated that PDGFRα and PAR2 coexpressions display a significant increase in colonic the smooth muscle layers in STZ-treated mice ((Figure 1A and C), $p < 0.05$, $n = 4$). Moreover, in order to further clarify the proliferation of GDGFRα+ cell in colon muscle tissue in diabetes, the Ki-67 expression in in colon muscle tissue was stated in STZ-treated mice. The results indicated that the PDGFRα and Ki-67 coexpressions showed a significant increase in STZ-treated mice ((Figure 1B and D), $p < 0.05$, $n = 4$).

We observed the expression of DGFRα positive cells with reference to the experimental methods of Iino et al19, and explored the difference of PDGFRα expression in colon smooth muscle layer of the control and diabetic mice. In STZ-treated diabetic mice the PDGFRα protein was found to be significantly increased to 117 ± 5% (Fig. 2A, $p < 0.05$, $n = 5$) in the colonic smooth layer. As well as the results of RT-PCR showed that the expression of PDGFRα gene was raised to 120 ± 3% ((Figure 2C), $p < 0.05$, $n = 5$) in STZ-treated diabetic mice. The expression of PAR2 protein and gene were also measured, and the results showed that the PAR2 protein and F2rl1 gene (encodes the PAR2 protein) expressions were significantly increased to 135 ± 8% (Fig. 2B, $p < 0.05$, $n = 5$) and 127 ± 7% (Figure 2D), $p < 0.05$, $n = 5$), respectively, in the STZ-induced diabetic mice. These phenomenons demonstrated that diabetes significantly ascended coexprssions of the PDGFRα and PAR2 in the colonic muscle layer.

4.2. Effects of PAR2 Agonist on Colonic Length and Smooth Muscle Weight

PAR2 signaling is involved in cell proliferation and migration, such as promoting vascular endothelial cell proliferation/migration and increasing proangiogenic factors [20, 21]. Whether PAR2 signaling contributes to PDGFRα+ cell proliferation in diabetic mice is unclear. After two weeks, the mice were pretreated with 2-F-L-a as in the reference [22], a PAR2 agonist, and changes in the colonic length and smooth muscle weight were measured. In the mice in the 2-F-L-a-treated group, the colons were significantly elongated, and colonic lengths were increased to 65.27 ± 0.30 mm from 53.33 ± 0.67 mm of control group ((Figure 3Aa and 3B), $p < 0.05$, $n = 15$). The weight of the colonic smooth muscle from the 2-F-L-a-treated mice was significantly increased, with an average weight of 61.47 ± 1.04 mg compared with an average weight of 43.93 ± 0.59 mg ($n = 15$; $p < 0.05$; (Figure 3C)) in the control mice. These results suggest that PAR2 may be involved in diabetes-induced colon PDGFRα cell proliferation.

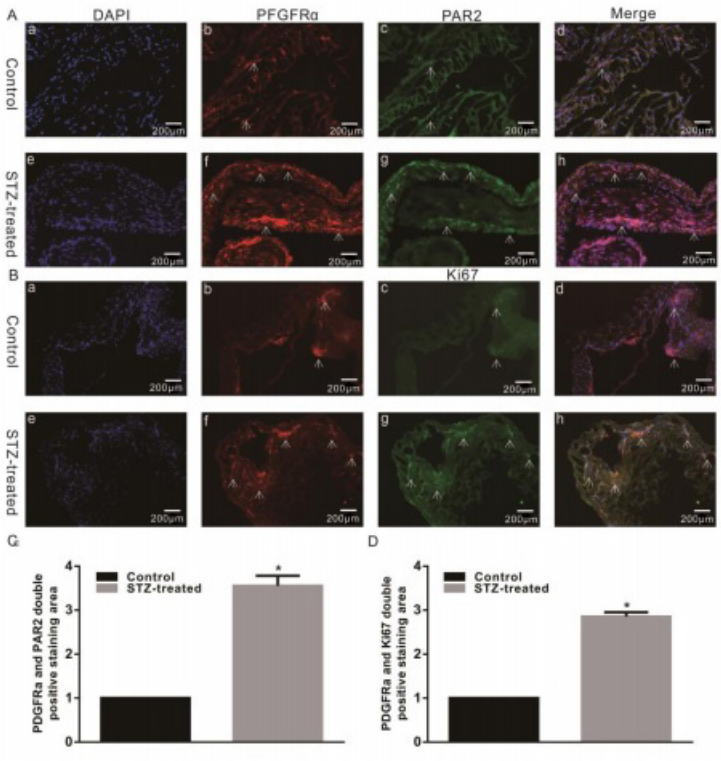

Figure 1: The expression of PAR2, PDGFRα and Ki-67 in the colon muscle tissues of STZ-induced diabetic mice. Immunofluorescence staining substantiate different Ki-67 and PDGFRα coexpression levels (A) and PDGFRα and PAR2 coexpression levels (B) in the colon smooth muscle layers between the control and STZ-induced diabetic mice (bar = 100 m). C and D: The double-positive staining region was detected applying Image J ($n = 4$; $^{*}p < 0.05$).

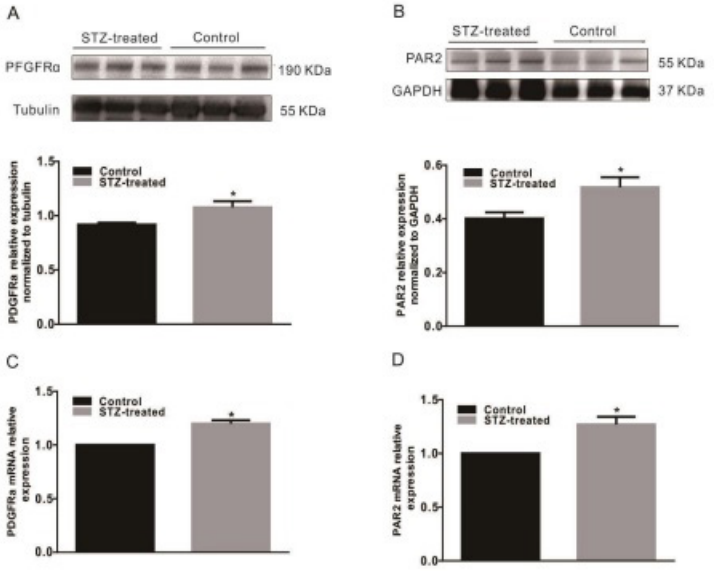

Figure 2: The expression levels of PDGFRα and PAR2 in the colonic muscle tissues of diabetic mice. Western blot analysis of PDGFRα (A) and PAR2 (B) in normal and STZ-treated diabetic mice. Ratio of PDGFRα and PAR2 to tubulin or GAPDH compared with the control mice. (n = 5, $^{*}p < 0.05$). The expression of PDGFRα (C) and PAR2 (D) were used quantitative RT-PCR analysis in the colon muscle layers of the normal and STZ-induced diabetic mice. The results were normalized to GAPDH expression and the normal mice (% of GAPDH and normalized to the data of the normal mice; PAR2, $^{*}p < 0.05$, n = 5; PDGFRα, $^{*}p < 0.05$, n = 5).

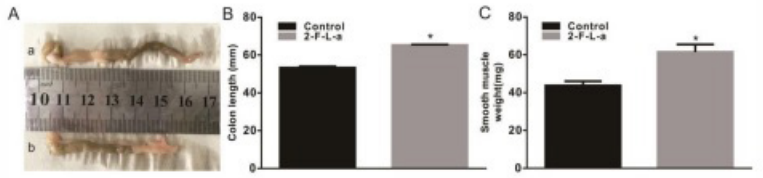

Figure 3: Change in colonic length in 2-F-L-a-treated mice. A: Comparison of the colon length between the control and 2-F-L-a-treated mice. B: Summarized data showing the colon length in the control and 2-F-L-a-treated mice ($^{*}p < 0.05$; n = 15). C: Summarized data showing the smooth muscle weight in the control and STZ-treated mice. The data are shown as the mean ± SEM. ($^{*}p < 0.05$; n = 15).

4.3. PAR2 Agonist Increased colonic Pdgfrα Expression in Diabetic Mice

To determine the relationship between PAR2 and diabetes-induced PDGFRα+ cell proliferation, the coexpression of PDGFRα and PAR2 was observed in the colonic muscle tissues of 2-F-L-a-treated mice. The results indicated that the PDGFRα and PAR2expressions were notably increased in 2-F-L-a-treated mice. To specify the expression levels of PAR2 and PDGFRα in the smooth muscle layers, the double-positive staining region was detected using Image J software and showed significantly increased coexpression of PDGFRα and PAR2 in the 2-F-L-a-treated mice PDGFRα and PAR2 coexpression levels was assessed by measuring double positive staining area by Image J software, and the results showed that the coexpression of PDGFRα and PAR2 was much higher in 2-F-L-a-treated group than that in control group (Fig. 4A and C, n=4, p< 0.05). ((Figure 4A and C), n = 4, $p < 0.05$). Furthermore, at different stages of the cell cycle, Ki-67 recognized cell proliferation indicators thus, Ki-67 expression was observed in colon muscle tissue to assess PDGFRα positive cell proliferation status in 2- F-L-a treated mice. The expressions of colonic PDGFRα and Ki-67 in 2- F-L-a treated mice were significantly increased, and the evaluation of double positive staining area showed that the co-expression of PDGFRα and Ki-67 was evidently increased ((Figure 4B and D), n = 4, $p < 0.05$).

In addition, the protein and mRNA expressions of PDGFRα and PAR2 in the colonic muscle tissue of 2-F-L-a-treated mice were evaluated. Compared with the control mice, the 2-F-L-a-treated mice showed evidently up-regulated in protein and mRNA expressions of PDGFRα and PAR2 (Figure 5A, B, C, D), $p < 0.05$, n = 4). These results suggest that PAR2 may be involved in the proliferation of colonic PDGFRα positive cells in mice.

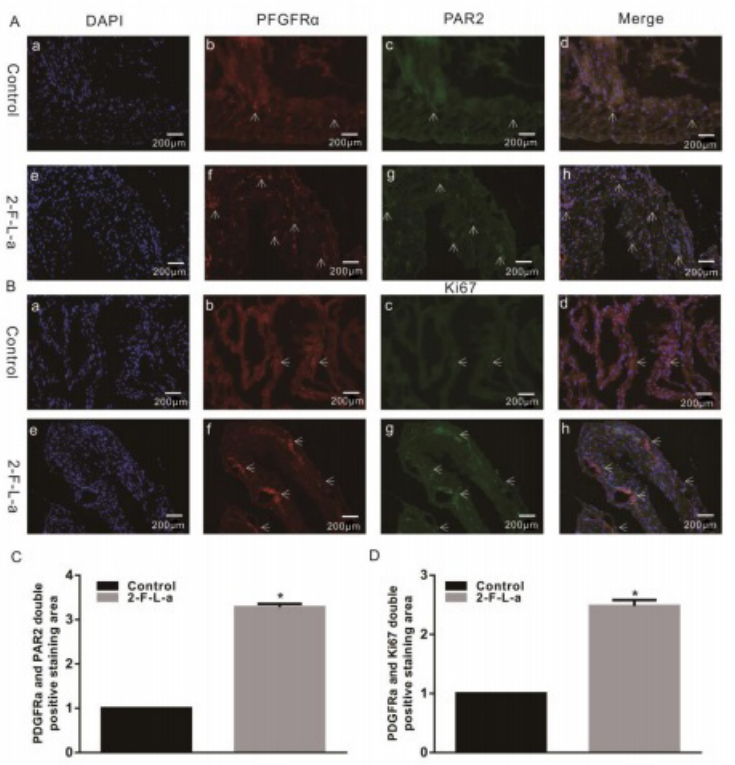

Figure 4: PDGFRα, Ki-67 and PAR2 expression in the 2-F-L-a-treated colonic muscle tissues. A and B: Immunofluorescence staining revealed different PDGFRα and Ki-67 coexpression levels and PDGFRα and PAR2 coexpression levels in the colon smooth muscle layers between the control and 2-F-L-a-treated mice (bar = 100 μm). The double-positive staining region was detected applying ImageJ software. (2-F-L-a: PAR2 agonist; n = 4; $^{*}p < 0.05$).

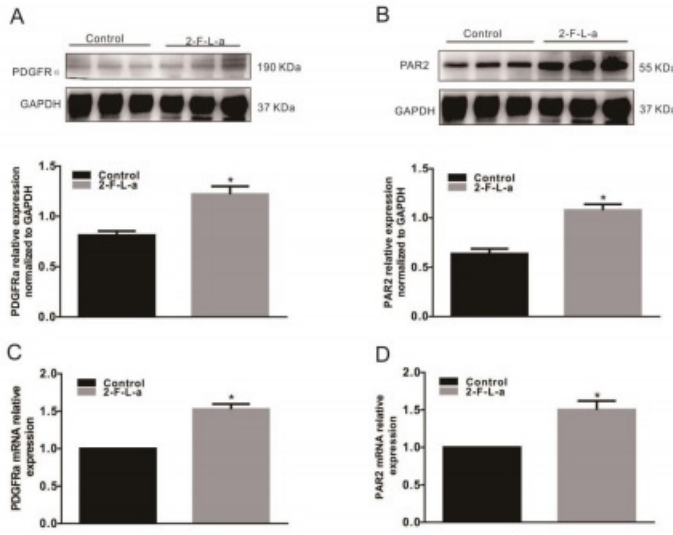

Figure 5: PDGFRα and PAR2 expression levels in the colon muscle tissues of the 2-F-L-a-treated mice. The expression of PDGFRα (A) and PAR2 (B) were analysed using western blot in the normal and 2-F-L-a-induced mice. GAPDH is shown as loading control, and densitometric values are shown. (2-F-L-a: PAR2 agonist; n = 5; $^{*}p < 0.05$). The expression of PDGFRα (C) and PAR2 (D) were used quantitative RT-PCR analysis in the colon muscle layers of the normal and 2-F-L-a-induced mice. The results were normalized to GAPDH expression and the normal mice (% of GAPDH and normalized to the data of the normal mice; 2-F-L-a: PAR2 agonist; n = 4; $^{*}p < 0.05$).

4.4. The Effect of PAR2 Agonist on Expression of PI3K/Akt in Colon of Diabetic Mice

As the main downstream effector of growth factor PI3K/Akt pathway is involved in many intracellular biological functions. To investigate whether PI3K/Akt signaling pathway mediates this process which PAR2-induced PDGFRα cell proliferation in STZ-treated diabetic colon, in the next experiment the effect of PAR2 agonists on the colonic PI3K/Akt signaling pathway was investigated in diabetic

mice. The outcomes sustained the expressions of phosphatidylinositol-3-kinase protein and mRNA were significantly decreased in STZ-induced diabetic mice ((Figure 6A, B), n = 6, $p < 0.05$). The total and phosphorylated protein levels of Akt were significantly downregulated in the colonic muscle tissues of the diabetic group ((Figure 6C, D), $p < 0.05$, n = 6). Similarly, in the 2-F-L-a-treated group the expression of PI3K protein was also importantly reduced (Figure 7B), n = 6, $p < 0.05$), and the total and phosphorylated Akt protein levels were evidently down regulated in the colonic muscle tissues ((Figure 7A, C), $p < 0.05$, n = 6). These data suggest that PAR2 is involved in diabetes-induced colon PDGFRα cell proliferation processes which may be mediated by PI3K/Akt signaling pathways.

4.5 The Effect of PAR2 Agonist on Pdgfrα+ NIH/3T3 Cell Proliferation

PDGFRα cells are called "fibroblast-like cells "(FLCs) by morphologists, and NIH/3T3 cells are established from mouse embryonic fibroblasts, so NIH/3T3 cells express PDGFRα cells too. Moreover, the PAR2 ((Figure 8A), n = 6) and PDGFRα proteins (Figure 8C) are expressed in NIH/3T3 cells. Therefore, NIH/3T3 cells are suitable for use as an in vitro model to examine the relationship between PAR2 and PDGFRα+ cell proliferation. NIH/3T3 cells cultured in high glucose were incubated with the PAR2 agonist 2-F-L-a at 1 μmol/L for 24 h, and the NIH/3T3 cells exhibited significant growth; however, when NIH/3T3 cells were incubated with 1 μmol/L 2-F-L-a and LY294002, a PAR2 antagonist, for 24 h, the 2-F-L-a-induced NIH/3T3 cell growth was significantly inhibited (Figure 8B), n = 6, $p < 0.05$).Similarly, after treated with 1μmol/L2-F-L-a, a PAR2 agonist, the expression of PDGFRα proteins was significantly increase, while treated with 1μmol/L2-F-L-a plus LY294002 for 24 h, the expression of PDGFRα proteins was significantly reduced in whole cell extracts ((Figure 8C), D, n = 6, $p < 0.05$). These results suggest that PAR2 promotes PDGFRα+ NIH/3T3 cell proliferation.

Next, the protein expression levels of PI3K, Akt, and p-Akt in NIH/3T3 cells were found to be significantly decreased by treatment with 1 μmol/L 2-F-L-a, while treatment with 2-F-L-a and LY294002 for 24 h significantly increased the protein expression levels of PI3K, Akt, and p-Akt in NIH/3T3 cells ((Figure 9), n = 4, $p < 0.05$). These results suggest that PAR2 participate in the process of NIH/3T3 cell proliferation, which is mediated by PI3K/Akt signals.

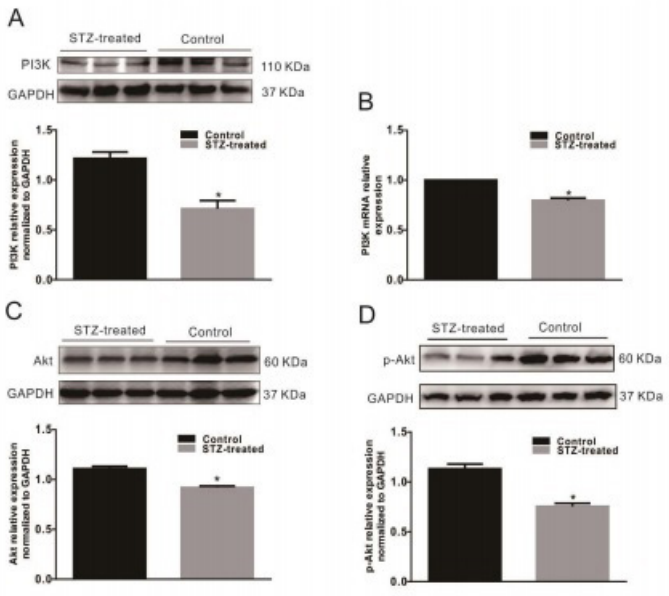

Figure 6: PI3K/Akt expression in colonic muscle tissues from diabetic mice. A, C, D: The expression of PI3K/Akt were analysed using western blot in the normal and STZ-induced diabetic mice. GAPDH is shown as loading control, and densitometric values are shown. (n = 5, $^{*}p < 0.05$). B: The expression of PI3K were used quantitative RT-PCR analysis in the colon muscle layers of the normal and STZ-induced diabetic mice. The results were normalized to GAPDH expression and the normal mice. (% of GAPDH and normalized to data of the normal mice; $^{*}p < 0.05$; n = 5).

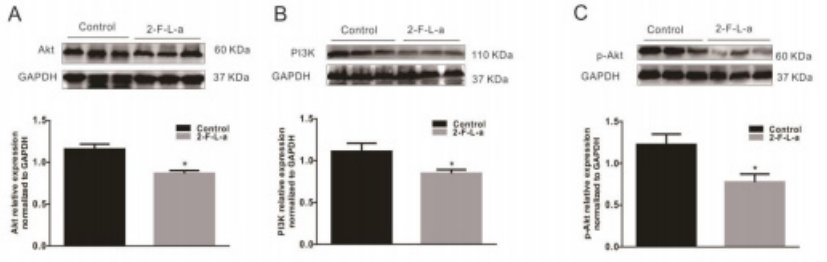

Figure 7: PI3K/Akt expression in colonic muscle tissues of 2-F-L-a-induced mice. A, B and C: Western blot analysis of PI3K, Akt, and p-Akt expression in the control and 2-F-L-a-treated mice. The data were analyzed using densitometric quantification (% of GAPDH and normalized to the data of the control mice; n = 5, $^{*}p < 0.05$).

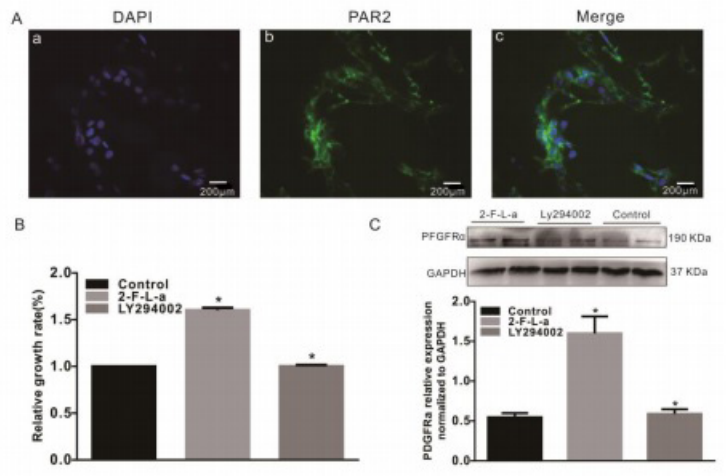

Figure 8: PAR2 expression in NIH/3T3 cells. A: Immunofluorescence staining revealed the PAR2 expression levels (bar = 100 μm) in NIH/3T3 cells. B: LY294002 inhibited the 2-F-L-a-induced proliferation of NIH/3T3 cells. The graph summarizes the data showing the relative growth rates of the control, 2-F-L-a-treated and 2-F-L-a plus LY294002-treated NIH/3T3 cells. C: Western blot analysis of PDGFRα protein expression in the control, 2-F-L-a-treated and 2-F-L-a plus LY294002-treated NIH/3T3 cells. The graph summarizes the data showing PDGFRα protein expression. (2-F-L-a: PAR2 agonist; LY294002: PI3K antagonist; n = 6; $^{*}p < 0.05$).

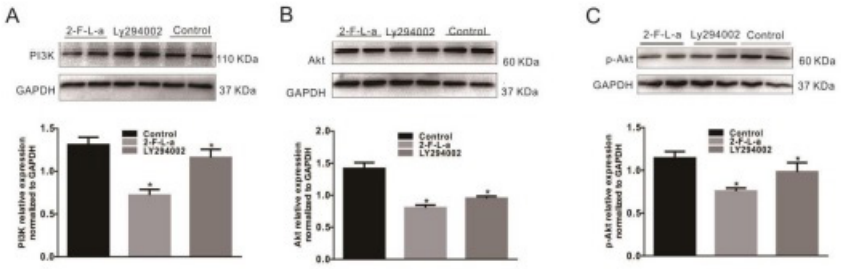

Figure 9: PAR2 expression in NIH/3T3 cells. Immunofluorescence staining revealed the expression levels of PAR2 in NIH/3T3 cells (n = 6). LY294002 inhibited the 2-F-L-a-induced proliferation of NIH/3T3 cells. The graph summarizes the data showing the relative growth rates of the control, 2-F-L-a-treated and 2-F-L-a plus LY294002-treated NIH/3T3 cells. (2-F-L-a: PAR2 agonist; LY294002: PI3K antagonist; n = 6; $^{*}p < 0.05$).

5. Discussion

In recent reports, colonic dysmotility has been shown to occur in diabetes patients, and many patients exhibit constipation [23, 24]. The patterns of colonic motility and transit are regulated by enteric nervous system and interstitial cells, such as ICCs, PDGFRα+ cells. Purinergic nerve is an important inhibitory nerve in colon smooth muscle, while purinergic neurotransmitters activate SK3 channels through P2Y1 receptors on PDGFRα cells and mediate the inhibitory effect of purinergic nerve on colon smooth muscle [25]. The expression of PAR2 in PDGFRα cells is much higher in colon than in ICC and smooth muscle cells, however, in normal smooth muscle, PAR2 is activated by trypsin, which can lead to APAMIN sensitive outward potassium current activation16. Our previous study found that the expression of PDGFRα+ cells increased significantly as well as the up-regulation of purine neurotransmitter / P2Y1 / SK3 in diabetic colon, and which caused the colonic slow transit [5]. However, in the present there are no reports that diabetes can affect colon PDGFRα positive cell proliferation by PAR2.The purpose of this work is to study whether PAR2 are involved in the proliferation of PDGFRα cells under diabetic conditions.

Regulatory disorders of PAR-2 activation can lead to a large number of common human diseases, especially due to the ability of PAR-2 receptors to induce key inflammatory signaling pathways [26]. Moreover, Ki-67 is a nuclear antigen that is generally used as an indicator of cell proliferation, since Ki-67 is expressed only at the G1, S and G2 mitotic phases of the cell cycle, but not at the G0 resting phase [27]. In this research, we first explored the interaction between PAR2 and colon PDGFRα expression in diabetic mice and preliminarily judged whether it PAR2 affect PDGFRα cells. The results showed that the PDGFRα and Ki-67 co-expression in diabetes and PAR2 agonist groups significantly increased compared with the control group (Figure 1 and 4). Furthermore, the expression levels of PDGFRα and PAR2mRNA in the diabetic and PAR2 agonist-treated mice exhibited significantly upregulated (Fig. 2 and Fig. 5), the PDGFRα and PAR2protein showed similar expression. The above results strongly suggest that increased co-expression of PDGFRα and PAR2 in the colon of diabetic or PAR2 agonist-treated mice may be associated with in the number of PDGFRα+ cells. Previous evidence indicates that the binding of a PAR2 agonist to PAR2 stimulates the cell proliferation mediated the PI3K/Akt signaling pathway [28, 29]. In pres-

ent study demonstrated that the expression levels of the PI3K, Akt, and p-Akt in the colonic muscle tissues were significantly decreased in both STZ- and PAR2-treated mice (Figure 6 and 7). Above data suggest that in STZ-induced mouse diabetic model, the proliferation of colonic PDGFRα+ cells increased significantly and accompanied by up-regulation of PAR2 expression, and PAR2 involved in PDGFRα+ cell proliferation through the PI3K/Akt signaling pathway leading to an imbalance in the function of ICCs and PDGFRα+ cells in SIP, which induced diabetic slow transit constipation.

We propose that PAR2 signaling is linked to cell homeostasis and mitogenic signaling pathways in the NIH/3T3 fibroblast cell line. After NIH/3T3 cells were cultured in high glucose and incubated with a PAR2 agonist for 24 h, the cells exhibited significant growth; however, incubation of the NIH/3T3 cells with the PAR2 agonist and LY294002, a PAR2 antagonist, significantly inhibited the growth-promoting effect (Figure 8). Similarly, the expression of the PDGFRα protein in NIH/3T3 cells was significantly increased by PAR2 agonist treatment, while the expression the PDGFRα protein was significantly decreased by PAR2 agonist and LY294002 treatment (Figure 8). These data suggest that PAR2 may be involved in NIH/3T3 cell proliferation and PDGFRα expression, as PAR2 is associated with increased protease-mediated hydrolysis, both in vitro and in vivo experiments. In response to protease hydrolysis, the PAR2 proteins were activated, thus contributing to a decrease in PI3K/Akt activity. The decrease in PI3K/Akt activity was associated with FOXO dephosphorylation, and dephosphorylated FOXO translocated into the nucleus acts as a transcription factor30-33. Next, it was observed that the PI3K Akt and p-Akt protein expressions of NIH/3T3 cells treated with PAR2 agonist were markedly decreased, while the PI3K Akt and p-Akt protein expressions of the NIH/3T3 cells treated with PAR2 agonist and LY294002, a PAR2 antagonist were markedly increased (Figure 9). These data suggest that PAR2 is involved in the process of NIH/3T3 cell proliferation, which is mediated by PI3K/Akt signals.

In summary, diabetes induces the proliferation of PDGFRα+ cells in the colon, leading to the imbalance of ICC and PDGFRα+ cells in the colonic SIP, and which leads to diabetic slow transit constipation. The hyper function of the colonic PAR2 induced by diabetes are involved in the process of colonic PDGFRα+ cells proliferation, and which mediated by PI3K/Akt signaling pathway. It is also possible that PAR2 upregulation may be due to the inflammatory stress caused by hyperglycemia. The colonic transit is regulated by the enteric nerve, and this process is mediated by interstitial cells such as ICC and PDGFRα+ cells. The diabetes-induced increase in PAR2 can promote colonic PDGFRα+ cells proliferation, as well as can stimulate SK3 channel activation on the cell membrane of the PDGFRα+ cells, which may lead to colonic motility disorders. Therefore, PAR2 and PDGFRα+ cells may be new targets for clinical treatment of diabetic colonic transit disorders.

6. Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant Nos. 31671192 and 31871158) and the Zhejiang Provincial Natural Science Foundation of China (No. LY20C11003).

References

1. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE, et al. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract. 2014; 103: 137-49.
2. Feldman M, Schiller LR. Disorders of gastrointestinal motility associated with diabetes mellitus. Ann Intern Med. 1983; 98: 378-84.
3. Ordog T. Interstitial cells of Cajal in diabetic gastroenteropathy. Neurogastroenterol Motil. 2008; 20: 8-18.
4. Talley NJ, Young L, Bytzer P, Hammer J, Leemon M, Jones M, et al. Impact of chronic gastrointestinal symptoms in diabetes mellitus on health-related quality of life. Am J Gastroenterology. 2001; 96: 71-5.
5. Song NN, Lu HL, Lu C. Diabetes-induced colonic slow transit mediated by the up-regulation of PDGFRα+ cells/SK3 in streptozotocin-induced diabetic mice. Neurogastroenterol Motil. 2018; 30: e13326.
6. Iino S, Nojyo Y. Immunohistochemical demonstration of c-Kit-negative fibroblast-like cells in murine gastrointestinal musculature. Arch Histol Cytol. 2009; 72: 107-15.
7. Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal motility--insights from smooth muscle biology. Nature reviews. Gastroenterology & hepatology. 2012; 9: 633-45.
8. Sanders KM, Zhu MH, Britton F, Koh SD, Ward SM. Anoctamins and gastrointestinal smooth muscle excitability. Experimental physiology 2012; 97: 200-6.
9. Blair PJ, Rhee PL, Sanders KM, Ward SM. The Significance of Interstitial Cells in Neurogastroenterology. J Neurogastroenterol Motil. 2014; 20: 294-317.
10. Koh SD, Ward SM, Sanders KM. Ionic conductances regulating the excitability of colonic smooth muscles. Neurogastroenterol Motil. 2012; 24: 705–18.
11. Jimenez M. Platelet-derived growth factor receptor-α-positive cells: new players in nerve-mediated purinergic responses in the colon. J Physiol. 2015; 593: 1765–6.
12. Lu HL, Zhang CM, Song NN. Colonic PDGFRα Overexpression Accompanied Forkhead Transcription Factor FOXO3 Up-Regulation in STZ-Induced Diabetic Mice. Cell Physiol Biochem. 2017; 43: 158-71.
13. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front Endocrinol (Lausanne). 2014; 5: 67.
14. Vergnolle N. Clinical Relevance of Proteinase Activated Receptors (PARS) In The Gut. Gut. 2005; 54: 867–74.
15. Sato K, Ninomiya H, Ohkura S, Ozaki H, Nasu T. Impairment of PAR-2-mediated relaxation system in colonic smooth muscle after intestinal inflammation. Br J Pharmacol. 2006; 148: 200-7.
16. Sung TS, Kim HU, Kim JH, Lu H, Sanders KM, Koh SD, et al. Protease-activated receptors modulate excitability of murine colonic smooth

muscles by differential effects on interstitial cells. J Physiol. 2015; 593: 1169-81.

17. Kawabata A, Matsunami M, Sekiguchi F. Gastrointestinal roles for proteinase-activated receptors in health and disease. Br J Pharmacol. 2008; 153: S230-40.
18. Wei M, Liu Y, Zheng M, Wang L, Ma F, Qi Y, et al. Upregulation of Protease-Activated Receptor 2 Promotes Proliferation and Migration of Human Vascular Smooth Muscle Cells (VSMCs). Med Sci Monit. 2019; 25:8854-62.
19. Ino S, Horiguchi K, Horiguchi S, Nojyo Y. c-Kit-negative fibroblast-like cells express platelet-derived growth factor receptor alpha in the murine gastrointestinal musculature. Histochem Cell Biol. 2009; 131: 691-702.
20. Zhu T, Sennlaub F, Beauchamp MH. Proangiogenic effects of protease-activated receptor 2 are tumor necrosis factor-alpha and consecutively Tie2 dependent. Arterioscler Thromb Vasc Biol 2006; 26: p744-50.
21. Rderiu G, Espinosa S, Pena E, Aledo R, Badimon L. PAR2-SMAD3 in microvascular endothelial cells is indispensable for vascular stability via tissue factor signaling. J Mol Cell Biol. 2016; 8: p255-70.
22. McLarty JL, Meléndez GC, Brower GL, Janicki JS, Levick SP. Tryptase/Protease-activated receptor 2 interactions induce selective mitogen-activated protein kinase signaling and collagen synthesis by cardiac fibroblasts. Hypertension 2011; 58(2): 264-270.
23. Camilleri M, Ford AC, Mawe GM, et al. Chronic constipation. Nat Rev Dis Primers 2017; 3: 17095.
24. Lembo A, Camilleri M. Chronic Constipation. N Engl J Med 2003; 349: 1360-1368.
25. Baker S. A, Hennig GW, Salter AK, Kurahashi M, Ward SM, Sanders KM. Distribution and Ca(2+) signalling of fibroblast-like (PDGFR(+)) cells in the murine gastric fundus. J Physiol 2013; 591: 6193-6208.
26. Coughlin SR, Camerer E. PARticipation in inflammation. J Clin Invest 2003; 111: 25-27.
27. Yuan JP, Wang LW, Qu AP, et al. Quantum dots-based quantitative and in situ multiple imaging on ki67 and cytokeratin to improve ki67 assessment in breast cancer. PLoS One 2015; 10: e0122734.
28. Du C, Zhang T, Xiao X, Shi Y, Duan H, Ren Y. Protease-activated receptor-2 promotes kidney tubular epithelial inflammation by inhibiting autophagy via the PI3K/Akt/mTOR signalling pathway. Biochem J 2017; 474(16): p2733-2747.
29. Kawabata A. [Physiological functions of protease-activated receptor-2]. Nihon Yakurigaku Zasshi 2003; 121(6): p411-20.
30. Kawabata A, Oono Y, Yonezawa D, et al. 2-Furoyl-LIGRL-NH2, a potent agonist for proteinase-activated receptor-2, as a gastric mucosal cytoprotective agent in mice. Br J Pharmacol 2005; 144: 212-9.
31. Dobson M, Ramakrishnan G, Ma S, Kaplun L, Balan V, Fridman R, Tzivion G. Bimodal regulation of FoxO3 by AKT and 14-3-3. Biochim Biophys Acta 2011; 1813: 1453-64.
32. Accili, D. and K.C. Arden. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004; 117: 421-6.
33. Lee, S. and H.H. Dong. FoxO integration of insulin signaling with glucose and lipid metabolism. J Endocrinol 2017; 233: R67-r79.