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Original Article

SCF/c-KIT signaling promotes mucus secretion of colonic goblet cells and development of mucinous colorectal adenocarcinoma

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Abstract: Mucinous colorectal adenocarcinoma (MCA) is characterized by a great mount of extracellular mucus fundamentally composed of Mucin2 (MUC2) which is significantly correlated with the high malignancy and strong invasive ability of MCA. However, rare is known about the underlying mechanism of the mucus accumulation in MCA. Our latest study demonstrated that SCF/c-KIT signaling was highly activated in MCA patients and mouse model, which up-regulated MUC2 transcription. In the present study, we paid a special interest in whether and how SCF/c-KIT signaling promoted mucus secretion by using wild-type (WT) C57BL mice and their littermates who harbor mutational *c-kit* gene ($Wads^{m/m}$), clinical colorectal cancer (CRC) samples, as well as human CRC cell lines. Our results clearly showed that the inner mucus layer of colon was thinner and the intracellular mucin residual was more in $Wads^{m/m}$ mice than those in WT mice by Alcian blue and PAS staining, suggesting that the mucus secretion process was crippled when SCF/c-KIT signaling was hypo-activated. Inhibiting SCF/c-KIT signaling by Imatinib also resulted in weakened mucus secretion in WT mice. Intraperitoneal administration of MANS which competitively inhibits the activity of the vesicular transport protein MARCKS efficiently reduced mucus secretion in colonic goblet cells of WT mice. Significantly, phosphorylated MARCKS (p-MARCKS) was overtly decreased in colonic mucosa of $Wads^{m/m}$ mice compared with WT mice, indicating that SCF/c-KIT signaling-regulated mucus secretion was probably mediated by MARCKS activation. Similar results were obtained in MCA patients and mouse model. Moreover, SCF/c-KIT signaling was activated or inhibited in HT-29 and LS174T CRC cells, which potently increased or decreased MARCKS activity, respectively. Finally, we found that PKCδ, a known kinase for MARCKS, was activated in WT and MCA mice along with MARCKS. Inhibition or activation of SCF/c-KIT signaling resulted in decreased or increased PKCδ activity respectively *in vitro*. In conclusion, we demonstrated that SCF/c-KIT signaling can promote the mucus secretion by activating PKCδ-MARCKS, which provided a new insight into understanding the mechanism of mucus secretion of goblet cells and MCA development.

Keywords: Goblet cell, c-KIT, MARCKS, mucinous colorectal adenocarcinoma, mucus secretion

Introduction

Mucinous colorectal adenocarcinoma (MCA), accounting for 15~20% of colorectal cancer (CRC), is characterized by a great mount of extracellular mucus (> 50% of tumor area) [1]. Compared with regular CRC (non-MCA), MCA is poorly differentiated and highly malignant. Patients suffering from MCA often show higher risk of metastasis, unsatisfactory treatment effect after surgery and chemotherapy, and poorer prognosis [1, 2]. Previous literatures revealed that the production of massive extracellular mucus, fundamentally composed of Mucin2 (MUC2), could be a key process during MCA development [3, 4]. However, rare is known about the underlying mechanism of the mucus accumulation in MCA.

The c-KIT receptor (CD117) is an important member of type III receptor tyrosine kinase (RTK) family encoded by the oncogene *c-kit*. C-KIT, expressed in the intestinal epithelial cells, contributed to the stem cell niche in the murine colon crypt base [5]. C-KIT signaling occurs when it binds its only known ligand,

stem cell factor (SCF), thereby, promotes cell growth, survival, differentiation, migration and secretion in different biological contexts [6-8]. Clinically, highly expressed RTKs and their downstream signal transduction molecules have been seen in a variety of mucinous adenocarcinoma, such as the high expression of insulin-like growth factor receptor 1 (IGF-1R) in lung mucinous adenocarcinoma and human epidermal growth factor receptor-2 (HER-2) in primary mucinous epithelial ovarian cancer [9, 10]. Our latest study demonstrated that SCF/c-KIT signaling was highly activated in MCA patients and mouse model compared with non-MCA controls, which up-regulated MUC2 transcription [personal communication].

Besides the transcriptional regulation on MUC2, the accumulation of extracellular mucus in MCA might be due to an excessive secretion of mucus. However, modulation of the mucus secretion remains unclear. Myristoylated Alanine Rich C-Kinase Substrate (MARCKS) is a ubiquitously expressed, rod-shaped protein containing 3 domains: an N-terminal myristoylated domain which mediates binding to membranes, a basic effector domain (ED) harboring the protein kinase C (PKC) phosphorylation sites as well as binding sites for actin and calmodulin, and a highly conserved MH2 domain of unknown function [11]. Within the airway epithelium, phosphorylated MARCKS (p-MARCKS) by PKCδ has emerged as a key factor driving exocytosis of mucin in goblet cells [12]. Generally, the inactived MARCKS binds to the inner leaflet of the plasma membrane, while p-MARCKS is freed from the membrane and translocated to the cytosol [13], which helps secretory granule release via an exocytotic process [14]. Yet, whether SCF/c-KIT signaling promoted mucus secretion in MCA has not been illustrated; and if yes, what role does MARCKS plays in the SCF/c-KIT-driven mucus secretion? In the present study, we showed that SCF/c-KIT signaling contributed to the mucus secretion by activating PKCδ-MARCKS using wild-type (WT) C57BL mice and their littermates who harbor mutational *c-kit* gene ($Wads^{m/m}$), clinical CRC samples, as well as human CRC cell lines.

Materials and methods

Mice

$Wads^{m/+}$ mice, which have a unique T-to-C transition mutation in *c-kit* gene [15], were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained in a specified pathogen free environment with controlled conditions of humidity (50 ± 10%), 12/12-h light/dark cycle and temperature (23 ± 2°C). WT and $Wads^{m/m}$ mice were obtained by mating $Wads^{m/+}$ parents as previously described [15]. Animal studies were carried out strictly under protocols approved by the Animal Care and Use Committee of Capital Medical University (Permit Number 2009-X-735). Every effort was made to minimize the number of mice used as well as their suffering.

MCA murine model

Mouse MCA model was established as previously described [16]. Briefly, each mouse received an intraperitoneal administration of azoxymethane (AOM, 10 mg/kg; Sigma-Aldrich, USA) and following 3 periods of intermittent 2.5% dextran sodium sulfate (DSS; MP Biomedicals, USA) in water. Controlled mice were injected with normal saline and had free access to water. MCA was successfully induced 37 weeks after the AOM injection.

Patients

The clinical CRC samples were collected immediately after surgical resection prior to any other therapeutic intervention at the Xuanwu Hospital Capital Medical University (Beijing, China). The CRC specimens were classified into MCA and non-MCA according to the amount of mucus defined by Alcian blue staining. All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki and the research was approved by the Clinical Research Ethics Committee of the Xuanwu Hospital Capital Medical University (Permit Number 2013-X-036, 20 July 2013).

Alcian blue/PAS staining

The distal colon of mice were removed and immediately submerged in Methanol-Carnoy's fixative at 4°C for 3 h followed by 100% methanol for 30 min, 100% ethanol for 20 min, and xylene for 15 min. Fixed tissues were embedded in paraffin and cut into 7 μm sections using a paraffin microtome (Leica RM 2135, Leica, Germany). Sections were stained with Alcian blue/PAS.

Immunofluorescent staining

Frozen sections (5 μm) were cut from tissues embedded in optimal cutting temperature compound (OCT; Sakura Tissue-Tek, USA) using a cryostat (Leica CM3050S, Leica). Sections were fixed with 100% of acetone for 30 min at 4°C or 4% of paraformaldehyde for 30 min at 25°C. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min. Then the sections were incubated with corresponding primary antibody at 4°C overnight followed by incubation with Cy3/488-conjugated secondary antibody for 1 h at 25°C in the dark. Sections were mounted with mounting medium with DAPI (Zhongshanjinqiao Biotechnology, Beijing, China) and visualized by a fluorescence microscope (Nikon, Eclipse Ni, Japan). Sections incubated with an isotype control antibody and omission of primary antibody was used as negative controls. All antibodies used in this study are listed in Supplementary Table 1.

Western blot

Total proteins were extracted using RIPA lysis buffer containing protease inhibitors (Applygen, Beijing, China) and phosphatase inhibitors (Sigma-Aldrich). 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed and proteins were transferred to PVDF membranes (Millipore, USA). After blocking with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk or 5% BSA for 1 h, membranes were incubated with corresponding primary antibody at 4°C overnight, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA) 1 h at 25°C. Anti-β-actin was used as internal control. Proteins were detected with ECL chemiluminescence and their intensity was analyzed with Image J software (NIH).

Imatinib administration

Imatinib (BioVision, USA) was orally administered at a final concentration of 0.5 mg/g/d for 7 days.

MANS administration

MANS peptide (identical to the first 24 amino acids of the N-terminus of MARCKS; myristic acid-GAQFSKTAAKGEAAAERPGEAAVA) or missense control RNS peptide (myristic acid-GTAPAAEGAGAEVKRASAEAKQAF) (Sangon Biotech, Shanghai, China) were administered at a final concentration of 80 μM (8 mg/kg). Mucus secretion was triggered 15 min later by intraperitoneal injection of pilocarpine (100 mg/kg; Abcam, USA) for an additional 10 min before sampling.

Cell culture

Human CRC cell lines (HT-29 and LS174T) were purchased from American Tissue Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Life technologies, USA) and 1% penicillin/streptomycin (Life technologies). Cells were grown at 37°C in the presence of 5% CO_2. Cells were treated with Imatinib (2 μM; BioVision, USA), or recombinant human SCF (rhSCF; 50 ng/mL; R&D System, USA).

Over-expression of c-KIT

To perform lentivirus mediated over-expression of c-KIT, HT-29 and LS174T cells were seeded in a 6-well plate and infected with Lv-*c-kit* (GeneChem, Shanghai, China) when reaching 30% confluence. Infection efficiency was evaluated by observing GFP expression using an inverted fluorescence microscope (Leica DMI-3000B, Leica).

Statistical analysis

Results were presented as mean ± SEM and analyzed by Student's t-test with the SPSS 17.0 software (USA). *P* value of < 0.05 was considered statistically significant.

Results

SCF/c-KIT signaling promoted mucus secretion of colonic mucosa

Because the mucinous cancer-related MUC2 was basically the same as that in normal goblet cells in protein conformation without any mutation [3, 17], we used WT mice and *c-kit* loss-of-functional mutant littermates, $Wads^{m/m}$ mice, to compare the excreted mucus covering colonic mucosa and the residual within goblet cells. C-KIT was hypo-expressed in the colonic

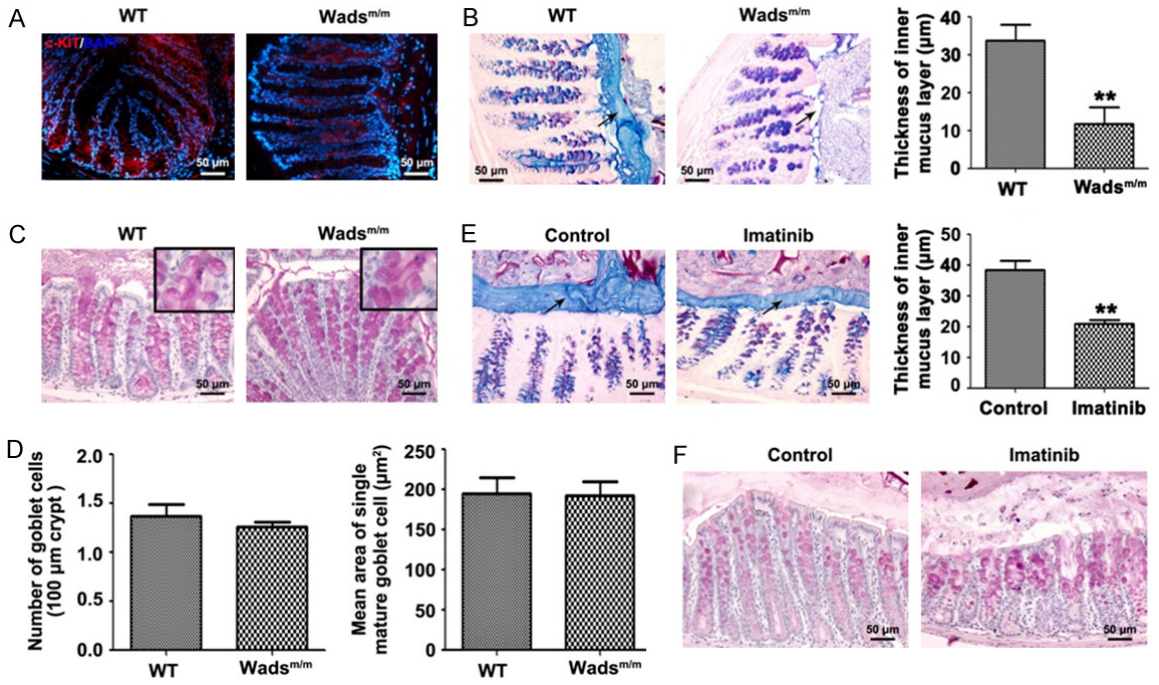

Figure 1. Inactivity of SCF/c-KIT signaling decreased mucus secretion. A. Immunofluorescent staining showing c-KIT expression in distal colon of WT and Wads$^{m/m}$ mice. C-KIT was mainly expressed in the basal crypt of colon. The immunoreactive intensity of c-KIT was stronger in WT mice than that in Wads$^{m/m}$ mice. B. Alcian blue and PAS double staining showing inner mucus layer (arrow) in distal colon of WT and Wads$^{m/m}$ mice. The inner mucus layer in WT mice was continuous and densely compacted while it was incomplete and loose in Wads$^{m/m}$ mice. Significantly, the inner mucus layer was thinner in Wads$^{m/m}$ mice (n=5) than that in WT mice (n=5) (**$P < 0.001$). C. PAS staining showing goblet cells in distal colon of WT and Wads$^{m/m}$ mice. The intracellular mucin residual was apparently greater in Wads$^{m/m}$ mice than that in WT mice. D. There was neither visible difference in goblet cell number between WT (n=5) and Wads$^{m/m}$ (n=5) mice, nor mean area of single goblet cell. E. Alcian blue and PAS double staining showing inner mucus layer (arrow) in distal colon of WT mice administered with Imatinib (0.5 mg/g/d for 7 days). The thickness of inner mucus layer became thin after the administration with Imatinib (n=5) (**$P < 0.001$). F. PAS staining showing goblet cells in distal colon of WT mice administered with Imatinib. There was more intracellular mucin residual in mice administered with Imatinib.

mucosa of Wads$^{m/m}$ mice (**Figures 1A** and **3A**). An intriguing result was that the colonic mucosa of Wads$^{m/m}$ mice was lack of a thick continuous overlaying inner mucus layer compared with WT mice (**Figure 1B**); while the mucin resudual within goblet cells of Wads$^{m/m}$ mice was more than that of WT mice (**Figure 1C**). However, there were no differences in the number of goblet cells and mean area of single mature goblet cell between WT and Wads$^{m/m}$ mice (**Figure 1D**). These results suggested that SCF/c-KIT signaling might play a significant role in mucus secretion of colonic goblet cells. To further confirm the role of SCF/c-KIT signaling, the WT mice were orally administered with Imatinib (0.5 mg/g/d) for 7 days to block the activity of SCF/c-KIT signaling. As expected, mice treated with Imatinib had thinner inner mucus layer and more residual of intracellular mucus (**Figure 1E** and **1F**). Therefore, we suggested that SCF/c-KIT activity was essential for the mucus secretion in colonic goblet cells.

MARCKS accelerated mucus secretion in mouse colon

Next, we searched for how SCF/c-KIT signaling played the role in promoting mucus secretion. MARCKS is a well-known molecular driving exocytosis of mucin in colonic goblet cells [18]. Here, we confirmed its role by the use of MARCKS-related peptide, MANS, which can competitively inhibit the attachment of MARCKS to membranes of intracellular mucin granules and inhibit mucin secretion upon secretagogue stimulation [19, 20]. WT mice received intraperitoneal administration of MANS, missense peptide (RNS) and normal saline (NS), followed

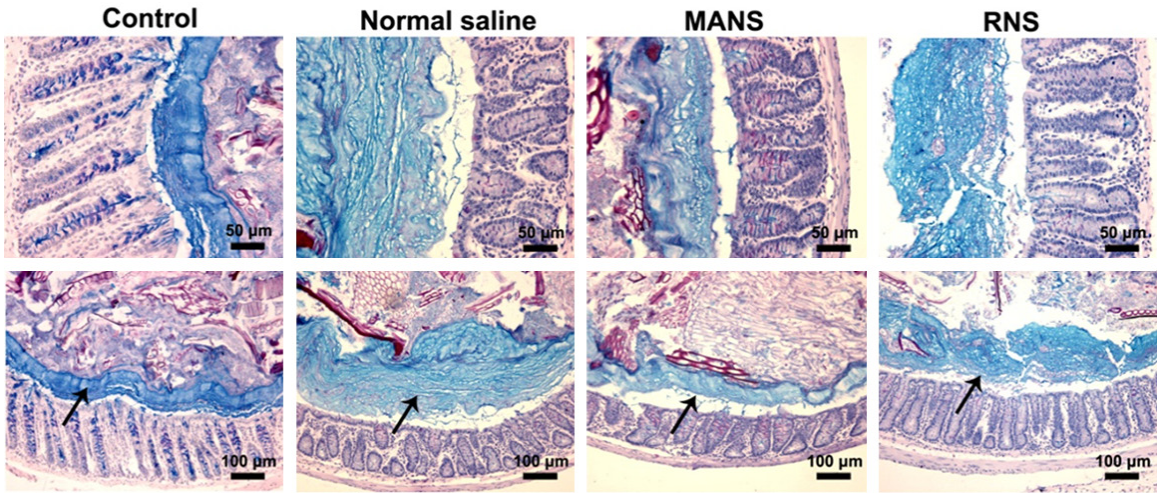

Figure 2. MARCKS accelerated mucus secretion in mouse colon. Alcian blue and PAS staining showing goblet cells and inner mucus layer (arrow) in distal colon of WT mice treated with normal saline, MANS peptide or RNS peptide and additional pilocarpine. WT mice received no any treatment were set as controls. Pilocarpine stimulated mucus secretion a lot in normal saline and RNS mice, indicated by the thick inner mucus layer and nearly empty cytoplasm of goblet cells. However, the effect of pilocarpine was deprived by MANS, indicated by the thin inner mucus layer and filled mucin residual in goblet cells.

by pilocarpine administration 15 min later, and a no any administration group as control. In groups of NS, nearly all of the mucus in goblet cells was released into lumen upon pilocarpine administration. MANS administration noticeably abrogated the release of mucus resulting in a thinned inner mucus layer; whereas RNS administration had no such effect (**Figure 2**). These results confirmed that MARCKS exactly accelerated mucus secretion in mouse colon.

SCF/c-KIT signaling activated MARCKS in vivo and in vitro

The above results inspired us to seek for the relationship between SCF/c-KIT signaling and MARCKS. As shown in **Figure 3A**, Wads$^{m/m}$ mice had a decreased phosphorylation of MARCKS (p-MARCKS) and translocation from membrane to cytoplasmic compartment compared with WT mice (**Figure 3A**). Furthermore, in MCA patients and mice p-MARCKS was significantly increased along with the highly expressed c-KIT and MUC2 compared with non-MCA, though the expression of MARCKS was not altered (**Figure 3B** and **3C**). The inactivity of MARCKS also occurred in WT mice treated with Imatinib compared to controls indicated by sharply decreased p-MARCKS level (**Figure 4A**). To further verify these observations *in vivo*, we cultured HT-29 and LS174T CRC cells which had a potential to secrete mucus and treated them with Imatinib. It turned out that blockage of SCF/c-KIT activity by Imatinib evidently inhibited phosphorylation of MARCKS in a time-dependent manner in HT-29 and LS174T cells (**Figure 4B**). In contrast, activating c-KIT by exogenous rhSCF or over-expression of c-KIT via lentivirus mediation could potently increase p-MARCKS expression in HT-29 and LS174T cells (**Figure 4C** and **4D**). Collectively, these results indicated that SCF/c-KIT signaling was implicated in mucus secretion by regulating MARCKS activity.

Activation of PKCδ was regulated by SCF/c-KIT signaling in vivo and in vitro

It has been reported that MARCKS is phosphorylated and activated by PKCδ since it harbors PKC phosphorylation sites [21] and RTKs were involved in the activation of PKCδ [22]. Therefore, whether the activation of PKCδ was under the control of SCF/c-KIT signaling was investigated in the current study. Wads$^{m/m}$ mice and Imatinib-treated mice showed a decreased phosphorylation of PKCδ (p-PKCδ) compared with WT and control mice, respectively (**Figure 5A** and **5B**). In MCA mice which had hyper-activated SCF/c-KIT signaling, p-PKCδ was increased as expected compared with non-MCA mice (**Figure 5C**). Consistent with the observa-

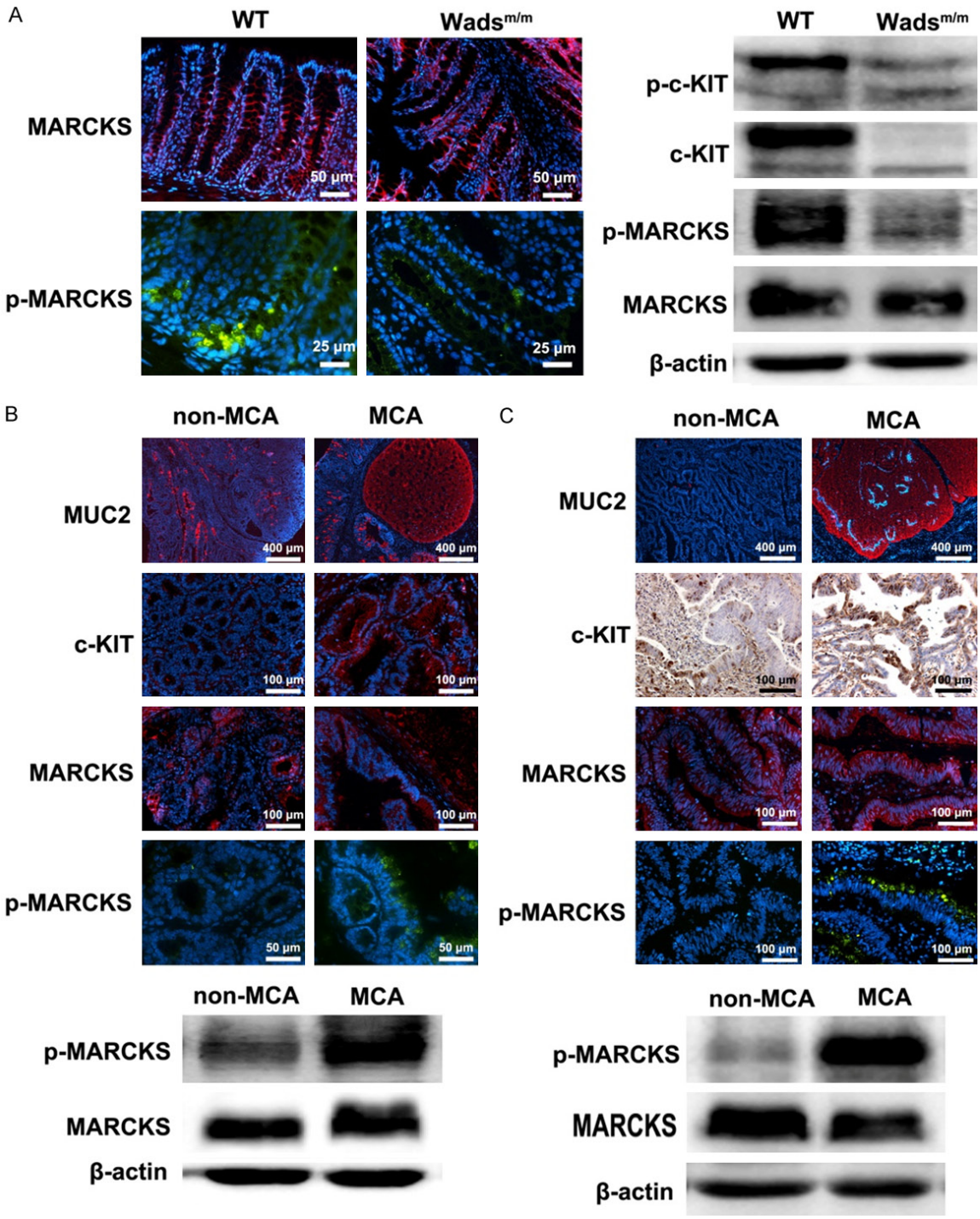

Figure 3. Activation of MARCKS was associated with SCF/c-KIT signaling. The expressions of c-KIT, MUC2, MARCKS and p-MARCKS were assessed by immunohistochemical staining and Western blot in human and mouse colonic mucosa. (A) P-MARCKS level was lower in Wads$^{m/m}$ mice which are lack of SCF/c-KIT activity than that in WT mice. (B) MCA mice (n=5) and (C) patients (n=5) had overtly increased p-MARCKS as well as c-KIT and MUC2 compared with non-MCA. But the total MARCKS was mainly the same between MCA and non-MCA tissues.

tions *in vivo*, blockage of SCF/c-KIT activity by Imatinib evidently inhibited the phosphorylation of PKCδ in a time-dependent manner in HT-29 and LS174T cells (**Figure 5D**). While, over-activating c-KIT by exogenous rhSCF or over-expression of c-KIT via lentivirus media-

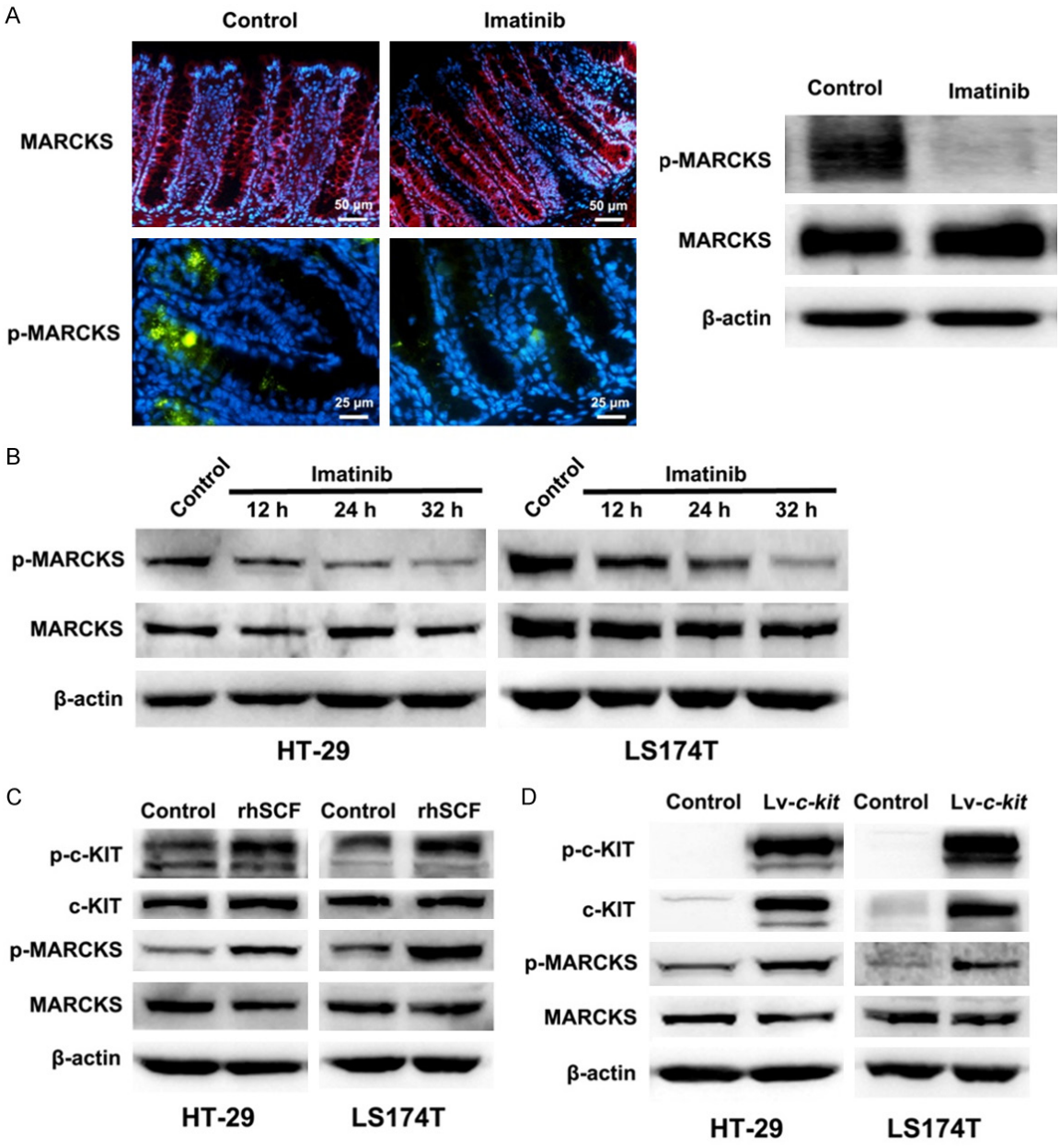

Figure 4. SCF/c-KIT signaling activated MARCKS *in vivo* and *in vitro*. (A) Imatinib (0.5 mg/g/d for 7 days) treated mice showed a decreased p-MARCKS in the colonic epithelium compared with controls. (B) Cultured HT-29 and LS174T cells treated with Imatinib had declining p-MARCKS in a time-dependent manner. Activation of c-KIT in HT-29 and LS174T cells either by rhSCF (C) or over-expression (D) brought about increased p-MARCKS.

tion could potently increased p-PKCδ expression in HT-29 and LS174T cells (**Figure 5E** and **5F**). Results above demonstrated that SCF/c-KIT signaling could regulate PKCδ activity.

Discussion

The primary defense against microbial and pathogen penetration into the intestinal lamina propria is the single layer of epithelial cells and its associated protective mucus layer. Goblet cells within intestinal epithelium produce and secrete mucins, predominantly MUC2, into the intestinal lumen, thereby forming the mucus layer [23]. MUC2 biosynthesis involves protein dimerization, glycosylation, oligomerization, and dense packing of these large netlike structures into secretory granules of the goblet cell

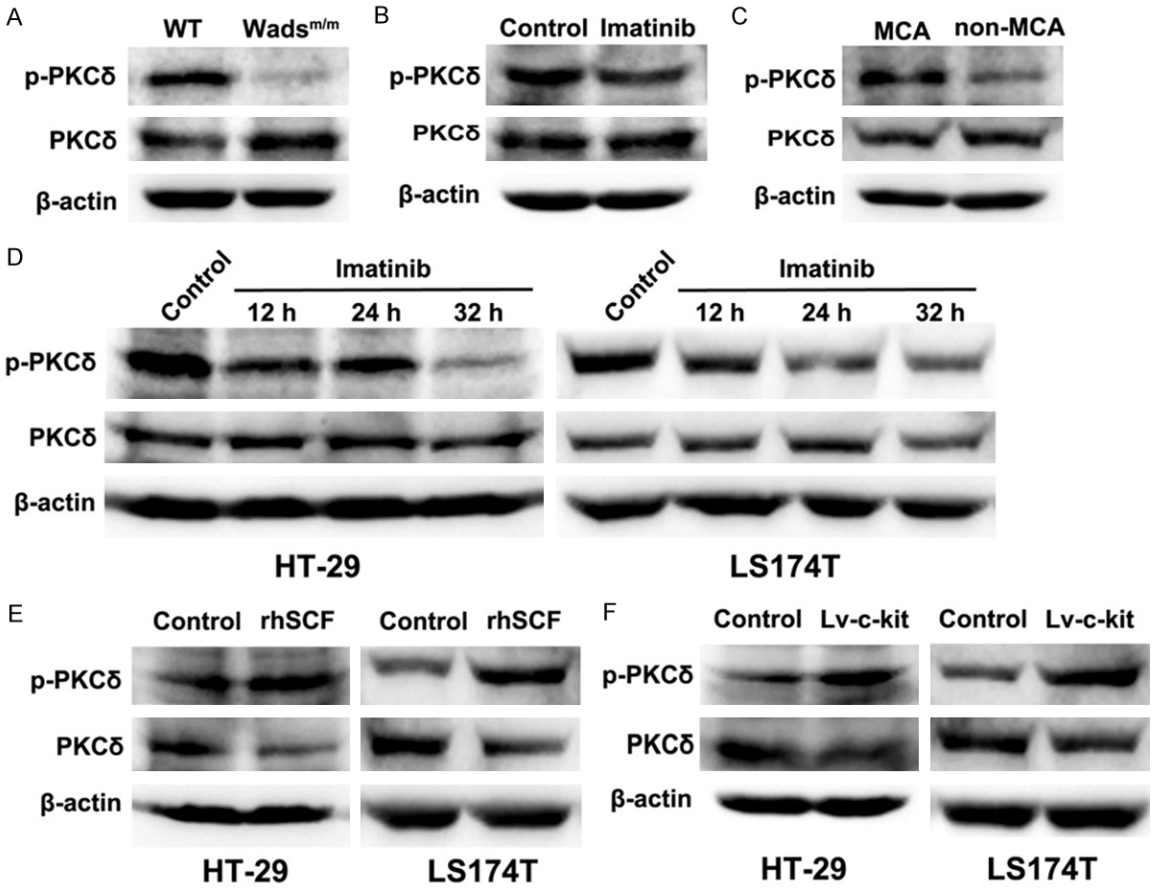

Figure 5. Activation of PKCδ was regulated by SCF/c-KIT signaling *in vivo* and *in vitro*. P-PKCδ level was lower in Wads$^{m/m}$ mice (A) and Imatinib (0.5 mg/g/d for 7 days) treated mice (B) than that in WT mice and control, respectively. (C) MCA mice (n=5) had overtly increased p-PKCδ compared with non-MCA mice (n=5). (D) HT-29 and LS174T cells treated with Imatinib had declining p-PKCδ in a time-dependent manner. Activation of c-KIT in HT-29 and LS174T cells either by rhSCF (E) or over-expression (F) brought about increased p-PKCδ.

[24]. Mucin-containing granules are stored within theca which separates mucin granules from the rest of the cytoplasm and gives mature goblet cells their distinctive shape [25]. Exocytosis of mucin occurs when apically oriented mucin granules fuse with the plasma membrane in a complex but not understood process [24, 25]. The intestinal mucus layer consists of two stratified layers and plays a key role in the maintenance of intestinal homeostasis [26]. In contrast to the loose matrix and microbiota containing outer mucus layer, the inner mucus layer composition is dense and devoid of the microbiota [26] and functions as a barrier, which serves to minimize microbial translocation and prevent excessive immune activation. It can be assumed, therefore, the destruction of goblet cells and/or inner mucus layer exposed to any deleterious factors would cause intestinal diseases even neoplasm.

C-KIT is expressed in the basal crypt of colon and confined to goblet and Paneth cells, indicating a potential involvement of c-KIT in their functions. We recently revealed that c-KIT, activated by its ligand SCF, could up-regulate Atoh1 transcriptional factor which further increased MUC2 expression [personal communication]. On the other hand, exocytosis of the MUC2-containing granules into intestinal lumen is another pivotal process during the formation of mucus layer. In the present study, we released the exciting result that SCF/c-KIT signaling was able to provoke mucus secretion and form a thick, densely packed inner mucus layer on the surface of colonic mucosa. In addition, we excluded the possibility that the lack of inner mucus layer under the circumstance of inactivated SCF/c-KIT signaling was due to the loss of goblet cells. We further demonstrated that the role of SCF/c-KIT signaling in mucus

secretion was through phosphorylating MARCKS, a key factor driving exocytosis of mucin in goblet cells [12, 18]. Aside from SCF/c-KIT signaling, MARCKS was previously confirmed to be provoked by PKCδ which was a downstream of PI3K/AKT [18]. Here, we demonstrated that PKCδ activity could be mediated by SCF/c-KIT signaling. Thus, we proposed a network in which SCF/c-KIT signaling could activate MARCKS through PKCδ.

Adequate SCF/c-KIT activity is necessary for MUC2 expression and mucin exocytosis in normal colon. However, we could not neglect the fact that in malignant MCA, exorbitant SCF/c-KIT activity stimulated excessive MUC2-containing mucus secretion also through activating MARCKS. As a result, MCA cells were endued with high malignancy and strong invasive ability. Unphosphorylated MARCKS has been described as a tumor suppressor in CRC, as well as a series of other tumors, by promoting apoptosis of tumor cells [27]. In contrast, phosphorylated MARCKS by SCF/c-KIT signaling seemed as a tumor promoter via conferring resistance to MARCKS-related apoptosis and boosting mucus secretion.

In conclusion, SCF/c-KIT signaling promoted mucus secretion through phosphorylating MARCKS in colonic goblet cells and MCA cells. Our results provided new insights into understanding the mechanism of mucus secretion of goblet cells and the MCA development.

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Antibodies and its application concentration

Antibodies	Immunohistoche-mical staining	Western blot analysis
Rabbit anti-MARCKS (Proteintech, USA)	1/200	1/1000
Goat anti-p-MARCKS (Santa Cruz, USA)	1/50	1/200
Rabbit anti-PKCδ (Santa Cruz, USA)		1/500
Rabbit anti-p-PKCδ (Santa Cruz, USA)		1/500
Rabbit anti-MUC2 (Santa Cruz, USA)	1/200	
Rat anti-c-kit (eBioscience, USA)	1/300	
Rabbit anti-c-kit (Dako, Denmark)	1/200	
Rabbit anti-c-kit (Cell Signaling Technology, USA)		1/1000
Rabbit anti-p-c-kit (Cell Signaling Technology, USA)		1/1000
Mouse anti-beta-actin (Santa Cruz, USA)		1/3000