Protein Kinase C-δ-Mediated Recycling of Active KIT in Colon Cancer

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Abstract

**Purpose:** Abnormal signaling through receptor tyrosine kinase (RTK) moieties is important in tumorigenesis and drug targeting of colorectal cancers. Wild-type KIT (WT-KIT), a RTK that is activated upon binding with stem cell factor (SCF), is highly expressed in some colon cancers; however, little is known about the functional role of SCF-dependent KIT activation in colon cancer pathogenesis. We aimed to elucidate the conditions and roles of WT-KIT activation in colon cancer tumorigenesis.

**Experimental Design:** Colorectal cancers with KIT expression were characterized by immunoblotting and immunohistochemistry. The biologic alterations after KIT-SCF binding were analyzed with or without protein kinase C (PKC) activation.

**Results:** We found that WT-KIT was expressed in a subset of colon cancer cell lines and was activated by SCF, leading to activation of downstream AKT and extracellular signal-regulated kinase (ERK) signaling pathways. We also showed that KIT expression gradually decreased, after prolonged SCF stimulation, due to lysosomal degradation. Degradation of WT-KIT after SCF binding was significantly rescued when PKC was activated. We also showed the involvement of activated PKC-δ in the recycling of WT-KIT. We further showed that a subset of colorectal cancers exhibit expressions of both WT-KIT and activated PKC-δ and that expression of KIT is correlated with poor patient survival ($P = 0.004$).

**Conclusions:** Continuous downstream signal activation after KIT-SCF binding is accomplished through PKC-δ–mediated recycling of KIT. This sustained KIT activation may contribute to tumor progression in a subset of colon cancers with KIT expression and might provide the rationale for a therapeutic approach targeting KIT.

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Introduction

The genetic aberrations of colon cancers have been well characterized. Recent colon cancer genome analysis studies showed that most colorectal cancers show similar patterns of genomic alteration, and mutations of *APC*, *TP53*, *SMAD4*, *PIK3CA*, and *KRAS* were frequently identified (1). These mutations contribute to the development of biologically aggressive colorectal carcinomas and are directly linked to dysregulation of signaling pathways involving Wnt/β-catenin, phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) in colon cancers (2).

Among the signaling pathways that are active in colorectal cancers, activation of signaling through the receptor tyrosine kinase (RTK) moieties of growth factor receptors plays a crucial role in colon cancer tumorigenesis and drug targeting. Activation of EGF receptor (EGFR) is particularly relevant in colorectal cancers. EGFR overexpression occurs in 60% to 80% of colorectal cancers and cetuximab, a monoclonal antibody to EGFR, has been shown to be clinically effective in colorectal cancers (3). KIT is a RTK that is expressed in some epithelial cell lineages during embryogenesis. KIT and its ligand, stem cell factor (SCF), are essential for the maturation of some primitive cells during embryonic development, and aberrant expression of KIT and SCF has been reported in human malignant tumor cells derived from epithelial cell lineages that express KIT during embryogenesis such as breast, lung, and prostate (4–6). In addition, previous studies showed that some human colorectal cancers express high levels of KIT and SCF relative to normal mucosa cells (7, 8).

Activation of KIT in tumors is achieved in two different ways. Activation through the acquisition of activating mutations is common and has been reported in gastrointestinal
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Translational Relevance
Abnormal signaling through receptor tyrosine kinase (RTK) moieties plays a crucial role in colon cancer tumorigenesis. Wild-type KIT (WT-KIT), a RTK that is activated upon binding with stem cell factor (SCF), is highly expressed in a subset of colorectal cancers. We found that activated WT-KIT gradually decreased through lysosomal degradation after SCF binding. We showed that PKC-δ binds to WT-KIT and is involved in KIT recycling. We also showed that expression of WT-KIT and activated PKC-δ are concomitantly present in some colorectal cancer tissues, and KIT expression correlates with poor patient survival. Our findings suggest that sustained WT-KIT activation through PKC-δ-mediated WT-KIT recycling may contribute to rapid colorectal cancer progression and provides a rationale for anti-KIT therapy in a subset of colorectal cancers with WT-KIT expression.

Materials and Methods
Cell lines and culture
DLD-1, HCT116, SNUC4, Colo320DM, HeLa, and Ls174T were purchased from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). HT29, HCT8, HMC-1, and LoVo were purchased from American Type Culture Collection. GIST882 has been reported previously (13). DLD-1, HCT116, SNUC4, Colo320DM, and Ls174T were selected from 11 colon cancer cell lines after initial screening of KIT expression by reverse transcription-PCR (RT-PCR). DLD-1, Colo320DM, and Ls174T cells were selected as KIT-expressing colon cancer cell lines, whereas HCT116 and SNUC4 cells were selected as control cell lines lacking KIT expression. In addition, the GIST882 cell line (derived from GIST with KIT-K642E mutation) was used as a positive control; HeLa cells were used as a negative control for KIT expression.

Construction of expression vectors
An expression vector for KIT cDNA containing a FLAG tag was constructed using pCMV vector and KIT coding regions amplified by PCR using cDNA from DLD-1 cells. The cDNA of Rab-11 was amplified from HEK293 cells and was constructed using the same method as that for the KIT expression vector. The generation of PKC constructs was described previously (14).

Western blotting and immunoprecipitation
Whole-cell lysates were prepared using passive lysis buffer (Promega) with a protease inhibitor cocktail (Roche). The membranes were incubated with primary antibodies against GAPDH (Trevigen); FLAG (Sigma-Aldrich); KIT (Dako); ERK, phospho-ERK, HA, (Santa Cruz Biotechnology); phospho-KIT (Invitrogen); and AKT, phospho-AKT, PKC-δ, phospho-PKC-δ (Cell Signaling Technology) for 2 hours at room temperature. Western blot images were analyzed with a LAS-4000 Mini camera (Fujifilm). For immunoprecipitation, lysates were precleared and immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma-Aldrich) at 4°C.

Immunofluorescence
Cells grown on slides were rinsed with PBS, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized in 0.3% Triton X-100 in PBS. The slides were incubated with primary antibody for 1 hour and incubated for 50 minutes with the appropriate fluorescent-labeled secondary antibody (Invitrogen). All images were obtained using a LSM700 confocal microscope (Carl Zeiss).

Quantitative RT-PCR
RT-PCR was done using AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems). The quantitative reverse transcriptase-PCR (qRT-PCR) was conducted using the ABI PRISM 7500 Sequence Detector (Applied Biosystems) and SYBR Premix Ex Taq II (TaKaRa). The amount of target mRNA was normalized to that of GAPDH mRNA. The sequences of the primers used are listed in Supplementary Table S1.

Cell proliferation assay
DLD-1 cells expressing KIT were washed with PBS, and incubated with cell media containing imatinib (50 μmol/L), as well as SCF (50 ng/mL) and/or phospholipid 12-myristate 13-acetate (PMA; 300 nmol/L). The number of cells was manually counted 3 days after the drug treatment, and the morphology of the cells was examined under a microscope. Every experiment was independently conducted in triplicate, and the mean number was used for further analysis.

Cell invasion assay
Cell invasion assay was conducted using the Chemicon QCM Cell Invasion Assay Kit (Millipore) according to the
manufacturer's instructions. Briefly, serum-free media were added into the interior of inserts to rehydrate the extracellular matrix layer, and replaced with serum-free media containing $1.5 \times 10^5$ cells transfected with siNC or siKIT. Then, SCF and/or PMA, as well as imatinib, were added to the cells. Media containing 10% FBS were then added to the lower chamber. Cells were incubated for 48 hours and the invaded cells were stained. Stained cells were counted under a microscope.

**FACS analysis**

Colo320DM and DLD-1 cells treated with SCF and/or PMA were detached using 5 mmol/L EDTA in PBS, and subsequently, fixed with 4% formaldehyde. Incubated with the appropriate amounts of antibody for 1 hour, samples were analyzed after three washes by FACSCalibur (BD Biosciences). Each experiment was conducted in triplicate.

**Patients and tissue samples**

Colorectal cancer tissue samples from 250 patients with primary colorectal cancers of stages I to IV were used in this study. The specimens were obtained from the archives of the Department of Pathology, Yonsei University (Seoul, Korea) and from the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation of the Ministry of Science and Technology. Colon cancer tissues were subjected to immunohistochemical analysis for KIT using a tissue microarray. All patients had undergone colorectal resection between 2004 and 2006 and fresh snap-frozen samples were obtained immediately at the time of surgery. The median follow-up time after surgery was 59.2 months. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University of College of Medicine.

**Statistical analysis**

The survival rate of patients with colon cancer according to KIT expression was analyzed by the Kaplan–Meier method, and differences between groups were evaluated by the log-rank test. Pearson $\chi^2$ tests were used for comparison of KIT expression and relevant clinicopathologic parameters. Student $t$ tests were conducted to analyze continuous data. All tests were two-tailed. All P values less than 0.05 were considered significant.

**Results**

**Identification of WT-KIT expression in colon cancer cell lines**

We examined KIT expression in 2 control cell lines (GIST882 as a positive control and HeLa as a negative control) and 5 colon cancer cell lines (DLD-1, HCT116, SNUC4, Colo320DM, and LS174T). KIT mRNA expression was identified in 3 colorectal cancer cell lines (DLD-1, Colo320DM, and LS174T) by RT-PCR, qRT-PCR, and KIT protein expression in these 3 cell lines correlated with the mRNA levels (Fig. 1A). No mutations were found in exons 9, 11, 13, and 17, which are sites of previously identified KIT mutations (data not shown). Loss of KIT expression after treatment with KIT-specific siRNA was confirmed (Fig. 1B). These findings indicate that WT-KIT is strongly expressed in a subset of colorectal cancer cell lines.

**SCF induces KIT activation and subsequently activates AKT and ERK signaling pathways**

We next examined whether WT-KIT is activated after SCF treatment of DLD-1, Colo320DM, and LS174T cell lines. In all 3 cell lines, treatment with SCF induced KIT activation in a dose-dependent manner. SCF also induced activation of the well-known downstream molecules, AKT and ERK, that are related to cell survival and proliferation, respectively, as assessed by Western blotting measuring the phosphorylated forms of these proteins (Fig. 1C and Supplementary Fig. S1). Activation of AKT and ERK after SCF treatment was abolished by KIT siRNA, showing that activation of AKT and ERK is dependent on activated KIT (Fig. 1C). In contrast with the cell lines with WT-KIT expression, SCF treatment did not affect downstream signaling pathways in GIST882 (KIT-K642E-mutant GIST) and HMC-1 (KIT-D816V-mutant mast cell leukemia) cells that express mutant KIT (Supplementary Fig. S2). These findings confirm that SCF activates WT-KIT and downstream signaling pathways in colon cancer cell lines but has no effect on mutant KIT expressed in other cell lines. We next evaluated the time course of SCF stimulation by examining the status of KIT activation, 5 and 15 minutes after SCF treatment and found that levels of phospho-KIT (p-KIT), phospho-AKT (p-AKT), and phospho-ERK (p-ERK) gradually increased over this period (Fig. 1D). However, the expression of KIT was markedly decreased 15 minutes after SCF treatment (Fig. 1D and Supplementary Fig. S3).

**Activated KIT proteins are degraded by the lysosomal degradation pathway**

Having shown the downregulation of WT-KIT protein 15 minutes after SCF treatment, we measured the mRNA and protein levels of KIT during a 2-hour period after SCF treatment. KIT mRNA expression levels in DLD-1, Colo320DM, and LS174T cell lines were constant during this time, whereas the levels of KIT protein were markedly decreased at 1 hour after SCF treatment (Fig. 2A). Also, fluorescence-activated cell sorting (FACS) analysis of KIT after SCF treatment showed that the proportion of tumor cells expressing KIT decreased from 50.9% to 21.6% after SCF treatment (Supplementary Fig. S4). The expression of p-KIT was markedly increased 10 minutes after SCF treatment, but was barely detectable at 1 and 2 hours (Fig. 2A). In contrast with the gradual downregulation of WT-KIT proteins after SCF treatment, there was no change in the expression of mutant KIT or p-KIT proteins in GIST882 and HMC-1 cells (Supplementary Fig. S5).

It is well known that RTKs are internalized into vesicles and transferred via early and late endosomes for degradation in the lysosome upon the appropriate stimulus. Normally, this endocytotic process is constantly ongoing in the
cell. To confirm whether lysosomal degradation is responsible for the gradual decrease in WT-KIT after SCF binding, we designed a rescue assay using inhibitors known to block 2 major protein degradation pathways: Bafilomycin A1 was used to block lysosomal degradation and MG132 was used to block the proteasomal pathway. Cells were treated with SCF alone or respective inhibitors for 1 hour and expression of KIT protein was analyzed. The results showed that KIT proteins were barely rescued after inhibition of proteasomal degradation (Fig. 2B), whereas there was significant rescue of KIT protein (up to 100% compared with the control) after Bafilomycin A1 treatment, indicating that the downregulation of activated WT-KIT is mainly dependent on the lysosomal degradation pathway. In addition, we also found that p-AKT and p-ERK levels also increased after Bafilomycin A1 treatment (Fig. 2C). We next validated the lysosomal degradation of KIT by analyzing the intracellular location of KIT after SCF binding. In the resting state, KIT proteins were mainly distributed on the plasma membrane of DLD-1 cells. After SCF stimulation, rapid internalization of KIT was observed (Fig. 2D). To identify the location of KIT after SCF stimulation, we compared the localization of KIT and an endosome marker lysosomal associated membrane protein 1 (LAMP-1). Without SCF stimulation, KIT was rarely colocalized with LAMP-1; however, the majority of KIT proteins clearly showed colocalization with LAMP-1 in cells.
treated with SCF (Fig. 2D). Taken together, these findings indicate that WT-KIT is activated by SCF binding, internalized, and finally targeted to the lysosomes for degradation.

**PKC activation rescues KIT from lysosomal degradation**

Given the rapid degradation of WT-KIT in colon cancer cell lines after SCF treatment, we speculated on the roles of SCF-KIT activation in colorectal cancer tumorigenesis. If SCF-KIT activation plays an important role in colorectal cancer tumorigenesis, constant activation of SCF-KIT would be required. Because WT-KIT proteins are degraded in the lysosome after binding of SCF, we hypothesized that activated WT-KIT proteins are recycled in tumor cells and thus constantly contribute to colorectal cancer tumorigenesis. We further suspected that PKC activation might be involved in the recycling of WT-KIT protein because it has been reported that other RTK receptors such as EGFR and platelet-derived growth factor (PDGF) β-receptor are recycled by PKC activation (15, 16). To test the involvement of PKC in the regulation of KIT, we treated the three KIT-expressing cell lines with PMA, a PKC activation factor, after SCF activation. We found that the level of KIT proteins was dramatically rescued after PMA treatment, and that activated forms of KIT, AKT, and ERK were stabilized even 2 hours after SCF treatment in all 3 cell lines (Fig. 3A).
and Colo320DM cells were concomitantly treated with PMA and G06983, a PKC inhibitor, KIT and p-KIT were no longer stabilized (Fig. 3B). We additionally conducted FACS analysis for p-AKT and p-ERK in the presence of SCF, PMA alone, and concomitant treatment of SCF and PMA in Colo320DM cells. Among the results therefrom, we showed that cell populations expressing p-AKT increased from 3.86% to 29.8% upon SCF and PMA cotreatment; the populations of tumor cells expressing p-ERK markedly increased from 1.25% to 85.2% in the cells cotreated with SCF and PMA (Supplementary Fig. S6).

To confirm that activation of PKC rescues KIT from degradation, we evaluated the intracellular location of KIT with or without PKC activation. Before SCF stimulation, most of the KIT proteins were localized at the plasma membrane in Ls174T cell. After SCF stimulation, cytoplasmic translocation of KIT was evident and only a scant amount of KIT was present in the plasma membrane. When the cells were concomitantly treated with SCF and PMA, more KIT protein was detected on the plasma membrane than in cells treated with SCF only, suggesting that PMA-mediated PKC activation leads to recycling of KIT proteins. In addition, we observed very little colocalization of KIT proteins with LAMP-1 in the cells cotreated with SCF and PMA (Fig. 3C). To validate the endosomal recycling of KIT after PMA stimulation, we analyzed the colocalization of KIT and Rab11, a well-known recycling endosome marker. When Ls174T cells were treated with SCF alone, very little colocalization of Rab11 and KIT was identified; however, in cells treated concomitantly with SCF and PMA, a large proportion of KIT proteins colocalized with Rab11 (Fig. 3D). We conducted additional FACS analysis to confirm the mediation of KIT recycling by Rab11. To do so, we evaluated membranous KIT expression by FACS after transfection of wild-type form (Rab11-WT) or dominant negative form of Rab11 (Rab11-S25N) in DLD-1 cells. We found rare recycle
of KIT in DLD-1 cells transfected with the dominant negative form of Rab11, compared with the wild-type form of Rab11 transfection (Supplementary Fig. S7). These findings indicate that PKC activation plays a role in sustained KIT activation by inhibiting KIT degradation and instead recycling the KIT proteins.

**PKC-δ directly binds and rescues WT-KIT**

Having identified a role of PKC in constant SCF-KIT activation in colon cancer cells, we investigated which isoform of PKC contributes to KIT recycling, by immunoprecipitation assays, using synthetic KIT and PKC proteins. *In vitro* binding assays conducted after treatment with SCF and PMA showed that WT-KIT directly and exclusively binds to PKC-δ (Fig. 4A). To further examine the interaction between KIT and PKC-δ, we conducted binding assays in the presence or absence of SCF and/or PMA. Binding of KIT to PKC-δ was only observed after treatment with both SCF and PMA, indicating that activated PKC-δ is involved in KIT recycling (Fig. 4B). Interestingly, the localization of KIT and PKC-δ was exclusively SCF and PMA dependent; no colocalization between KIT and PKC-δ was found in cells treated with SCF or PMA alone (Fig. 4C). To confirm that activation of PKC-δ can rescue KIT, cells were transfected with control siRNA or PKC-δ-specific siRNA before treatment with SCF and PMA. The rescue of KIT was substantially reduced from 61% to 8% in the cells transfected with siPKC-δ compared with control cells (Fig. 4D), indicating that KIT recycling depends on activated PKC-δ.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** PKC-δ directly binds WT-KIT and rescues KIT from degradation. A, synthetic WT-KIT protein directly bound to synthetic PKC-δ protein *in vitro*. HA-PKC isoenzyme constructs were cotransfected with Flag-KIT into HeLa cells. Cells were pretreated with 300 nmol/L PMA and incubated with SCF for 2 hours. B, binding between KIT and PKC-δ was evident only in cells treated with SCF and PMA. C, HA-PKC-δ was cotransfected with Flag-KIT into HeLa cells. Cells were pretreated with 300 nmol/L PMA and incubated with or without SCF for 1 hour. Cells were stained for KIT (green), HA-PKC-δ (red) and DAPI (blue). In cells treated with SCF and PMA, colocalization of PKC-δ and KIT was evident (yellow). D, inhibition of PKC-δ resulted in the loss of rescued KIT in DLD-1 cells treated with SCF and PMA. DLD-1 cells transfected with control siRNA or siPKC-δ were pretreated with 300 nmol/L PMA and incubated with or without SCF for 2 hours.
PKC activation enhances colon cancer cell proliferation and invasion

To evaluate the tumorigenesis effects of PKC activation in KIT-expressing colon cancer cells, we chose one KIT-expressing colon cancer cell line (DLD-1), to perform a cell proliferation and invasion assay after 3 days with or without PKC activation. Treatment of SCF and PMA induced p-KIT, p-AKT, and p-ERK expression. The increased expressions of p-KIT and p-AKT were mostly inhibited by imatinib, whereas the expression of p-ERK was rarely inhibited (Supplementary Fig. S8). We next evaluated the inhibitory effects of imatinib on proliferation and invasion of colorectal cancer cells. When the cells were cotreated with SCF and PMA, a marked increase (64% increase compared with the control) in tumor cell number was recorded. Treatment of imatinib in cells treated with SCF and PMA reduced the number of tumor cells (Supplementary Fig. S9A). In the cell invasion assay, DLD-1 cells treated with SCF and PMA exhibited greater cell invasion than the control cells. However, the addition of imatinib to the cells cotreated with SCF and PMA dramatically reduced cell invasion. Knockdown of KIT also markedly blocked the invasion ability of DLD-1 cells (Supplementary Fig. S9B).

KIT and PKC-δ are activated in KIT-expressing colon cancer tissues

Although the expression of KIT in colon cancer cell lines has previously been reported, KIT expression in colon cancer tissues was not well characterized. We evaluated KIT expression in 250 colorectal cancer tissues by immunohistochemistry and detected expression in 47 (18.8%) cases. In Western blot analysis of 22 of the 47 KIT-immunopositive cases, 18 cases (81.8%) showed strong KIT expression compared with the normal matched mucosa (Fig. 5A). In contrast, Western blotting did not show increased KIT expression in 10 KIT-immunonegative colon cancers (data not shown). We also showed that most of the KIT protein expressed in colon cancer tissues is activated by detecting p-KIT expression in 16 of the 22 KIT-immunopositive colon tissues (72.7%). The expression of KIT and activated KIT in tumor tissues was significantly higher than in normal tissue (Fig. 5B and C). In addition, the expression of phospho-PKC-δ (p-PKC-δ) was increased in the colorectal cancer tissues with KIT expression and there was a linear correlation between KIT expression and p-PKC-δ expression (Pearson r = 0.5119, Spearman r = 0.445, P < 0.001; Fig. 5D).

Clinicopathologic characteristics of colon cancers with KIT expression

Finally, we analyzed the clinicopathologic characteristics of colorectal cancers with KIT expression among 250 colorectal cancers using tissue microarray. Expression of KIT was not identified in normal colonic mucosa and most of the colorectal cancers tested. The majority of tumors with KIT expression showed expression in the tumor cell cytoplasm (Fig. 6A). The amount of KIT expression was analyzed using the ImageJ program (http://rsbweb.nih.gov/ij/; NIH, Bethesda, MD) and categorized as KIT expression or no expression (Fig. 6B). There was no significant correlation between KIT expression and clinicopathologic variables was found except for increased serum CEA level in colorectal cancers with KIT expression (Supplementary Table S2). We also evaluated KIT as a prognostic factor and found that the expression of KIT correlated with...
poor survival. The overall cumulative survival rates for patients with KIT expression \((n = 47)\) and without KIT expression \((n = 203)\) were 68.1% and 85.7%, respectively \((P = 0.004; \text{Fig. 6C})\). When we analyzed the correlation between KIT expression and patient survival for the sub-group of patients with stage II and III disease, the overall cumulative survival rates for stage II and III patients with KIT expression \((n = 34)\) and without KIT expression \((n = 161)\) were 79.4% and 92.5%, respectively \((P = 0.017, \text{Fig. 6D})\).

**Discussion**

KIT, a member of the RTK family, is a highly oncogenic tyrosine kinase that is involved in the activation of major signal transduction pathways. The best-known downstream signal transduction pathways of KIT are PI3K-AKT, Ras-ERK, and JAK-STAT \((11, 17, 18)\). As we have described previously, KIT can be activated in two ways: WT-KIT is activated by binding of SCF, whereas mutant KIT is constantly activated in the absence of SCF \((11, 18, 19)\).

Although expression of WT-KIT has been reported in many tumors, including colorectal cancers \((7, 19–21)\), its role in tumorigenesis and the therapeutic efficacy of inhibition of activated KIT have not been well characterized. If activated WT-KIT plays a role in tumorigenesis, it is reasonable to assume that the downstream signaling pathway of activated WT-KIT would be the same or similar to that of mutant KIT and the activation would be constant, as for mutant KIT. SCF is the only known ligand for WT-KIT \((18)\). To achieve the constant activation of the signaling pathway from WT-KIT, continuous stimulation of SCF is necessary. As reported in previous studies, exogenous SCF facilitates tumor growth and angiogenesis \((22–24)\), and expression of SCF is upregulated by hypoxia in breast cancer cells \((25)\). We found absence or very low levels of SCF mRNA in three colorectal cancer cell lines with KIT expression (Supplementary Fig. S10). Accordingly, the sources of SCF in colorectal cancers are expected to be diverse, and continuous SCF stimulation can be achieved in some specific tumor environments. The present study showed that the signaling pathways downstream of the activation of WT-KIT, after SCF binding, are almost the same as those activated through mutant KIT \((11, 19)\), and that imatinib exerts antiproliferative and antiinvasive effects \((19, 26)\). Nevertheless, WT-KIT degradation after SCF binding has been reported \((27)\). Therefore, continuous SCF stimulation in tumor cells and activation of an intracellular mechanism for the inhibition of WT-KIT degradation may be required for WT-KIT mediated tumorigenesis in colorectal cancers with KIT expression.

In this study, we provide the first evidence that WT-KIT can be constantly activated through recycling of KIT. When we continuously stimulated KIT-expressing cell lines with...
SCF, the expression of KIT decreased according to the time of SCF treatment, and activation of the downstream signaling pathways was also decreased. Although these findings suggest a physiologic role of WT-KIT activation, they raise questions about the role of SCF-KIT activation in tumorigenesis. Although we observed degradation of activated KIT in colon cancer cell lines, we hypothesized that KIT could be recycled in the tumor cells and thus be continuously active after SCF treatment. Some other RTKs are known to be rapidly recycled back to the membrane in cancer cells instead of being degraded. For example, activation of PKC-α is a critical step in sorting the PDGF β-receptor towards Rab4a-dependent recycling (16). Recent studies also suggest that recycling of PDGFR drives the invasion of glioblastoma cells, and recycling of EGFR coupled with Δ51 integrin induces the migration of ovarian cancer cells (28, 29). However, little is known about recycling of KIT and the impact of KIT-SCF activation on colorectal cancer progression. We therefore investigated the possibility that KIT is recycled in KIT-expressing colon cancer cell lines. In the case of EGFR recycling, PKC is known to be involved in the inhibition of EGFR degradation (15). We initially activated PKC by treating colon cancer cells with PMA and found that KIT was not degraded after prolonged SCF stimulation but instead constantly activated the downstream signaling pathways. These findings indicate that KIT-SCF might contribute to colon cancer tumorigenesis through PKC activation and subsequent KIT recycling. We further showed that PKC-δ is responsible for KIT recycling by showing direct binding of KIT and PKC-δ in vitro and concomitant overexpression of KIT and PKC-δ in a subset of colon cancer tissues. PKCs play important roles in the regulation of proliferation, tumor promotion, apoptosis, and angiogenesis (30, 31). Various factors such as growth factors, tumor promoters, chemotherapeutic agents, and ras protein induce activation of PKCs (32–34). Activation of PKC-δ by substance P-induced proinflammatory signaling in human colonocytes (35) and by a hypoxic microenvironment has been reported (36). Therefore, it is likely that endogenous PKC-δ is activated and functions in KIT recycling in the microenvironment of colorectal cancers. Our findings indicate that colorectal cancers expressing WT-KIT can constantly generate activated SCF-KIT signaling as a result of KIT recycling (Supplementary Fig. S11).

In addition to showing expression of endogenous KIT and activated PKC-δ in colorectal cancers, we found that patients with colon cancer with KIT-expressing tumors showed a worse prognosis than patients without KIT expression. One previous report also showed worse prognostic correlation with KIT expression in colon cancers (37), but several studies found no prognostic correlations (38, 39). These findings might result from the variable and low incidence of KIT expression in colon cancers, marked intratumoral heterogeneity of KIT expression in colon cancers, requirement of continuous SCF stimulation to the KIT expressing tumors, and varying concomitant activations of endogenous KIT and PKC-δ in human colorectal cancers. These considerations make it difficult, if not impossible, to carry out an in vivo validation study of the antitumor effect of KIT inhibitors in colon cancers expressing WT-KIT and exemplify why the results for tyrosine kinase inhibitors in clinical trials in colon cancers are unimpressive, except for regorafenib, a multi tyrosine kinase inhibitor (40). However, continuous downstream signal activation through SCF binding to WT-KIT may be present in some colorectal cancers, and these cancers are expected to exhibit rapid progression. Because the downstream signaling pathways of KIT-SCF binding are same or similar to those of activated mutant KIT, activation after KIT-SCF binding can be inhibited at posttranscriptional and/or posttranslational levels. We previously showed that specific inhibition of KIT by transfection with microRNA decreased induction of p-AKT and p-ERK by SCF stimulation in cells expressing WT-KIT (13). These findings suggest that inhibition of KIT mRNA might be a novel therapeutic tool in tumor cells expressing KIT. We also showed the in vitro antitumoral effects of imatinib in KIT-expressing tumor cells cotreated with SCF and PKC activator. Therefore, posttranslational inhibition of KIT by imatinib treatment could be applied in a subset of colon cancers expressing WT-KIT and activated PKC-δ. In addition to these approaches of direct inhibition of activated KIT, it is possible that activated WT-KIT could be regulated by interrupting the recycling pathway. Future studies into modulating PKC-δ activity in tumors expressing WT-KIT might provide novel therapeutic tools for cancer treatment.

In conclusion, we propose KIT-SCF activation and PKC-δ-induced KIT recycling as a novel mechanism of tumor progression in colon cancers with WT-KIT expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W.K. Kim, Hoguen Kim
Development of methodology: Minseon Park, W.K. Kim
Acquisition of data (provided animal, acquired and managed patients, provided facilities, etc.): W.K. Kim, Minhee Park, H.J. Nam, Hunsik Kim, Hoguen Kim
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