Protein Kinase C-δ-mediated recycling of active KIT in colon cancer

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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 (CRCs). We found that activated WT-KIT gradually decreased through lysosomal degradation after SCF binding. We demonstrated that PKC-δ binds to WT-KIT and is involved in KIT recycling. We also demonstrated that expression of WT-KIT and activated PKC-δ are concomitantly present in some CRC 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 CRC progression, and provides a rationale for anti-KIT therapy in a subset of CRCs with WT-KIT expression.
Abstract

**Purpose:** Abnormal signaling through receptor tyrosine kinase (RTK) moieties is important in tumorigenesis and drug targeting of colorectal cancers (CRCs). 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:** CRCs with KIT expression were characterized by immunoblotting and immunohistochemistry. The biological 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 ERK signaling pathways. We also demonstrated 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 demonstrated the involvement of activated PKC-δ in the recycling of WT-KIT. We further showed that a subset of CRCs 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.
Introduction

The genetic aberrations of colon cancers have been well characterized. Recent colon cancer genome analysis studies demonstrated 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, PI3K, and MAPK in colon cancers(2).

Among the signaling pathways that are active in colorectal cancers (CRCs), 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 epidermal growth factor receptor (EGFR) is particularly relevant in CRCs. EGFR overexpression occurs in 60 to 80% of CRCs and Cetuximab, a monoclonal antibody to EGFR, has been shown to be clinically effective in CRCs (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 demonstrated that some human CRCs 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 stromal tumor (GIST), acute myelogenous leukemia (AML), and mastocytosis (4). The molecular mechanisms of this type of activation are well established and imatinib, which targets activated KIT molecules, is effective in the control of tumors with KIT mutations (9, 10). The other pathway involves ligand-dependent activation of wild type KIT (WT-KIT). To date, SCF is the only identified ligand that binds to KIT. Binding of SCF to WT-KIT leads to receptor dimerization and induces activation of downstream signals (11, 12). Although the molecular characteristics of KIT activation after SCF binding are well established, little is known about the functional roles of SCF-induced WT-KIT activation in cancers.

In this study, we analyzed the biological alteration of KIT after SCF binding in WT-KIT expressing colorectal cancer cells as well as its impact on downstream signaling. Based on our findings, we propose that sustained activation of downstream signals after KIT and SCF binding can be accomplished by PKC-δ-mediated recycling of WT-KIT.

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 (ATCC). GIST882 was previously reported (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-polymerase chain reaction (RT-PCR). DLD-1, Colo320DM, and Ls174T cells were selected as KIT-expressing colon cancer cell lines, while HCT116 and SNUC4 cells were selected as control cell lines lacking KIT expression. In addition, the GIST882 cell line (derived from gastrointestinal stromal tumor 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, Madison, WI, USA) with a protease inhibitor cocktail (Roche, Meylan, France). The membranes were incubated with primary antibodies against GAPDH (Trevigen, Gaithersburg, MD, USA); FLAG (Sigma-Aldrich, Poole, Dorset, UK); KIT (Dako, Cambridge, UK); ERK, phospho-ERK, HA, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-KIT (Invitrogen); and AKT, phospho-AKT, PKC-δ, phospho-PKC-δ(Cell Signaling, Danvers, MA, USA) for 2 hours at room temperature. Western blot images were analyzed with a LAS-4000 Mini camera (Fujifilm, Tokyo, Japan). 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 phosphate-buffered saline (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, Jena, Germany).

**Quantitative RT-PCR**

RT-PCR was performed using AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed using the ABI PRISM 7500 Sequence Detector (Applied Biosystems) and SYBR Premix Ex Taq II (TaKaRa, Tokyo, Japan). 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 uM), as well as SCF (50 ng/mL) and/or PMA (300 nM). 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 performed using the Chemicon QCM Cell Invasion Assay Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, serum-free media were added into the interior of inserts to rehydrate the ECM layer, and replaced with serum-free media containing 1.5×10^5 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 hr 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 mM EDTA in PBS, and subsequently, fixed with 4% formaldehyde. Incubated with the appropriate amounts of antibody for 1 hr, samples were analyzed after three washes by FACS Calibur (BD Biosciences). Each experiment was conducted in triplicate.

**Patients and tissue samples**

CRC tissue samples from 250 patients with primary CRCs 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 colon cancer patients according to KIT expression was analyzed by the Kaplan-Meier method, and differences between groups were evaluated by the log-rank test. Pearson’s Chi-square tests were used for comparison of KIT expression and relevant clinicopathological parameters. Student's t-tests were performed to analyze continuous data. All tests were two-tailed. All p-values <0.05 were considered significant.

Results
Identification of WT-KIT expression in colon cancer cell lines
We examined KIT expression in two control cell lines (GIST882 as a positive control and HeLa as a negative control) and five colon cancer cell lines (DLD-1, HCT116, SNUC4, Colo320DM, and Ls174T). KIT mRNA expression was identified in three CRC cell lines (DLD-1, Colo320DM, and Ls174T) by RT-PCR, qRT-PCR and KIT protein expression in these three 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 following treatment with KIT-specific siRNA was confirmed (Fig. 1B). These findings indicate that WT-KIT is strongly expressed in a subset of CRC 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 three 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, demonstrating that activation of AKT and ERK is dependent on activated KIT (Fig. 1C). In contrast to 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 demonstrated down-regulation 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 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 to the gradual down-regulation 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 two 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 were 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 to the control) after Bafilomycin A1 treatment, indicating that the down-regulation of activated WT-KIT is mainly dependent on the lysosomal degradation pathway. Additionally, 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 LAMP-1 (lysosomal associated membrane protein 1). Without SCF stimulation, KIT was rarely co-localized with LAMP-1; however, the majority of KIT proteins clearly showed co-localization 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 CRC tumorigenesis. If SCF-KIT activation plays an important role in CRC 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 CRC tumorigenesis. We further suspected that protein kinase C (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 phorbol 12-myristate 13-acetate (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 three cell lines (Fig. 3A). When DLD-1 and Colo320DM cells were concomitantly treated with PMA and Gö6983, a PKC inhibitor, KIT and p-KIT were no longer stabilized (Fig. 3B). We additionally performed 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 demonstrated that cell populations expressing p-AKT increased from 3.86 % to 29.8 % upon SCF and PMA co-treatment; the populations of tumor cells expressing p-ERK markedly increased from 1.25 % to 85.2% in the cells co-treated 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 co-localization of KIT proteins with LAMP-1 in the cells co-treated with SCF and PMA (Fig. 3C). To validate the endosomal recycling of KIT after PMA stimulation, we analyzed the co-localization of KIT and Rab11, a well-known recycling endosome marker. When Ls174T cells were treated with SCF alone, very little co-localization of Rab11 and KIT was identified; however, in cells treated concomitantly with SCF and PMA, a large proportion of KIT proteins co-localized with Rab11 (Fig. 3D). We performed 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 to 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 performed 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 performed 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 co-localization 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.

**PKC activation enhances colon cancer cell proliferation and invasion**
In order 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), in which 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, while the expression of p-ERK was rarely inhibited (Supplementary Fig. S8). We next evaluated the inhibitory effects of imatinib on proliferation and invasion of CRC cells. When the cells were co-treated with SCF and PMA, a marked increase (64% increase compared to 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 co-treated 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 CRC 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 demonstrated 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). Additionally, the expression of phospho-PKC-δ (p-PKC-δ) was increased in the CRC tissues with KIT expression and there was a linear correlation between KIT expression and p-PKC-δ expression (Pearson r = .5119, Spearman r = .445, P < .001; Fig. 5D).

**Clinicopathologic characteristics of colon cancers with KIT expression**

Lastly, we analyzed the clinicopathologic characteristics of CRCs with KIT expression among 250 CRCs using tissue microarray. Expression of KIT was not identified in normal colonic mucosa and most of the CRCs 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/; National Institutes of Health,
Bethesda, MD), and categorized as KIT expression or no expression (Fig. 6B). The clinicopathologic characteristics of the 250 patients with CRCs according to KIT expression are described in Supplementary Table S2. No significant correlation between KIT expression and clinicopathologic variables was found except for increased serum CEA level in CRCs 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=.004; Fig. 6C). When we analyzed the correlation between KIT expression and patient survival for the subgroup 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=.017, 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 PI3 kinase-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 CRCs (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), and expression of SCF is up-regulated by hypoxia in breast cancer cells (23). We found absent or very low levels of SCF mRNA in three CRC cell lines with KIT expression (Supplementary Fig. S10). Accordingly, the sources of SCF in CRCs are expected to be diverse, and continuous SCF stimulation can be achieved in some specific tumor environments. The present study demonstrated 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 anti-proliferative and anti-invasive effects (19, 24). Nevertheless, WT-KIT degradation after SCF binding has been reported (25). 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 CRCs 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 physiological role of WT-KIT activation, they raise questions regarding 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 α5β1 integrin induces the migration of ovarian cancer cells (26, 27). 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 demonstrated 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 (28, 29). Various factors such as growth factors, tumor promoters, chemotherapeutic agents, and ras protein induce activation of PKCs (30-32). Activation of PKC-δ by substance P-induced proinflammatory signaling in human colonocytes (33) and by a hypoxic microenvironment has been reported (34). Therefore, it is likely that endogenous PKC-δ is activated and functions in KIT recycling in the microenvironment of CRCs. Our findings indicate that CRCs expressing WT-KIT can constantly generate activated SCF-KIT signaling as a result of KIT recycling (Supplementary Fig.S11).
In addition to demonstrating expression of endogenous KIT and activated PKC-δ in CRCs tissues, we found that colon cancer patients 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 (35), but several studies found no prognostic correlations (36, 37). These findings might result from the variable and low incidence of KIT expression in colon cancers, marked intra-tumoral 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 CRCs. These considerations make it difficult, if not impossible, to perform an in vivo validation study of the anti-tumor 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 (38). However, continuous downstream signal activation through SCF binding to WT-KIT may be present in some CRCs, 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 post-transcriptional and/or post-translational 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 demonstrated the in vitro anti-tumoral effects of imatinib in KIT expressing tumor cells co-treated with SCF and PKC activator. Therefore, post-translational 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.

Acknowledgements
Misun Park and Won Kyu Kim contributed equally to this article.
Figure Legends

Figure 1. KIT expression and SCF treatment induced activation of downstream signaling pathways in colon cancer cells. A, RT-PCR and real-time PCR analysis of KIT gene expression in GIST 882 (positive control), HeLa (negative control), SNUC4, HCT116, DLD-1, Colo320DM, and Ls174T cells. KIT mRNA was expressed in DLD-1, Colo320DM, and Ls174T cell and KIT protein expression correlated with mRNA expression. Data are shown as mean ± standard deviation (SD; n=3) and GAPDH was used as a loading control. B, Inhibition of KIT expression by siKIT. KIT mRNA and protein were measured by real-time PCR and Western blotting. GAPDH was used as a loading control. *P < .05. C, Analysis of phospho-KIT (p-KIT), phospho-AKT (p-AKT), and phospho-ERK (p-ERK) expression after SCF treatment. None of the induced phospho-proteins was detected after KIT knockdown by siKIT. D, Increased levels of p-KIT, p-AKT, and p-ERK were observed 5 and 15 minutes after SCF treatment. In contrast, the level of KIT protein was decreased 15 minutes after SCF treatment.

Figure 2. Activated KIT proteins are degraded through the lysosomal pathway. A, mRNA and protein expression levels of KIT in DLD-1, Colo320DM, and Ls174T cells after SCF treatment were analyzed at the indicated times by real-time PCR and Western blotting. Data are shown as mean ± SD (n=3). The mRNA levels of KIT were constant in all three cell lines during the experiment, however KIT protein was barely detectable 60 and 120 minutes after SCF treatment. B, Treatment with MG132 for 60 minutes had no effect on SCF-induced KIT degradation in all three cell lines. C, Treatment with Bafilomycin A1 (Baf) rescued the KIT protein level and stabled levels of p-KIT, p-AKT, and p-ERK in all three cell lines. D, Immunostaining of KIT and LAMP1 in DLD-1 cells treated with or without SCF. DLD-1 cells were stimulated by SCF for 1 hour prior to immunostaining for KIT (green), LAMP-1 (red), and staining with DAPI (blue). Co-localization of KIT and LAMP-1 is evident as a yellow signal in cells treated with SCF.

Figure 3. PKC activation rescues KIT from lysosomal degradation. A, To activate PKC, cells were pretreated with 300nM PMA for 30 minutes and then incubated with or without SCF for 120 minutes. All three cell lines showed expression of KIT, p-KIT, p-AKT, and p-ERK after treatment with SCF and PMA. B, Rescue of KIT expression was not detected in the cells treated with Gö6983, a PKC inhibitor, in DLD-1 and Colo320DM cells. C and D, Ls174T cells were pretreated with 300nM PMA and incubated with or without SCF for 1 hour. Cells were stained for KIT (green), DAPI (blue), and LAMP-1 (red) (C) or Rab11 (red) (D). In cells
treated concomitantly with SCF and PMA, membrane expression of KIT was constantly detected (C), while co-localization of LAMP-1 and KIT was rarely detected. Co-localization of KIT and Rab11 was evident in the cells treated with SCF and PMA (D).

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 co-transfected with Flag-KIT into HeLa cells. Cells were pretreated with 300nM 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 co-transfected with Flag-KIT into HeLa cells. Cells were pretreated with 300nM 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, co-localization 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 300nM PMA and incubated with or without SCF for 2 hours.

Figure 5. KIT and PKC-δ are concomitantly activated in colon cancer tissues. A, Western blotting of KIT, p-KIT, PKC-δ, p-PKC-δ and GAPDH expression in human colon cancer tissues (T) and matched non-tumorous colonic mucosal tissues (N). B and C, Expression of KIT (B) and p-KIT (C) in 22 KIT positive colon cancer tissues (T) compared with non-tumorous colonic mucosal tissues (N). Statistical analysis was performed with a paired t-test (P<.0001 for KIT and P =.0005 for p-KIT). D, Quantification of Western blotting of KIT and p-PCK-δ showed that expression of KIT correlated with expression of p-PCK-δ (Pearson r=.5119, Spearman r=.445, P<.001).

Figure 6. Clinicopathologic characteristics of colon cancers with KIT expression. A, Immunohistochemical analysis of KIT protein expression in colon cancer tissues. Normal colonic mucosa (Normal) and most of the colon cancer tissues tested did not express KIT protein (Negative). Expression of KIT protein was evident in some colon cancers (Weak). In such cases, KIT protein was detected in the membrane and cytoplasm of tumor cells (Strong). B, The amount of KIT expression was analyzed by using the ImageJ program and categorized as KIT positive and negative. C, Overall survival according to KIT expression in colorectal cancers (n=250). The survival rate of colon cancer patients according to KIT expression was analyzed by the Kaplan-Meier method. Colon cancer patients with KIT expression showed poorer prognosis than patients whose tumors did not express KIT. D, overall survival curve according to KIT expression for patients with stages II and III colorectal...
cancers (n=195).
References


31. Gliki G, Wheeler-Jones C, Zachary I. Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3'-kinase-mediates PKC delta...


Figure 1

A. RT-PCR and qRT-PCR showing expression levels of KIT and GAPDH in different cell lines.

B. Bar graph depicting KIT mRNA levels in DLD-1, Colo320DM, and Ls174T cells transfected with siNC or siKIT.

C. Western blots for KIT, p-KIT, p-AKT, and p-ERK in DLD-1, Colo320DM, and Ls174T cells treated with SCF.

D. Western blots for KIT, p-KIT, p-AKT, and p-ERK in DLD-1, Colo320DM, and Ls174T cells treated with SCF at 0, 5, and 15 minutes.
Figure 2

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qRT-PCR

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KIT mRNA

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Figure 5

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- KIT
- p-KIT
- p-PKC δ
- PKC δ
- GAPDH

B

Paired t test
P < .0001

C

Paired t test
P = .0005

D

Pearson r = .5119
Spearman r = .445
P < .001
Protein Kinase C-δ-mediated recycling of active KIT in colon cancer

Misun Park, Won Kyu Kim, Meiying Song, et al.

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