STK31 maintains the undifferentiated state of colon cancer cells

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The expression of serine/threonine kinase (STK) family is frequently altered in human cancers. However, the functions of these kinases in cancer development remain elusive. Here, we report that STK31 is robustly and heterogeneously expressed in colon cancer tissues and plays a critical role in determining the differentiation state of colon cancer cells. Knockdown of or overexpression of STK31 induced or inhibited differentiation of colon cancer cells, respectively. Deletion of the STK domain abolished the inhibiting effect of STK31. Associated with differentiation, knockdown of STK31 resulted in significant suppression of tumorigenicity both in vitro and in vivo. Genome microarray analysis showed that knockdown of STK31 altered the expression profile of genes that are known to be involved in germ cell and cancer differentiation. Taken together, these results suggest that STK31 is able to control the differentiation state of colon cancer cells, which critically depends on its STK domain. The present findings may shed light on the new therapeutic approach against cancer by targeting STK31 and cancer differentiation.

Introduction

Cells within a tumor often exhibit variations in biological properties, including the differentiation status, tumorigenicity and therapeutic resistance. These variables in tumors are referred to as tumor heterogeneity (1–3). Intratumor heterogeneity has long been observed in colon cancers (4,5). Recently, using a high throughput single cell PCR transcription analysis platform, the heterogeneity in colon cancer has been shown to be a corollary of multi-lineage differentiation (6), indicating the presence of cellular hierarchy in colon cancers.

Differentiation of colon cancer cells may give rise to enterocytes in the absorptive lineage or enteroidendocrine cells and goblet cells in the secretory lineage (7). TGF-β and BMP pathways are two well-known cascades that regulate colon differentiation. While TGF-β shows a strong cytostatic response in intestinal culture (in vitro) (8), BMP inhibits intestinal stem cells renewal (in vivo) (9). These counteracting pathways share the common SMADs protein family as the intracellular messengers. Although the regulatory machinery in colon cancer differentiation has been postulated to recapitulate the signaling mechanisms in normal stem cell counterpart (10), the involvement of reactivated oncogenes in regulating colon-cancer differentiation through intrinsic mechanism remains elusive.

Abbreviations: STK, serine/threonine kinase; RT-PCR, Reverse transcription polymerase chain reaction.

Tyrosine kinases and serine/threonine kinases (STKs) are two major classes of kinases in the human kinome. Although extensive studies have been focused on the functions and therapeutic potential of tyrosine kinases in cancers (11,12), a recent study revealed that the expression of STKs is frequently altered in human cancers, suggesting that STKs may play an important role during cancer development. STK31 is a highly conserved member of the STK family and the Tudor Royal domain (Xu et al., 2004). Interestingly, in addition to its specific expression in the testis (13), STK31 is also expressed in gastrointestinal cancer by promoter hypomethylation, suggesting its potential role in cancer development (14). Despite all these interesting findings, the biological function of STK31 in gastrointestinal cancers has not been investigated. We undertook the present study to investigate the functional role of STK31 and we showed that STK31 plays a critical role in maintaining the undifferentiated state of colon cancer cells, which critically depends on its STK domain.

Materials and methods

Constructs

Full-length STK31 was cloned into pEGFP-C2 vectors (Clontech, Mountain View, CA) and verified by sequencing. For domain deletion constructs, 101–160 a.a. and 809–872 a.a. were deleted in Atudor and Akinase, respectively, by overlapping extension PCR method (15). For RNAi experiment, miR RNA is designed using web-based software (Invitrogen, Carlsbad, CA). Design targeting bacterial LacZ was used as control. Annealed oligos were cloned into pcDNA6.2 G/W/EmGF-miR vector (Invitrogen, Carlsbad, CA) and verified by sequencing. Primer and oligo sequences are listed in Supplementary Table 1, available at Carcinogenesis Online.

Gene expressions

RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Gene expressions were studied by RT-PCR or real-time PCR using specific primers (Supplementary Table 1, available at Carcinogenesis Online). SYBR green master mix and 7500 Fast Real-Time PCR Systems were used for real-time PCR (Applied Biosystems, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase was used as internal control.

For microarray analysis, RNA from Caco2 STK31 knockdown and control miR was labeled with Cy3 and Cy5, with dye swapped in duplicate experiment. Labeled cDNA were hybridized to Agilent human whole genome microarray (Agilent, Santa Clara, CA). Data were analyzed by GeneSpring 10.0 software (Agilent, Santa Clara, CA).

Expressions of candidate genes in germ cells were obtained from GermSAGE database (16).

Clinical samples

In tissue array experiment, colon adenocarcinoma tissue array was purchased from US Biomax (US Biomax Inc., Rockville, MD). The tissue array contains 110 colon cancer patients and 10 normal tissues. The 110 patients included 22 well-to-moderately differentiated tumors (Grade 1 or 1–2); 59 moderately differentiated tumors (Grade 2); 22 poorly differentiated tumors (Grade 3) and 7 undifferentiated tumors. The signal intensities were classified into three categories: (− or 0)—no expression; (+ or 1)—weak expression and (++ or 2)—strong expression. Since normal tissue sections have signal intensities ≤1, cancer sections with signal intensities >1 were assigned to low-expression group, whereas those ≥1 were assigned to high-expression group. Heterogeneous expression was defined by the presence of at least 5% of cells with similar morphology showing differences in signal intensities.

Frozen colon cancer samples were collected from Shenzhen Second People’s Hospital, the first affiliated hospital of Shenzhen University. Eight patients were recruited with signed informed consent.

Immunostaining and cell imaging

For immunostaining and cell imaging, Caco2 and SW1116 cells were grown on coverslips for 48 h and fixed with 4% ice-cooled paraformaldehyde. In both immunofluorescence and immunohistochemical staining experiments, tissue array or cells were stained with antibody against STK31 (1:100, Abnova, @ The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
Fig. 1. Expression of STK31 in colon cancers. (A) Immunohistochemical staining revealed that STK31 protein was highly expressed in colon cancer patient samples (representative figures are shown), whereas weak signals were observed in normal tissues. Perinuclear granular staining (dashed circle) was observed.
Clones were selected by Blasticidin (Invitrogen, Carlsbad, CA) following manufacturer protocol. In miR RNAi experiment, stably transfected SW1116 cells were maintained in RPMI 1640 supplemented with 10% FBS.

Caco2 cells were maintained in DMEM supplemented with 10% FBS.

Santa Cruz Biotech, Santa Cruz, CA).

(1:500, both from Cell Signaling, Danvers, MA) and anti-β-tubulin. Antibodies used in this study were anti-STK31 (1:1000, Abnova, Taipei, Taiwan), anti-cleaved caspase-3, anti-cleaved PARP (1:500, both from Cell Signaling, Danvers, MA) and anti-β-tubulin, (1:2000, Santa Cruz Biotech, Santa Cruz, CA).

Western blot

Cells were lysed in RIPA buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS) with protease inhibitor cocktail. Proteins were resolved and blotted onto nitrocellulose membrane and probed with indicated antibodies. Antibodies used in this study were anti-STK31 (1:1000, Abnova, Taipei, Taiwan), anti-cleaved caspase-3, anti-cleaved PARP (1:500, both from Cell Signaling, Danvers, MA) and anti-β-tubulin, (1:2000, Santa Cruz Biotech, Santa Cruz, CA).

Cultures and transfection

Caco2 cells were maintained in DMEM supplemented with 10% FBS. SW1116 cells were maintained in RPMI 1640 supplemented with 10% FBS. All cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer protocol. In miRNAi experiment, stably transfected clones were selected by Blasticidin (Invitrogen, Carlsbad, CA).

In cell-count experiment, 1 × 10^6 cells per well were seeded into a 24-well plate. Cells were trypsinized and counted by trypan blue exclusion at indicated time point.

Flow cytometry

Stable transfectants were seeded into 35-mm dishes at three densities: 1 × 10^4 per dish (sub-confluent), 6 × 10^4 (confluent) and 1 × 10^5 cells (post-confluent). Cells were grown for 2 days and collected for flow cytometry. Cells were fixed in chilled 70% ethanol for 30 min on ice. Fixed cell were stained 5 μg/ml propidium iodide (Sigma, St. Louis, MO) and 10 μg/ml RNase A for 2 h at room temperature. All samples were analyzed by FacsCalibur flow cytometer (BD Biosciences, New Jersey, NJ). Data were analyzed by FCS express V3 software (Denovo software, Los Angeles, CA).

Differenntiation assay

In spontaneous differentiation, Caco2 cells were cultured for 14 days with medium changed every 3 days. In sodium butyrate-induced differentiation, Caco2 and SW1116 cells were treated with 2 mM sodium butyrate (Sigma, St. Louis, MO). Cells were collected for molecular studies at 24—48 h post-treatment.

For alkaline phosphatase activity assay, cells were lysed in lysis buffer (1x PBS; 5 mM EDTA; 1% Triton X-100 with PMSF and PImix). Protein lystate was loaded into NPNP substrate solution, incubated for 30 min and terminated by stopping solution. Optical densities were measured at 405 nm.

Soft agar colony formation assay

A total of 2–4 × 10^4 cells were mixed with top agar (0.3%) and overlaid onto bottom agar (0.7%). Colonies were allowed to form for at least 2 weeks followed by staining with 0.005% crystal violet. Number of colonies per plate was counted. Statistical data were obtained from three biological replicates.

Xenograft assay

Animals were provided by Laboratory Animal Service Centre, CUHK. The protocols were approved by animal ethics committee, LASEC, CUHK (09/005/MIS). For Caco2 cells, 6 × 10^6 cells were injected subcutaneously into 5–6-week-old female NOD/SCID mice. For SW1116 cells, 1 × 10^6 cells were injected subcutaneously into 7–8-week-old female athymic nude mice. The mice were then killed after 4–6 weeks. Tumor weight, tumor size and the number of mice that developed solid tumor were determined.

Statistical analysis

Statistical analyses were performed by Prism 3.0. Results are presented as mean ± standard error mean (SEM) or standard deviation (SD). Data were compared by student’s t test, Chi Square (χ^2) or one-way ANOVA analysis. A P value <0.05 was considered statistically significant.

Results

Reactivation of STK31 in multiple cancers

Previous study has demonstrated the promoter hypomethylation and reactivation of STK31 in gastrointestinal cancer by PCR and immunohistochemistry (14). To examine the reactivation of STK31 in cancer development, we studied the expression profile of STK31 in a cohort of cancers. Our RT-PCR results showed that STK31 mRNA was reactivated in nasopharyngeal and liver cancer cell lines (Supplementary Figure S1, available at Carcinogenesis Online). The reactivation of STK31 in a board spectrum of cancers suggests that it may play an important role in cancer development. To consolidate the reactivation of full-length STK31 in colon cancer, we analyzed the protein expression of STK31 by both immunohistochemistry and western blot. We purchased an antibody raised against human full-length STK31 protein (Abnova) and validated the specificity of the antibody by western blot in STK31-transfected HEK293 cells. The antibody specifically recognizes full-length STK31 protein (116kDa; Supplementary Figure 2, available at Carcinogenesis Online). Then, we examined the reactivation of STK31 in a cohort of colon cancer samples by immunohistochemistry using the validated antibody. Colon tumor tissue array analysis showed enhanced STK31 expression in the cytoplasm of 57% (63 of 110) human colon adenocarcinoma specimens (Figure 1A; Supplementary Table 2A, available at Carcinogenesis Online). Weak signals were detected in cancer-adjacent and normal colonic tissues. Reactivation of STK31 was not always homogeneous within a tumor, with 22 out of 63 STK31-reactivated patients’ samples showing varied signal intensities in at least 5% of the cancer cells in the samples. The expression of STK31 appeared to be less heterogeneous in moderately to poorly differentiated cancers (31% heterogeneous expression) when compared with well-differentiated cancers (50% heterogeneous expression: P = 0.28); however, the difference was not statistically significant (Supplementary Table 2B, available at Carcinogenesis Online). While the reactivation of STK31 was not correlated to gender and histological grade (P = 0.25 and 0.23, respectively), patients with STK31 reactivation were slightly younger (P = 0.16; Supplementary Table 2B, available at Carcinogenesis Online), which is consistent with the previous report (14). Interestingly, perinuclear granular signals were observed in some of the tumor samples (Figure 1A red circle), which is reminiscent of the localization of its mouse homolog—Stk31—in the RNA processing body of the germ cells (17). To further validate the reactivation of STK31 in a quantitative way, we examined the expression of STK31 by real time-PCR. The results showed that the expression of STK31 transcripts were significantly increased in 5 out of 8 patients (P < 0.05) compared with normal tissue (n = 8; Figure 1B), confirming the reactivation of STK31 in colon cancer.

We next examined the expression and localization of endogenous STK31 in two colon cancer cell lines—Caco2 and SW1116—by immunofluorescent staining. Consistent with the IHC results, STK31 was localized in the cytoplasm (Figure 1C). Of note, expression of STK31 was heterogeneous in these two cancer cell lines. Particularly, in Caco2 colonies, high STK31 expression levels were found at the edge of colonies, which become decreased or undetectable in the central part of the colonies, where cells were compacted and were in contact with each other. Given that Caco2 cells are prone to undergo spontaneous post-confluence differentiation (18), the marked decrease in STK31 expression in the confluent cell population suggests that the expression of STK31 is downregulated in a differentiated state. To further demonstrate the correlation between the expression of STK31 and the differentiation of colon cancer cells, Caco2 cells were treated...
Fig. 2. Knockdown of STK31 alters cell morphology and suppresses cell proliferation and cell cycle progression in both Caco2 and SW1116 cells. STK31 RNAi construct was stably transfected into Caco2 and SW1116 cells. (A) Western blot of STK31 showed that the RNAi design could knock down STK31 in both cell lines.
with sodium butyrate, a metabolite that is known to induce differentiation of colon cancer cells. After sodium butyrate treatment, the activity of the brush border enzyme—alkaline phosphatase—was significantly increased (Figure 1D) while the protein expression of STK31 was markedly downregulated (Figure 1E). This result suggested a negative regulation of STK31 on differentiation of colon cancer cells.

**Knockdown of STK31 alters cell morphology and cell cycle progression**

Differentiation of colon adenocarcinoma cells is characterized by cell flattening (19) and growth retardation (20,21). To test whether STK31 is involved in regulating cell differentiation, we designed and constructed an RNAi for stable knockdown of STK31 in Caco2 and SW1116 cells (Figure 2A). Growth and morphology of the gene-manipulated cells were monitored over time. While knockdown of STK31 did not alter morphological or growth properties in subconfluent cultures (Supplementary Figure 3, available at Carcinogenesis Online), cells with knocked-down STK31 became significantly more flattened and spread out on culture dish upon cell confluence as indicated by the average area taken by a cell (P < 0.01; Figure 2B). The flattened morphology could not be attributed to the increase in cell size as indicated by flow cytometric analysis (Supplementary Figure 4, available at Carcinogenesis Online). More importantly, knockdown of STK31 resulted in a significant decrease in cell proliferation in both cell lines upon confluence (P < 0.05; Figure 2C). Further analysis on cell cycle progression in Caco2 cells revealed that knockdown of STK31 resulted in G1 arrest in both confluence and post-confluence (P < 0.05; Figure 2D). These results indicate that knockdown of STK31 has the potential to enhance differentiation in both colon adenocarcinoma cell lines.

**Knockdown of STK31 enhances differentiation of colon cancer cells**

When grown in appropriate culture conditions or treated with inducers, colon cancer cells from primary tumors can differentiate into two lineage-restricted cell types after in vitro expansion—the enterocytes in the absorptive lineage and the goblet cells in the secretive lineage (7,22). Caco2 cells are capable to undergo enteroctytic differentiation that is marked by the formation of dome and the expression of villin (an enteroctye marker), whereas SW1116 cells are capable to undergo goblet-like differentiation that is identified by expression of mucin (a goblet cell marker; 7,23,24). Since transition from undifferentiated to differentiated states in these two cell lines seems to recapitulate the growth arrest and commitment of lineage-specific differentiation of colonic cancer cells, we further investigated the potential roles of STK31 in regulating differentiation of the colon carcinoma cell phenotype in these two cell lines.

In spontaneous differentiation of Caco2 cells (25), knockdown of STK31 increased both dome (P < 0.05) and pleomorphic glandular formations (P < 0.001; Figure 3A), which are indicative of mature phenotypes of colon cancer cells (22). In keeping with the morphological changes, expression of villin was ~2–3-fold higher in STK31 knockdown cells compared with that in control cells in both spontaneous and sodium butyrate–induced differentiations (P < 0.05; Figure 3B). Besides, in sodium butyrate–induced differentiation of SW1116 cells, knockdown of STK31 resulted in significant increase in the number of cells with vacuolated cytoplasm (P < 0.001; Figure 3C), a morphological characteristic of goblet-like differentiation (7,26). Although differentiation marked by vacuolated cytoplasm had been associated with senescence, it should not have been the case here because no massive cell death or senescence was observed (Supplementary Figure 5, available at Carcinogenesis Online). In addition, expression of mucin was significantly higher (~2–3-fold) in STK31 knockdown cells compared with that in control cells (P < 0.01; Figure 3D). These results suggest that STK31 may be required for maintaining the undifferentiated state of the two colon cancer cell lines.

**STK domain of STK31 is required for maintaining the undifferentiated state**

To confirm the role of STK31 in maintaining the undifferentiated state of colon cancer cells, we examined whether the overexpression of STK31 would inhibit differentiation. To further identify the functional domains on STK31 responsible for regulating differentiation, we constructed window–deletion mutants in two conserved domain, the N-terminal tudor domain (ΔTudor) and the C-terminal kinase domain (ΔKinase), of STK31 (Figure 4A). Since the transfection efficiency of Caco2 cells is low, we used SW1116 cells for transient transfection experiments. To induce differentiation, sodium butyrate was added to the transfectants 24h post-transfection. Overexpression of STK31 and the two mutants was confirmed by western blot (Figure 4B). Differentiation status of transient transfectants was determined by the expression of goblet cell differentiation marker—mucin. As expected, the overexpression of STK31 significantly decreased the expression of mucin in both untreated and butyrate-treated conditions (Figure 4C, P < 0.001 and P < 0.01, respectively), indicating that the STK31-overexpressed groups are less differentiated in both conditions. This result confirmed the role of STK31 in maintaining the undifferentiation state of colon cancer cells. In domain deletion experiment, the expression of mucin in ΔTudor mutant-transfected cells was similar to that of full-length STK31 transfectant, suggesting that tudor domain was dispensable in regulating the differentiation of cancer cells (Figure 4C). Interestingly, deleting the STK domain partially reversed the inhibition of differentiation in untreated condition while completely abolishing the effect of STK31 overexpression on butyrate-induced differentiation (Figure 4C). These results suggest that the STK domain of STK31 is required for regulating the differentiation of colon cancer.

**Knockdown of STK31 suppresses tumorigenicity in vitro and in vivo**

The observed inhibition in cell proliferation and enhanced differentiation in STK31 knockdown colon cancer cells prompted us to assess the tumorigenicity of STK31 knockdown colon cancer cells in vitro and in vivo by soft agar colony formation assay and mice xenograft assay, respectively. As shown in Figure 5A, the knockdown of STK31 significantly decreased the number of colonies formed in soft agar in both CaCo2 and SW1116 cells (P < 0.05). In SW1116 xenografts, although all mice developed solid tumors, the size of tumors in STK31 knockdown group was significantly smaller than those in the control group (P < 0.05; Figure 5B). Moreover, histochemical staining showed that STK31 knockdown tumors displayed a more differentiated phenotype characterized by the formation of a glandular structure (Figure 5C). Similar results were obtained in Caco2 xenograft experiment where knockdown of STK31 decreased the number of mice developing solid tumors (Supplementary Figure 6A, available at Carcinogenesis Online). Furthermore, while sections of tumor formed by control miR Caco2 cells demonstrated typical well-differentiated adenocarcinoma morphology, tumor formed by STK31 knockdown Caco2 cells showed remarkable karyorhexis and karyolysis, indicating the occurrence of necrosis (Supplementary Figure 6B, available at Carcinogenesis Online). Together, these results indicate
Fig. 3. Knockdown of STK31 enhances differentiation of Caco2 and SW1116 cells. 6 × 10⁴ stable transfected Caco2 and 4 × 10⁴ stable transfected SW1116 cells/well of a 24-well plate were cultured in a serum-containing medium for 14 days for spontaneous differentiation or treated.
with 2 mM sodium butyrate (NaBT) for 48 h. (A) Knockdown of STK31 enhanced enterocytic differentiation in Caco2 cells as reflected by significant increase in number of dome (dash circles) and glandular crypt formation (arrows). Magnification: ×100. Quantification of data is shown on the right panel. (B) Real time-PCR showed that expression of villin, a known enterocyte marker, was up-regulated in STK31 knockdown Caco2 cells that underwent sodium butyrate–induced differentiation (right) compared with control cells (left). Data are presented as mean ± SEM; t–test, **P < 0.01, ***P < 0.001. (C) Knockdown of STK31 enhanced goblet-like differentiation in SW1116 cells as indicated by significant increase in the number of cells showing vacuole formation in cytoplasm (arrows). Magnification: ×100 (left panel) and ×200 (right panel). Quantification of data is shown on the right panel. (D) Real time-PCR showed that expression of mucin, a known goblet cell marker, was up-regulated in STK31 knockdown SW1116 cells that underwent sodium butyrate–induced differentiation. Data are presented as mean ± SEM; t–test, **P < 0.01, ***P < 0.001.

Fig. 4. Effect of domain deletion and overexpression of STK31 on SW1116 differentiation. (A) Schematic diagram on the window deletion of STK31. (B) Western blot showing the overexpression of full-length and domain-deleted STK31 in SW1116 cells. The size of Δtudor and Δkinase are slightly smaller than that of the full-length protein. (C) Real time-PCR results showing the expression of goblet cell marker, mucin, was downregulated in STK31-overexpressed SW1116 cells under either untreated or sodium butyrate–induced differentiation condition. Δtudor-expressed cells showed similar mucin expression compared with that of full-length STK31 overexpressed cells. Deleting the STK domain completely abolished the down-regulation of mucin in sodium butyrate–induced condition. Data are presented as mean ± SEM; One-way analysis of variance, *P < 0.05, **P < 0.01, ***P < 0.001.

that the knockdown of STK31 decreases tumorigenicity both in vitro and in vivo by promoting differentiation of colon cancer cells.

Differential gene expression in STK31 knockdown-mediated colon cancer differentiation

We next asked how STK31 could regulate the differentiation status of colon cancer. To answer this question, we compared the transcriptome of STK31 knockdown and control Caco2 cells using whole genome microarrays, and identified 95 genes that were significantly altered (≥2-fold). Interestingly, among the 95 genes regulated by STK31, 10 genes (KIT, NRG1, HDGFRP3, SMAD1, AR, GJA1, CCND2, TREFR1, FABP5 and SCIN) have been implicated both in tumorigenesis and in spermatogenesis (Supplementary Table 3, available at Carcinogenesis Online). To validate that genes identified by microarray study are bona fide targets of STK31, we performed real time-PCR to assess the expression levels of genes that (i) their function in tumorigenesis has been reported and with a 10-fold difference in microarray study or (ii) are known to implicate in both spermatogenesis and tumorigenesis. Our results showed that AR, FGF12, GJA1, HDGFRP3, LUM, NRG1, and SMAD1 were significantly downregulated in STK31 knockdown cells from both Caco2 and SW1116 cell lines (Figure 6A and 6B). Downregulation of KIT was only observed in STK31 knockdown Caco2 cells, but not in SW1116 cells probably because SW1116 do not express this receptor. Differential expression of the other two
Fig. 5. Knockdown of STK31 decreases tumorigenicity with enhanced differentiation. (A) Knockdown of STK31 in both Caco2 and SW1116 cell lines decreases the number of colonies formed in soft agar colony formation assay. Quantification of data is shown below. (B) 1 × 10^6 STK31 knockdown SW1116 cells were subcutaneously grafted into athymic nude mice. Mice were killed 2 weeks later and the tumors were collected and dissected for histochemical staining. Knockdown of STK31 in SW1116 cells decreased the size of tumor formed (n = 8). (C) Tumor xenograft formed by STK31 knockdown SW1116 cells showed a higher differentiation status compared with that formed by control cells as indicated by the presence of glandular cavities (arrow and inset; scale bar: 100 µm). Data are presented as mean ± SEM; t test, *P < 0.05, ***P < 0.001.

genes—ARL11 and PTPN13—was also observed in Caco2 cells only (Figure 6A and 6B).

Discussion
The present study has demonstrated that STK31 is highly expressed in multiple cancers, including colon cancer. Suppression of STK31 induces differentiation and inhibits tumorigenicity of colon cancer cells, both in vitro and in vivo. In general, tumors are heterogeneous in terms of their surface antigen expression, cell morphology (differentiation stages), proliferation, metastatic capacity, tumorigenicity and therapeutic resistance (1,2), indicating a cellular hierarchy consisting of cancer cells at various stages of abnormal differentiation. This cellular hierarchy is thought to be tightly regulated by homeostasis between proliferation and differentiation (27); however, the detailed regulatory mechanism remains unclear. Here, we have demonstrated that the reactivation of STK31 is crucial in maintaining the undifferentiated state of colon cancer cells. The heterogeneous expression pattern of STK31 in both cancer cell lines and primary tumors, together with the enhanced differentiation upon STK31 knockdown, strongly suggests its possible role in maintaining the primitive state of its expressing cells and thus the cellular hierarchy within the colon cancer cell lines or primary tumors. Moreover, the suppressed cancer growth associated with differentiation in vivo further lends compelling support for the anti-differentiation role of STK31 in colon cancer.

STK31 contains two highly conserved domains: a tudor domain and a STK domain. Of interest, we found that the STK domain is required for regulating the differentiation of colon cancer cells while the tudor domain is dispensable for the process. It has been reported that the expression of STKs is frequently altered in human cancers (28). Importantly, the majority of the STKs are reactivated or up-regulated in cancers, suggesting that gaining the activities of STK may favor tumor development. In the present study, we have demonstrated for the first time that the STK domain of STK31 is required for maintaining the undifferentiation state of colon cancer, providing strong support to the importance of STK in regulating tumor development. Since a variety of inhibitors of STKs is available (29), they may be used for cancer therapy in targeting reactivated or up-regulated STKs, including STK31.

The restricted expression in normal testis and aberrant expression in many tumors indicates that STK31 can be classified as cancer/testis gene. It has been suggested that spermatogenesis and tumorigenesis share significant similarities in biological and biochemical processes (30), which may be attributable to the reactivation of cancer/testis genes. Previous reports and our preliminary data revealed that STK31 expression was confined to spermatogonia in testis (13), indicating its pivotal role in germ cell differentiation. Therefore, we speculate that the regulation in colon cancer differentiation may resemble that in germ cell differentiation. In support of this notion, our gene expression profiling studies revealed that ~40% (39/95) of the STK31 target genes identified in colon cancer cells were expressed in germ cells. It is particularly noteworthy that several important autocrine/paracrine systems known to be involved in spermatogenesis (including KIT/SCF, BMPs/SMADs, NRG1/ErbB and possibly hepatoma-derived growth factor HDGF/HDGFRP3) are regulated by STK31 in colon cancer cells. Moreover, most of the STK31 targets (KIT, SMAD, NRG1) in colon cancer cells are specifically expressed in spermato- gonia in testis, in which STK31 is normally expressed, and has been implicated in the germ cell differentiation. During spermatogenesis, the receptor tyrosine kinase, KIT, and its ligand, stem cell factor (SCF), are crucial for spermatogonial proliferation and differentiation (31). Knockout of KIT has been shown to abolish spermatogonial
stem-cell differentiation, which results in sterility (32). Signaling by KIT also plays an important role in cellular transformation and differentiation through multiple signaling cascades, including STAT3, PI3K, PLC and MAPK (33–36). Of particular relevance to this study, human colorectal tumors express c-kit and activation of c-kit protected human colon adenocarcinoma cells against apoptosis enhanced their invasive potential (37). In our study, the downregulation of KIT by STK31 knockdown suggests that repression of KIT may be functionally important in STK31-mediated regulation of differentiation and tumorigenicity in colon cancer. Apart from KIT, BMP signaling plays critical roles both in colon cancer progression (38) and in spermatogonial differentiation (39). Particularly, SMAD1-mediated signaling cascades have been implicated in anchorage-independent growth (40) and tumorigenicity (41). Consistent with these findings, we have found that SMAD1 was downregulated by STK31 knockdown in the two colon cancer cell lines (Figure 6), indicating that SMAD1 is another possible target of STK31 contributing to its suppressive effects on tumorigenicity in vivo.

In conclusion, our study suggests that reactivation of STK31 in colon cancer maintains the undifferentiated state of colon cancer cells. Acquisition of this anti-differentiation ability could be one of the driving forces of tumorigenesis in colon cancer development. The current findings are of important clinical implications. Traditional cancer therapy has limitations with chemoresistance and cancer relapse, which are largely responsible for the treatment failure (10). With better understating on the molecular mechanisms underlying cellular heterogeneity and hierarchy in tumors, new therapeutic approach targeting undifferentiated tumorigenic tumors cells has evolved. The so-called differentiation therapy attenuates the tumorigenicity of cells with high differentiation potential through enforced commitment into defined lineages (42,43). Given its demonstrated role in regulating differentiation, together with its expression in a broad spectrum of cancers and specific expression in normal testis, STK31 represents a potent and promising target for the development of differentiation-directed therapy in colon cancer treatment.

Supplementary material
Supplementary Tables 1–3 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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References


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