RET is a potential tumor suppressor gene in colorectal cancer

Yanxin Luo[*,1,4], **Karen D. Tsuchiya**[*,2,3,4], **Dong Il Park**[4,5], **Rebecca Fausel**[4,6], **Samornmas Kanngurn**[4,7], **Piri Welcsh**[8], **Slavomir Dzieciatkowski**[4], **Jianping Wang**[1], and **William M. Grady**[4,6]

[1]Department of Colorectal Surgery, The Sixth Affiliated Hospital, Sun Yat-Sen University Guangzhou, P.R. China [2]Dept of Laboratory Medicine and Pathology, Seattle Children's Hospital, Seattle, WA [3]Dept of Laboratory Medicine, University of Washington Medical School, Seattle, WA [4]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA [5]Dept. of Gastroenterology, Kangbuk Samsung Hospital, Sungkyunkwak University, Seoul, Korea [6]Division of Gastroenterology, University of Washington Medical School, Seattle, WA [7]Tumor Biology Research Unit and Dept of Pathology, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand [8]Division of Medical Genetics, University of Washington Medical School, Seattle, WA

Abstract

Cancer arises as the consequence of mutations and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes. Through a genome-wide screen for methylated genes in colon neoplasms, we identified aberrantly methylated *RET* in colorectal cancer. *RET*, a transmembrane receptor tyrosine kinase and a receptor for the GDNF-family ligands, was one of the first oncogenes to be identified and has been shown to be an oncogene in thyroid cancer and pheochromocytoma. However, unexpectedly, we found *RET* is methylated in 27% of colon adenomas and in 63% of colorectal cancers, and now provide evidence that *RET* has tumor suppressor activity in colon cancer. The aberrant methylation of *RET* correlates with decreased *RET* expression, whereas the restoration of RET in colorectal cancer cell lines results in apoptosis. Furthermore, in support of a tumor suppressor function of RET, mutant *RET* has also been found in primary colorectal cancer. We now show that these mutations inactivate *RET*, which is consistent with *RET* being a tumor suppressor gene in the colon. These findings suggest that the aberrant methylation of *RET* and the mutational inactivation of *RET* promote colorectal cancer formation and that *RET* can serve as a tumor suppressor gene in the colon. Moreover, the increased frequency of methylated *RET* in colon cancers compared to adenomas suggests RET inactivation is involved in the progression of colon adenomas to cancer.

Corresponding author: William M. Grady, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N. D4-100, Seattle, WA 98109, phone: 206-667-1107, fax: 206-667-2917 wgrady@fhcrc.org.
[*]These authors contributed equally to this work.

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We declare there are no financial or commercial conflicts of interest in this study.

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Introduction

Epigenetic alterations, particularly the aberrant hypermethylation of CpG islands in gene promoters, have been found to be common in colorectal cancer and to affect hundreds to thousands of genes in the average colorectal cancer genome (1-4). The aberrant methylation of these genes can silence their expression and can promote tumor formation through the inactivation of tumor suppressor genes that control a variety of cellular processes including proliferation, and apoptosis, among others (5-7). It is widely assumed that only a small number of these epigenetic changes and mutations are needed for tumor formation (i.e. "driver genes"] with the remainder being bystander events (i.e. "passenger genes") (3, 8-10). Thus, the determination of which mutated genes and which methylated genes in cancer genomes are driver genes is an area of intense research interest given its significance for understanding the pathogenesis of colorectal cancer (11, 12).

In order to identify driver genes in the colon cancer genome, we initially conducted a genome-wide survey for methylated genes in colon cancer cell lines in order to find candidate colon cancer driver genes affected by epigenetic alterations. In this genome-wide survey we found hundreds of novel methylated genes (See supplemental data, **Table S1**). Unanticipated, we identified *RET* in this survey. The discovery of methylated *RET* in colorectal cancers was unanticipated given that mutant *RET* is a well-established oncogene in thyroid cancer (13). Thus, our finding raised the question of what role methylated *RET* is playing in the biology of colorectal cancer given that the published literature has shown an oncogenic role for *RET* in thyroid cancer. We elected to investigate the possibility that *RET* could be a conditional tumor suppressor gene in the colon not only based on the identification of aberrantly methylated *RET* in colon cancers but also based on the previous identification of *RET* mutations in colorectal cancer (10) (COSMIC database).

Although cancer related genes are classically defined as being either tumor suppressor genes or oncogenes, some genes appear to be both tumor suppressor genes and oncogenes, depending on the context of the mutant gene. For example, *TGFBR2* (the gene for the transforming growth factor beta receptor type II) and other genes in the TGF-ß signaling pathway, have been shown to have paradoxical roles in carcinogenesis. *TGFBR2* has been shown to be a tumor suppressor gene in the colon but an oncogene in the breast (14-18). We postulated that a similar situation may exist for *RET* with it being an oncogene in the thyroid gland and a tumor suppressor gene in the colon.

Furthermore, the biological function of RET also lends itself to the possibility of having contrasting effects on cancer cells arising from different tissues (19) *RET* encodes a transmembrane tyrosine kinase receptor that has three isoforms, long (RET51), intermediate (RET43), and short (RET9), which differ by having 51, 43, and 9 amino acids in the carboxy terminus. RET51 and RET9 are the most commonly expressed isoforms and have different effects on the maturation and development of the kidneys and gut, presumably due to a Grb2

binding site being present in RET51 but not in RET9 (19-22). Furthermore, RET is a member of the GDNF family receptor complex and is a receptor for the ligands GDNF, artemin (ARTN), neurturin (NRTN), and persephin (PSPN)(23). RET binds these ligands as part of a multi-subunit receptor complex that includes the GDNF Family Receptor alpha (GFR-alpha) proteins, which leads to activation of receptor tyrosine kinases (24) in a variety of signaling pathways including the MAPK, JNK, p38MAPK, and PLC-gamma pathways (19, 25-28). Interestingly, there is also controversial data that suggests RET may be a dependence receptor, which can induce apoptosis in certain cell types if it is not bound to a ligand (29, 30). If this is the case in the colon, it would imply that RET can induce cleavage of a death domain peptide and cause apoptosis if its ligands are not expressed in the colon. Moreover, the GDNF ligands can induce Src-family activation, and activation of Fos, and CREB when RET is absent through a receptor complex composed only of GFR-alpha, suggesting that loss of RET may lead to the activation of atypical signaling pathways, which could play an additional role in cancer formation (31, 32).

The importance of *RET* in the gastrointestinal tract is revealed in part by its role in Hirschsprung's disease and by the phenotype of *Ret* null and *Gdnf* null mice, which have defects in enteric innervation and can develop colitis (20, 33-35). RET normally regulates both cell proliferation and apoptosis in the gut, making it a plausible conditional tumor suppressor gene. Thus, three key pieces of evidence, 1) the biological functions of RET in the colon, 2) the recent discovery of mutant *RET* in colorectal cancer, and 2) our finding of methylated *RET* in colorectal cancer suggest *RET* could be a tumor suppressor gene in the colon and led us to investigate this possibility in more detail(10). Through an assessment of the functional consequences of mutant *RET* and methylated *RET* in colorectal cancer, we now provide evidence that *RET* is a probable conditional tumor suppressor gene in colorectal cancer.

Results

RET is aberrantly methylated in colon adenomas and colon adenocarcinomas

We initially carried out a genome wide screen for methylated loci in colon cancer cell lines using the MCA (Methylated CpG Island Amplification) method (See Methods and Shen et al (36)). We found hundreds of methylated genes in this screen, including methylated *RET*. (Supplemental Data, **Table S1**) We selected *RET* for further analysis given its role in the biology of the colon (19). We first assessed the methylation status of *RET* in early and more advanced adenomas as well as in adenocarcinomas. For this initial analysis, we developed an end-point Methylation Specific PCR (MSP) assay, which can determine the presence or absence of methylated *RET* in the tumor samples, but which cannot quantitate the amount of methylation. We found aberrantly methylated *RET* in 7% of normal colon mucosa (N=14), 0% of early adenomas (N=8), 30% of advanced adenomas (N=10), and 69% of adenocarcinomas (N=16) (p=0.0045 adenomas vs. adenocarcinomas, Chi-square analysis). (**Figure 1**)

After finding *RET* to be frequently methylated in advanced adenomas and adenocarcinomas using the endpoint MSP assay, we developed a quantitative MethyLight assay to more precisely assess methylated *RET* in the colon. MethyLight assays allow a more accurate

assessment of the amount of aberrantly methylated DNA in a sample. This is of importance in the assessment of aberrant methylation in tumors because most normal tissues have a certain amount of low-level methylated DNA present, and the amount that is present varies based on the locus being assessed (37, 38). Consequently, in order to determine if an aberrant degree of DNA methylation is present in tumors, the quantitative MethyLight assay is the more accurate than an endpoint PCR assay because a baseline threshold for gene methylation in normal tissue can be set. We fixed the detection threshold value for methylated *RET* at a PMR (percentage methylated ratio) of 4 as previously published (39). We then conducted a validation study of our initial results by using the RET MethyLight assay to determine the methylation status of *RET* in normal colon, colon adenomas, and colon adenocarcinomas. Analysis of a second independent set of tissues revealed aberrant methylation in 0% of normal colon mucosa (N=30), 26.7% of colon adenomas (N=30), and 63.3% of colon adenocarcinomas (N=30; $p = 0.079$ and $p < 0.0001$, normal colon mucosa vs. adenomas and normal colon mucosa vs. colon cancer, respectively.). Of the 30 cancer cases, six (20%) were identified to be CpG Island Methylator Phenotype (CIMP) tumors, which was used as a reference for the overall methylation state in the genome of the specific cancers and identifies those cancers with a high proportion of methylated genes (40). Aberrantly methylated-*RET* was found in 4 of 6 CIMP tumors and in 15 of 24 non-CIMP tumors ($P = 0.78$, 2-sided chi-square test). Our findings demonstrate that the methylation of *RET* is a neoplasm-specific event in CRC and shows that it is not restricted to CIMP tumors. This result suggests that methylated *RET* is not strictly the consequence of random DNA methylation occurring throughout the genome in which case it would be expected to be more frequent in CIMP tumors. The results of the MSP assays were confirmed by bisulfite sequencing. (Supplemental Data, **Figure S1**)

The aberrant methylation of *RET* silences *RET* expression in colorectal cancer

The colorectal cancer cell lines, SW48, RKO (CpG Island Methylator Phenotype, CIMP); and AAC1/SB10 (microsatellite stable, MSS) carry methylated *RET*. Two MSS cell lines, Vaco411 and SW480, carry unmethylated *RET*, and HCT116 (microsatellite unstable, MSI) carries both methylated and unmethylated *RET*. These cell lines were treated with the DNMT1 inhibitor 5-aza-2'-deoxycytidine (5-AZA) to determine if *RET* methylation silences *RET* mRNA expression. *RET* expression was absent or minimally expressed at baseline in AAC1/SB10, SW48, Vaco411, RKO and SW480, and was expressed at a high level at baseline in HCT116. The lack of expression of *RET* in Vaco411 and SW480 is presumably secondary to factors other than DNA that regulate gene expression. SW48 and RKO demonstrated a significant increase in expression (>5X) after 5-AZA, whereas the other cell lines demonstrated no significant increase in RET expression after 5-AZA. SW480 (unmethylated *RET*) showed a <5X increase, which is presumably secondary to nonspecific effects of 5-AZA. (**Figure 2A**) We also determined *RET* mRNA expression in primary colon adenomas and cancers that were assessed for methylated *RET*. *RET* expression was significantly decreased in those tumors that carried methylated *RET* compared to the tumors that carried unmethylated *RET* with mean expression levels of 1.04 ± 0.30 vs 2.51 ± 0.56, respectively (p= 0.0279, n = 41; **Figure 2B**).

Expression of RET and its ligands, artemin (*ARTN*), neurturin (*NTRN*) and GDNF in normal colon mucosa and colorectal neoplasms

Assessment of the relative expression levels of *RET* mRNA in primary colon tissues showed *RET* mRNA to be higher in normal colon mucosa (mean relative expression 2.20 ± 0.30, N=22) and early adenomas (mean relative expression 3.56 ± 0.85, N=11) than in advanced adenomas (mean relative expression 1.06 ± 0.4, N=9) and adenocarcinomas. *RET* expression was lowest in the colon adenocarcinomas (mean relative expression 1.06 ± 0.35, N=20, $P = 0.0057$, one-way ANOVA). (**Figure 2C**) Validation of these results with absolute quantitative RT-PCR for *RET* confirmed that *RET* was more highly expressed in normal colon mucosa than in the adenocarcinoma ($8.43 \times 10^8 \pm 4.05 \times 10^8$ molecules/µg RNA versus $2.29 \times 10^8 \pm 2.77 \times 10^8$ molecules/µg; $P = 0.014$, 2-sided student *t* test). (**Figure 2D**)

As noted in the introduction, RET is a receptor for the four glial derived neurotrophic factors family ligands, GDNF, neurturin (NRTN), artemin (ARTN), or persephin (PSPN). Thus, we assessed the expression of *GDNF, ARTN*, and *NRTN*, the primary ligands for RET, in normal colon mucosa, colon cancer cell lines and in colon neoplasms, in order to determine the effect of loss of RET in the colon. (We did not assess the expression of persephin since it is only expressed in the retina (41).) We found that *GDNF* is not expressed in any of the colon cancer cell lines (HCT116, SW480, V400, SW48, or AAC1/SB10). (Supplemental data, **Figure S2**) *ARTN* and *NRTN* were expressed at the highest levels in the cell lines SW480 and HCT116, and there was no or low expression in the other cell lines. Of interest, the cell lines that do not express *NRTN* or *ARTN* are the same cell lines that do not express RET. (**Figure 3A and 3B**) We next assessed the expression of *GDNF, NRTN* and *ARTN* in tissue samples from normal colon, colon adenomas, and adenocarcinomas. Similar to what was observed in the colon cancer cell lines, the majority of the primary colon adenocarcinomas do not express GDNF, but do express ARTN and NRTN. (**Figure 3C**) The lack of expression of GDNF in colon adenocarcinomas raises the possibility that GDNF, but not ARTN or NRTN, may have tumor suppressor activity in the colon. This tumor suppressor activity is not necessarily related to RET, but could be related to RET, if RET is a dependence receptor for GDNF. In the latter possibility, the loss of GDNF in the adenomas would create selective pressure for inactivation of RET by either mutation or aberrant methylation in order to avoid the induction of apoptosis caused by RET when GDNF is absent (42).

The in situ expression of RET and GDNF was also assessed in normal colon mucosa (N=10), colon adenomas (N=10), and colon adenocarcinomas (N=10) using immunohistochemistry. We found the normal colon mucosa expresses both RET and GDNF, whereas the colon adenomas and colon cancers have little if any detectable expression in the majority of cases. RET expression in the colorectal cancers was significantly less than that in the normal colon mucosa (mean IHC final score: 8.4 ± 0.40 versus 3.8 ± 4.9, $P < 0.0001$, 2-sided student t test). Similarly, we only detected GDNF expression in the normal colon mucosa (mean IHC score: 7.2 ± 0.66) and found no or weak GDNF expression in the cancers (mean IHC score: 2.0 ± 0.70, $P < 0.0001$, 2-sided student t test) (**Figure 4**). Of interest, we did detect RET expression in some adenocarcinomas that carried unmethylated

RET, which is consistent with the methylation of *RET* repressing its expression (Supplemental Data, **Figure S3 and Table S2**).

GFRα- receptor expression in colorectal cancer

The functional RET receptor complex includes RET and one of four glycosylphosphatidylinositol-anchored co-receptors, designated GDNF-α receptors, GFRα1, GFRα2, GFRα3, and GFRα4. Given that RET, GDNF, ARTN, and NRTN are expressed in the colon, (**Figure 3**), we assessed the expression of the GFRα's in the colon cancer cell lines and in primary colon tissues. We found GFRα3 expression in a subset of the colon cancer cell lines, whereas expression of GFRα1 or GFRα2 is absent in these cell lines. In contrast in primary colon tissue, both GFRα1 and GFRα3 are present in a subset of tumors, with GFRα1 being the predominant receptor detected. (**Figure 5A, B**, Data for GFRα1 expression in colon cancer cell lines not shown). Thus, in the primary colon and colon neoplasms, it appears that GFRα1 and/or GFRα3 are potential binding partners with RET to form a receptor complex for the GDNF ligands.

RET can induce apoptosis in colorectal cancer cell lines

In light of data in some cell line systems that RET can induce apoptosis when not engaged with a ligand, we carried out a series of studies to determine if RET expression in the colon cancer cell lines would induce apoptosis (13, 42). Thus, in order to assess the effect of *RET* expression on colon cancer, the two most commonly expressed isoforms of *RET, RET51* and *RET9*, were reconstituted in the RKO and SW48 cell lines, which both carry methylated *RET* and do not express RET. (The reconstituted RET mRNA and protein levels are shown in **Supplemental Data, Figure S4**). These cell lines were then assessed for apoptosis (43). In SW48 and RKO, *RET51* reconstitution increased apoptosis 4-6 X. The addition of GDNF (100 ng/mL) suppressed the apoptosis induced by *RET51*. (**Figure 6A** and **B**) *RET51* did not induce apoptosis in the SW480 cell line, which expresses *RET* at baseline levels (**Figure 6C**). The apoptosis inducing effect of ligand-free RET occurred in cell lines stably transfected as well as transiently transfected with *RET* (Supplemental Data, **Figure S5**). These studies support the role of *RET* as a tumor suppressor gene in the colon.

Somatic *RET* mutations also occur in primary colorectal cancer and inactivate RET

Our discovery of aberrantly methylated *RET* in colorectal adenomas and adenocarcinomas led us to assess the COSMIC (Catalog Of Somatic Mutations In Cancer) database for *RET* mutations in colorectal cancer. The COSMIC database has catalogued missense *RET* mutations in primary colorectal cancers, and, in fact, *RET* mutations are among the most common mutations found in colorectal cancer (10). In order to determine the effect of the mutant *RET* genes on the behavior of colorectal cancers, we constructed plasmids that express the mutant *RET* genes listed in COSMIC-*RET-V145G, RET-R360W* or *RET-G593E*. The mutant *RET* genes were then transfected into the SW48 colon cancer cell line. Transduction of the mutant *RET* genes *RET-V145G* and *G593E* into the SW48 cells, did not induce apoptosis (**Figure 7**), but the wild-type *RET* gene and the *RET-R360W* mutant gene did induce apoptosis. Thus, 2 out of 3 mutations in *RET* are inactivating mutations with regards to apoptosis. (Of note, the mutation status of all the constructs was confirmed by

direct sequencing (**Supplemental Data, Figure S6**)). These findings suggest *RET* is a tumor suppressor gene in the colon that is inactivated by both epigenetic and genetic mechanisms. The identification of both methylated *RET* and mutant *RET* in colorectal cancers provides evidence that inactivation of RET promotes tumor formation in the colon.

RET suppresses soft agar colony formation of colon cancer cell lines

Soft agar colony formation assays were used as an in vitro assessment of the effect of loss of RET on the tumorigeneticity of colorectal cancer cell lines. This assay is considered one of the most reliable assays for assessing tumorigenecity in vitro (44). The RKO cell line was transduced with *RET51* or a control vector (either empty vector or vector expressing an inactive form of RET, *RET R758T*) and plated into soft agar. RKO transduced with *RET* showed a nearly 5-fold reduction in colony number compared to RKO transduced with control vectors. The addition of GDNF (100ng/ml) into the soft agar prevented RET-induced suppression of colonies. We also observed that the addition of GDNF to both RKO and SW48 induced phosphorylation of RET at Y1062. (Supplemental Figure S9) Notably, ARTN did not rescue the suppression of colony formation ability of RET-transfected RKO cells. Furthermore, *RET* transduction did not affect the colony formation ability of SW480 cells, which express RET (**Figure 8A and 8B**) (Supplemental Data, **Figure S7**). We also assessed the effect of treatment with GDNF, ARTN, and NRTRN on RET Y1062 phosphorylation in cells expressing transfected RET51. We found that the transfected RET has basal phosphorylation and that GDNF, but not ARTN or NRTN, increased the phosphorylation level (Supplemental data, Figure S9B).

In order to further assess the effect of loss of *RET* on colon cancer, HCT116 cells were transfected with RET-siRNA to knockdown RET (**Figure 8C**). Down-regulation of RET reduced apoptosis as well as enhanced soft agar colony formation in siRET-transfected HCT116 cells. Interestingly, knockdown of RET expression in MCF7, which is a breast cancer cell line and in which *RET* is reported to function as an oncogene, can induce apoptosis and suppress soft agar colony formation in the siRET-transfected MCF7 cells (**Figure 8C**). Taken together, these studies demonstrate that RET can suppress the tumorigenecity of colon cancer cells and that this appears to be a tissue specific effect of RET. (The knockdown effects were validated by detecting the RET mRNA expression using qRT-PCR (Supplemental Data, **Figure S8**).)

RET inactivation leads to perturbed MAPK signaling

RET has been reported to induce apoptosis in cells through the release of a pro-apoptotic fragment after caspase 3 cleavage, and may additionally induce apoptosis through the regulation of a variety of signaling pathways, including the MAPK-ERK, NF-κB and PI3K pathways, among others (34). We observed that SW48 reconstituted with *RET51* shows activation of the MAPK/ERK pathway independent of GDNF expression. (**Figure 9A**) Of interest, in the SW480 cell line, which carries unmethylated *RET* and expresses *RET* at baseline transduction of *RET51* had no effect on activation of the MAPK/ERK pathway. (**Figure 9B**) Importantly, we also observed that RET-induced apoptosis was inhibited in SW48 by the MAPK inhibitor U0126, demonstrating that RET mediates its pro-apoptotic effects through the MAPK signaling pathway in addition to caspase 3 cleavage. (**Figure 9C**)

As mentioned above and consistent with these results we found that ectopically expressed RET in SW48 and RKO cells was activated at Tyr1062 site, which was stimulated by GDNF treatment. (**Supplemental Data, Figure S9**). We found no expression of RET or phosphorylated RET in the untransfected cell lines.

Discussion

Colorectal cancer results from the concurrent effects of gene mutations and epigenetic alterations on colon epithelial cells. These mutations and epigenetic alterations affect a wide variety of genes that are involved with regulating cell behavior related to proliferation, apoptosis, cell motility, metabolism, as well as other processes. Through a genome wide screen for methylated genes in colorectal cancer, we identified *RET* as being subject to epigenetic silencing in colon adenomas and adenocarcinomas. We have now provided evidence that the aberrant methylation of *RET* promotes the formation of colorectal cancer by releasing colon cancer cells from the apoptosis-inducing effects of *RET*. Our studies demonstrate a novel function for *RET* as a colon cancer tumor suppressor gene. Our finding was not predicted in light of the clear demonstration of *RET*'s role as an oncogene in thyroid cancer (34). In papillary thyroid cancer, *RET* mutations that activate the tyrosine kinase domain of RET and consequently induce the MAPK pathway, PI3K pathway, and STAT pathway, as well as other signaling pathways are common (13). In addition, therapy directed at RET inhibition has shown modest clinical efficacy against thyroid cancer. Although many of the trials are in early clinical phases and have used multikinase inhibitors that inhibit VEGFR, PDGFR, or BRAF as well as RET, these results support the role of RET as an oncogene in thyroid cancer (45). The difference in RET's role in thyroid cancer vs. colon cancer presumably reflects fundamental differences in signaling networks between cell types (45). Indeed, our observation of frequent *RET* methylation as well as inactivating *RET* mutations in colorectal cancers suggests that RET inactivation rather than activation is favorable for cancer formation in the colon. Also, of interest is the fact that although RET inactivation has been observed to cause Hirschsprung's disease, a developmental disease in which regions of the intestinal tract lack ganglion cells, its role as a tumor suppressor gene in cancer has not been clearly demonstrated to date (34). Of note, individuals with Hirshsprung's disease are not predisposed to colon cancer possibly because of the differences in the effects of constitutional mutations vs. somatic mutations in the colon. A similar situation has been observed for mutations in the transforming growth factor ß receptor, which when mutated in the germline causes Marfan's syndrome type II, which is not associated with a predisposition to cancer, but when *TGFBR2* is somatically mutated in the colon epithelium, it plays a role in colon cancer formation (46). In light of our findings and RET's established role as an oncogene in the thyroid, we reasoned that RET function may depend on its context and that it may be able to act as a conditional tumor suppressor gene in the colon (30).

Our studies demonstrate that RET is a probable tumor suppressor gene in the colon and, thus, appears to be a member of a recently identified class of conditional colon cancer tumor suppressor genes that include DCC and UNC5C as well as others (47-49). The role of *RET* as a tumor suppressor gene in the colon is not only supported by the recent discovery of it being hypermethylated in cancer (3, 50) but also by the discovery of *RET* as a candidate

cancer gene (CAN gene) in the colon cancer genome (10). Heterozygous missense mutations (V145G, R360W, and G593E) have been found in colon cancers and are estimated to occur in approximately 10% of colon cancers. We have shown that the V145G and G593E mutations inactivate *RET* consistent with *RET* being a tumor suppressor gene in the colon.

The discovery of *RET* as a tumor suppressor gene in the colon also raises the interesting possibility that the GDNF ligands could serve as oncogenes in the colon. There is evidence that ARTN can be oncogenic in breast and lung cancer (51, 52). Our results would suggest that, if the GDNFs can function as tumor promoting genes in the colon, the strongest candidate ligands would be ARTN and NRTN rather than GDNF given the lack of expression of GDNF observed in colorectal cancers. However, given the high frequency of RET inactivation in colon cancers (>60%), if ARTN or NRTN were to act as oncogenes in the colon, it would likely be germane only in the subset of colorectal cancers that express RET or would need to have oncogenic effects in a RET independent fashion.

In summary, we have identified *RET* as a novel tumor suppressor gene in the colon and have provided evidence that it can induce apoptosis and suppress anchorage independent growth in colon cancer cells when expressed in the absence of GDNF. The nature of the tumor suppressor effect of RET was clearly shown by *RET* reconstitution and knock-down experiments. Moreover, our functional studies of RET mutations found in primary colorectal cancers provide genetic evidence in addition to our epigenetic evidence that RET is a tumor suppressor gene in the colon. RET appears to be a tumor suppressor gene for colorectal cancer that is both commonly epigenetically silenced in colon cancer and mutated in colorectal cancer. Our findings suggest the activation of RET can induce the death of colon cancer cells in the setting of epigenetic therapies and may be one mechanism for their effects on colorectal cancer cells.

Materials and Methods

Tissue samples

Colon adenomas and adenocarcinomas were collected from patients treated at Vanderbilt University Medical School, which includes the Department of Veterans Affairs Tennessee Valley Health Care System, Vanderbilt University Medical Center and Meharry Medical Center (Nashville, TN) and at University Hospitals of Cleveland (Cleveland, OH) following protocols approved by the Institutional Review Board of each institution. The samples were provided by MKW or were obtained through the Cooperative Human Tissue Network site based at Vanderbilt University Medical Center. All tissue samples were formalin-fixed and paraffin-embedded tissue blocks obtained from the pathology archives and were collected randomly based on tissue availability. The cases included colon adenomas, primary colon adenocarcinomas, and samples of normal colon mucosa obtained from colon resection specimens from patients who had diverticular disease. In order to assess for the possibility of the sample source having an effect on the detection of mRNA or methylated DNA, the mean values and range of values was compared between samples based on collection site. No significant difference was observed between the groups.

Extraction of DNA/RNA and description of cell line RNA panel

Genomic DNA and RNA was extracted using published protocols (Grady et al., 1998; Grady et al., 2008) RNA was extracted from formalin-fixed tissue using the RNeasy FFPE kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

Methylated CpG Island Amplification and Microarrays (MCAM) and initial validation studies of MCA results

MCAM experiments were performed using cell lines VACO 235, VACO 411, AA/C1, and AA/C1/SB10 that were cultured as previously described (53, 54). VACO 235 and AA/C1 were established from colon adenomas, and lack characteristics associated with malignancy (55, 56). VACO 411 and AA/C1/SB10 are derivatives of VACO 235 and AA/C1, respectively, that have acquired malignant features. Reference DNA for MCAM was obtained from peripheral blood leukocytes from a healthy female donor. Methylated genes identified by MCAM of cell line DNA were subsequently analyzed in primary colon tumors (adenomas and adenocarcinomas) and non-neoplastic colon mucosa from specimens obtained from the Cooperative Human Tissue Network (CHTN) and University Hospitals of Cleveland (generous gift of Sanford Markowitz). Tumor and non-neoplastic colon mucosa were enriched by macrodissection using an H&E stained frozen section as a guide.

MCA on the DNA from the cell lines was performed as described previously (Estecio 2007). RMCA24 and RMCA12 PCR adaptors (Toyota 1999) were used for all MCA experiments. PCR products were labeled with Cy3-dUTP or Cy5-dUTP (GE Healthcare) using the Bioprime Array CGH Genomic Labeling System (Invitrogen). Two micrograms of PCR product were mixed with 35 μL of 2.5x random primer solution in a total volume of 75 μL, incubated at 95 °C for 5 min and immediately cooled on ice. The following components were mixed together and then added to the DNA/random primer mixture: 8.2 μL of dUTP mix (1.2/0.6 mM), 1.5 μL of Cy3 or Cy5-dUTP (1 mM), 1.5 μL Klenow (40 U/ μL), 1.8 μL water. The labeling reaction was incubated at 37 °C for three hours, then purified using the purification columns provided in the Bioprime kit according to manufacturer instructions. Five micrograms each of labeled test and reference DNA were combined and water was added to a give a volume of 150 μL. Fifty microliters of human Cot-1 DNA (1.0 mg/ml; Invitrogen), 50 μL 1x Agilent blocking agent (Agilent Technologies), and 250 μL 2x Agilent hybridization buffer were then added. This mixture was hybridized to Agilent 44K human proximal promoter arrays according to the manufacturer's instructions. These arrays were designed based on the hg 17 assembly and provided coverage of approximately 17,000 transcripts. For the analysis, we filtering out fragments with SmaI sites > 3 Kb apart, which resulted in 8731 genes being represented by informative probes, with 2272 represented by one probe and 6459 genes represented by >1 probe). Probe coverage extended from 0.8 Kb upstream to 0.2 Kb downstream of the transcription start site, with an average of 4.5 probes per transcript. The hybridized slides were scanned using either a GenePIX 4000B (Molecular Devices,) or Agilent Technologies scanner. Data was extracted from scanned images using Agilent Feature Extraction v. 9.1 software for slides scanned using an Agilent scanner. Data from images obtained using slides scanned on the GenePix scanner were extracted using GenePix Pro software. Array data was filtered using GDFilter software and analyzed using GeneTraffic (Iobion).

Potentially methylated genes detected by MCAM were initially identified based on the presence of at least one probe with a normalized log2 ratio 1.3 (Estecio, 2007). Candidate genes were selected for futher analysis based on a combination of the number of probes for a given gene with a log2 ratio 1.3 in more than one cell line, presence of a CpG island near the 5' end of the gene, and the biologic function of the gene.

Methylation Specific PCR (MSP)

The DNA was modified with sodium bisulfite for use in methylation specific PCR (MSP) assays as previously described (Grady et al., 2000). MSP primers were designed to amplify the methylated and unmethylated alleles for *RET* and methylation-specific PCR assay conditions were determined so that specific reaction products were obtained from each respective set of primers. Each PCR reaction mix consisted of a total volume of 20 μL composed of PCR buffer (Qiagen, Valencia, CA), 200 pM deoxynucleotide triphosphate mix (Applied Biosystems, Foster City, CA), 500 nM of each primer (Sigma Genosys, The Woodlands, TX), 1 unit of HotStar Taq DNA polymerase (Qiagen), and bisulfite-modified DNA. The primers sequences are as follows: Methyl-specific forward primer: 5'-TATGGCGTAGTAAGCGTTTCGC-3', reverse primer: 5'-TGAAAATAAAACCCAAAAACCCGAA-3'; Unmethyl specific forward primer: 5'-TTTTTGAGAAATGGTGTAGTAAGTGTTTTGT-3 ' and reverse primer: 5'-TCAAAAATAAAACCCAAAAACCCAAA-3'. All these primers have a universal primer sequence on the 5' end, which is 5'-GCGGTCCCAAAAGGGTCAG-3' in order to improve the specificity of the MSP reaction. The thermocycler conditions are 94 °C × 15 min, 45 × (94 °C × 30 sec, 62 °C × 30 sec, 72 °C × 30 sec), 72 °C × 10 min. All the samples were subjected to at least two independent rounds of sodium bisulfite treatment and MSP assays. Control samples from cells with known methylated and unmethylated *RET* were included in each MSP assay to confirm the technical success of the assays. The MSP products were subjected to horizontal gel electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized with UV transillumination using an Eagle Eye Imaging system (Stratagene, La Jolla, CA).

For sodium bisulfite sequencing, this was carried out as previously published (Grady et al., 2008). The primer sequences are as follows: forward primer: 5'-TGAAAAAGGTTATTTTGTTTT-3 ', and reverse primer: 5'-CTTACAATCCCTACCTTTTACCCTT-3'. The sequencing thermocycler reaction conditions are as follows: 94 °C ×15 min, 40 × (94 °C × 30 sec, 54 °C × 30 sec, 72 °C × 30 sec), 72 °C × 10 min.

MethyLight RET assay

Quantitative MSP ("MethyLight") assays were carried out using on bisulfite treated DNA following published protocols (Eads et al., 1999). The methylated *RET* qMSP assay was performed using an Opticon 2 System (Bio Rad, USA). The Primer/Probe sequences for methylated *RET* were designed using ABI PrimerExpress software Version 5.0 and were as follows: Methyl-specific forward primer: 5'-GAAAGGGTAAAAGGTAGGGATTGTAA-3', methyl specific reverse primer: 5'-GCGCACCTACGCAAAACC-3'; probe sequence: 5'-CGCGTATCGGGTAGGA-3'. The

probe was labeled with FAM. The reaction mix consisted of 0.45 μL primers (20 μM), 0.03 μL probe (100 μM), 1.5 mM $MgCl_2$, 200 pM dNTPs, HotStart Taq 1.5 U. The thermocycler conditions are as follows: 95 °C × 15 min, 45 × (94 °C × 30 sec, 62 °C × 30 sec). A published qMSP assay for repetitive Alu sequence ("AluC4") was used to normalize for the amount of input DNA (Weisenberger et al., 2006). All samples were run in triplicate at least twice. The methylation status of the samples was determined using the scoring scheme proposed by Eads et al (Eads et al., 2000).

Quantitative RT-PCR

RNA was extracted from cells from the cell lines using Trizol (Invitrogen), OH) following the manufacturer's recommended protocol. One microgram of total RNA from each sample was reverse transcribed using oligo d(T) priming and Superscript-II reverse-transcriptase (Invitrogen). TaqMan On-Demand primers and probes were used to determine the relative expression levels of RET (Hs01120027_m1), ARTN (Hs00365083_m1), NRTN (Hs00177922_m1), GDNF (Hs01047952_m1), and GFRα1 (Hs00237133_m1, ABI), GFRα2 (Hs00394700_m1, ABI) and GFRα3 (Hs01125112_m1, ABI) (Applied Biosystems, Foster City, CA) and 18S in all samples (Assay number Hs99999901_s1, Applied Biosystems). The precise assessment of RET expression analysis was determined by using the RET51 expression vector to create a standard curve, which allowed for the measurement of the absolute number of cDNA molecules in the colonic epithelial cells. *GUSB* (Assay ID: Hs99999908_m1) was used as an internal loading control. The reactions were run in triplicate in the Opticon II detection system (BioRad, Hercules, CA).

Cells, Transfection Procedures, and Plasmid Constructs

The cell lines SW48, RKO, SW480, and V400 were grown following published protocols (Markowitz et al., 1995; Rojas et al., 2008). Transfection of SW48, RKO, SW480, or V400 was performed using FuGENE®6 Transfection Reagent (Roche Molecular Biochemicals) following the manufacturer's protocol. The pcDNA3-based vectors containing *RET9, RET51-K758R* and *RET51* were a gift of Dr. Maria Grazia Borello, Milan Italy). *RET51-K758R* is a mutant *RET51* (lysine (K) → arginine (R) substitute at amino acid position 758) kinase domain and was used as a negative control plasmid. The plasmids containing *RET51-V145G, RET-R360W* and *RET51-G593E* were constructed based on the wild-type *RET51* expression vector by using GENEART site-direct mutagenesis system (Invitrogen) following the manufacturer's protocol. An expression vector expressing BAX was a kind gift from Dr. Hockenbery at Fred Hutchinson Cancer Research Center. The pcDNA3 vector was used as an empty vector control plasmid. The studies using transfected cell lines were carried out in triplicate. In the studies involving GDNF (GDNF 0.1μg/mL; R&D Systems, Inc.), neurterin (NRTN) (Leinco Technologics Inc.) and artemin (ARTN) (R&D Systems), these were added to the culture medium 24 hours after transfection. In order to determine the optimal concentration of GDNF to use in these studies, a dose-response curve was generated using concentrations of 25, 50 or 100 ng/mL GDNF. Transfected RKO or SW480 cells were grown for 10 ~ 14 days in media containing Geneticin (G418; at 2400 μg/mL for both cell lineages; Invitrogen) to select for cells that were stably transfected with the pcDNA3-based plasmids. The stably transfected cells then were then maintained in culture using media containing G418 (1200 μg/mL).

siRNA transfection of cell lines

On-Target Plus Smart pool RET siRNA (L-003170-00-0005) or siGENOME non-targeting control siRNA #3 (a negative control siRNA with at least four mismatches to any human, rat, or mouse gene; both from Thermo Scientific-Dharmacon, Waltham, MA) were prepared for transfection according to the manufacturer's instructions. Transfection of HCT116 or MCF7 cells was performed using DharmaFECT Set of 4 siRNA Transfection Reagents (Fisher) following the manufacturer's protocol. For the HCT116 cells, 3×10^5 cells were seeded in each well of a 24-well plate and 2 μL of transfection reagent #1 was used for each transfection. For the MCF7 cells, 5×10^4 cells were seeded in each well of a 24-well plate and 1 μL of transfection reagent #1 was used for each transfection. Five hundred μL of either siRNA transfection mix was added to both HCT116 and MCF7 cells seeded in wells of a 24-well plate. The cells were incubated for 48 hours at 37°C in 5% CO_2 prior to being harvested for further assays.

Cell Death Analysis and Caspase Activity Measurement

Cell death was analyzed using the Cell Death Detection ELISA[PLUS] (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, 48 hours after transfection or 24 hours after treatment with GDNF or a selective ERK inhibitor, the cells were lysed, and then the lysates were analyzed.

Caspase-3 and caspase-7 activity were measured by using the Caspase-Glo® 3/7 Assay (Promega) following the manufacturer's protocol. Relative caspase activation was calculated as the ratio between the caspase activity of the transfected cells and that measured in nontransfected cells or cells transfected with a control vector. Serum-free starvation or Bax-induced apoptosis was also used as a positive control for the apoptosis studies. These studies were carried out in cell lines both transiently and stably transfected with *RET*.

Treatment with 5-aza-2'-deoxycitidine

The cell lines were grown for 48 hours before treatment with 5-aza-2'-deoxycytidine (5-aza-2-dC) (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in DMSO. The cells were treated with 5-aza-2-dC (1 μM final concentration) for 48 hours. The cells were then incubated for an additional 24 hours before harvesting. DNA was extracted and analyzed by bisulfite treatment followed by MSP using *RET* primers as described above. RNA was isolated from identically treated cultures for qRT-PCR analysis. The control samples were treated with the DMSO vehicle only.

Western blotting

We used rabbit anti-RET monoclonal antibody (3223, Cell Signaling Technology) to assess the RET protein expression levels and used anti-phospho-RET (Tyr905, 3221, Cell Signaling Technology) antibody to detect endogenous levels of phosphorylated RET at tyrosine 905. For assessing signaling pathway activation after RET51-transecfection, phospho-Akt (S473), phospho-NF-κB (S536) and phospho-Erk (Thr202/Tyr204) levels in SW48 (methylated RET) and SW80 (unmethylated RET) cells were measured by Western blot using anti-phospho-Akt (Ser473, Cell Signaling Technology), anti-phospho-NF-κB

(Ser536, Cell Signaling Technology) and anti- phospho-Erk1/2 (Thr202/Tyr204, Cell Signaling Technology) after 24 hours of culture in serum-free media following the transfection or GDNF stimulation of the cell lines as described above, or after treatment with a selective pathway inhibitor.

Immunohistolochemistry assay

Formalin-fixed, paraffin-embedded sections of adenocarcinomas, adenomas, and matched normal colonic mucosa tissues were subjected to immunostaining using a rabbit anti-human RET monoclonal antibody (HPA008356, Sigma-Aldrich) or a rabbit anti-human GDNF polyclonal antibody (SC-328, Santa cruz). Briefly, tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval by boiling in sodium citrate buffer (10 mmol/L, pH 6.0). The sections were then incubated for 30 minutes with the RET (1:250) antibody at room temperature or for 60 minutes with the GDNF primary antibody (1:250) at room temperature, washed incubated with an anti-rabbit IgG secondary antibody and then strepavidin-biotin complex and then stained with 3,3-diaminobenzidine. After visualization of immunoreactivity, the sections were counterstained with hematoxylin and mounted. The stains were scored according to following criteria: (a) percentage of immunoreactive cells, 1 = 0–30%, 2 = >30%–70%, 3 = >70%; and (b) staining intensity, 1 = weak, 2 = moderate and 3 = strong. The final score generated used to score the slide was (a) × (b).

Soft agar colony formation assay

Six thousand cells were seeded into a 6 cm dish containing soft agar media (DMEM + 10%FBS+1200 μg/mL G418) as previously described (57). The Cells were incubated at 37°C for 7~14 days at which time the colonies were manually counted. All experiments were conducted in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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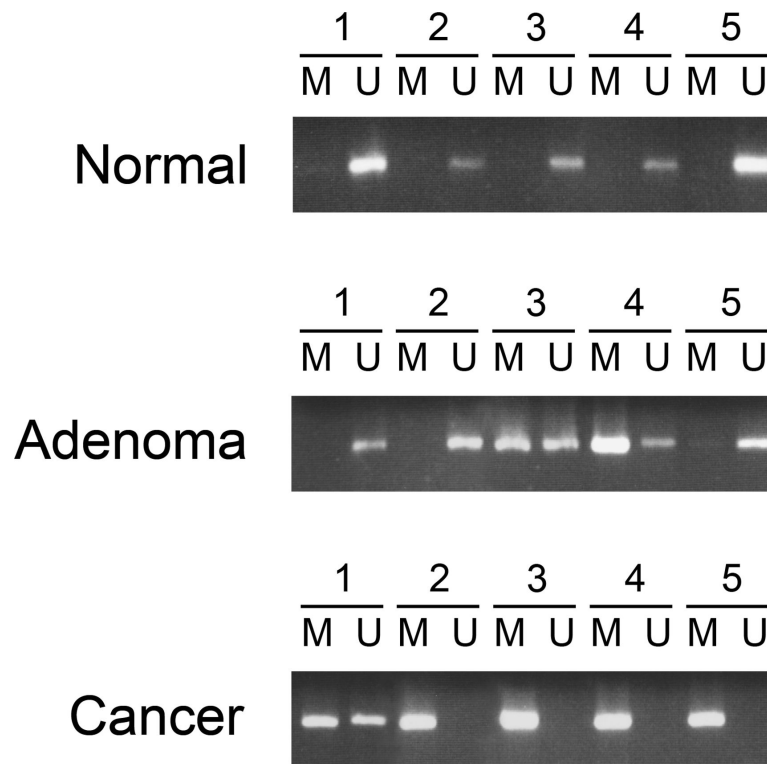
Figure 1.
Assessment of *RET* methylation status in representative normal colon, colorectal adenomas, and colorectal cancers. End-point *RET* MSP results from representative cases are shown. M=methylated, U=unmethylated. The case numbers are designated across the top of each gel photo.
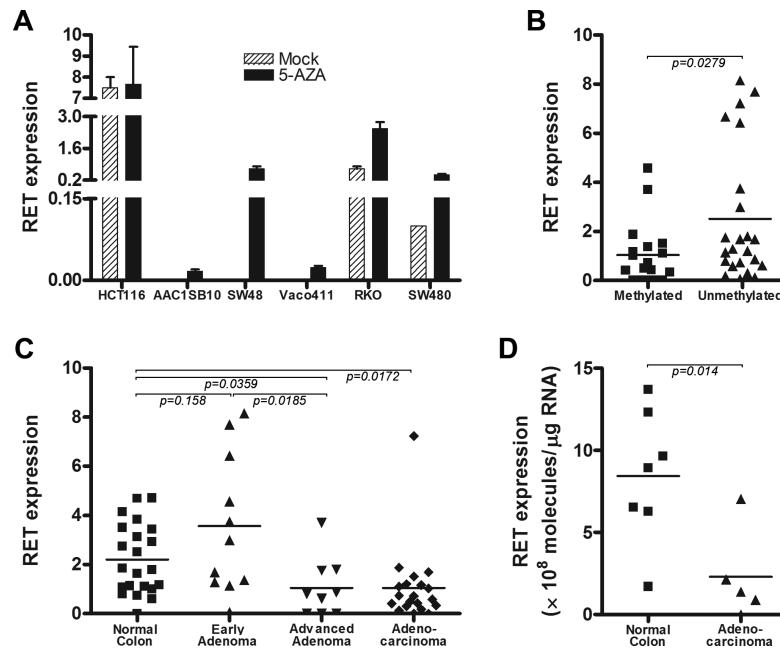
Figure 2.
A. *RET* expression after treatment with 5-aza-2'-deoxycytidine (5-AZA). Colon cancer cell lines that carry methylated *RET* (SW48, RKO, AAC1/SB10), methylated and unmethylated *RET* (HCT116), and unmethylated *RET* (V411 and SW480) were treated with 5-AZA or vehicle alone and then assessed for *RET* expression using qRT-PCR. *RET* is minimally expressed at baseline in all the cell lines except HCT116. The expression of *RET* is increased >5X after 5-AZA treatment in the SW48 and RKO cell lines. **B.** *RET* mRNA expression levels were significantly lower in colorectal tumors (either cancer or adenoma) with methylated *RET* as compared to those carried unmethylated *RET* (mean expression: 1.04 ± 0.30 and 2.51 ± 0.56, respectively, $P = 0.0279$, 2-sided student t test). **C.** Expression of *RET* mRNA in primary normal colon mucosa, colon adenomas, and colon adenocarcinomas. When compared to normal colon mucosa, *RET* is significantly reduced in the colon cancers but not in the colon adenomas (p= 0.0057, ANOVA). **D.** Absolute quantitative measurement of *RET* mRNA in primary normal colon mucosa and adenocarcinomas. Consistent with the relative mRNA expreesion levels measured in 2C, *RET* is more highly expressed in normal colonic epithelia cells as compared to the adenocarcinomas. ($P = 0.014$, 2-sided student t test).
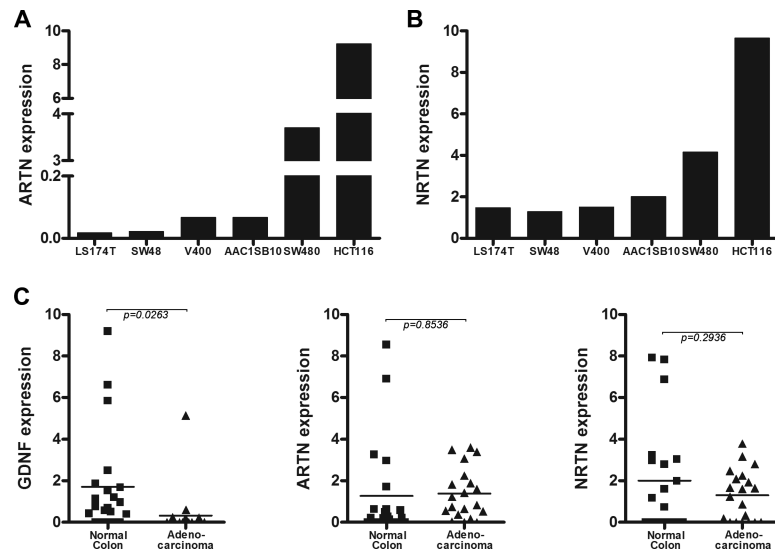
Figure 3.
Expression of RET ligands in colorectal cancer cell lines and primary tissues. GDNF was not expressed in any of the colorectal cancer cell lines. (This data is shown in Supplemental Data, **Figure S2**.) **A**. Expression of *ARTN* in colon cancer cell lines. **B.** Expression of *NRTN* in colon cancer cell lines. The expression of these ligands is highly variable between cell lines with the highest expression being present in the cell lines HCT116 and SW480, which express RET. **C.** Expression of the GDNF ligands in normal colon mucosa and colon neoplasms. GDNF is expressed at higher levels in the normal colon compared to colon cancer ($P = 0.0263$), which is in contrast to ARTN, which is expressed at the same level or higher in colorectal cancers ($P = 0.854$), and NRTN, which is expressed at the same level in normal colon and colorectal cancers ($P = 0.2936$). All expression levels were determined using qRT-PCR. The units are relative units calculated after normalization with GUSB, which is a loading control. (A 2-sided student t test was used for the statistical analysis of these results.).
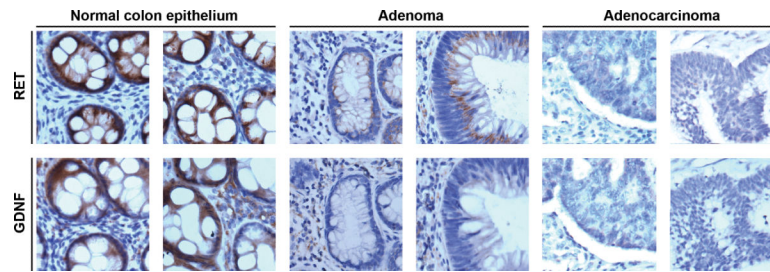
Figure 4.
Expression of RET and GDNF in normal colon mucosa, adenomas and adenocarcinomas, assessed by immunohistochemistry. Representative cases assessed for both RET and GDNF expression are shown in the upper panel and lower panels separately. RET and GDNF are expressed in the normal colon epithelium cells, however, decreased or absent RET and no GDNF is detectable in the adenocarcinomas. The representative adenocarcinoma cases shown all carry methylated *RET*. Each image is from a different tissue sample (200X).
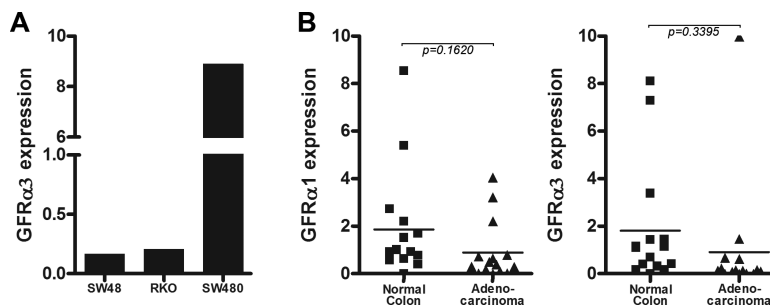
Figure 5.
Expression of GFRα1 and GFRα3 mRNA in (A) colorectal cancer cell lines and (B) primary normal colon mucosa and colon adenocarcinomas. **A**. GFRα3 is highly expressed in SW480, which also expresses RET, compared to SW48 and RKO, which do not express RET. None of the cell lines expressed GFRα1. **B**. Expression levels were determined by quantitative RT-PCR and show decreased GFRα1 and GFRα3 expression in colorectal cancers compared to normal colon mucosa, although the difference is not statistically significant between normal colon and colorectal cancer ($P = 0.1620$ for GFRα1 and $P = 0.340$ for GFRα3, 2-sided student t test). The units are relative units calculated after normalization with *GUSB* expression, which is a loading control.
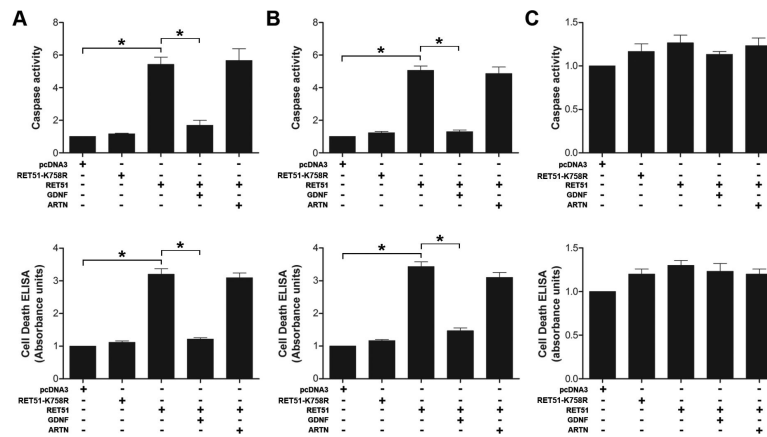
Figure 6.
Assessment of apoptosis after reconstitution with RET in SW48, (A) RKO (B), and SW480 (C). RET induces caspase activity in SW48 and RKO, and GDNF inhibits this effect in both cell lines. Both SW48 and RKO carry methylated *RET*. Apoptosis was assessed in the cell lines 48 hours after transfection with *RET51*. GDNF (100 ng/mL), but not ARTN (100 ng/mL) decreased the amount of apoptosis in SW48 (**A**) and RKO (**B**). **C.** *RET51* transfection did not induce apoptosis in SW480, which carries unmethylated *RET*. All these experiments were performed in triplicate and carried out using the Caspase-Glo 3/7 assay (Promega; top panel) or Cell Death Detection ELISA assay (Roche; bottom panel). The Caspase activity and ELISA assay results are shown as fold changes compared to the vector only group. pcDNA3 was used as the control vector to normalize for nonspecific effects of the transfection on apoptosis. *RET51-K758R*, which lacks kinase activity, was also used as a second control for these studies. The asterisks indicate statistically significant differences, *P* < 0.05 as determined by a 2-sided Mann-Whitney rank sum test.
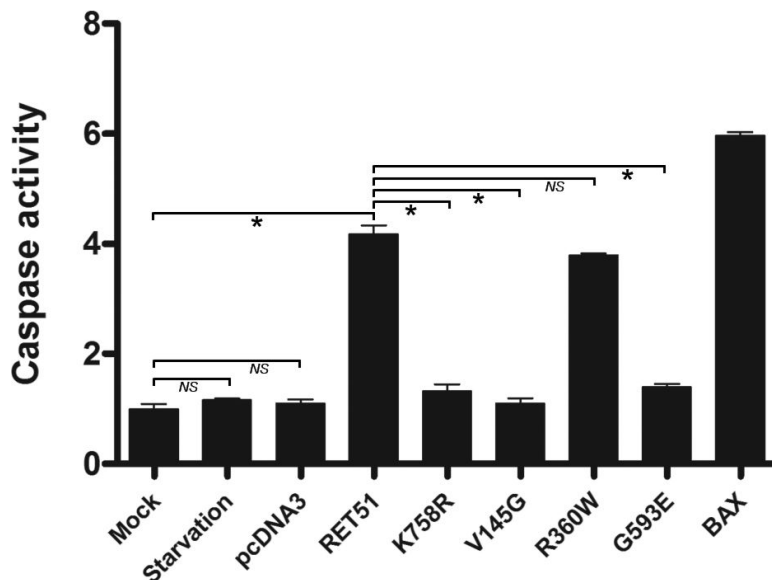
Figure 7.
Assessment of apoptosis after reconstitution with human somatic mutated *RET* in SW48. Apoptosis was assessed in the SW48 cells 48 hours after transfection with parental *RET51*, *RET51-V145G* (V145G), *RET51-R360W* (R360W) or *RET51-G593E* (G593E). The last 3 mutations were found in human colon cancer samples in previous studies (10). V145G and G593E did not induce apoptosis after transduction, which was consistently caused by the reintroduction of *RET51* expression. pcDNA3 was used as the control vector to normalize for nonspecific effects of the transfection on apoptosis. Bax was used as a positive control for apoptosis assays. *RET51-K758R* (K758R), which lacks kinase activity, was also used as a control for these studies. The asterisks indicate statistically significant differences, *P* < 0.05 as determined by a 2-sided Mann-Whitney rank sum test.
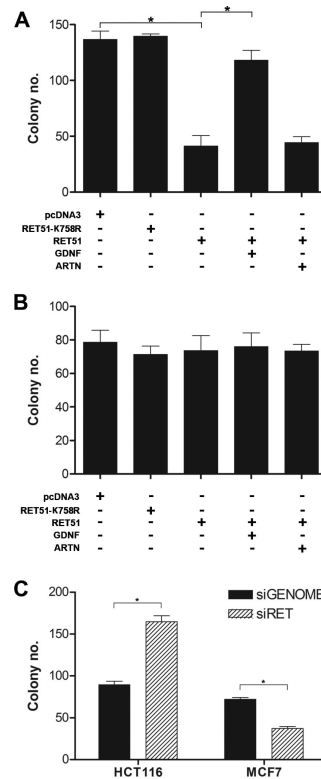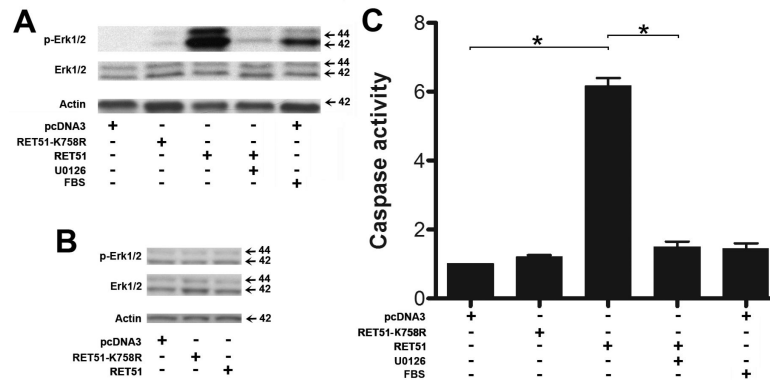
Figure 8.

Soft agar colony formation with RKO (**A**; carries methylated *RET*) and SW480 (**B**; carries unmethylated *RET*) cells after transfection with pcDNA3, *RET K758R* or *RET51*. RET inhibits colony formation in RKO but not SW480. Treatment of the *RET* transduced RKO with GDNF (100ng/ml) but not ARTN (100ng/ml) blocks the effect of RET on colony formation. (**C**) Knock-down of RET expression by RET-targeted siRNA promotes the colony formation ability of HCT116 cells, whereas, it suppresses the colony formation ability of the breast cancer cell line MCF7, which is consistent with *RET* acting as a tumor suppressor gene in colorectal cancer but as an oncogene in other types of cancer. Results are shown as the mean colony numbers from three independent experiments. The asterisks indicate statistically significant differences, $P < 0.05$ as determined by 2-sided student t test.

Figure 9.
Western blot analysis of phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 in colorectal cancer cells after transfection with *RET51* and the control vectors pcDNA 3.0 and pcDNA3-*RET K758R*, which is an inactive form of RET. **A**. *RET51* transfection induces the phosphorylation of ERK in cell line SW48, which carries methylated *RET*. RET51-induced phosphorylation of ERK is suppressed in cells treated with U0126 (10 μM). **B**. *RET51* transfection does not affect ERK phophorylation in SW480, which carries unmethylated *RET* and expresses RET. The empty vector pcDNA3 and a vector containing RET51-K758R, which is a kinase-inactive receptor, do not affect ERK phosphorylation. Protein loading was normalized using actin. Each experiment was done in triplicate and a representative immunoblot is shown. **C.** Inhibition of MAPK activity after *RET* reconstitution in SW48 decreases *RET* induced apoptosis. Cell death induction in the CRC cell line SW48 was quantified 48 hours after transfection with *RET51* using the Caspase-Glo 3/7 assay. The selective MAPK/ERK pathway inhibitor, U0126 (10 μM), significantly decreases apoptosis in the SW48 cell line transfected with *RET51*. 16% FBS was used to stimulate the cells before protein harvest for 30 minutes as an extracellular stimuli control for the activation of Erk1/2. All experiments were performed triplicate, and the results shown are fold changes compared to the empty vector control. The asterisks indicate statistically significant differences, $P < 0.05$ as determined by a 2-sided Mann-Whitney rank sum test.