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TGF- β receptor inactivation and mutant *Kras* induce intestinal neoplasms in mice via a β -catenin independent pathway

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Abstract

Background & Aims—During colorectal cancer pathogenesis, mutations and epigenetic events cause neoplastic behavior in epithelial cells by deregulating the Wnt, Ras–Raf–ERK, and transforming growth factor (TGF)- β signaling pathways, among others. The TGF- β signaling pathway is often inactivated in colon cancer cells by mutations in the gene encoding the TGF- β receptor *TGFBR2*. The Ras–Raf–ERK pathway is frequently upregulated in colon cancer via mutational activation of *KRAS* or *BRAF*. We assessed how these pathways interact *in vivo* and affect formation of colorectal tumors.

Methods—We analyzed intestinal tumors that arose in mice that express an oncogenic (active) form of *Kras* and that have *Tgfr2* mutations—2 common genetic events observed in human colorectal tumors. *LSL-KrasG12D* mice were crossed with mice with *Villin-Cre;Tgfr2E2flx/E2flx* mice, which do not express *Tgfr2* in the intestinal epithelium.

Results—Neither inactivation of *Tgfr2* nor expression of oncogenic *Kras* alone was sufficient to induce formation of intestinal neoplasms. Histologic abnormalities arose in mice that expressed *Kras*, but only the combination of *Tgfr2* inactivation and *Kras* activation led to intestinal neoplasms and metastases. The cancers arose via a β -catenin-independent mechanism; the epidermal growth

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factor signaling pathway was also activated. Cells in the resulting tumors proliferated at higher rates, expressed decreased levels of p15, and expressed increased levels of cyclin D1 and cdk4, compared to control cells.

Conclusions—A combination of inactivation of the TGF- β signaling pathway and expression of oncogenic *Kras* leads to formation of invasive intestinal neoplasms through a β -catenin-independent pathway; these adenocarcinomas have the capacity to metastasize.

Keywords

colon cancer; transforming growth factor beta; KRAS; transformation; epidermal growth factor

Introduction

Colon cancer develops through a histologic progression sequence, called the polyp→cancer sequence, as the result of the accumulation of genetic and epigenetic alterations in intestinal epithelial cells. The classic model of colon cancer formation is that the initiation of colon adenomas results from activation of the Wnt signaling pathway, secondary to mutations in genes such as *APC* ^{1, 2}. The progression of the adenomas then results from activation of the MAPK and/or PI3K signaling pathways through oncogenic mutations in *KRAS*, *PIK3CA*, etc. ^{2, 3}. The next step of malignant transformation of the adenomas to cancer is accompanied by mutations in genes such as *TP53* or *TGFBR2* ^{4, 5}. Notably, the effects of mutations of some of these genes, such as *KRAS* and *TP53*, appear to be productive for cancer formation only in the context of preceding mutations ^{6, 7}. This context dependence of the mutations on cancer formation also appears to be true of the TGF- β signaling pathway and has important implications regarding our understanding of how *TGFBR2* mutations affect cancer cells ^{5, 8, 9}.

The importance of TGF- β signaling inactivation in colon cancer is highlighted by the high frequency of resistance to transforming growth factor β (TGF- β), a multifunctional cytokine that can act as a tumor suppressor, that is observed in colon cancer ¹⁰. TGF- β mediates its effects on cells through a cell surface receptor that consists of two obligate serine-threonine kinase components, TGF- β receptor type I (TGFBR1) and type II (TGFBR2). In colon cancer, mutation of *TGFBR2* is a common mechanism for inactivating the TGF- β signaling pathway ¹⁰. The mutational inactivation of *TGFBR2* results in deregulation of a multitude of cellular processes that may effect tumorigenesis including: 1) proliferation and differentiation, 2) apoptosis, 3) angiogenesis, 4) extracellular matrix remodeling, 5) chromosomal stability, 6) local immune cell responses, and 7) senescence ^{2, 11}. A major question that remains to be answered is what mechanism(s) dictates which of this myriad of TGF- β regulated processes are important in the pathogenesis of cancers that acquire TGF- β resistance.

In addition, the TGF- β signaling pathway has been shown to interact with the key signaling pathways that are often deregulated in colon cancer, the Wnt- β -catenin, Ras-Raf, and PI3K pathways, and the interaction of these pathways may be a major factor that determines the biological consequences of TGF- β signaling inactivation in the cancer cells. However, the effect of TGF- β signaling loss in the context of mutations of *KRAS*, *APC*, and *PIK3CA* is largely unknown ^{12, 13}. We have previously demonstrated an *in vivo* interaction between mutant *Apc* and *Tgfbr2* loss on the malignant transformation of intestinal adenomas ⁵. We have now generated an *in vivo* model to assess the interaction between TGF- β signaling inactivation and oncogenic *Kras* in intestinal cancer formation. We have observed that tumors arise in these mice in a β -catenin independent fashion and that activation of the EGF signaling pathway may contribute to this process. Furthermore, deregulation of cell proliferation appears to be a

prominent biological event that affects tumorigenesis in this model and is associated with increased expression of cdk4 and cyclin D1 and with decreased expression of p15.

Materials and Methods

Generation and characterization of *Villin-Cre;Tgfr2^{E2flx/E2flx}* (called *Tgfr2^{IEKO}*), *LSL-Kras^{G12D/wt}* and *Apc^{1638N/wt}* mice

The generation of the following genetically engineered mice has been previously described: *Villin-Cre;Tgfr2^{E2flx/E2flx}* (*Tgfr2^{IEKO}*, also termed VcTT), *Villin-Cre;Tgfr2^{E2flx/E2flx};Apc^{1638N/wt}* (*Tgfr2^{IEKO};Apc^{1638N/wt}*, also termed AVcTT), and *LSL-Kras^{G12D}* 5, 14–16. These mice were mated to generate the following compound genotypes: *Tgfr2^{IEKO};LSL-Kras^{G12D/wt}* (also termed KVcTT), *LSL-Kras^{G12D/wt};Villin-Cre;Tgfr2^{wt/wt}* (also termed KVcT^{wt/wt}) *LSL-Kras^{G12D/wt};Tgfr2^{E2flx/E2flx}* (also termed KTT) and were fed *ad libitum* with a standard rodent diet. Prior to generating the mice with the compound genotypes, the mice were backcrossed onto mice that were 100% C57Bl6 for three generations to obtain mice that are >90% C57Bl6 on average. Animals were monitored daily and sacrificed upon signs of distress. The mice were genotyped using published protocols⁵. Mice with the genotype *Tgfr2^{IEKO};LSL-Kras^{G12D/wt}* were harvested at an average of 20 weeks of age along with their age matched controls to evaluate the intestines and assess for neoplasms. Handling of the *Apc^{1638N/wt};Tgfr2^{IEKO}* mice has been described⁵. The studies were all approved by the local IACUC.

Tissue processing

Animal handling, tissue handling, and fixation of tissues was performed using previously described protocols^{5, 8}.

Cell lines

The following human colorectal cancer cell lines were grown in 10% FBS/DMEM: SW480 (mutant *KRAS^{G12V}* and TGF- β resistant), HKe-3, and HCT-116 (mutant *TGFBR2*, and mutant *KRAS^{G13D}*)¹⁷. HKe-3 is a derivative cell line from HCT116 that carries one wild-type *KRAS* allele only¹⁸.

For the studies with the EGFR and TGF- β receptor inhibitors, the cells were plated at approximately 70–80% confluence and grown in DMEM + 1% FBS overnight. The cells were then treated with either the EGFR inhibitor (10 μ M) (AG1478; Calbiochem; #658552) or TGF- β RI Inhibitor III (300nM) (616453; Calbiochem), or DMSO alone.

Western blotting

The cell lines and tissues were lysed using sonication and RIPA lysis buffer supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO). The intestinal mucosa was obtained by scraping PBS rinsed intestines gently with a glass slide. Total protein content was determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Immunoblotting was carried out using 20 μ g of tissue protein or 30 μ g of cell line protein with antibodies listed in Supplemental Table 1. The secondary antibodies used were goat anti-rabbit IgG HRP and donkey anti-goat IgG HRP (Santa Cruz). Luminol (Sigma) chemiluminescence and autoradiography were used for detection of the secondary antibody.

Gene Transfection

Cell lines were transfected with the *TGFBR2* vectors as previously described¹⁰.

Quantitative reverse transcription-PCR

TaqMan gene expression assays (Assays-on-Demand; Applied Biosystems) were used for *Areg*, *Ereg*, *EREG*, *ErbB1*, *ErbB3*, *ErbB4* and 18s RNA (Mm00483241_m1; Mm00437583_m1; Mm00514794_m1; Hs00154995_m1; Mm01187872_m1; Mm01159999_m1; Mm01256806_m1 respectively) as previously published ⁵.

Immunohistochemistry staining for cleaved caspase 3, Ki67, cytokeratin 19, β -catenin, cyclin D1 and p53 and special staining

The immunostaining expression was evaluated and scored as previously described ^{5, 8}. Please see supplemental data for details.

Periodic Acid Schiff (newcomers) staining (PAS) and Alcian blue staining was performed in the Experimental Histopathology Core at the Fred Hutchinson Cancer Research Center (Seattle, WA) following routine lab protocol, which is available upon request.

Results

The activation of *Kras* results in increased proliferation in the intestinal epithelium but no malignancies

In order to assess the *in vivo* effects of TGF- β signaling and KRAS-RAF-MAPK signaling deregulation on the intestinal epithelium, mice that carried the Villin-cre transgene were bred with mice carrying *LSL-Kras*^{G12D} or *Tgfr2*^{E2flx} to yield the following mice: *LSL-Kras*^{G12D/wt}; *Villin-Cre*; *Tgfr2*^{wt/wt} (KVcT^{wt/wt}), *LSL-Kras*^{G12D/wt}; *Villin-Cre*; *Tgfr2*^{E2flx/E2flx} (KVcTT), and *Tgfr2*^{flx/flx}; *LSL-Kras*^{G12D/wt} (KTT). The KVcTT mice display an increase in the colonic crypt length and an expansion of the proliferative compartment in the crypt compared to the KTT mice (Figure 1). An increase in the crypt length and Ki67 labeling index is present in the mice carrying the activated *LSL-Kras*^{G12D} allele. The crypt length and labeling indices in the KTT, KVcT^{wt/wt}, and KVcTT mice were 24 $\pm$ 2 cells vs. 41 $\pm$ 2 cells vs. 36 $\pm$ 2 cells ($p=4.35\times10^{-5}$ KTT vs. KVcT^{wt/wt}; $p=0.0001$ KTT vs. KVcTT, student's t-test) and 41 $\pm$ 6% vs. 65 $\pm$ 6% vs. 60 $\pm$ 3%, respectively ($p=0.005$ KTT vs. KVcT^{wt/wt}, $p=0.004$ KTT vs. KVcTT, student's T-test). We also observed a branching morphology of the crypts and an alteration in the cytodifferentiation of the intestinal epithelium in the KVcTT and KVcT^{wt/wt} mice with an increase in the proportion of mucin producing cells in the crypts. The branching morphology is most pronounced in the KVcT^{wt/wt} mice and is attenuated in the KVcTT mice for unclear reasons. PAS and Alcian blue special stains highlight the increased proportion of mucin producing cells in the intestinal epithelium, which can be seen with the PAS stains and indicates these cells are making neutral and not acidic mucopolysaccharides. (Supplemental Figure 1). Assessment for paneth cells with lysozyme staining and for neuroendocrine cells with chromogranin A staining revealed a near absence of paneth cells in the small intestine and colon of the KVcTT mice and decreased paneth cells in the small intestine and colon of the KVcT^{wt/wt} mice compared to the KTT mice. There was no difference in neuroendocrine cells between the different genotypes of mice. (Supplemental Figure 2).

Tgfr2 inactivation cooperates with *Kras* activation to cause small intestinal and colon neoplasms that arise via a β -catenin independent mechanism

In prior studies, we observed mice that lack TGFBR2 in their intestinal epithelium rarely develop tumors when observed up to 52 weeks of age ⁵. Now, we also have found mice that express the oncogenic *Kras*^{G12D} allele (*Villin-Cre*; *LSL-Kras*^{G12D}; *Tgfr2*^{wt/wt}) do not develop tumors. (Table 1) In contrast, mice that carry both activated *Kras* and lack Tgfr2 (KVcTT) commonly developed both small intestinal and colonic neoplasms by 22 weeks of age.

Approximately 70% of the KVcTT mice developed intestinal tumors by this age. (Table 1, Figure 2) In contrast, none of the KVcT^{wt/wt} or KTT mice developed tumors by 22 weeks of age. The majority of the tumors in the KVcTT mice were adenocarcinomas, and there was no difference in the proportion of adenomas to adenocarcinomas between the small intestine and colon. As neither *Kras* mutation nor *Tgfr2* deletion appear to be sufficient to initiate intestinal tumor formation, we assessed the activation state of the Wnt signaling pathway in the tumors to determine if this pathway was being activated and initiating these tumors. In the normal intestinal epithelium, β -catenin appropriately localizes to the cytoplasmic membrane. Assessment of β -catenin in the tumors revealed nuclear β -catenin in only 8% of the tumors (N=13) suggesting that the majority of the tumors arose via a β -catenin/Wnt independent mechanism. In contrast, analysis of intestinal tumors arising in a *Apc*^{1638N/wt};*Tgfr2*^{IEKO} mouse model generated previously revealed 80% of the tumors (N=10) display nuclear or cytoplasmic localization of β -catenin (data not shown)⁵. (Figure 2). Thus, although Wnt activation can be found in some tumors arising in the KVcTT mice, the majority of tumors arise via a β -catenin independent process. We also assessed the status of another canonical gene in colon cancer formation, *Trp53*. Immunostaining for p53 in the normal epithelium revealed few p53 positive cells as would be expected based on known expression patterns in wild type mice and also revealed increased p53 expression (>20% of nuclei immunoreactive) in 60% of the tumors (N=10) suggesting that p53 deregulation is common in these tumors, in contrast to the infrequent Wnt pathway activation observed. (Figure 2) Also, of note, COX2 is overexpressed in 63% of tumors (N=7/11) arising in the KVcTT mice and PGE2 is significantly higher in the tumors compared to the normal intestinal epithelium, despite the lack of nuclear β -catenin (Supplemental Figure 3).

Erk and Akt phosphorylation in the intestinal epithelium and intestinal tumors in the LSL-*Kras*^{G12D};*Tgfr2*^{IEKO} (KVcTT) mouse

ERK phosphorylation is present in the intestinal epithelium of mice carrying the expressed activated *Kras*^{G12D} allele regardless of TGFBR2 status and is minimally present in mice with intact *Tgfr2* and wild-type *Kras* alleles (KTT). (Figure 3) ERK phosphorylation is also present in >80% (N=5) of the neoplasms in the LSL-*Kras*^{G12D};*Tgfr2*^{IEKO} mice compared to <50% of the tumors arising in the *Apc*^{1638N/wt};*Tgfr2*^{IEKO} mice (N=4). Activation of the PI3K-AKT pathway is also common in the intestinal epithelium of all the genotypes of mice regardless of the *Kras* or *Tgfr2* status of the mice. Tumors arising in the KVcTT, AVcTT, and ATT mice display evidence of PI3K pathway activation, with the lowest proportion of tumors showing PI3K-AKT activation being those from the KVcTT mice (40% (N=5) vs. 100% of the AVcTT (N=5) or ATT (N=4) tumors). These results suggest that activation of the MAPK-ERK pathway, but not the AKT pathway is a consequence of the cooperation of mutant *Kras* with loss of *Tgfr2* (Figure 3) In contrast, no increased p70s6 kinase activity (by measurement of phosphorylation of p70S6kinaseThr389) is present in the intestinal epithelium and neoplasms arising in the KVcTT mice (Supplemental Figure 4)

Tgfr2 inactivation and Kras activation cooperate in tumor formation through the deregulation of proliferation but not through effects on apoptosis or genomic stability

The formation of intestinal neoplasms in the KVcTT mice demonstrates that the deregulation of these two pathways can cooperate in vivo to promote cancer formation. However, as the entire intestinal epithelium lacks *Tgfr2* and expresses activated *Kras*^{G12D}, it is also clear that additional somatic events are needed for tumor formation to occur. Thus, we assessed several hallmark behaviors implicated in cancer formation to gain insight into the somatic processes occurring that lead to tumor formation in the KVcTT mice¹⁹. Ki67 labeling is higher in the tumors arising in the KVcTT mouse model than in the *Villin-Cre*;*Apc*^{1638N/wt};*Tgfr2*^{E2flx/E2flx} (AVcTT) or *Apc*^{1638N/wt};*Tgfr2*^{E2flx/E2flx} (ATT) mice (47% +/-7% vs. 19% +/-4% vs. 25% +/-4%; p=0.0003 and 0.006 comparing KVcTT vs. ATT

and KVcTT vs. AVcTT, respectively.)⁵. (Figure 4) Notably, there was no evidence of attenuated apoptosis in the tumors as measured by immunostaining for cleaved caspase 3 (data not shown) or in senescence as assessed by the expression of DEC1 and DcR2 in the tumors arising in the KVcTT mice²⁰. (Supplemental Figure 5) Likewise, array CGH results on four tumors from independent mice did not support the hypothesis that genomic instability is a contributing mechanism in the pathogenesis of these tumors. Loss of TGF- β signaling in keratinocytes has been shown to result in aneuploidy; however, we did not observe any aneuploidy in the adenocarcinomas arising in the KVcTT mice^{21, 22}. There was also no evidence for segmental chromosomal gains or losses in these tumors, other than small, intragenic copy number changes of unclear significance (data not shown)²³.

Increased proliferation in the adenocarcinomas correlates with suppressed p15 expression and increased cyclin D1 and cdk4 expression

Assessment of cyclins, cyclin-dependent kinases and cdk inhibitors known to be regulated by TGF- β revealed that p15 expression is decreased in the tumors arising in the KVcTT mice and not in the AVcTT or ATT mice. (Figure 5) Notably, the expression of p21 is substantially elevated in the AVcTT and ATT mouse tumors, which is not observed in the tumors in the KVcTT mice and which may explain the difference in proliferation between the tumors in the KVcTT mice compared to the AVcTT mice (Figure 5) Cdk4 and cyclin D1 expression is increased in the tumors arising in the KVcTT, AVcTT, and ATT mouse models when compared to normal colonic mucosa (Figure 5, Supplemental Fig 6). The decreased expression of p15 is a predicted consequence of loss of TGF- β signaling and is seen in the model of intestinal cancer formation driven by oncogenic *Kras* and TGF- β signaling inactivation but not in the *Apc*^{T638N/wt}; *Villin-Cre*; *Tgfb2*^{flx/flx} model of intestinal cancer²⁴.

Oncogenic *Kras*^{G12D} and TGF- β signaling inactivation results in increased epiregulin expression and activates EGFR signaling

We next assessed for possible paracrine or autocrine events that would promote both the increased proliferation and activation of the PI3K-AKT pathway observed in the KVcTT mice. Expression of the EGF ligands that are overexpressed in human colorectal cancer, epiregulin (*Ereg*), and amphiregulin (*Areg*), was assessed^{25, 26}. Increased mRNA levels of *Ereg* (Figure 6A) but not *Areg* (data not shown) are observed in the tumors compared to the normal mucosa ($p < 0.0001$, Mann Whitney test). With regards to the EGF receptors, expression of *ErbB1* is increased in the normal mucosa and tumors in the KVcTT mice when compared to the VcTT and KVcT^{wt/wt} mice (*, $p < 0.05$; ** $p < 0.01$) (A trend towards increased expression of *ErbB1* in the normal mucosa and tumors of the KVcTT mice compared to the KTT mouse normal mucosa is also present but is not statistically significant.) (Figure 6B) Conversely, low-level expression of *ErbB3* and no expression of *ErbB4* was detected in the mucosa or tumors of any of the models (data not shown). In order to determine whether the cooperation between activated *Kras*^{G12D} and inactivated TGF- β signaling is the mechanism causing increased epiregulin expression, we assessed the expression of *EREG* in the HCT116 microsatellite unstable colon cancer cell line and derivative line HKe-3, which lacks mutant *KRAS*18. The HCT116 and HKe-3 lines both lack a functional TGFBR2 and thus were reconstituted with TGFBR2 using the *TGFBR2* 2+8 transgene, which is stable in the setting of microsatellite instability²⁷. (Figure 6C) Epiregulin expression is highest in HCT116, which has a mutant *KRAS* allele and no functional TGFBR2, and decreased after the reconstitution of *TGFBR2* or deletion of mutant *KRAS*. The cooperation between TGF- β and *KRAS* to induce *EREG* expression was also observed in the colorectal cancer cell line SW480 (intact TGFBR2 and mutant *KRAS*) in which treatment with a TGFBR1 inhibitor results in a modest increase in *EREG* expression. (Figure 6D)

Effect of activated *Kras* and TGF- β signaling loss on the metastatic behavior of intestinal tumors

In addition to having primary intestinal neoplasms in the small intestine and colon, approximately 15% of the mice (N=20) developed grossly obvious metastatic lesions in the regional lymph nodes or lungs. Assessment of recombined *Tgfb2* and cytokeratin 19 confirmed that these tumors were derived from intestinal epithelium. (Figure 7) Thus, it appears that loss of TGF- β signaling and oncogenic *Kras* cooperate to create a permissive state for metastatic behavior. It is also apparent that the deregulation of both pathways is not sufficient to induce tumor metastases given the low incidence of metastatic tumor formation. Additional somatic events are likely needed in order for the primary tumor to metastasize in this model system.

Discussion

We have demonstrated in an *in vivo* model that mutant *Kras* and TGF- β receptor inactivation can cooperate to induce intestinal adenocarcinomas. The tumors that arise in this model system display substantial desmoplasia and mucinous changes and have the capacity to metastasize. The pathogenesis of these tumors is independent of the Wnt signaling pathway and is associated with activation of the EGF signaling pathway presumably by autocrine epiregulin expression. When the hallmark behaviors of cancer are considered, increased proliferation and deregulation of the G1-S cell cycle checkpoint appear to be the predominant biological effects that may drive tumor formation in the setting of oncogenic *Kras* and inactivated TGFBR2¹⁹. However, it is also clear that increased proliferation alone is not sufficient to cause tumor formation in light of the increased proliferation seen in the normal mucosa of the KVC^{T^{wt/wt}} and KVC^{TT} mice and prior studies that have demonstrated increased intestinal epithelial cell proliferation secondary to expression of *Kras*^{G12D} with no occurrence of intestinal adenocarcinomas^{6, 28, 29}. Thus, the occurrence of tumors in the KVC^{TT} mice may be a consequence of increased proliferation in combination with decreased expression of the cdk inhibitor p15 and activated cdk4, which are known to regulate apoptosis, senescence, and cell growth control as well as proliferation^{30, 31}.

Our results from the *Villin-Cre;Tgfb2^{E2flx/E2flx} (Tgfb2^{IEKO})* mice and the *Villin-Cre;LSL-Kras^{G12D}* mice demonstrate that *Tgfb2* null intestinal epithelium *in vivo* is not highly susceptible to spontaneous tumor formation nor is epithelium that carries an oncogenic mutant *Kras* expressed at endogenous levels, which is consistent with prior studies^{6, 7}. Thus, neither activation of *Kras* and the MAPK-ERK pathway nor inactivation of the TGF- β signaling pathway alone appear to be sufficient to initiate and promote tumor formation. However, the concurrence of *Kras* mutation and *Tgfb2* deletion promotes the formation of adenocarcinomas in the intestines. These results are consistent with *Tgfb2* acting as a tumor suppressor gene in the intestines but only having obvious tumor suppressing effects in the context of other deregulated signaling pathways, such as the Wnt-APC- β -catenin pathway⁵. Furthermore, we can conclude from this mouse model that this effect is cell autonomous and not a consequence of impaired TGF- β signaling in T-cells or stromal cells, which has been shown to affect tumor formation in mouse models^{32, 33}. It is also likely that at least some of the tumor promoting effect is secondary to autocrine and/or paracrine effects mediated through the EGFR pathway. The induction of tumor promoting ligands by deregulated TGF- β signaling has been observed in other cancer mouse models and may be one of the common mechanisms through which TGF- β signaling inactivation contributes to cancer formation^{9, 34}. Epiregulin expression is increased in the tumor cells of advanced adenomas and may be one of the mechanisms through which TGFBR2 loss mediates the malignant transformation of colon adenomas²⁵.

A consequence of activation of *Kras* has been shown to be increased MAPK activity, which has been observed in some models of intestinal tumorigenesis that employ oncogenic *Kras*,

but not in others^{6, 7, 35}. It is not clear why this discrepancy has been noted, but a partial explanation is that the differences may be secondary to specific effects of different mutant alleles of *Kras* or *Apc*. In the KVcTT mice, we have observed activation of the MAPK-ERK pathway in both the tumors and normal mucosa of the mice. In contrast, we have observed both suppression and activation of the PI3K-AKT pathway in the tumors in the KVcTT mice. Interestingly, we observe activated AKT in the majority of tumors arising in the AVcTT mice suggesting that the effect observed in the KVcTT mice is not specific for concurrent KRAS-MAPK and TGF- β signaling pathway deregulation. The activation of the PI3K pathway is common in human colon cancer, and the observation that this pathway is activated in the KVcTT and AVcTT mice suggests these models recapitulate this aspect of human intestinal cancer³. Of interest, as with the MAPK-ERK pathway, there is heterogeneity between the activation state of the PI3K and the mTOR pathways in different intestinal neoplasm models that employ oncogenic *Kras*, and the mechanisms causing the heterogeneity are not known^{6, 7, 35}.

A predominant biological consequence of the cooperation between oncogenic *Kras* and loss of *Tgfr2* is enhanced proliferation in the tumors^{7, 35}. The increased proliferation occurs in the context of reduced p15 expression with no change in the expression of p21 or p16 (data not shown). We observed no increase in proliferation in the intestinal tumors arising in the AVcTT mice possibly because of compensatory increased p21 expression. In addition, our results and previously published studies suggest the increased proliferation in the KVcTT mice may be partially related to increased EGFR signal pathway activation that is a result of increased epiregulin and *Erbb1* expression³⁶. These results suggest that tumors that carry *KRAS* mutations and *TGFBR2* mutations may be dependent on EGF signaling and susceptible to therapies targeting this pathway^{25, 37}. In light of the observation of lymph node metastases and metastatic tumor to the lungs in a subset of the KVcTT mice it is possible that the cooperation between oncogenic *Kras* and *Tgfr2* inactivation promotes metastatic behavior. Thus, therapies directed at the EGFR may be effective for inhibiting tumor metastases in patients with colorectal cancer that carry mutant *TGFBR2* and mutant *KRAS*.

In summary, we have demonstrated in an *in vivo* model system that loss of *Tgfr2* in the intestinal epithelium contributes to intestinal cancer formation by cooperating with mutant *Kras* to induce the formation of adenocarcinomas and metastases. The results of these studies using the *Villin-Cre;Tgfr2^{E2flx/E2flx}* mice provide evidence from an *in vivo* model system that inactivation of *TGFBR2* has a pathogenic role in the formation of human colon cancers and cooperates with *KRAS* mutation to promote the progression of intestinal adenocarcinomas to metastatic disease.

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Abbreviations used are

TGF- β transforming growth factor- β
TGFBR2 transforming growth factor- β receptor type II

PCR polymerase chain reaction

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Figure 1.

Photomicrographs of representative examples of distal colon epithelium from the KTT, KVcT^{wt/wt}, and KVcTT mice (age: 20 weeks). **A,C,E.:** H&E stained sections reveal increased goblet cells and crypt length in the KVcT^{wt/wt} and KVcTT mice compared to the KTT mice. **B, D, F.:** Ki67 immunostaining of the KTT, KVcT^{wt/wt}, and KVcTT mice reveals more Ki67 labeled cells in the KVcT^{wt/wt} and KVcTT mice compared to the KTT mice. (magnification 100×)

Figure 2.

A, B H&E stained tissue sections from tumors arising in the small intestine (**A.**) and colon (**B.**) (100x magnification). The tumors display marked desmoplasia and mucinous features, including signet ring cells and mucin lakes (arrow and asterisk, respectively). **C, D.** β -catenin immunostaining of the normal epithelium (**C.** colon, **D.** small intestine) in the KVcTT mice reveals cytoplasmic membrane distribution, as expected. **E, F.** p53 immunostaining in normal intestinal epithelium (**E.** colon, **F.** small intestine) in the KVcTT mice reveals occasional cells with nuclear staining in the base and luminal surface of the epithelium in the colon and small intestine with the majority of cells showing no staining. **G.** β -catenin cellular localization in the neoplasms arising in the KVcTT mice. Immunostaining for β -catenin reveals that <10% of

the KVcTT tumors show nuclear β -catenin {compared to 80% of the AVcTT tumors (data not shown)}. (Arrow indicates neoplastic gland with β -catenin expression.) **H.** Representative example of p53 immunostaining of KVcTT tumor (100X respectively). Increased nuclear p53 nuclear immunoreactivity is present in the tumors. The arrow indicates cells that show nuclear p53 immunoreactivity.

Figure 3.

Expression of phosphorylated ERK1/2 (pThr202/Tyr204), total ERK1/2, phosphorylated AKT (pSer473), and total AKT in the normal intestinal mucosa of the KTT, KVcTT, and KVcT^{wt/wt} mice and in the intestinal neoplasms of the KVcTT, AVcTT and ATT mice. Phosphorylated ERK1/2 is present in the adenocarcinomas (n=5) and mucosa (n=3) of KVcTT mice as well as in KVcT^{wt/wt} epithelium (n=3). Phosphorylated ERK1/2 is present in approximately 60–70% of the neoplasms (N=9) in AVcTT and ATT mice regardless of whether *Tgfb2* is inactivated. Similarly, AKT phosphorylation is present in 40–100% of the tumors in the mouse models, and is present in the majority of the normal mucosa samples of the KTT, KVcT^{wt/wt}, and KVcTT mice.

Figure 4. Ki67 immunostaining in the KVcTT vs. AVcTT neoplasms reveals significantly increased proliferation in the KVcTT tumors compared to the AVcTT tumors. **A–B.** Representative examples of tumors from the AVcTT mice (100x and 200X). **C–D.** Representative examples of tumors from the KVcTT mice (100X and 200X).

Figure 5.

Immunoblotting of protein lysates extracted from the normal intestinal mucosa from the KTT, KVcTT, and KVcT^{wt/wt} mice and neoplasms from the KVcTT, AVcTT, and ATT mice for p15, p21, cyclin D1 and cdk4. The expression of p15 is reduced in the tumors from the KVcTT mice compared to the normal mucosa and to the AVcTT and ATT tumors. Furthermore, p21 expression is the same or decreased in the KVcTT tumors compared to the normal mucosa and is substantially less than the majority of the AVcTT and ATT tumors. Cyclin D1 and cdk4 expression is increased in all the tumors when compared to the normal mucosa. The “X” indicates that no protein lysate was available from this sample to perform this study. Actin was used to control for protein loading.

Figure 6.

Ereg and *Erbb1* expression in the intestinal mucosa of the VcTT, KTT, KVcT^{wt/wt} and KVcTT mice and in the adenocarcinomas from the KVcTT mice. **A.** Epiregulin (*Ereg*) expression measured by qRT-PCR is increased in the neoplasms compared to the normal mucosa. (*, *** Mann Whitney test, $p < 0.0001$) **B.** *Erbb1* (expression measured by qRT-PCR) is increased in the neoplasms compared to the normal mucosa with the statistically significant differences shown. (* $p < 0.05$, ** $p < 0.01$ Mann Whitney test) REU=Relative Expression Unit **C.** *EREG* expression in the HCT116 colon cancer cell line is increased by oncogenic *KRAS* (*KRAS*^{G13D}) and *TGFBR2* inactivation. Quantitative RT-PCR analysis shows *EREG* mRNA expression is higher in parental HCT116 (*KRAS*^{G13D} and biallelic *TGFBR2* *BATRII* mutations) than in HCT116 with reconstituted *TGFBR2* (HCT116 + *TGFBR2* 2+8), with only wild-type *KRAS* (HKe-3), or wild-type *KRAS* and *TGFBR2* reconstitution (HKe-3 + *TGFBR2* 2+8). The deletion of the mutant *KRAS* allele has a more pronounced effect on *EREG* expression than does *TGFBR2* reconstitution (* $p < 0.01$, student's t-test), although the differences between all the groups are statistically significant including between HKe-3 and HKe-3+*TGFBR2* 2+8. (** $p = 0.03$, student's t-test) **D.** *EREG* mRNA expression is increased in the SW480 colon cancer cell line (mutant *KRAS* and wild-type *TGFBR2*) after treatment with TGF- β RI Inhibitor III (300nM) (616453; Calbiochem).

Figure 7. Metastatic tumors in the *Tgfbr2*^{IEKO}; *LSL-Kras*^{G12D/wt} (KVcTT) mouse. **A.** Gross appearance of enlarged lymph nodes that were shown to contain metastatic tumor. The enlarged lymph nodes are indicated by arrows. **B.** Photomicrograph of H&E stained lymph node metastasis (100X) **C.** Photomicrograph of H&E stained lung metastasis (100X). **D.** Cytokeratin immunostaining of metastatic lesions in the lymph node (100X, insert 400X). **E.** Results of PCR-based assays demonstrating the recombination of the *Tgfbr2*^{E2flx} allele in representative lung metastatic tumors (#1 and #2), and mesenteric lymph node (LN #3a and #3b) metastases. The positive and negative control DNA samples are from an AVcTT epithelial cell line and “normal” mesenteric lymph nodes from an age-matched KVcTT mouse that did not have grossly evident metastatic disease.

Table 1

Tumor incidence in KVCt^{wt/wt} and KVCtTT mice

Genotype	Number of mice with tumors	Average number of tumors per mouse [^]	Total number of tumors (Small intestine:colon)	Total number of adenomas (small intestine:colon) [*]	Total number of adenocarcinomas (small intestine:colon) [*]
<i>Villin-Cre; LSL-Kras^{G12D}; Tgfb²^{wt/wt}</i> (KVCt ^{wt/wt}) (n=20)	0	0	0:0	0:0	0
<i>LSL-Kras^{G12D}; Tgfb²^{ΔEKO}</i> (KVCtTT) (n=21)	15	2.4	3:8	8:17	36

* p=0.0005, student's T-test

[^] Only mice with tumors included in this calculation.