Abstract

*SMAD4* is localized to chromosome 18q21, a frequent site for loss of heterozygosity in advanced stage colon cancers. Although Smad4 is regarded as a signaling mediator of the TGFβ signaling pathway, its role as a major suppressor of colorectal cancer progression and the molecular events underlying this phenomenon remain elusive. Here, we describe the establishment and use of colon cancer cell line model systems to dissect the functional roles of TGFβ and Smad4 inactivation in the manifestation of a malignant phenotype. We found that loss of function of Smad4 and retention of intact TGFβ receptors could synergistically increase the levels of VEGF, a major proangiogenic factor. Pharmacologic inhibition studies suggest that overactivation of the TGFβ-induced MEK-Erk and p38-MAPK (mitogen-activated protein kinase) auxiliary pathways are involved in the induction of VEGF expression in *SMAD4* null cells. Overall, *SMAD4* deficiency was responsible for the enhanced migration of colon cancer cells with a corresponding increase in matrix metalloprotease 9 enhanced hypoxia-induced GLUT1 expression, increased aerobic glycolysis, and resistance to 5-fluorouracil-mediated apoptosis. Interestingly, Smad4 specifically interacts with hypoxia-inducible factor (HIF) 1α under hypoxic conditions providing a molecular basis for the differential regulation of target genes to suppress a malignant phenotype. In summary, our results define a molecular mechanism that explains how loss of the tumor suppressor Smad4 promotes colorectal cancer progression. These findings are also consistent with targeting TGFβ-induced auxiliary pathways, such as MEK-Erk and p38-MAPK and the glycolytic cascade, in *SMAD4*-deficient tumors as attractive strategies for therapeutic intervention.

Introduction

Colon cancer is the third most frequently diagnosed cancer and the second leading cause of cancer deaths in the United States, accounting for more than 50,000 cancer deaths per year (1). There has been significant progress in understanding the familial predisposition to colon cancer and it has been exploited as an excellent model to understand the multistep progression of human cancer (2, 3). On the other hand, as the majority of colon cancer cases are of sporadic origin and often diagnosed at an advanced stage, it remains a major form of cancer fatality. There has been little progress made in elucidating the molecular basis for the conversion of a benign form of the cancer to a more malignant and metastatic form, which accounts for the majority of colon cancer deaths. Thus, the delineation of the key genetic and epigenetic alterations that promote malignancy of colon cancer is important not only for prognosis and clinical surveillance of affected individuals but also for devising treatment strategies to block the dissemination of cancer cells and to effectively eradicate tumors.

Resistance to growth inhibition by TGFβ is common in a variety of human cancers, emphasizing the importance of intracellular pathways mediated by this polypeptide to the neoplastic process (4, 5). Early investigations to understand the molecular basis of this resistance were concentrated at the level of TGFβ receptors and uncovered, lack of expression (6–8) and inactivation by point mutations of the TGFβ receptor type II (RII; refs. 9–11). Subsequently, evidence for TGFβ receptor type I (RI) mutations was also reported (12). A major breakthrough in understanding the genetic basis of TGFβ insensitivity to growth emerged with the isolation of the *SMAD4* gene as a target tumor suppressor gene localized to chromosome 18q21.1 in pancreatic carcinomas (13). Because loss of heterozygosity (LOH) at chromosome 18q has long been established as a late event...
during colon cancer progression (2), our studies were the first to report that SMAD4 mutations or deletions occurred in 30% of colon cancers that exhibited LOH for chromosome 18q (14). Additional confirmations in numerous follow-up studies also showed that a high frequency of LOH at 18q was associated with an increase in the frequency of SMAD4, and less frequently SMAD2 or DCC mutations (14–17).

When tumors corresponding to different stages of colon cancer were interrogated for SMAD4 inactivation arising from deletions or point mutations, there was a strong correlation between the higher frequency of SMAD4 gene mutations and distant metastases relative to nonmetastatic forms of colon cancer (14, 15, 18–21). Additional credence was also derived from studies with mouse models where a dramatic increase in malignant progression of intestinal polyps in cis-compound heterozygotes (i.e., APC+/− SMAD4+/−) compared with the simple APC+/− heterozygotes was observed (22, 23). Overall, studies using both human tumors and animal models corroborated the notion that disabling TGFB signaling pathway at the level of Smad4 may be a critical late event in multistep colon cancer progression.

Here, we provide molecular evidence supporting that genetic defects in SMAD4 and increased TGFB levels in colon cancer cells are associated with transition to malignancy with the acquisition of angiogenic and metastatic potential. These findings form a molecular basis for the creation of model systems harboring a SMAD4 defect to aid in the discovery of biomarkers and therapeutic targets for colon cancer.

Materials and Methods

Cell lines and culture

Isogenic HCT116 SMAD4+/+ and SMAD4−/− colon cancer cell lines [a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD)] were maintained in McCoy’s 5A medium supplemented with 0.4 mg/mL G418, 0.1 mg/mL hygromycin B and 10% FBS. SW620 colon cancer cell line and used for further assays.

Antibodies and reagents

The following antibodies and reagents were used in this study: VEGF (BD Biosciences), Smad4 (Santa Cruz), anti-HA (Roche), β-actin and anti-Flag (Sigma), Smad2, P-Smad2, Erk, P-Erk (p42/44), Akt, P-Akt, p38MAPK (mitogen-activated protein kinase), p-p38MAPK and cleaved caspase-3 (Cell Signaling) and GLUT1 (Abcam). We also used protein A/G agarose beads (Santa Cruz), inhibitors for MEK (PD98059) and p38 MAPK (SB203580; Calbiochem) and 5′-fluorouracil (5′-FU; Sigma).

Plasmid construction

To generate the pBabe-puro-TGFβRII-HA plasmid, TGFβRII-HA cDNA was excised from pCEP4-Zeo/Hyg-TGFβRII-HA plasmid (24), using BamHI/HindIII digestion followed by Klenow enzyme reaction to generate a blunt-end DNA fragment and then ligated into SmaII-digested, pBabe-puro vector. To generate the pBabe-puro-Smad4-Flag plasmid, Smad4-Flag cDNA was excised from a PRK5-Smad4-Flag plasmid (25) using EcoRI/HindIII digestion followed by Klenow enzyme reaction and then ligated into SmaII-digested pBabe-puro vector. All plasmids were verified by DNA sequencing.

Viral production and infection of target cells

Retrovirus was generated by cotransfection of pBabe-puro empty vector or pBabe-puro-Smad4-Flag or pBabe-puro-TGFβRII-HA along with pSV-S-G (envelope) and pSV-S-GP (packaging) plasmids in 293FT cells. Target cells were infected overnight with 4 mL of virus-containing medium in the presence of 10 μg/mL polybrene. The next day, cells were cultured in fresh medium and allowed to grow for another 24 hours. After replacing with fresh medium, cells were selected with 2 μg/mL puromycin for 7 to 10 days, pooled, and used for further assays.

Western blotting

Western blot analysis was performed as previously described (26).

Transient transfections and luciferase reporter assays

Cells were seeded in 12-well plates (Corning) overnight prior to transfection. Transfections of firefly luciferase reporter and Renilla luciferase (internal control) plasmids were performed using Fugene (Roche). Transfected cells were allowed to grow overnight prior to TGFB treatments. Firefly luciferase reporter activity was measured with a dual luciferase reporter assay kit (Promega), according to the manufacturer’s protocol, using a Monolight 3010 luminometer (BD Biosciences) at 570 nm. Expression was calculated as the ratio of arbitrary firefly luciferase units normalized to Renilla luciferase. These experiments were independently repeated three times and each treatment consisted of triplicate samples.

Drug and inhibitor treatments

HCT116 cells were seeded in 6-well or 12-well plates 24 hours prior to any treatment. Cells were pretreated 30 minutes before the beginning of each experiment with 20 μmol/L of MEK inhibitor (PD98059), 20 μmol/L of p38 MAPK inhibitor (SB203580; Calbiochem) or 1 μg/mL 5′fluorouracil (Sigma).

Wound-healing assays

Cells were grown to confluency and a wound was introduced using a sterile Q-tip. The ability of cells to migrate was monitored at different time points using a light microscope. Images were captured using a Nikon E4300 digital camera to monitor the cell migration rate.

ELISA assays

Cells were seeded and allowed to grow for 24 hours. Culture medium was replaced with serum-free medium and cells were allowed to grow for another 36 hours. After collecting the conditioned medium, cells were washed again with 1 mL of
serum-free medium, pH 5.0, to enhance the release of VEGF bound to the VEGF receptors on the cell membrane. This medium was pooled with the previously harvested conditioned medium and concentrated 5 times by centrifugation (7,500 × g for 15 minutes) using an Amicon 50K filter unit (Millipore). Secreted VEGF was quantified using a human VEGF Quantikine ELISA Kit (R&D) according to the manufacturer’s protocol.

**Lactate assay**

Equal numbers of HCT116 SMAD4+/+ and SMAD4−/− cells were plated and allowed to grow for 24 hours under normoxic conditions. The amount of lactate in the culture media secreted by the cells was measured using a lactate assay kit (Biovision), according to the manufacturer’s protocol.

**Oxygen consumption assay**

The oxygen consumption rates were measured as described in Supplementary Methods.

**Zymogram assays**

Conditioned medium from cells was collected as described above. The activity of matrix metalloproteinases (MMP) was assessed by resolving the concentrated conditioned media on 10% gelatin native zymogram gels (Novex) followed by Coomassie blue staining.

**Protein coimmunoprecipitation**

Coimmunoprecipitation experiments were performed following cotransfection of PRK5-Smad4-Flag along with pCDNA3-HIF1αAA (hypoxygen-inducible factor) or pCDNA3-HIF2αAA vectors in HCT116 cells. Cells were cultured under 1% O2 conditions for 5 hours and then were lysed in ice-cold RIPA (radioimmunoprecipitation assay) buffer (50 mmol/L of Tris-HCl, pH 7.4, 150 mmol/L of NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mmol/L of EDTA), containing protease and phosphatase inhibitors (Roche). Immunoprecipitation was performed using either anti-mouse IgG or anti-Flag antibodies in 300 μL total cell lysate using 30 μL protein A/G-agarose beads followed by overnight incubation at 4°C. The immune complexes were washed 5 times with 1 mL lysis buffer and analyzed by Western blotting.

**Statistical analysis**

Two-tailed paired t test was performed for statistical analysis of luciferase assays. A P value of less than 0.05 [indicated by the asterisk (*) in respective figs.] was considered statistically significant. Error bars represent ± SE values.

**Results**

To elucidate the molecular mechanisms that drive colon cancer progression to malignancy and metastasis, we hypothesized that loss of Smad4 function along with TGFβ overexpression and intact TGFβRII contribute to the acquisition of malignant properties of colon cancer cells. Here, we describe the use of model cell lines to dissect the molecular basis for angiogenic and metastatic phenotypic properties resulting from SMAD4 deficiency that promote colon cancer progression.

**Development and characterization of colon cancer cell line model systems**

To test our hypothesis, we first generated appropriate colon cancer cell line model systems. We used 2 independent colon cancer cell lines, HCT116 and SW620, to examine the contribution of SMAD4 defect in colon cancer.

First, we took advantage of a pair of isogenic HCT116 cells that are either SMAD4 proficient (+/+ +) or deficient (−/−), due to somatic deletions of both SMAD4 alleles engineered by homologous recombination (24). Because these isogenic HCT116 cell lines harbor a mutation in the TGFβRII, which inactivates its kinase activity (27), we stably restored the expression of wild-type TGFβRII using retroviral gene transduction. The following stable colon cancer cell lines were generated: HCT116 SMAD4+/+ pBabe and pBabe-TGFβRII-HA as well as the isogenic SMAD4−/− pBabe and pBabe-TGFβRII-HA (Fig. 1A).

Secondly, we stably restored the expression of wild-type Smad4 in the SW620 colon cancer cell line, with previously reported metastatic potential (28), as these cells harbor a deletion and a nonsense mutation in each of the 2 SMAD4 alleles. In both systems, TGFβRII and Smad4 expression were verified by Western blotting (Fig. 1A and C) and the restoration of an intact TGFβ signaling pathway was confirmed by Smad-binding element luciferase (SBE4-Luc) reporter assays (Fig. 1B and D). Treatment of HCT116 SMAD4+/+ pBabe and pBabe-TGFβRII-HA and SW620-pBSmad4 cells with TGFβ resulted in transactivation of the luciferase reporter. These steps enabled us to generate 2 isogenic pairs of in vitro model systems ideal to study the relationship between TGFβ signaling and/or SMAD4 status and the malignant properties of colon cancer cells.

**Smad4 suppresses VEGF expression in colon cancer cells**

To investigate the expression of genes involved in the biological effects of Smad4-mediated suppression of colorectal tumorigenesis, we first examined the effects of Smad4 on the expression of VEGF, a well-established regulator of angiogenesis and metastasis, overexpressed in a wide variety of human tumors (29, 30).

We performed VEGF-Luc reporter assays in the HCT116 cell line model system to assess the effects of Smad4 and TGFβRII status on VEGF transcription. On treatment with TGFβ following serum starvation, HCT116 SMAD4+/+ cells with restored TGFβRII expression exhibited increased VEGF promoter activity compared with the SMAD4+/+ cells (Fig. 2A, i). These results were also consistent with the VEGF protein levels (Fig. 2A, ii). To independently confirm these findings, we also used the SW620 system. As predicted, restoring Smad4 expression in these cells resulted in significantly reduced VEGF promoter activity (Fig. 2B, i) and corresponding reduction in VEGF protein levels (Fig. 2B, ii).

Because VEGF is a secreted growth factor which can mediate the angiogenic program of tumors in an autocrine and paracrine fashion, we hypothesized that SMAD4-deficient
cells secrete more VEGF compared with SMAD4-proficient cells. ELISA assays confirmed that restoration of Smad4 expression in SW620 caused the suppression of VEGF secretion (Fig. 2C). Overall, these studies demonstrated that Smad4 suppresses VEGF expression in the colon cancer cells.

**Activation of auxiliary signaling pathways by TGFβ results in VEGF upregulation in SMAD4-deficient cells**

It is well known that TGFβ can potently activate Smad-dependent as well as Smad-independent signaling pathways (31). Therefore, we hypothesized that the effects of Smad4 loss on VEGF expression might be mediated through activation of auxiliary signaling pathways. To test this, we examined the effects of Smad4 and TGFβRII status on the kinetics of TGFβ-activated signaling pathways. The 4 groups of HCT116 cells (proficient and deficient in Smad4 and with or without TGFβRII restoration) were serum-starved overnight and then treated with TGFβ for various time points as indicated in Figure 3. The kinetics of the major downstream TGFβ-activated signaling pathways that have been shown to be involved in cancer progression was determined by Western blotting. We observed increased phosphorylated MAPK and Smad2 in the presence of RII indicating the likely reconstitution of auxiliary signaling pathways. Interestingly, TGFβ treatment caused prolonged activation of the MEK-Erk pathway in the SMAD4-deficient cells compared with the SMAD4-proficient cells in a TGFβRII status-independent manner (Fig. 3A and B). Furthermore, the retention of wild-type TGFβRII appeared to be necessary for the TGFβ-induced activation of the p38-MAPK pathway in both SMAD4-proficient and SMAD4-deficient cells and exhibited a much earlier activation in the SMAD4-deficient cells compared with SMAD4-proficient cells in response to TGFβ (Fig. 3A and B). Although the MEK-Erk pathway remained consistently overactive, a similar early activation of the p38-MAPK pathway was also observed in the SMAD4-deficient SW620 cells in response to TGFβ (Supplementary Fig. S1). The hyperactivity of the MEK-Erk pathway in both SMAD4-deficient and SMAD4-proficient SW620 cells may be derived from other genetic differences between SW620 and HCT116.

Both MEK-Erk and p38-MAPK pathways have been implicated in the regulation of VEGF expression in cancer cells (32, 33). As our data suggested that these pathways become overactive in SMAD4-deficient colon cancer cells in response to TGFβ, we decided to test whether VEGF upregulation is mediated through these signaling cascades. We found that pharmacologic inhibition of MEK-Erk and p38-MAPK pathways in SMAD4-deficient cells suppressed VEGF promoter activity, as indicated by VEGF-Luc reporter assays (Fig. 3C and D). Consistent with our signaling pathway kinetics data, treatment with the MEK inhibitor suppressed VEGF activation in both SMAD4-proficient and SMAD4-deficient cells (Fig. 3C), whereas p38-MAPK inhibition suppressed VEGF expression only in the SMAD4-deficient cells (Fig. 3D). In conclusion, these studies suggest that SMAD4 defect in the presence of functional TGFβRII results in an increase in VEGF expression caused, at least in part, by TGFβ-induced overactivation of the MEK-Erk and p38-MAPK signaling pathways.
SMAD4 defect causes increases in cell motility and matrix metalloprotease 9 activity

To evaluate the effects of Smad4 deficiency on the migratory properties of colon cancer cells, we performed wound-healing assays. We found that HCT116 SMAD4/−/− cells migrated into the cell-free areas and completely closed the wound in 40 hours, whereas the migration rate of HCT116 SMAD4+/+ cells was significantly reduced (Fig. 4). Interestingly, the accelerated migration of SMAD4-deficient cells appeared to be independent of the status of TGFβRII (Fig. 4A and B). Because SMAD4 defect was found to enhance TGFβ-induced Erk phosphorylation (Fig. 3A), we hypothesized that overactivation of this pathway might be involved in the acquisition of promigratory properties. Consistent with this notion, treatment of SMAD4/−/− cells with MEK inhibitor suppressed the cell migration (Fig. 4A and B). These data suggest that SMAD4 defect enhances the migration rate of HCT116 cells and that activation of the MEK-Erk pathway might be involved in this process.
critical step required for the metastatic dissemination. It could be aided not only by the acquisition of a more migratory phenotype, but also through the upregulation of MMP enzymes involved in the degradation of the extracellular matrix. To test whether SMAD4 status affects the activity of such enzymes, we performed zymogram assays using conditioned media from the parental SMAD4-deficient and SMAD4-reconstituted SW620 cells. Restoration of Smad4 expression suppressed the matrix metalloprotease 9 (MMP9) activity in these cells (Fig. 4C) supporting the notion that Smad4 acts to inhibit both the migratory and invasive properties of colon cancer cells.

SMAD4 defect suppresses hypoxia-induced cell death, induces aerobic glycolysis, and promotes 5'-fluorouracil resistance of colon cancer cells

Because increased glycolytic rates have been correlated with chemoresistance of colon cancer cells (34), we hypothesized that loss of Smad4 might affect the expression of enzymes associated with the glycolytic pathway under hypoxic conditions. Smad4 Inactivation Promotes Malignancy in Colon Cancer
conditions that mimic the microenvironment of advanced tumors. Indeed, SMAD4-deficient cells exhibited higher levels of the major glucose transporter GLUT1, but not hexokinase, when cultured under normoxic or hypoxic conditions, compared with SMAD4-proficient cells (Fig. 5A, i). In addition, SMAD4-deficient cells secrete significantly higher levels of lactate compared with SMAD4+/+ cells (Fig. 5A, ii) indicating an enhanced rate of aerobic glycolysis. Interestingly, we also found that Smad4 physically interacts with HIF1α, but not HIF2α, under hypoxic conditions (Fig. 5B) suggesting that it may negatively regulate HIF1α-mediated GLUT1 expression. Furthermore, this phenomenon was also not associated with altered oxygen consumption rate (Supplementary Fig. S2) indicating that mitochondrial function and oxidative respiration is not involved. Consistent with these findings, SMAD4-null cells were resistant to hypoxia-induced cell death compared with their wild-type counterparts (Fig. 5C). Overall, these observations suggested that the increase in GLUT1 protein levels, due to SMAD4 loss, may be correlated to an increased rate of aerobic glycolysis and survival under hypoxic conditions.

On the basis of these observations and the literature suggesting that chromosome 18q loss results in resistance to a commonly used drug for colorectal cancer treatment, 5'-fluorouracil (5'-FU; ref. 35), we hypothesized that SMAD4 deficiency might be responsible for this effect. Treatment of HCT116 SMAD4+/+ cells with 5'-FU for 72 hours resulted in profound induction of apoptosis, corroborated by the presence of cleaved caspase-3 (Fig. 5D, i and ii). On the contrary, there was almost undetectable level of apoptosis in SMAD4−/− cells suggesting that SMAD4 defect results in the acquisition of 5'-FU resistance in colon cancer (Fig. 5D, i and ii).

**Discussion**

TGFβ overexpression and SMAD4 mutations or deletions have been directly correlated with colon cancer metastasis. Several pathologic and genetic studies suggested that chromosome 18q loss is a critical event during colorectal cancer progression and that the SMAD4 tumor suppressor is the primary target for inactivation (2, 14). Subsequent reports have established that allelic loss of chromosome 18q is directly correlated with liver metastasis of colorectal cancer and poor prognosis (36, 37). Despite the strong genetic evidence for the association between SMAD4 inactivation and advanced stage of colon cancer, the molecular basis remains elusive.

To examine if SMAD4 inactivation is a major switch that favors tumor malignancy and propensity for angiogenesis and metastasis of colon cancer, we elected to use cell line model
Figure 5. SMAD4 deficiency correlates with increased GLUT1 levels and resistance to hypoxia-induced cell death and 5'-fluorouracil treatment. A, i, loss of SMAD4 increases GLUT1 protein levels. Western blotting for detection of GLUT1 protein levels in protein lysates isolated from HCT116 SMAD4+/+ and SMAD4−/− grown under normoxic (21% O2) or hypoxic (1% O2) conditions for 24 hours. A, ii, lactate secretion from HCT116 SMAD4+/+ and SMAD4−/− cells growing under normoxic conditions. B, Smad4 physically interacts with HIF1α, but not with HIF2α, under hypoxic conditions. HCT116 SMAD4+/+ cells were transiently cotransfected with PRK5-SMAD4-Flag and pCDNA3-HIF1αAA vectors or PRK5-SMAD4-Flag and pCDNA3-HIF2αAA vectors, respectively, for 16 hours and cultured under hypoxic conditions for an additional 5 hours. Total cell lysates were immunoprecipitated with mouse IgG antibody (mock) or mouse anti-Flag antibody and immunoprecipitates were analyzed by Western blotting to detect either HIF1α or HIF2α. C, representative examples of light microscopy images (i) and Western blotting for detection of the cleaved PARP (ii) in HCT116 SMAD4+/+ and SMAD4−/− grown in normoxic (21% O2) or hypoxic conditions (1% O2). D, representative examples of light microscopy images (i) and Western blotting for detection of the cleaved caspase-3 (Asp 175; ii) from total cell lysates of HCT116 SMAD4+/+ and SMAD4−/− cultures which were treated with either mock (DMSO) or 5'-fluorouracil (5'-FU; 1 μg/mL) for 72 hours.
systems to investigate both the molecular basis and cellular properties associated with SMAD4 inactivation and concurrent increase in the TGFβ levels, conditions that mimic advanced stage colorectal tumors. Because the pairs of cell lines studied are genetically identical, except for their SMAD4 status, we reasoned that comparing the properties and gene expression patterns should help to better understand the role of SMAD4 in tumor malignancy.

Here, we show that Smad4 loss enhances VEGF expression synergistically with increased levels of TGFβ, whereas expression of Smad4 suppresses VEGF levels in colon cancer cells. These results are consistent with a previous report using the pancreatic cancer cell line, Hs766T, harboring homozygous deletion in both SMAD4 alleles, in which the restoration of Smad4 expression was found to suppress angiogenesis and xenograft tumor growth by inhibiting VEGF expression (38). We also found that SMAD4 deficiency prolonged TGFβ-mediated Erk phosphorylation and activation in HCT116 cells. The fact that Erk signaling is initially activated by TGFβ and eventually turned off at 24 hours in SMAD4+/− cells, suggests that a phosphatase may act to revert phosphorylation to the basal levels. Our results are also consistent with hyperactivation of Ras-mediated Erk signaling and progression into undifferentiated carcinoma upon inhibition of Smad4 in transformed keratinocytes (39).

Interestingly, our data also showed that increased TGFβ-mediated activation of MEK-Erk and p38-MAPK pathways combined with SMAD4 loss, at least in part, mediates VEGF upregulation. This is in agreement with studies showing that Erk kinase is required for VEGF upregulation in colon carcinoma cells upon serum starvation (32) as well as that p38-MAPK activation by heregulin-β-1 is required for VEGF induction in endothelial cells (33). Our studies also found that SMAD4 inactivation in colon cancer cells enhances their migratory and invasive properties consistent with a previous report showing that restoration of Smad4 expression reversed the invasive phenotype of pancreatic cancer cells (40).

Clinical studies have shown that patients retaining heterozygosity at the 18q locus benefit significantly better from treatment with 5′-fluorouracil than patients with LOH at this site (34). Moreover, chromosome 18q loss and absence of TGFβRII mutations were found to correlate with low survival rates in patients treated with adjuvant chemotherapy (41). These clinical data are consistent with our findings using HCT116 cells harboring SMAD4 loss and intact TGFβRII status, which cooperate to induce VEGF expression. Other studies also showed a direct correlation between low levels of Smad4 in tumors and worse outcome following surgery and treatment with 5′-fluorouracil in colon cancer patients (42).

Figure 6. SMAD4 inactivation promotes transition to malignancy in colon cancer. Transition of premalignant colon cancer cells to malignancy is blocked by functional Smad4 due to inhibition of transcription factors (TF), such as the HIF1α, or other molecular events that are activated downstream of oncogenic signaling pathways and cross-talking TGFβ signaling events involved in promoting malignant properties. The inactivation of Smad4 during colon cancer progression removes the block in transition from the premalignant to the malignant stage by allowing accumulation of factors such as GLUT1 and VEGF.
Elevated glycolytic rates, even under normoxic conditions, also known as the "Warburg effect" (43), have been correlated with the acquisition of chemoresistance in cancer cells (44, 45) and HIF1α is established as a major transcriptional regulator of the glucose transporter GLUT1 (46). Interestingly, we found that SMAD4-deficient cells exhibit increased levels of GLUT1 expression and lactate secretion as well as resistance to 5-FU–mediated apoptosis. Because SMAD4 deficiency did not affect oxidative respiration (Supplementary Fig. S1), we conclude that increased glycolysis aided by the robust glucose transport contributes to the enhanced survival of these cells. The fact that there was physical interaction between Smad4 and HIF1α suggests a mechanistic basis for these observations. On the basis of these findings, we propose that Smad4 may negatively regulate HIF1α-induced GLUT1 expression and the rate of aerobic glycolysis, providing a molecular link to explain the acquisition of chemoresistance in colorectal tumors harboring chromosome 18q deficiency (Fig. 6).

In summary, our studies provide direct evidence for a molecular basis to explain an association between a Smad4 defect and progression to malignant colon cancer (Fig. 6). The model systems described here may help to uncover novel biomarkers for advanced stage colon cancer to improve prognostic evaluations and identify effective targets for therapeutic intervention.

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

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Smad4 Inactivation Promotes Malignancy and Drug Resistance of Colon Cancer

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