Wnt5a promotes human colon cancer cell migration and invasion but does not augment intestinal tumorigenesis in Apc1638N mice

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Whereas aberrant activation of canonical Wnt/β-catenin signaling underlies the majority of colorectal cancer cases, the contribution of non-canonical Wnt signaling is unclear. As enhanced expression of the most extensively studied non-canonical Wnt ligand WNT5A is observed in various diseases including colon cancer, WNT5A is gaining attention nowadays. Numerous in vitro studies suggest modulating capacities of WNT5A on proliferation, differentiation, migration and invasion, affecting tumor and non-mutant cells. However, a possible contribution of WNT5A to colorectal cancer remains to be elucidated. We have analyzed WNT5A expression in colorectal cancer profiling data sets, altered WNT5A expression in colon cancer cells and used our inducible Wnt5a transgenic mouse model to gain more insight into the role of WNT5A in intestinal cancer. We observed that increased WNT5A expression is associated with poor prognosis of colorectal cancer patients. WNT5A knockdown in human colon cancer cells caused reduced directional migration, deregulated focal adhesion site formation and reduced invasion, whereas Wnt5a administration promoted the directional migration of colon cancer cells. Despite these observed protumorigenic activities of WNT5A, the induction of Wnt5a expression in intestinal tumors of Apc1638N mice was not sufficient to augment malignancy or metastasis by itself. In conclusion, WNT5A promotes adhesion sites to form in a focal fashion and promotes the directional migration and invasion of colon cancer cells. Although these activities appear insufficient by themselves to augment malignancy or metastasis in Apc1638N mice, they might explain the poor colon cancer prognosis associated with enhanced WNT5A expression.

Introduction

Whereas canonical Wnt/β-catenin signaling is well known to be a major player in intestinal cancer (1,2), the role of non-canonical Wnt signaling herein is poorly understood. Wnt5a is the most extensively studied non-canonical Wnt ligand, as it has gained substantial attention over the last years, being implicated in various human diseases including a multitude of cancer types, inflammatory diseases and metabolic disorders (3–7). Wnt5a is especially known to be an important regulator of oriented cell movements and structure outgrowth during embryonic development; however, a possible contribution to postnatal processes remains unclear (8–10). A wide variety of in vitro studies have suggested tumor-promoting activities of Wnt5a during multiple cellular processes, including growth, differentiation, epithelial-to-mesenchymal transition, angiogenesis, migration and invasion, thereby affecting both tumorigenic and non-mutant cells (3,5,7,11–13). Clear protumorigenic roles for Wnt5a have been shown for melanoma and gastric cancer, in which Wnt5a enhances migration and invasion of tumor cells (13–17). On the other hand, loss of Wnt5a protein is associated with high-risk neuroblastoma and leukemia, suggesting a tumor-suppressing role of Wnt5a in these tumor types (4,7,18,19). The contribution of Wnt5a expression to colon cancer, however, is poorly understood. Enhanced expression of Wnt5a RNA in human and mouse intestinal tumors has been reported consistently, notably produced by the stromal cells of the tumor and following an augmenting trend during progression from normal intestine through adenoma to carcinoma (20–24). However, contradictory results have been reported describing reduced expression of Wnt5a protein or gene silencing in the epithelial compartment of a subset of colorectal tumors (25,26). Despite the relevance of Wnt5a as suggested by associative Wnt5a expression and in vitro studies, solid evidence for a significant impact of Wnt5a on intestinal cancer is lacking. We aimed to gain insight into the contribution of Wnt5a to intestinal cancer. Therefore, we analyzed WNT5A expression profiling data of colon cancer patients and colon cancer cell lines. Next, we generated stable WNT5A knockdown and overexpression in human colorectal cancer cell lines and examined the consequences for their migratory and invasive properties. Moreover, we investigated the impact of increased Wnt5a expression during Apc-driven intestinal tumor development in vivo, using our previously generated inducible Wnt5a transgenic mouse model (8). Our data suggest that enhanced WNT5A expression is associated with poor prognosis in colorectal cancer patients. We found that in human colon cancer cells, WNT5A promotes directional cell migration, probably through regulating the focal formation of adhesion sites and invasion. However, we observed no discernible consequences on intestinal tumorigenesis when enhanced Wnt5a expression was induced in Apc1638N mice, providing evidence that increased Wnt5a by itself is not sufficient to induce invasion and metastasis of adenomas that develop in the genetically modified Apc1638N mouse model.

Materials and methods

RNA expression profiles

Expression profiles of human colorectal cancer cell lines were obtained from two publically available data sets, i.e. GSE10843 and woost-00041, the latter available from the caArray data portal (28). Expression profiles from colorectal cancer cell lines were obtained from two publically available data sets, i.e. GSE10843 and woost-00041, the latter available from the caArray data portal (28). Expression profiles from colorectal cancer cell lines were obtained from two publically available data sets, i.e. GSE10843 and woost-00041, the latter available from the caArray data portal (28).

Cell culture and treatments

Human colon cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich) and 1% penicillin/streptomycin (p/s) (Gibco). Stable human WNT5A knockdown and non-targeting control SW480 cells were generated using Sigma Mission shRNA pLKO-puro lentiviral vectors, WNT5A shRNA-1 CCCTGACACTTTGTGTGCAA and WNT5A shRNA-2 CACACAGCAGTACAGGAGAAG. A non-targeting shRNA CAACGAGTAGAAGAGCAGCAA was used as control. Cells were selected and maintained in DMEM with 10% FBS, 1% p/s and 2 μg/ml puromycin (Sigma). Stable Wnt5a-expressing HCT116 cells were established by lentiviral transduction with a PLVX-IRES-tdTomato vector (Clontech) in which murine Wnt5a complementary DNA (cDNA) was inserted. Next, tdTomato-positive and -negative cells were separated by fluorescence-activated cell sorting and proper Wnt5a expression was validated in the tdTomato-positive cells (Supplementary
Figure S1C, available at Carcinogenesis Online. Two continuous growing cell lines from Apc1638N adenomas (i.e. 1638N-T1 and 1638N-T3) had been established previously, using the protocol described by Paraskeva et al. (29). These were cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in DMEM supplemented with 20% FBS, Insulin/Transferrin/Selenium Solution (Gibco) and 1% ps. To maintain cell viability, these cells were passaged as small clumps using dispase at 2.5 units/ml (BD Biosciences). All cell lines were grown at 37°C and 5% CO₂. Control and Wnt5a stimulations were performed with either 25% of L-control or L-Wnt5a conditioned medium.

Immunoblotting
Subconfluent cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol and incubated for 10 min at 95°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE) (30). Antibodies are indicated in Supplementary Table I, available at Carcinogenesis Online. Quantification was performed using Odyssey LI-COR software.

RNA isolation, cDNA synthesis and quantitative PCR
RNA was isolated from cultured cells followed by cDNA generation using iScript cDNA Synthesis Kit(Bio-Rad). Quantitative PCR was performed using Sensimir SYBR Green (Bioline) or TaqMan (in case of Axin2/Actb) Gene Expression Assays (Applied Biosystems) and ran in the iQ5 Cycler PCR machine (Bio-Rad). Primers were used WNT5A forward 5′-CTACGAGAGTGCTCGCATCC-3′ and reverse 5′-AGGCCACATACGCGCAGGTTG-3′, and GAPDH forward 5′-GCATTGCCTCACCAGACCC-3′ and reverse 5′-CACCACCCTGGTGCGTATG-3′. Expression levels were corrected for expression of GAPDH or Actb, averaged and presented as fold changes.

MTT proliferation assay
SW480 cells were seeded 6 × 10⁴ cells per M24 well and measured at indicated time points by adding 100 μl 5 mg/ml MTT per well, 30 min incubation at 37°C and replacement of the medium by 100 μl dimethyl sulfoxide. Absorbance (550 nm) was measured and averaged. Assays were performed three times in duplicate.

β-Catenin reporter assay
β-Catenin reporter assay was performed as described previously (30), using the Wnt-responsive element reporter kindly provided by Dr Georges Rawadi (Galapagos, Paris, France). This β-catenin-responsive construct encodes the firefly luciferase reporter gene under the control of a minimal collagenase promoter preceded by six tandem repeats of the TCF/LEF transcriptional response element, for which we also generated a mutant-responsive element-luciferase variant. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Assays were performed three times in triplicate.

Migration assay
The migration assay was conducted as described previously (31), with minor modifications. Briefly, a coverslip was inserted into an Axtofluor incubation chamber (Molecular Probes) and sterilized. Coverslips were coated with gelatin (1 mg/ml) or fibronectin (10 μg/ml) and incubated for 1 h at 37°C, prior to cell seeding. A removable circular migration barrier was inserted into the chamber, which averts cell growth in the center of the coated coverslip. 5 × 10⁵ HCT116 or 2 × 10⁵ SW480 cells were seeded in DMEM, 10% FBS, 1% ps (and 2 μg/ml puromycin in case of SW480 cells) around the barrier and the rings were incubated at 37°C for 24 h, thereby generating a confluent monolayer in the periphery and a cell-free area in the center of the coverslip. Post 24 h, the migration barrier was removed, the cells were washed twice and medium was refreshed. Images of migrating cells were captured every 12 min, for 24 h, using a ×100 30 Plan-Neofluar objective lens (Carl Zeiss). Migration was monitored using time-lapse microscopy on Axioskop 100M inverted microscopes, equipped with AxioCam MRC digital cameras (Carl Zeiss B.V., Sliedrecht, Netherlands). Time-lapse movies were used to establish and quantify parameters of cell migration. The total or absolute movement of the cells in 24 h was termed ‘total distance of migration’ and includes random cell movement. The net directional movement of cells to the cell-free center of the coverslip was termed ‘effective distance of migration’, representing directed migration rather than random migration. Migration efficiency was calculated as the percentage of ‘effective distance of migration’ over the ‘total distance of migration’. Migration velocity was calculated by dividing total distance of migration by time. Parameters are also indicated in Supplementary Figure S1A, available at Carcinogenesis Online. Three independent migration assays were performed and 10 cells were tracked, per assay and condition, and P-values were calculated with the Mann–Whitney U test. All cell-tracking measurements were conducted using AxioVision 4.5 software. Track diagram images were processed in Adobe Illustrator CS3 (Adobe Systems Inc, San Jose, CA).

Cell dispersion assay
Cytodex-3 microcarrier beads (Sigma–Aldrich) were mixed with cells considering a density of 25 cells per bead. The suspension was incubated at 37°C for 24 h with gentle mixing every 2 h to ensure complete coating of the beads. Coated beads were embedded in 1.6 mg/ml collagen gel (collagen: modified Eagle’s medium:7.5% w/v NaHCO₃, in the ratio 8:1:1) in a 12-well plate such that each well had approximately 300 beads. Plates were incubated at 37°C for 2 h for the beads to settle in the gel and the polymerized gels were covered with 500 μl DMEM, 10% FBS, 1% ps and 2 μg/ml puromycin. Cell dispersion was followed with a x100/0.30 Plan-Neofluar objective lens (Carl Zeiss) and measured as the maximum migrated distance from the surface of the bead into the collagen gel. All measurements were performed using AxioVision 4.5 software and assays were performed three times in duplicate. Two-way analysis of variance was performed to calculate P-values.

Immunofluorescence
Subconfluent cells cultured on gelatin-coated glass coverslips were fixed 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.15% Triton-X100 in PBS and blocked in 1% BSA, 0.05% Tween-20 in PBS. Primary antibodies were incubated O/N at 4°C followed by visualization using secondary antibody and Vectashield (Vector Laboratories) containing 4’,6-diamidino-2-phenylindole 1:1000 (Invitrogen). Antibodies are indicated in Supplementary Table II, available at Carcinogenesis Online. Immunofluorescent images were taken using an Axiovert 100M microscope with ×63 Oil-PLUAR lens (Carl Zeiss) and a ORCA II ER camera (Hamamatsu Photonics Systems).

Adhesion assay
Cells were seeded at a density of 5 × 10⁵ per well in 96-well plates. At each time point cells were fixed with 10% trichloroacetic acid and stained with sulforhodamine B (SRB). After washing with 1% acetic acid, plates were dried at 50°C and the dye was solubilized in 10 mM Tris buffer (Sigma–Aldrich). The absorbance was measured using a microplate reader (VICTOR 1420, Wallac, Turku, Finland) at 510 nm.

Transgenic mouse models and in vivo doxycyline administration
Tet-O-Wnt5a mice (8) were interbred with hnRNP-rta2S-M2 mice (32), obtaining double-transgenic Tet-O-Wnt5a/rta2S-M2 mice. Subsequently, Tet-O-Wnt5a/rta2S-M2 mice were crossed with Apc1638N mice, generating ApcΔWNT5A/Tet-O-Wnt5a/rta2S-M2 mice and corresponding control animals (33,34). From 3–8 months of age, 0.2 mg/ml doxycycline with 5% sucrose was administered via drinking water, directly followed by examination of the mice. Animal experiments were approved by the Institute’s Animals Ethics Committee and performed according to Dutch legislation.

Tissue processing and histology
Mouse intestinal tumor tissues were washed in PBS and fixed overnight in 4% paraformaldehyde at 4°C. Paraffin embedding, hematoxylin and eosin and periodic acid-Schiff stainings and immunohistochemistry were performed according to routine protocols. More details with regard to antigen retrieval and antibodies used in immunohistochemistry are shown in Supplementary Table III, available at Carcinogenesis Online.

Results
WNT5A expression is associated with poor prognosis of colon cancer
Although the contribution of WNT5A expression to colon cancer is poorly understood, enhanced expression of WNT5A has been reported consistently in human and mouse intestinal tumors, following an augmenting trend during progression from normal intestine through adenoma to carcinoma (20–24). We analyzed WNT5A expression in a publically available RNA expression data set of human colorectal tumors (27). This analysis revealed an increased WNT5A expression in cases showing early recurrence or metastasis, indicating that WNT5A associates with poor prognosis in colon cancer patients (Figure 1A). Subsequently, we analyzed WNT5A RNA expression in human colon cancer cell lines from two publically available expression profiling data sets (28). This revealed that SW480 cells show strongly enhanced levels of WNT5A compared with other cell lines (Figure 1B). In parallel, we analyzed WNT5A protein levels of the different colon cancer cell lines, and we were able to detect WNT5A protein in SW480 cells only (Figure 1C, upper band). Notably, SW480 cells have been derived from a primary adenocarcinoma of a patient that suffered from recurrence with widespread metastasis (35). Together
these findings indicate that enhanced WNT5A expression is associated with colon cancer malignancy.

**WNT5A promotes directional migration and invasion but not proliferation of colon cancer cells**

To dissect the mechanisms through which WNT5A might contribute to colon cancer, we generated stable WNT5A knockdown SW480 cell lines. Two different WNT5A short hairpin RNA (shRNA) constructs were used and cells were compared with simultaneously generated control non-targeting shRNA SW480 cells. Successful knockdown of endogenous WNT5A was verified on protein and RNA level in both WNT5A shRNA lines (Figure 2A and B). Cell proliferation was determined using an MTT assay, revealing no gross differences in SW480 cells induced by WNT5A knockdown (Figure 2C). Subsequently, β-catenin luciferase assay showed that WNT5A knockdown does not influence the intrinsic Wnt/β-catenin signaling of SW480 cells (Figure 2D). Together this suggests that WNT5A does not influence growth-related properties of SW480 cells.

We investigated the role of WNT5A in migration of human colon cancer cells using a previously described ring-barrier system (31). In short, cells migrate to a reproducibly sized cell-free area in the center of the well and time-lapse photography enables precise tracking of individual cells. We observed reduced migration of WNT5A knockdown SW480 cells toward the cell-free area on both gelatin and fibronectin coatings (Figure 3A; Supplementary Figure S1B, available at Carcinogenesis Online). Quantification revealed that WNT5A knockdown slightly reduced total migration and migration velocity (Figure 3B; Supplementary Figure S1B, available at Carcinogenesis Online). Moreover, the effective migration and thereby migration efficiency were drastically reduced by WNT5A knockdown in SW480 cells (Figure 3B; Supplementary Figure S1B, available at Carcinogenesis Online). As effective migration is defined as the directional movement of the cells to the cell-free center, distinguishable from random cell movement (Supplementary Figure S1A, available at Carcinogenesis Online), this indicates that WNT5A is especially important for directional cell migration. Accordingly, stimulation of WNT5A knockdown SW480 cells with exogenous Wnt5a ligand clearly promoted their migration efficiency, thus rescuing the directional migration defect (Figure 3C and D). To further validate this finding, we generated HCT116 colon cancer cells stably expressing Wnt5a (Supplementary Figure S1C, available at Carcinogenesis Online).
at *Carcinogenesis* Online). Consistently, stable Wnt5a expression in HCT116 cells clearly promoted the migration efficiency of these cells (Figure 3E and F). Taken together, these data demonstrate that Wnt5a promotes the directional migration of human colon cancer cells.

Next, we examined the influence of Wnt5a on three-dimensional cell dispersion, representing the invasive capacity. Beads coated with either SW480 or HCT116 cells and settled in collagen gels were tracked at multiple time points and the distance migrated by the cells from the surface of the bead into the surrounding matrix was quantified (Figure 4). WNT5A knockdown SW480 cells were clearly reduced in their capacity to migrate through the collagen gel (Figure 4A and B). However, HCT116 cells tended to migrate throughout the gel in sheets instead of individually, mostly remaining associated with the beads and presenting as a poorly dispersing cell line. This dispersion behavior was not significantly affected by the presence of Wnt5a, although a slight increase in dispersion distance was noticed (Figure 4C and D). Thus, Wnt5a promotes the directional migration of both SW480 and HCT116 colon cancer cells, and additionally, Wnt5a promotes the invasive capacities of SW480 cells.

### WNT5A regulates focal adhesion of colon cancer cells

Cellular migration requires tightly regulated dynamics of assembly and disassembly of focal adhesion sites, as these serve as traction sites during migration. This involves the local activation of focal adhesion kinase (FAK) and its downstream partner paxillin by phosphorylation. To gain more insight into the involvement of WNT5A in the migration of colon cancer cells, we examined the adhesion characteristics of the SW480 cells. First, we envisaged the cellular adhesions sites by staining with phallolidin (detecting F-actin). Control SW480 cells displayed clear focal phallolidin staining reflecting focal adhesion sites (Figure 5A). However, SW480 cells with WNT5A knockdown revealed phallolidin staining that was more evenly distributed along the entire cell membrane instead of the focal expression observed in control cells (Figure 5A). Staining for p-FAK and p-paxillin further supported this observation. Hence, more focally located, punctate expression of these proteins was observed at the cell membrane in control SW480 cells than in WNT5A knockdown SW480 cells (Figure 5B and C). Overall levels of p-FAK appear to show a minor increase, whereas p-paxillin is more clearly (1.5- to 2-fold) increased in WNT5A knockdown cells (Figure 5D). This increase in p-FAK and p-paxillin levels is even more pronounced (2.5-fold for both) when SW480 cells are subjected to serum starvation (Supplementary Figure S1D, available at *Carcinogenesis* Online). Altogether, these data show that SW480 WNT5A knockdown cells exhibit less focal and more enlarged regions of adhesion, compared with control SW480 cells. We assessed whether this has consequences for the initial adhesion of cells to their substrate. As shown in Figure 5E, the amount of cells that adhered at indicated time points is not significantly affected by knockdown of WNT5A either without coating or on fibronectin. Altogether, these data indicate that WNT5A is not involved in cell-substrate adhesion in a quantitative manner but rather regulates adhesion in a spatial manner by promoting focal adhesion above randomly distributed adhesion.

**Induced Wnt5a expression does not affect intestinal tumorigenesis in Apc1638N mice**

As our data indicate that WNT5A is associated with colon cancer cell migration, invasion and malignancy, we aimed to investigate the effects of Wnt5a modulation in a genetically modified mouse model for intestinal cancer. *Apc1638N* mice develop gastrointestinal tumors spontaneously and extra-intestinal lesions including desmoids and cutaneous cysts, without exhibiting metastasis to distant organs (34). In mouse and human intestinal tumors, enhanced Wnt5a RNA expression has been observed (20–24). We performed Wnt5a immunohistochemical stainings on *Apc1638N* intestinal tumors. The Wnt5a antibody is known to detect Wnt5a protein only when overexpressed and we have shown previously that no Wnt5a protein can be detected in the non-tumorigenic, healthy mouse intestine (8). In *Apc1638N* mice, we were able to confirm enhanced stromal Wnt5a expression in a subset of the intestinal tumors on protein level (Figure 6A). To investigate the *in vivo* effects of increased Wnt5a expression on intestinal tumorigenesis, we used the inducible transgenic Wnt5a mouse model that we generated previously (8). TetO-Wnt5a:hnRNP-rtTA (Wnt5a\(^{rtTA}\)) mice were crossbred with
Fig. 3. Two-dimensional migration analysis of human colon cancer cells, including migratory track overviews of individual cells and quantification of migration parameters. Cells migrated on gelatin during 24 h, locations being captured every 12 min (x = start position, cell-free areas are at the right side of dotted lines). (A and B) A slight decrease in total migration and migration velocity following WNT5A knockdown is observed in SW480 cells, while especially the effective migration and migration efficiency are reduced. (C and D) Stimulation of WNT5A knockdown SW480 cells with exogenous Wnt5a ligand rescues the disturbed migration efficiency. (E and F) Overexpression of Wnt5a in HCT116 cells promotes the migration efficiency of these colon cancer cells. *P < 0.05; **P < 0.001.
Apc1638N mice and transgenic Wnt5a expression was induced during tumor development. Abundant transgenic Wnt5a expression was confirmed in the developing tumors (Figure 6A). Functionality of the transgenic Wnt5a protein and its paracrine activity have been demonstrated in our previous report (8). Although Wnt5a appeared to reduce the protein expression of Wnt5a receptor Ror2, the availability of Ror2 protein was confirmed in these tumors (Supplementary Figure S2A and B, available at Carcinogenesis Online). Characteristic for the Apc1638N mouse model, the overall majority of tumors was identified in the proximal small intestine of Apc1638N and Apc1638N;Wnt5aind mice. Despite the abundance of Wnt5a in Apc1638N;Wnt5aind mice, the number of gastrointestinal tumors that developed was not altered compared with Apc1638N mice, and we also observed no effect on the size of the gastrointestinal tumors (Figure 6B). Histological tumor grading as hyperplasia/adenoma, dysplastic adenoma or adenosarcoma revealed that induced Wnt5a did not affect tumor malignancy (Figure 6C). Hence, in Apc1638N and Apc1638N;Wnt5aind mice, the majority of the tumors was identified as dysplastic adenoma. Concomitantly, no metastasis to distant organs was found in both groups. In parallel, extraintestinal lesions associated with Apc1638N mice were examined and the incidence of desmoids and cysts was found unaltered upon transgenic Wnt5a induction (Supplementary Figure S3, available at Carcinogenesis Online).

Apc1638N;Wnt5aind intestinal tumors showed no difference in size compared with corresponding Apc1638N intestinal tumors, suggesting no influence of Wnt5a on tumor-cell growth or proliferation. We further confirmed this by phospho-histone H3 staining, detecting no altered proliferation upon induction of transgenic Wnt5a protein (Figure 6D). Although β-catenin signaling can be influenced by Wnt5a (36,37), we observed unaltered membrane-associated and nuclear β-catenin following transgenic Wnt5a induction (Figure 6E). Also the presence of the β-catenin target Cyclin D1 was unaffected (Figure 6F). Further confirming the absence of an effect of Wnt5a on Wnt/β-catenin signaling, we demonstrated unaltered Axin2 messenger RNA expression levels in two cell lines derived from Apc1638N intestinal tumors upon exogenous Wnt5a stimulation (Figure 6G). These data are in line with the in vitro findings that Wnt5A knockdown in SW480 cells does not affect proliferation and β-catenin signaling of these adenomatous polyposis coli (APC) mutant tumor cells. Altogether, our data indicate that Wnt5a does not contribute significantly to formation and proliferation of Apc-driven intestinal tumors.

Wnt5a has been suggested to influence cell types of tumorigenic and non-mutant nature, which might have consequences for the composition of tumors. We assessed the presence of different intestinal cell types by staining for goblet cells by periodic acid-Schiff, enteroendocrine cells by synaptophysin and Paneth cells by lysozyme, all revealing no differences between both groups (Figure 7A–C).
Staining for smooth muscle actin indicated no alteration in the stromal composition of the intestinal tumors upon transgenic Wnt5a induction (Figure 7D). Also, epithelial E-cadherin was unaltered by induced Wnt5a, not suggesting any effect on epithelial-to-mesenchymal transition (Figure 7E). Taken together, despite the many processes in which Wnt5a has been implicated, our data demonstrate that Wnt5a does not affect the formation, growth, cellular characteristics or malignancy of Apc-driven intestinal tumors in mice, showing that Wnt5a by itself is not capable to initiate metastasis to distant organs.

Discussion

Given the controversial indications with regard to a possible role for WNT5A in colon cancer, we aimed to gain more insight herein. We investigated this using expression profiling data sets, alteration of WNT5A expression in human colon cancer cells and our previously generated inducible Wnt5a transgenic mouse model. Our data suggested that enhanced WNT5A expression is associated with poor prognosis in human colorectal cancer patients. We found that in human colon cancer cells, WNT5A promotes directional cell migration, probably through regulating the formation of focal adhesion sites, and invasion. However, no discernible consequences on intestinal tumorigenesis were observed when enhanced Wnt5a expression was induced in adenomas of Apc1638N mice, providing evidence that increased Wnt5a by itself is not sufficient to promote invasion and metastasis in the genetically modified Apc1638N mouse model.

Our WNT5A expression analysis indicated that enhanced WNT5A expression is associated with poor prognosis in human colorectal cancer patients. The location of WNT5A expression in these tumors is undefined and discussion exists considering the main source of WNT5A in colorectal tumors. Most reports described an increase in WNT5A RNA expression produced by the stromal cells of the tumor, following an augmenting trend during progression from normal intestine through adenoma to carcinoma (20–24). These studies suggest a tumor-promoting role for WNT5A. On the other hand, Dejmek et al. (25) described expression of WNT5A protein in the epithelial compartment of colorectal tumors and suggested that reduced epithelial WNT5A expression is associated with tumor progression. In this latter report, epithelial expression was detected using a home-made polyclonal antibody. However, epithelial WNT5A expression has not been validated by an independent report using alternative antibodies. We did not succeed in detecting WNT5A expression in human colorectal tumors using a generally used commercial Wnt5a antibody (data not shown). Supporting the reports indicating enhanced stromal WNT5A expression, we were able to verify enhanced endogenous Wnt5a protein expression in the stroma of Apc1638N mouse intestinal tumors (Figure 6A). Furthermore, the majority of the human colorectal cancer cell lines, all derived from the epithelial compartment, do not express WNT5A (Figure 1B and C); during mouse gut development, Wnt5a expression is typically restricted to the mesenchyme (38). Altogether, most indication exists for the upregulation of Wnt5a expression in the stromal compartment of intestinal tumors.

The majority of colorectal tumors acquire mutations resulting in aberrant activation of the Wnt/β-catenin signaling pathway (1). WNT5A has been attributed the capacity to modulate canonical Wnt/β-catenin signaling, mostly in an inhibiting manner (36,37,39,40). Although the exact mechanism is incompletely understood, Topol et al. (37) have indicated that the inhibitory action by WNT5A is dependent on intact APC. As most colorectal cancers acquire loss of function mutations in both copies of the APC gene, WNT5A is not expected to grossly affect intestinal tumor growth through modulation of β-catenin signaling within colorectal tumor cells. Accordingly, we observed that WNT5A knockdown in SW480 cells does not affect intrinsic Wnt/β-catenin signaling, and we observed no changes in staining for β-catenin and its target Cyclin D1 in Apc1638N intestinal tumors following transgenic Wnt5a expression. This was further confirmed by the unaltered...
expression of the β-catenin target gene Axin2 upon Wnt5a exposure to cell lines derived from these tumors. Our data are in line with those of Topol et al. (37), who observed unaffected Wnt/β-catenin signaling following ectopic Wnt5a expression in SW480 cells. Few exceptions have been reported demonstrating that WNT5A inhibits intrinsic β-catenin signaling in colorectal cancer cell lines. However, these cell lines express either full length APC (HCT116 and SW48) or a long truncated APC protein with a considerable level of residual activity (HT29) (26,37,41). Wnt/β-catenin signaling is an important determinant of intestinal cell proliferation and tumor initiation. Corresponding with the observed unaffected Wnt/β-catenin signaling in intestinal cancer cells, we observed no discernible alterations in intestinal tumor-cell proliferation following WNT5A knockdown in SW480 cells or Wnt5a induction in intestinal tumors in vivo. Accordingly, tumor initiation in the gastrointestinal tract and extra-intestinal tissues was not affected by induced Wnt5a expression.

In addition to unaltered tumor formation, induced Wnt5a caused no gross differences in tumor composition or in the degree of malignancy of the Apc1638N intestinal tumors. Moreover, no metastases to distant organs were observed. Although we clearly demonstrated that in vitro WNT5A promotes the formation of focal adhesions, directional migration and invasion of colon cancer cells, these activities of Wnt5a appear not sufficient to augment tumor malignancy or metastasis by itself in Apc1638N mice. Despite the relevance of Apc-mutant mouse models to study intestinal tumorigenesis in a representative in vivo context, to date no genetically modified mouse models for intestinal cancer have been reported that show robust metastasis to distant organs. One possible explanation for this lack of metastases is the absence of spontaneous somatic mutations in the Ras and P53 genes in Apc1638N intestinal tumors (42,43), which typically contribute to the progression of human colorectal cancers (44), and are also present in SW480 cells. We postulate that these additional mutations might be required to cooperate in inducing the tumor-promoting effect of enhanced Wnt5a expression. Proceeding on this line of reasoning, we provided evidence that WNT5A clearly promotes the invasion capacity of SW480 and HCT116 colon cancer cells. However, whereas WNT5A promotes the invasion capacity of SW480 cells, it did not affect that of HCT116 cells. Apparently, Wnt5a was not sufficient to provide these poorly dispersing HCT116 cells with invasive capacity, despite that Wnt5a clearly promoted their directional migration two dimensionally. Different mutation profiles and cellular characteristics most likely underlie this difference in dispersion capacity. Although both SW480 and HCT116 cells harbor KRAS mutations, SW480 cells carry P53 mutations in addition and express less E-cadherin, properties providing the SW480 cells with more intrinsic tumor progressive potential than HCT116 cells.
Wnt5a in intestinal cancer

Expanding the already existing data on WNT5A activity in cellular migration and invasion in various cell types(3,7,11–13,15–17), we now demonstrated a promoting role of WNT5A in the directional migration and invasion of colon cancer cells. Using SW480 and HCT116 colon cancer cells, we covered both mutation types underlying aberrant β-catenin signaling commonly observed in colorectal cancer, being APC and CTNNB1 mutant respectively. Also, these cell lines both express Ror2 protein endogenously (Supplementary Figure S2C, available at Carcinogenesis Online), leaving open the possibility that the migration defects might be Ror2-mediated. However, this remains to be verified for colon cancer cells specifically. Comparable tumor-promoting activities of WNT5A have also been reported for gastric cancer cells and melanoma cells(13,15–17). To elucidate how WNT5A regulates directional migration, we investigated adhesion characteristics of the cells. Although initial cell-substrate adhesion directly after seeding appears not grossly affected by WNT5A knockdown, our data show that WNT5A is needed for the local formation of cellular focal adhesion sites. Hence, in WNT5A knockdown SW480 cells, F-actin, p-FAK and p-paxillin present in a more diffusely distributed pattern, instead of focal adhesion sites. It has been indicated in other cell types that enlargement of cellular adhesion sites is associated with reduced turnover of adhesion sites and concomitantly, with reduced cellular migration (15,45,46). Since we observed that WNT5A promotes the focal formation of adhesion sites, this activity of WNT5A likely explains the reduced directional migration of Wnt5a knockdown colon cancer cells.

In conclusion, our study has confirmed enhanced stromal Wnt5a expression in mouse intestinal tumors and has shown an association between increased WNT5A expression and poor colon cancer prognosis. Induction of increased Wnt5a expression in Apc1638N mice did not alter intestinal tumorigenesis, indicating that induced Wnt5a is not sufficient by itself to augment tumor malignancy or induce metastasis during Apc-driven intestinal cancer in mice. However, our data demonstrated that WNT5A promotes adhesion sites to form focally and stimulates directional migration and invasion of colon cancer cells. These properties can obviously contribute to local invasion and metastasis and thereby to colon cancer progression. Above all, these WNT5A activities can explain the poor prognosis associated with increased WNT5A expression and propose WNT5A as a potential candidate target for therapy in colon cancer. A genetically modified mouse model of intestinal cancer that exhibits metastasis to distant organs would be very useful to further unravel the capacities of Wnt5a in intestinal cancer.

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

Conflict of Interest Statement: None declared.

References

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