Reelin-Dab1 Signaling System in HumanColorectal Cancer

José Manuel Serrano-Morales, María Dolores Vázquez-Carretero, María José Peral,* Anunciacion Ana Ilundáin, and Pablo García-Miranda

Departamento de Fisiolog´ıa, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain

Reelin is an extracellular matrix protein that plays a critical role in neuronal migration. Here we show that the mucosa of human colon expresses reelin, its receptors ApoER2 and VLDLR, and its effector protein Dab1.
Immunohistochemical analyses reveal that reelin expression is restricted to pericryptal myofibroblasts; Dab1 is detected at myofibroblasts, the apical domain of surface epithelial and crypt cells, and a strong linear staining is observed at the basement membrane; VLDLR and ApoER2 are in the cytoplasm of surface epithelium and myofibroblasts, and VLDLR is also detected in the cytoplasm of the crypt cells. Human colorectal cancer downregulates reelin without change in vimentin or N-cadherin mRNA levels. Decreased Reelin mRNA expression is accompanied by decreased HIC1 mRNA levels, increased mRNA levels of ApoER2 and DNMT1, increased reelin hypermethylation and no change in either Cask or TGF-b1 mRNAs, suggesting that reelin repression results from a DNMT1-mediated hypermethylation of the reelin gene promoter. Decreased HIC1 expression may repress reelin transcription via increasing ApoER2 transcription. We conclude that the mucosa of human colon expresses the reelin-Dab1 signaling system and that reelin is repressed in colorectal cancer before epithelial-mesenchymal transition has occurred. The significant down-regulation of reelin expression makes this gene a promising biomarker for colorectal cancers. © 2016 Wiley Periodicals, Inc.

Key words: reelin; ApoER2; colon; adenocarcinoma; cancer

INTRODUCTION

In brain the reelin signaling system is essential for proper positioning of migrating neurons during the development of the central nervous system [1], but its role in peripheral tissues is not well understood. We have previously reported that rat small intestine expresses reelin, its receptors VLDLR (very low density-lipoprotein receptor) and ApoER2 (apolipo- protein E receptor 2), and its cytosolic effector protein Dab1 (Disabled-1) [2] and that reelin deficiency alters the balance between cell proliferation, differentia- tion, and apoptosis of the crypt-villus unit in mice [3]. As reelin mRNA and protein expression is restricted to the subepithelial

myofibroblasts and the other com- ponents of the signaling system are localized to the epithelium cells and myofibroblasts [2], we suggested that the reelin released to the extracellular matrix by the myofibroblasts might act on the epithelial cells and regulate the dynamics of the crypt-villus unit. Mice lacking reelin present the following additional intestinal modifications: i) Reduced number of Paneth cells [3], cells that secrete antibacterial molecules into the crypts lumen, thus contributing to maintenance of the gastrointestinal barrier, though they have also been implicated in the development of inflammatory bowel disease [4]. ii) Expanded extra- cellular space of the adherens junctions and desmo- somes [3], which may favor exposure to external pathogens or/and indicate loosening of the junction. iii) Reduced intestinal expression of a large number of genes that code for proteins involved in the intestinal immune response, inflammation, and cancer pro- cesses [5]. All of these observations, together with the loss of control of epithelial cell proliferation, differ- entiation and apoptosis, prompted us to suggest that reelin may protect the intestine from the develop- ment of pathologies.

The purpose of the current work was to find out whether reelin plays a role in the development of colon pathologies. We have evaluated the expression of the reelin-Dab1 signaling system in the mucosa of the human normal colon and in human colorectal adenocarcinoma.

A preliminary report of some of these results was published as an abstract [6].

MATERIALS AND METHODS

Chemicals

The antibodies and dilutions used were: the anti-reelin (clone 142) obtained from Calbiochem, Darmstadt, Germany and anti-reelin (H-221) from Santa Cruz Biotechnology, Inc., Dallas, TX, both at 1:100 dilution for immunostaining assays, the anti-reelin (clone 142) obtained from Chemicon International Inc., Billeria, MA at 1:1000 dilution for Western blot, the anti-Dab1 from Chemicon International Inc. at 1:200 dilution, the anti-VLDLR and anti-ApoER2 from Santa Cruz Biotechnology, Inc. at 1:50 dilution, the anti-a-SMA (a-smooth muscle actin) from Sigma–Aldrich at 1:200 dilution, the anti- E-cadherin from BD Transduction Laboratories, UK at 1:200 dilution and the anti-GAPDH from Sigma– Aldrich at 1:6000 dilution. Unless otherwise stated, the other reagents used in the current study were obtained from Sigma–Aldrich, Spain.

Human Tissue Samples

Samples from colorectal adenocarcinoma and from normal colon, located away from the adenocarci- noma, were obtained from 11 patients, 4 females, and 7 males, between 50- and 80-year-old, who had undergone colon resection at

Valme Hospital, Sevilla, Spain, between 2009 and 2010. The tissues collected were immediately either frozen in liquid nitrogen for RNA extraction or fixed in PBS containing 4% paraformaldehyde overnight, at 48C, for immunohistochemical assays. Tissues were subjected to a pathological examination at the Hospital to confirm the diagnosis of colorectal adenocarcinoma. Representative pictures of 7 mm cryosections of normal and pathological colon, stained with haematoxylin, are showed in the Figure 1, where it is clear the disruption of the normal structure of the colon mucosa in the adenocarcinoma samples. The study was approved by the Ethic Committees of Sevilla University and of Nuestra Señora de Valme Hospital. Informed consent was obtained from all patients.

Relative Quantification of Real-Time PCR

Total RNA was isolated from the tissue samples using the method described by Chomczynski and Sacchi [7] and RT-PCR was performed on total RNA as previously described [8]. Sample purity was assessed by spectro- photometric measurement of $OD_{260/280}$. RNA integrity was analysed by visual inspection after electrophoresis on agarose gel in the presence of ethidium bromide. cDNA was synthesized from 1 mg of total RNA using the QuantiTect[1] reverse transcription kit (Qiagen, Hilden, Germany), as described by the manufacturer. The primers were chosen according to the human cDNA sequences entered in Genbank and designed using PerlPrimer program v1.1.14 (Parkville, Vic., Australia) (see Table 1). Real-time PCR was performed with 10 ml iQ^{TM}SYBR[1] Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA), 0.4 mM primers and 1 ml cDNA. Controls were carried out without cDNA. Amplifica- tion was run in a MiniOpticon^{TM} System (Bio-Rad) thermal cycler (948C/3 min; 35 cycles of 948C/40, 588C/40, and 728C/40 s, and 728C/2 min). Following amplification, a melting curve analysis was performed by heating the reactions from 65 to 958C in 18C intervals while monitoring fluorescence. Analysis confirmed a single PCR product at the predicted melting temperature. The PCR primers efficiencies ranged from 90% to 110%. The cycle at which each sample crossed a fluorescence threshold, Ct, was determined and the triplicate values for each cDNA were averaged. Analyses of real-time PCR were done using the comparative Ct method, with the Gene Expression Macro software supplied by Bio-Rad. b- actin served as reference gene and was used for samples normalization. The $2^{-D\acute{C}T}$ method was used to validate b-actin as internal control gene [9].

Immunostaining Assays

The cell localization of reelin, Dab1, VLDLR, ApoER2, and a-SMA proteins was performed by immunostaining assays as previously described [2]. Briefly, 7 mm thick cryosections of normal colon and colorectal cancer samples were cut and applied to Superfrost Plus slides. The slides were washed with PBS,

permeabilized with 1% Triton X-100 for 15 min and washed in PBS for 5 min three times. The sections were blocked with 5% bovine serum albumin (BSA), 3% fetal calf serum (FCS) in PBS for 1 h and incubated overnight at 48C with the indicated commercial primary antibodies, diluted in PBS containing 5% BSA and 3% FCS. Controls were carried out without primary antibody. Antibody binding was visualized with FITC-conjugated (Jackson Immuno Research Inc., West Grove, PA) either anti-rabbit IgG or anti- mouse IgG, Alexa Fluor 546 anti-mouse IgG (Life Technologies, Waltham, MA) or biotinylated secondary antibodies (Dako, Glostrup, Denmark), followed by immunoperoxidase staining using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) and 3,3^{0}-diaminobenzidine. Nuclei were visualized with Hoechst 33258. The slides were mounted and photographed with either a Zeiss Axioskop 40 microscope (Zeiss, Gottingen, Germany), equipped with a SPOT Insight V 3.5.4.1 digital camera (Diag- nostic Instrument, Inc., Sterling Heights, MI) or an Olympus BX61 microscope equipped with an Olym- pus DP73 camera. Acquired images were analysed by using Spot Advance 3.5.4.1 Program analysis (Diagnostic Instrument, Inc).

Western Assays

SDS–PAGE was performed on a 4–15% gradient precast polyacrylamide gel (BioRad) for reelin. The lysis buffer contained: 150 mM NaCl, 2 mM EDTA, 10 mM EGTA, 1% NP-40, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 10 mg/ml leupeptin, 50 mM Tris-HCl pH 7.5. Protein was extracted from the desired tissue as previously described [8]. Briefly, the tissue samples were homogenized in lysis buffer with a polytron homogenizer and incubated at 48C for 10 min on a rotating shaker, followed by centrifugation at 14,000*g* for 30 min. The resultant supernatant was dissolved in the Laemmli sample buffer. A total of 15 mg were loaded to each lane, electrophoresed, electrotransferred onto a nitrocellulose membrane and the immunoreactive bands were viewed using a chemiluminescence procedure (GE Healthcare, Little Chalfont, UK). Anti-GAPDH antibody was used to normalize band density values. The relative abundance of the bands was quantified using the Image J program version 1.46 (National Institutes for Health, http://rsb. info.nih.gov/ij/index.html). Protein was measured by the Bradford method [10] using gamma globulin as the standard.

Reelin Promoter Methylation Analysis

Methylation status of the 5^{0}CpG island of reelin was determined by methylation-specific PCR (MSP) as previously described by Stein et al. [11]. Genomic DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen) as described by the manufacturer. Five hundred nanogram of genomic DNA was modified by sodium bisulfate usinga Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA) and

diluted in 10 ml of elution buffer. Two microliter of sodium bisulfate modified DNA was used in each PCR reaction. Primer sequences for reelin MSP were GGGT GTT TTT TTA GGT TTG GTT (forward) and CCA AAA AAT TCC ACAAAA AAC AAC (reverse) for unmethylated reactions (156 base pairs) and GGC GTT TTT TTA GGT TTG GTC (forward) and CGA AAA ATT CCG CGA AAA ACG (reverse) for methylated reactions (155 base pairs). PCR was performed using MyFi DNA Polymerase (Bioline, UK) and the amplification was run in a MiniOpticon™ System (BioRad) thermal cycler (948C/1 min; 40 cycles of 948C/15, 608C/15, and 728C/30 s, and 728C/5 min).

Products were separated on 2% agarose gel and visualized by RedSafe (Intron Biotechnology) staining. Methylation levels were calculated as the ratio of the gray scale value of the methylated band to that of the combined methylated and unmethylated bands using the Image J program version 1.46 (National Institutes for Health).

Statistical Analysis

Data are presented either individually or as a mean S.E.M. Comparisons were evaluated by the two-tailed Student's *t*-test. Differences were set to be significant only for $P < 0.05$. Pearsonścorrelation and Spearmanś analysis for nonparametric correlation were performed using GraphPad Prism 6 software. Significant correlations were set for $P < 0.05$.

RESULTS

Reelin, Dab1, VLDLR, and ApoER2 Expression in Human Colon

The work was started examining the presence of reelin, Dab1, VLDLR, and ApoER2 mRNAs in the mucosa of human colon. a-SMA mRNA was evaluated as a marker of myofibroblasts. Real-time PCR results indicate that reelin, Dab1, VLDLR, and ApoER2 are expressed in the human colon (Figure 2A).

Reelin mRNA abundance plotted versus a-SMA mRNA levels (Figure 2B) reveals that the relationship between the two parameters follows a Pearsonś correlation (r 0.966, ¼ P 0.0004), indicating that reelin mRNA abundance increases as a-SMA mRNA abundance increases.

The cell localization of the reelin-Dab1 signaling system in the mucosa of human normal colon was determined by immunohistochemistry, using the corresponding primary antibody and 7 mm cryosections of human colon as described in Methods. Figure 3 shows that the specific signal produced by the anti-reelin antibody is observed only in pericryp-tal myofibroblasts, a cell layer underneath the epithelial cells that also reacts to a-SMA. Immuno- reaction to Dab1 protein is observed at the pericryptal myofibroblasts, the apical domain of surface epithelium, crypt cells, and a very strong staining is seen at the basement membrane. VLDLR and ApoER2 proteins are detected at the cytoplasm of surface epithelium, crypt cells, and

myofibroblasts. Specific labeling is not detected in the absence of the corresponding primary antibody. All these observations demonstrate that reelin expression in the mucosa of the human colon is restricted to the pericryptal myofibroblasts, whereas the other com- ponents of the reelin signaling system studied are located at the surface epithelium, crypt cells and myofibroblasts.

Localization of Reelin, Dab1, ApoER2, VLDLR, and E- Cadherin Proteins in Human Normal Colon and Colorectal Cancer

The location of reelin-Dab1 signaling system and that of E-cadherin was determined by immunohistochemistry on paired sections of normal colon and adjacent tumor. The results shown in Figure 4 reveal that reelin expression is restricted to pericryptal myofibroblasts,and is no longer detected in the adenocarcinoma. The other components of the reelin signaling system studied are expressed in the tumor, but the tissue is so disorganized that is not possible to establish a specific cell location. E-cadherin staining is mainly located at the lateral membranes of neighboring cells in normal colon and in those areas of the adenocarcinoma where the epithelial structure is maintained, but where the tissues are disorganized its cell location is not evident. Specific labeling is not observed in the absence of the corresponding primary antibody.

Expression of Reelin-Dab1 Signaling System Genes in Human Normal Colon and in Colorectal Cancer

To determine whether the lack of immunoreactive reelin signal found in the colon adenocarcinoma is due to a decrease in reelin gene transcription, we next deter- mined the reelin mRNA abundance as well as that of Dab1, VLDLR, or ApoER2. a-SMA mRNA was measured as a marker for myofibroblasts. Total RNA was obtained from normal and affected colon of the same patient. The results are given in Figure 5A as the relative mRNA abundance measured in the adenocarcinoma versus that measured in normal colon for each patient. Results reveal that the colorectal cancer markedly reduces reelin expression, being undetected in two patients; increases expression of ApoER2 and Dab1, and does not significantly modify expression of VLDLR. a-SMA mRNA abundance is slightly decreased.

We analyzed the correlation between reelin mRNA abundance and that of the other genes under study and found that reelin mRNA levels versus those of ApoER2 follow a negative Spearman's correlation, (r 0.55 and P 0.04), indicating that as ApoER2 expression increases that of reelin decreases (Figure 5B). No correlation was found between reelin expression and that of either Dab1, VLDLR (data not shown), or a-SMA (Figure 5B). The loss of the positive correlation between reelin and a-SMA mRNAs abundance found in normal colon indicates that the adenocarcinoma-related decrease in reelin expression results from

repression of reelin gene transcription.

Western Blot of Reelin

To determine whether the decrease in reelin gene expression leads to a decrease in reelin protein levels, we performed Western blot assays on protein extracted from paired normal colon and adenocarcinoma. The results (Figure 6) reveal that the band detected by the anti-reelin antibody has an apparent molecular mass of approx. 420 kDa that corresponds to the full-length reelin. The intensity of this band decreases in adenocarcinoma samples, corroborating the mRNA data.

Expression of E-Cadherin, Vimentin, and N-Cadherin mRNAs in Human Normal Colon and in Colorectal Cancer

To determine if epithelial–mesenchymal transition (EMT) has occurred in the human colon adenocarcinomas under study, we measured the abundance of E-cadherin, vimentin, and N-cadherin in normal and affected tissue. The results in Figure 7 reveal that the adenocarcinoma tissue has reduced levels of E-cadherin mRNA whereas neither vimentin nor N-cadherin mRNA levels are modified as compared with normal tissue. The results also reveal that, for each mRNA sample examined, the stromal contribution has not significantly changed.

Regulation of Reelin Gene Expression

To investigate the mechanism(s) underlying the colorectal cancer-related down regulation of reelin, the relative mRNA levels of Cask (calcium/calmodulin-dependent serine protein kinase 3), TGF-b1 (Transforming growth factor beta 1), DNMT1 (DNA (cytosine-5)-methyltransferase 1), and HIC1 (Hyper- methylated in cancer 1) were measured in normal and affected colon from the same patient. The results are given in Figure 8. No significant differences are observed in the mRNA levels of either Cask or TGF- b1 between the two types of tissues, but in colorectal cancer the abundance of DNMT1 mRNA is significantly increased and that of HIC1 is significantly reduced when compared with normal tissue.

We next determined whether the carcinoma- induced decrease in reelin expression was due to methylation of the reelin promoter. The results given in Figure 9 reveal that the normal tissue and the adenocarcinoma yielded both methylated and unmethylated products and that the methylated products were significantly higher in the adenocarcinoma than in normal tissue.

DISCUSSION

Colorectal adenocarcinoma, the primary cause of cancer death throughout the world, is a multifactorial disease involving the interaction between a large number of genes and their environment. It develops through a gradual accumulation of genetic and epigenetic changes, leading to the transformation of normal colonic mucosa into invasive cancer. Herein we show that the reelin expression is reduced in colorectal cancer.

The current work reveals that the mucosa of human colon expresses the reelin-Dab1 signaling system and that, as found in rodent small intestine [2], reelin protein is only detected at the pericryptal myofibro- blasts, the mucosal cells also reactive to anti-a-SMA antibody. Bo6ttner et al. [12] did not detect reelin at the myofibroblasts of human colon, it was detected at the enteric nerve plexuses of the muscle layer, a layer that has not been the subject of the current study. Dab1, VLDLR, and ApoER2 are present at the surface epithelium, crypts cells and myofibroblasts and a very strong immunoreactive signal of Dab1 is also be added to the list of extracellular matrix components produced by the myofibroblasts. The great repertoire of substances secreted by the subepithelial myofibroblasts allows them to orchestrate functions that range from control of epithelial renewal processes to peripheral immune tolerance [13,14] and to participate in the underlying pathophysiology that characterizes ulcerative colitis and colorectal cancer [14–16]. The current results suggest that colorectal cancer changes the phenotype of the myofibroblasts to cells expressing less reelin. Thus, colon adenocarcinoma reduces reelin mRNA and protein abundance, and eliminates the positive detected at the epithelial basement membrane that is located between epithelial cells and myofibroblasts [13]. Since Dab1 is a cytosolic protein, the latter signal must correspond to Dab1 present at the basal domain of the surface epithelium, at the myofibroblasts and/or other mesenchymal cells. Together these observations suggest that reelin could have paracrine actions on the epithelial cells and autocrine actions on myofibroblasts and that it could lineal correlation observed between a-SMA and reelin in normal tissue. Colorectal cancer also increases ApoER2 mRNA abundance and slightly increases that of Dab1. Reduction in reelin expression has been found in several cancers [11,17–20] and this loss has been associated with poor cancer prognosis and increased metastasis [17,19,20]. However, in esophageal and prostate cancers reelin expression was upregulated [21,22]. Up-regulation of ApoER2 has been observed in lung cancer [23] and Dab1 down- regulation has been found in several cancers, especially in brain and endometrial cancer [24].

Transcription factors and epigenetic mechanisms, such as methylation of the gene promoter region, regulate reelin expression [25]. Reports have shown that in esophageal carcinoma, Cask [26], and TGF- b1 [21] up-regulate and down-regulate, respectively, reelin transcription and the association of Cask to Tbr- 1 (T-

box brain1) is required to activate reelin gene expression in brain [27]. However, neither CASK nor TGF-b1 seems to mediate the adenocarcinoma-related reelin repression because it occurs without change in the mRNA levels of either factor. The downregulation of reelin is accompanied by both increased expression of the DNMT1 gene and hypermethylation of the reelin promoter region, suggesting that reelin repression results from DNMT1-dependent hypermethylation. These observations agree with those showing enhanced expression of DNMT1 in human colorectal cancer [28,29], reelin gene hypermethylation in cancers [11,18] and DNMT1-mediated reelin hyper- methylation in the brain of patients with schizophrenia [25,30–32].

Reelin repression is also accompanied by an increase in ApoER2 mRNA abundance and a decrease in that of HIC1. Reports have shown that reelin transcription is directly reduced by ApoER2 overexpression [33] and that HIC1 directly represses ApoER2 transcription whilst HIC1 absence up-regulates ApoER2 [34]. These observations suggest that in the colon, tumor-induced reduction in HIC1 expression might activate that of ApoER2, which in turn may suppress reelin expression.

The epithelial–mesenchymal transition (EMT) is a fundamental process of cancer invasion/metastasis, whereby, following a loss of E-cadherin from the cell–cell apical junctions, epithelial cells acquire expression of mesenchymal components, such as vimentin and N-cadherin, and manifest a migratory phenotype [35]. Several observations indicate that reelin repression is an early event that occurs before invasion and metastasis have taken place. Thus, i) a-SMA is up-regulated during invasion [36] because this includes the trans-differentiation of fibroblasts to myofibroblasts, but the adenocarcinoma-related reelin repression is accompanied by a slight decrease in a-SMA mRNA levels, indicating that the trans-differentiation of fibroblasts into myofibroblasts has not taken place to a great extent. ii) Reelin down- regulation occurs without change in TGF-b1 mRNA mean value, a factor that modulates the trans- differentiation of fibroblasts into myofibroblasts [36] and is associated with colorectal cancer progression in humans [37]. iii) Though the tumors have reduced E-cadherin mRNA levels there is not increase in the mesenchymal cell markers vimentin and N-cadherin.

In conclusion, as far as we know, this is the first report showing that the myofibroblasts of human colon mucosa produce reelin and that colorectal cancer represses reelin expression via DNMT1-dependent reelin gene hypermethylation. Increases expression of ApoER2 might also contribute to reelin down-regula- tion. Though the role of reelin in human colorectal cancer is still unknown, reelin expression could be used as a prognostic marker for such pathology.

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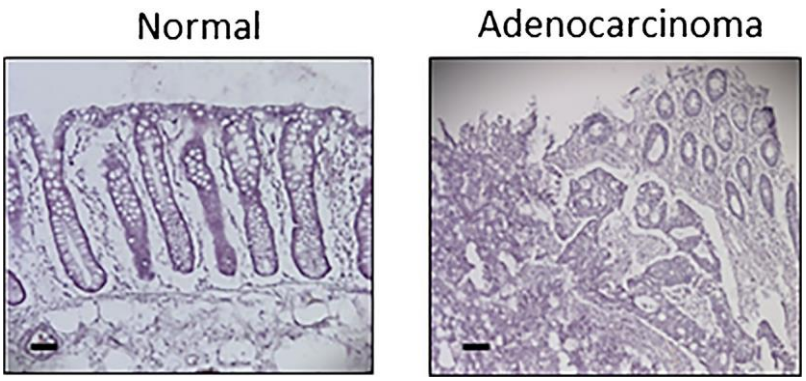

Figure 1. Images of normal and adenocarcinoma tissues from the human colon stained with haematoxylin. Scale bars represent 50 mm.

Table 1. Primers Sequences Used for Real-Time PCR

Gene	Genebank ID	Sense (5^0.. ..3^0)	Antisense (5^0..... 3^0)
Reelin	NM_005045	CCACGAGAACTGATTACCAC	ATTGTGCTGACATTGGAAGG
Dab1	NM_021080	CACACAAACTGTTATGCCTT	GTTTCATCATGGAATCTTGACATA
ApoER2	NM_000041	AGATGGGAGTGATGAAGCTG	GCAGTTCTTGGTCAGTAGGT
VLDLR	NM_003383	CCAGAACAGTGCCATATGAG	CCATTGCATACAAAGTTCCTG
a-SMA	XM_011540016	AGACTTTCAATGTCCCAGCC	CTCAGCAGTAGTAACGAAGGA
Cask	XM_011543997	TCTCCAGAAAGTGCTAACGG	ATGAAGTGTACCTTGCCTGTG
TGF-b1	NM_000660	AACCCACAACGAAATCTATGA	AAAGATAACCACTCTGGCGA
DNMT-1	NM_001130823	CAAGTTCTGCCTATCTTGTATCC	TGATGTTGAAAGTAAAGGCCTC
HIC1	NM_001098202	CGACGACTACAAGAGCAGCAGC	CAGGTTGTCACCGAAGCTCTC
E-Cadherin	NM_004360	TTTGAAGATTGCACCGGTCG	TCTCTTCTGTCTTCTGAGGC
Vimentin	NM_003380	GAGCTATGTGACCACGTCCA	TGAACTCAGTGTTGATGGCGT
N-Cadherin	NM_001792	AGTACAATATGAGAGCAGTGAG	CTGAATTGTCTTGGGAACAC

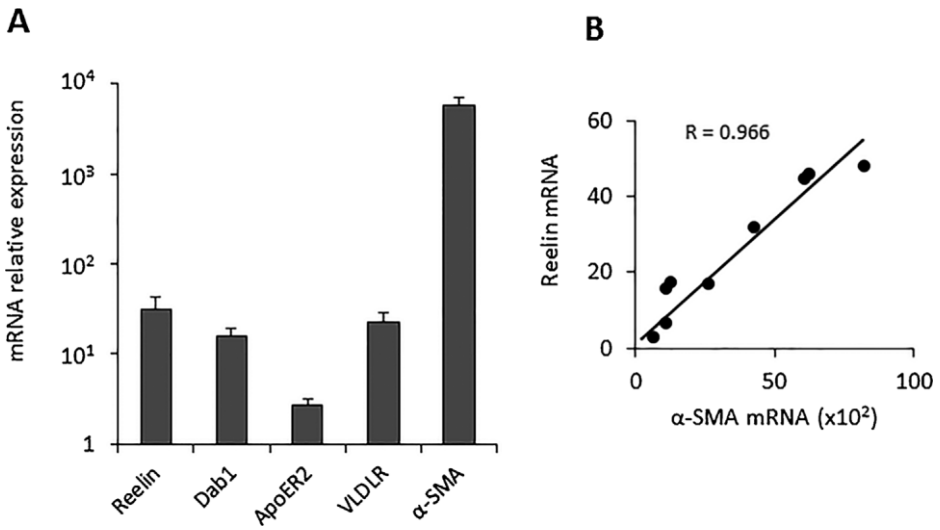

Figure 2. mRNA levels of reelin, Dab1, VLDLR, and ApoER2 in the mucosa of human normal colon. a-SMA was used as a marker of myofibroblasts. Normal tissue was obtained from 11 patients. RT-PCR was performed on total RNA isolated from the mucosa of the normal colon. (A) Relative mRNA abundance. The histograms represent the means S.E.M. of arbitrary units of mRNA abundance and are plotted on a logarithmic scale. (B) Plot of relative reelin mRNA abundance versus that of a-SMA, for each patient. Linear regression was obtained by Pearson's correlation, R ¼ 0.966.

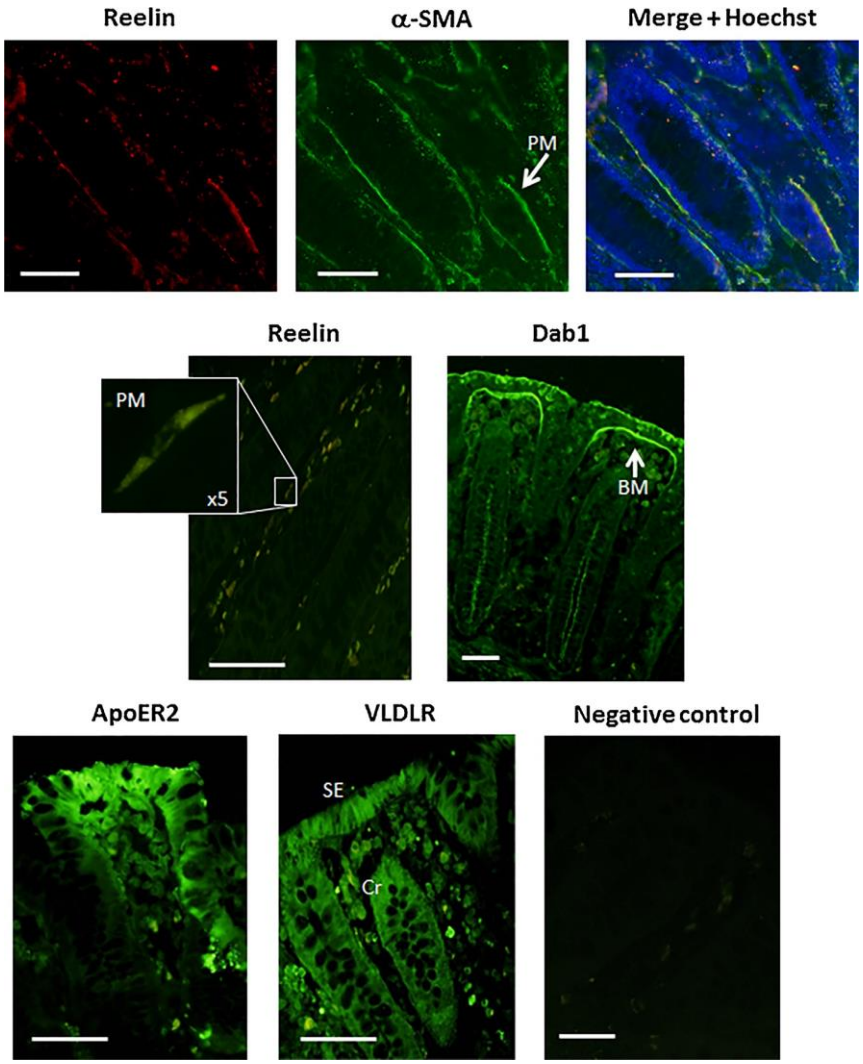

Figure 3. Immunolocalization of reelin, Dab1, VLDLR, and ApoER2 in the mucosa of the human normal colon. Seven micrometer colonsections were incubated with either anti-reelin (1:100 dilution), anti-Dab1 (1:200 dilution), anti-VLDLR (1:50 dilution), anti-ApoER2 (1:50 dilution), or anti-a-SMA (1:200 dilution) antibodies. The anti-reelin antibody H-221 was used for immunocolocalization. a-SMA was used as a marker of myofibroblasts. Nuclei were visualized with Hoechst. Scale bars represent 50 mm. The photographs are representative of three different assays. Negative controls without primary antibody were run in parallel. PM, perycriptal myofibroblasts; BM, basementmembrane; Cr, crypts; SE, surface epithelium.

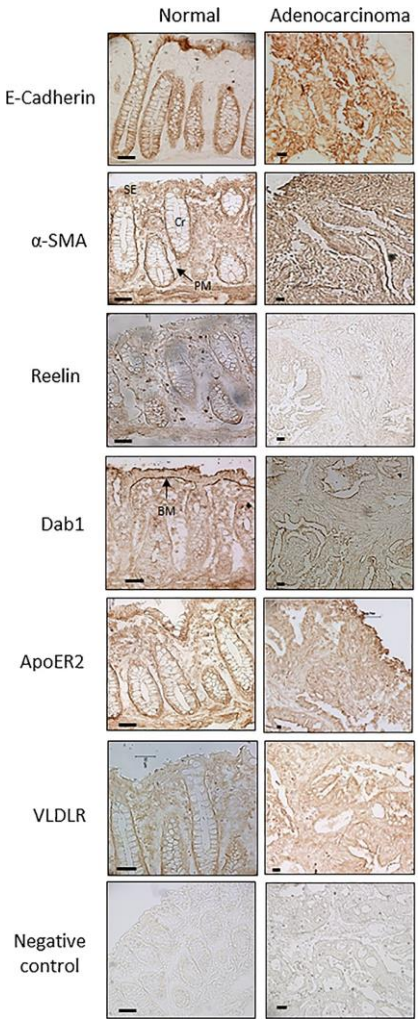

Figure 4. Immunolocalization of reelin, Dab1, ApoER2, and VLDLR proteins in human colorectal adenocarcinoma. Left panels correspond to normal colon mucosa and right panels to adenocarcinoma samples. Anti-E-cadherin antibody (1:200 dilution) was used to check the integrity of the epithelium. PM, perycriptal myofibroblasts; BM, basement membrane; Cr, crypts; SE, surface epithelium. Other details as in Figure 3

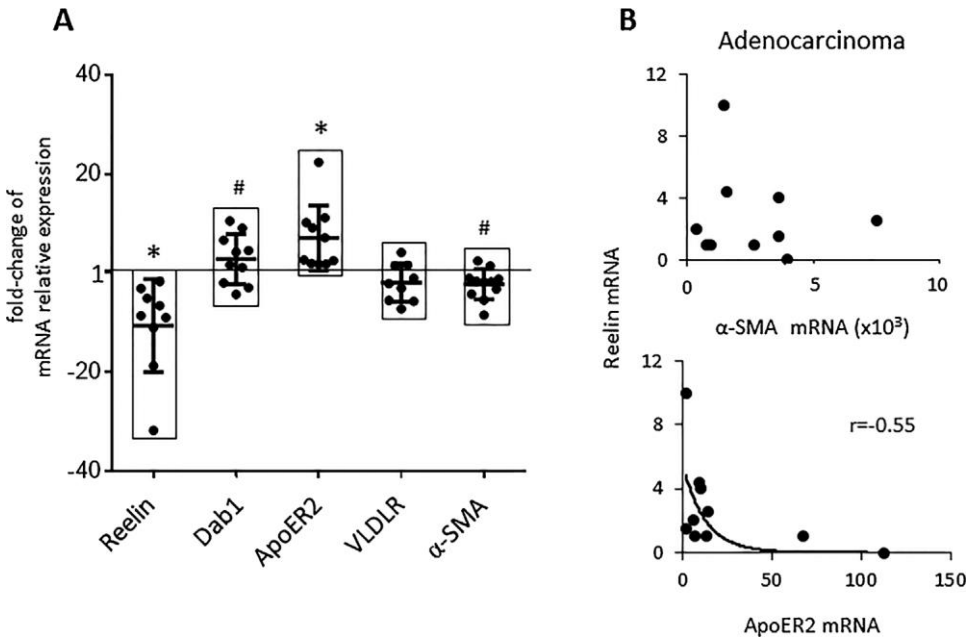

Figure 5. mRNA levels of reelin, Dab1, ApoER2, and VLDLR in human normal colon and in colorectal adenocarcinoma. (A) Ratio of relative mRNA levels in adenocarcinoma/ those in normal colon (fold change) for reelin, Dab1, ApoER2, VLDLR and a-SMA measured in each patient. $^{\omega}P < 0.001$, $^{\#}P < 0.05$, comparisons between the means S.E.M. of the mRNA abundance in adenocarcinoma versus that in normal tissue. (B) Reelin mRNA relative abundance plotted versus either that of a-SMA or ApoER2. Other details as in Figure 2.

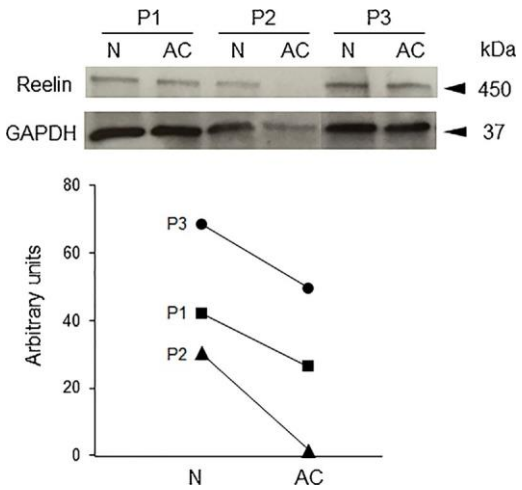

Figure 6. Western blot assays of reelin. Fifteen microgram protein extracted from normal (N) and adenocarcinoma (AC) of the same patient (P) were loaded in each lane. The blots were probed with either anti-reelin (1:1000) or anti-GAPDH (1:6000) antibodies as described in the Methods. Graph represents the relative abundance of full-length reelin normalized with GAPDH. The blot is representative of three assays.

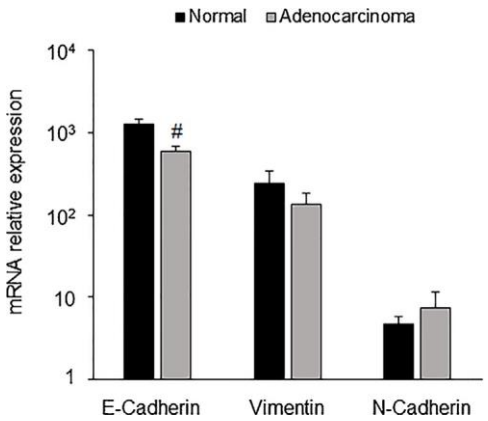

Figure 7. mRNA levels of E-cadherin, vimentin, and N-cadherin in human normal colon and in colorectal adenocarcinoma. $^{\#}P < 0.05$, comparisons between the means S.E.M. (n 4) of the mRNA abundance in adenocarcinoma versus that in normal tissue. Other details as in Figure 2. ¼

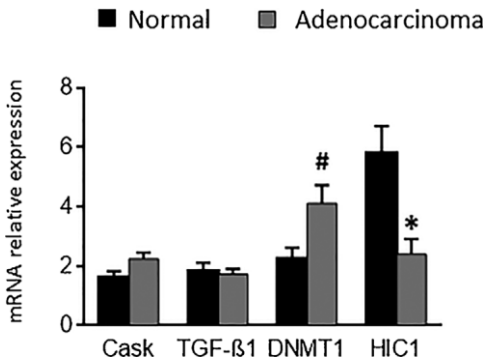

Figure 8. Regulation of reelin transcription. Relative mRNA abundance of Cask, TGF-b1, DNMT1, and HIC1 genes in normal colon and in adenocarcinoma samples. Means S.E.M. $^{\omega}P < 0.001$, $^{\#}P < 0.05$adenocarcinoma values compared with normal colon values.

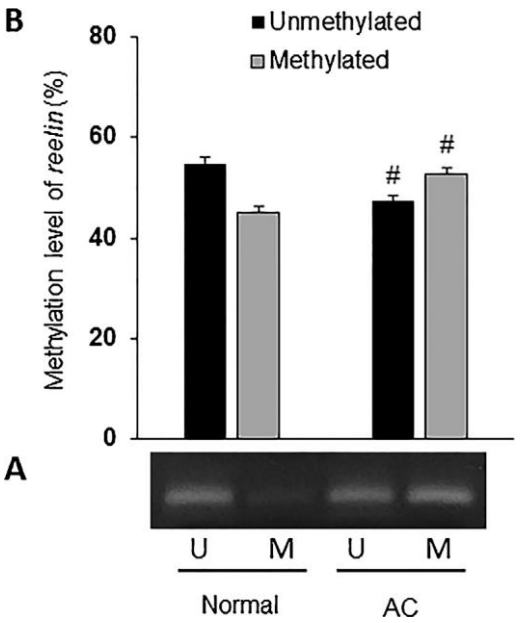

Figure 9. Reelin promoter methylation analyses. MSP analysis of reelin in adenocarcinoma (AC) and normal colon was carried out asindicated in Methods. (A) Density bands of methylated (M) and unmethylated (U) DNA. (B) Histograms are the mean values S.E.M of the methylation/unmethylated levels. Methylation levels were calculated as the ratio of the gray scale value of the methylated/ unmethylated band to that of the combined methylated and unmethylated bands. $^{\#}P < 0.05$ adenocarcinoma values compared with normal colon values. The plot is representative of three assays.