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Claudin-7 expression induces mesenchymal to epithelial transformation (MET) to inhibit colon tumorigenesis

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Abstract

In normal colon, claudin-7 is one of the highly expressed claudin proteins and its knockdown in mice results in altered epithelial cell homeostasis and neonatal death. Notably, dysregulation of the epithelial homeostasis potentiates oncogenic transformation and growth. However, the role of claudin-7 in the regulation of colon tumorigenesis remains poorly understood. Using a large colorectal cancer (CRC) patient database and mouse models of colon cancer, we found claudin-7 expression to be significantly downregulated in cancer samples. Most notably, forced claudin-7 expression in poorly differentiated and highly metastatic SW620 colon cancer cells induced epithelial characteristics and inhibited their growth in soft agar and tumor growth *in vivo*. By contrast, knockdown of claudin-7 in HT-29 or DLD-1 cells induced epithelial-to-mesenchymal transition (EMT), colony formation, xenograft-tumor growth in athymic mice and invasion. Importantly, a claudin-7 signature gene profile generated by overlapping the DEGs (differentially expressed genes in a high-throughput transcriptome analysis using claudin-7-manipulated cells) with human claudin-7 signature genes identified high-risk CRC patients. Furthermore, Rab25, a colon cancer suppressor and regulator of the polarized cell trafficking constituted one of the highly upregulated DEGs in claudin-7 overexpressing cells. Notably, silencing of Rab25 expression counteracted the effects of claudin-7 expression and not only increased proliferation and cell invasion but also increased the expression of p-Src and mitogen-activated protein kinase–extracellular signal–regulated kinase 1/2 that were suppressed upon claudin-7 overexpression. Of

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

interest, CRC cell lines, which exhibited decreased claudin-7 expression, also exhibited promoter DNA hypermethylation, a modification associated with transcriptional silencing. Taken together, our data demonstrate a previously undescribed role of claudin-7 as a colon cancer suppressor and suggest that loss of claudin-7 potentiates EMT to promote colon cancer, in a manner dependent on Rab25.

INTRODUCTION

Tight junctions (TJs) are the most apical components of the epithelial cell junctional complex and provide a form of cell–cell adhesion.[1,2] Disruption of the TJ function is associated with alterations in cellular polarity and cancer development.[3,4] In accordance, the expression of TJ-integral proteins, the claudins, is altered in multiple cancers compared with normal tissues.[5,6] Downregulation of the claudins in cancer, in general, is consistent with the disruption of TJs during tumorigenesis.[7] Among the claudin family of proteins, claudin-7 presents unique cellular distribution as it not only demonstrates apical but also basolateral membrane distribution in various epithelia, including the intestine. Notably, loss of claudin-7 expression reduces integrin α2 expression, changes its cellular distribution and disrupts the claudin-7/integrin α2/claudin-1 complexes that are present in normal intestinal epithelial cells.[8] The loss of claudin-7 expression is also associated with increased matrix metalloprotease expression, intestinal epithelial tissue damage, mucosal inflammation and death.[8]

Rab proteins regulate polarized trafficking to the cell membrane and therefore represent potential regulators of the neoplastic transition. Notably, studies have demonstrated a tumor-suppressor function for Rab25 in the intestinal neoplasia and its loss is associated with poorer patient prognosis.[9] Rab25 expression is also reduced in many breast tumors, and its loss in estrogen-receptor-negative breast cancer cell lines promotes aggression.[10,11]

In the current studies, using data from human patients and *in vitro* and *in vivo* data using colon cancer cells manipulated for claudin-7 expression, we demonstrate a colon cancer-suppressive role for claudin-7 and present evidence that loss of claudin-7 expression due to hypermethylation may help identify colon cancers that behave aggressively in patients. We further provide evidence that claudin-7 loss in colon cancer cells promotes mesenchymal traits through the regulation of Rab25 expression and promotes tumorigenesis. Taken together, our studies support a novel tumor-suppressor role of claudin-7 in the colon.

RESULTS

Claudin-7 shows altered and reduced expression in human colon cancer

To characterize the role of claudin-7 in colon tumor progression, we assessed its expression in a combined Moffitt Cancer Center/Vanderbilt Medical Center colon cancer expression array data set using 250 colorectal cancer (CRC) patient tumors, 6 adenomas and 10 normal adjacent tissue samples (demographics; Supplementary Table S1). Claudin-7 transcript levels were significantly decreased in adenomas and in all CRC stages compared with the normal adjacent mucosal specimen (Figure 1A), $P<0.001$. To determine whether claudin-7

protein levels also decreased similar to the mRNA level, we performed immunohistochemical staining in normal and CRC tissues to localize claudin-7 expression (11 samples). Claudin-7 was largely localized basolaterally in normal colonic mucosa as demonstrated previously (Figure 1B).[12] In contrast, tumor tissue revealed little or no detectable claudin-7 staining (72% negative cases: 8/11). These findings were well in accordance with our previous report, using immunoblot analysis, that claudin-7 decreases in CRC samples.[13] To further corroborate, we determined claudin-7 expression in the colon tumors from APC^{min} mice. Indeed, claudin-7 expression decreased drastically in the tumors and correlated with increased cytosolic β-catenin expression (Figure 1C). A similar correlation of decreased claudin-7 expression with increased cytosolic β-catenin was also observed in human CRC samples (Supplementary Figure S1). The findings above indicated that loss of claudin-7 expression associates with colon cancer progression.

Restoration of claudin-7 induces epithelial features in colon cancer cells

Above findings indicated altered and reduced expression of claudin-7 in CRC. We then asked whether claudin-7 has a causal role in modulating tumor phenotypes or is merely a bystander in the malignant transformation process. We used SW620 metastatic CRC cells that do not express claudin-7 as a model.[13] Using stable transfection, we generated vector-expressing $SW620^{control}$ and claudin-7-expressing SW620 cells (two clones: $SW620^{claudin\text{-}7(1)}$ and $SW620^{claudin\text{-}7(2)}$). Claudin-7 expressed basolaterally (Supplementary Figure S2A) and induced epithelial features in these poorly differentiated cells, as $SW620^{claudin\text{-}7}$ cells grew in flat, adherent sheets (Supplementary Figure S2B). Also, trans-epithelial resistance, a measure of mature cell–cell adhesion, significantly increased supporting formation of a mature and competent epithelium (Supplementary Figure S7). $SW620^{claudin\text{-}7}$ cells expressed E-cadherin while ZO-1 and β-catenin became predominantly membrane localized (Figure 2b). By contrast, the expression of vimentin, claudin-1, N-cadherin, Zeb1 and Snail-1 decreased sharply in $SW620^{claudin\text{-}7}$ cells (Figures 2a(i and ii)). Consistent with the epithelial characteristics, $SW620^{claudin\text{-}7}$ cells demonstrated marked downregulation of anchorage-independent growth by 60% ($P<0.001$; Figures 2c and d) as compared with control cells although the size of the colonies did not differ (Supplementary Figure S2C).

We further investigated whether claudin-7 expression suppresses cell invasion, a critical step in cancer cell metastasis, and we observed that there were more vector-transfected cells that migrated through the collagen than there were claudin-7-transfected cells ($P<0.001$; Figures 2e and f). Also, marked suppression of the proliferation and increased apoptosis accompanied claudin-7 expression in SW620 cells (Supplementary Figures S2D and E). Collectively, these findings demonstrated that claudin-7 expression can reverse mesenchymal growth characteristics of colon carcinoma cells.

Loss of claudin-7 induces mesenchymal features in epithelial cells

To confirm whether changes in claudin-7 localization and /or expression actively mediate epithelial-to-mesenchymal transition (EMT) in epithelial cells, we used a reciprocal approach by stably inhibiting claudin-7 expression in well-differentiated and claudin-7-expressing HT29 and DLD-1 colon cancer cells ($HT29^{shRNA}$ and $DLD\text{-}1^{shRNA}$). These cells

were again evaluated for epithelial–mesenchymal traits, which include (a) morphological characteristics, (b) membrane localization of the junctional proteins, (c) markers of EMT phenotype, (d) anchorage-independent growth and (e) cell invasion. Phase-contrast microscopy demonstrated spindle shapes of the $HT29^{shRNA}$ and $DLD\text{-}1^{shRNA}$ cells with long processes in comparison with the control cells (Supplementary Figure S3A). Also, vimentin, claudin-1, N-cadherin, Zeb-1 and Snail-1 expression increased, whereas E-cadherin expression was inhibited (Figures 3a(i and ii). As predicted, claudin-7 depletion led to an increase in proliferation while apoptosis decreased compared with the control cells (Supplementary Figure S3B).

Also, immunostaining demonstrated a decrease with loss of the membrane expression to redistribute in the cytoplasm of junctional proteins, including ZO-1, E-cadherin and β-catenin (Figure 3b and data not shown). Inhibition of claudin-7 expression in HT-29 and DLD-1 cells increased colony formation by 38% ($P<0.001$) and 41% ($P<0.001$), respectively (Figures 3c and d). Additionally, $HT29^{shRNA}$ cells displayed an increase in cell invasion by 59% ($P<0.001$) and by 70% in $DLD\text{-}1^{shRNA}$ cells as compared with the respective control cells (Figures 3e and f). Collectively, these observations indicated that claudin-7 negatively regulates EMT and tumorigenic programs in colon cancer cells. This finding correlates well with our published data that claudin-7 expression decreases markedly in cells overexpressing claudin-1 and the increased claudin-1 and decreased claudin-7 are associated with phenotypic features of EMT, increased colony formation and increased invasiveness.[13]

Claudin-7 expression reduced tumor xenograft growth in nude mice

To study whether claudin-7 can inhibit tumor growth *in vivo*, we injected $SW620^{control}$ or $SW620^{claudin\text{-}7}$ cells into the nude mice using subcutaneous xenograft tumor model ($n = 7$/group). As previously described, mice receiving the $SW620^{control}$ cells demonstrated tumor development 2 weeks postinjection, and the average tumor volume was $542.4 \pm 161.2\ cm^3$ after 4 weeks of growth (Figure 4a).[13] By contrast, tumors resulting from the injection of $SW620^{claudin\text{-}7}$ cells were significantly smaller with average volumes of $77.6 \pm 19.6\ cm^3$ after the same period of growth (Figure 4a). The tumor weight followed a similar pattern and was 50% lower ($P<0.05$) in mice injected with $SW620^{claudin\text{-}7}$ cells compared with those injected with $SW620^{control}$ cells (Figure 4b). Conversely, tumor volume and the weight increased significantly when claudin-7 expression was suppressed (Figures 4c and d). Specifically, the tumor volume was significantly less in the HT29 control cells $155.8 \pm 30.2\ cm^3$ ($HT29^{control}$) as compared with the HT29 cells in which claudin-7 was suppressed $464.8 \pm 108.4\ cm^3$ ($HT29^{shRNA}$) at 4 weeks after cell implantation. The resulting tumors were then evaluated for the expression of claudin-7 and E-cadherin, proliferation and apoptosis (Figures 4e(i and ii) and f (i and ii)). Importantly, there was an increased rate of apoptosis as determined by TUNEL (terminal deoxinucleotidyl transferase-mediated dUTP-fluorescein nick end labeling) staining and cleaved caspase-3 expression, while Ki-67 immunoreactivity was decreased in the tumors resulting from $SW620^{claudin\text{-}7}$ cells suggesting that claudin-7 expression restores a cell death program and inhibits proliferation. Similar to the *in vitro* findings, E-cadherin expression was robust in tumors resulting from $SW620^{claudin\text{-}7}$ cells; however, it remained markedly suppressed in $HT29^{shRNA}$ cell-

dependent tumors (Figure 4f). These data from *in vivo* xenograft tumor assays strongly supported the role of claudin-7 as a tumor suppressor.

Claudin-7 expression in human CRC and association with clinical outcomes

In the light of consistent and robust anti-tumorigenic effects of claudin-7 expression in colon cancer cells, we further determined how modulation of claudin-7 expression alters gene transcription using high-throughput transcriptome analysis to identify differentially expressed genes. RNA was isolated from exponentially growing control and claudin-7-manipulated cells under investigation and were subjected to transcriptome analysis on the Affymetrix HG-U133 Plus 2.0 platform (details in Materials and methods).[12] We queried our 250 patient CRC-patient database for claudin-7 expression to investigate potential associations with epithelial cell-specific genes. To do this, we examined differentially expressed genes that correlated with claudin-7 expression from the transcriptome analysis of 250 CRC patients.[12] An epithelial-specific gene expression profile of 113 specific probes mapped to 101 genes was generated by overlapping claudin-7 signature gene expression from claudin-7-manipulated cells with the claudin-7 signature gene expression from human samples. The resulting integrated claudin-7 gene signature was then subjected to unsupervised hierarchical cluster analysis using the 250 patient cohort data. The clustering analysis of claudin-7 signature gene expression revealed three distinct clusters (Figure 5a). We then hypothesized that the claudin-7-associated gene expression profile could identify high-risk CRC patients and tested this hypothesis by performing Kaplan–Meier analysis for patient clusters. Patients in cluster 2 (blue) were noted to have significantly better overall survival and disease-free survival as compared with the group of patients in clusters 3 (green) and 1 (red) (Figures 5b and c, $P = 0.004$, $P<0.001$, respectively).

The differential expression and the fold change of these 101 genes per cluster are displayed in Supplementary Table S2. Out of these 101 genes, we validated the change in the expression of a number of the genes that are known to be involved in colon cancer progression (Supplementary Figure S4). The expression of BMP-2, Rab25 and CD55 increased in association with claudin-7 overexpression, whereas Wasf3 and GNG4 were sharply down-regulated (Figure 5a and Supplementary Figure S5). Interestingly, the levels of Rab25 were the highest in cluster 2 patients who demonstrated better overall and disease-free survival, whereas the levels of Wasf3 and GNG4 were higher in the clusters associated with poor prognosis. Ingenuity pathway analysis also implicated Rab25 in the top network (data not shown).

Claudin-7 effects are mediated by Rab25 through extracellular signal–regulated kinase (ERK)/Src signaling

As Rab25 seem to be an important gene in the claudin-7 signature, next we wanted to determine the Rab25 mRNA and protein expression in claudin-7-manipulated cells. The quantitative PCR analysis also showed significant increase in the expression of Rab25 in $SW620^{claudin\text{-}7}$ compared with $SW620^{control}$ cells (Figure 6a). Immunoblot analysis confirmed upregulated expression of Rab25 in $SW620^{claudin\text{-}7}$ as compared with control cells (Figure 6b). Of note, the Rab family of proteins has an important role in membrane trafficking and polarity maintenance.[14,15] Notably, a key observation in our studies was the

marked changes in the membrane distribution of epithelial cell-specific proteins upon modulation of claudin-7 expression. Our findings corroborate recent studies demonstrating a tumor-suppressive function for Rab25 in the intestinal neoplasia.[9] In addition, to determine the signaling pathway involved in claudin-7 mediated effects on colon tumorigenesis, we determined the role of ERK, Src and JNK (c-Jun N-terminal kinase) activation. Although p-JNK expression was not affected, we observed marked decreases in p-ERK and p-Src levels in cells overexpressing claudin-7 (Figure 6c). To further determine the functional significance of these signaling pathways in claudin-7-induced phenotype, we inhibited the ERK1/2 (U0126; 10 uM) and Src (PP2; 10 uM) signaling. Interestingly, inhibition of the ERK signaling suppressed proliferation in both $HT29^{shRNA}$ and $DLD\text{-}1^{shRNA}$ cells. However, inhibition of the Src signaling had no effect upon proliferation (Supplementary Figures S6A (i and ii) and B (i and ii)) and thus suggested its role in other cellular functions, including invasion.

We then determined the role of Rab25 in claudin-7-dependent changes in the tumorigenic functions of colon cancer cells. In this regard, we silenced Rab25 expression in $SW620^{claudin\text{-}7}$ cells (high Rab25) using human Rab25-specific shRNA, which was confirmed by quantitative PCR (Figure 6d) and immunoblotting (Figure 6e). Inhibition of Rab25 expression resulted in increased p-Erk and p-Src expression (Figure 6e). Silencing of Rab25 also increased proliferation (Figure 6f) and invasion (Figure 6g) compared with control cells, despite constitutive claudin-7 overexpression, and thus supported the causal role of Rab25 in tumor-suppressive effects of claudin-7 expression through potential regulation of p-ERK and p-Src signaling.

Mechanism of the loss of claudin-7 expression in colon cancer

The presence of CG-dinucleotide-rich sequences in the promoter region of genes and their hypermethylation is a common feature in many cancers, resulting in transcriptional silencing of many genes.[16] We wanted to analyze the mechanism responsible for the loss of claudin-7 expression in SW620 cells (Figure 7a). The presence of CG-dinucleotide-rich sequences in the promoter region of genes is quite often a signature denoting that hypermethylation may be a potential mechanism for gene silencing.[17–19] The claudin-7 promoter contains a CpG-rich region extending from −20 to 900 bp upstream of the translational start site (accession no. 11425795). Therefore, we investigated the promoter region of the claudin-7 gene. Methylation-specific PCR analysis was performed on DNA from five colon cancer cell lines (SW480, SW620, HCT116, CaCO2 and HT29). Notably, SW620 cells that do not express claudin-7 expression demonstrated hyper-methylation at the claudin-7 promoter CpG sites (Figure 7b(i)). Conversely, claudin-7 promoter was unmethylated in claudin-7 expressing SW480, HCT116, CaCo2 and HT29 cells (Figures 7a and b(i and ii)). To test further, we subjected SW620 cells with 5-aza-2′-deoxycytidine (5-Aza-dC), a methylation inhibitor. Re-expression of claudin-7 mRNA transcript as well as protein expression in 5-Aza-treated cells further supported the role of hypermethylation in suppressing the claudin-7 expression in CRC (Figures 7b(ii) and c).

To further determine the specific role of the methylation machinery in the regulation of claudin-7 in colon cancer cells, we manipulated DNA methyltransferase 3a (DNMT3a).

Recent studies have shown that DNMT3a can regulate other claudin members and is sensitive to 5-aza-CdR.[20] In this regard, we transfected pcDNA3.DNMT3a plasmid construct in HCT15 and SW480 cells, which express low levels of DNMT3a and express claudin-7. Indeed, overexpression of DNMT3a led to a significantly decreased claudin-7 expression in both cell types (Figure 7d), suggesting key role for DNMT3a in the regulation of claudin-7 promoter methylation.

DISCUSSION

The escape of cancer cells from the primary tumor sites is a crucial step in invasion and metastasis, the major culprits behind the cancer-related deaths. The EMT is an important, however, complex phenomenon that determines the aggressiveness of cancer cells, including motility and invasion. Notably, this morphological transformation of cancerous cells is accompanied by various cellular processes; however, key importance of the alterations in cell–cell adhesion and cell matrix degradation in this regulation is well recognized.[21–23] Our findings strongly support this notion and highlight a key role of claudin-7 in the regulation of EMT in colonic epithelial cells. A colon tumor-suppressive role for claudin-7 is further supported by our findings that its expression is markedly suppressed in CRC patients as well as in colon tumors resulting from the mouse model of sporadic or inflammation-induced colon cancer. In accordance, forced claudin-7 expression in highly tumorigenic and poorly differentiated SW620 CRC cells negatively affected their ability to grow in soft agar and invade through the matrix and to induce tumor growth *in vivo*. By contrast, claudin-7 knockdown in HT29 and DLD-1 cells enhanced their tumorigenic abilities. A tumor-suppressive function for claudin-7 in colon cancer gets further support from our findings, suggesting hypermethylation may help suppress its expression in colon cancer cells. Hypermethylation of claudin-7 in colon cancer has been previously reported.[24] Similar hypermethylation of claudin-7 promoter to suppress its expression has been reported in breast cancer and squamous cell carcinoma.[25–29] Of note, hypermethylation is a cellular mechanism often employed by cancer cells to inactivate the expression of the tumor-suppressive genes.[30–32] Our data that claudin-7 was silenced during the early tumorigenesis is consistent with the notion that CpG island methylation in carcinoma occurs early during tumorigenesis.[33,34] Our data further suggest DNMT3a to be responsible for methylation to downregulate claudin-7 expression. Taken together, our current studies identify claudin-7 as a colon cancer-suppressive gene and suggest that the loss of its expression may promote EMT in colon cancer cells to promote aggressiveness.

Loss of the cell–cell adhesion in transformed cancer cells has long been studied that have now established E-cadherin, an adherent junction protein, as a robust epithelial marker and a ‘gold standard’ to define epithelial cell transformation.[35,36] Notably, our studies demonstrated a parallel correlation between claudin-7 and E-cadherin expression, as manipulation of claudin-7 expression in colon cancer cells had parallel effect upon E-cadherin expression in multiple colon cancer cell lines. Our findings get strong support from similar effect of claudin-7 expression upon E-cadherin expression in esophageal carcinoma cells in combination with suppressed cell invasion.[37] Notably, the effect of claudin-7 expression upon E-cadherin expression was unidirectional as manipulation of E-cadherin expression did not alter claudin-7 expression in the same cells.[37] Taken together, these

findings suggest a potential master regulator role for claudin-7 in the regulation of the differentiation in specific epithelial cell types. Of interest, claudin-7 is one of the highest expressed proteins in the colonic epithelium.[8,38] Such a postulation gains strong support from the fact that homozygous deletion of claudin-7 gene in mice leads to epithelial cell sloughing, inflammation and neonatal death.[8] Here it is also worthy to note that claudin-7, despite being a TJ-integral protein, is expressed in a basolateral manner in colonic epithelial cells along the cell–cell junction.[8,38] Further, claudin-7 physically associates with the α2-integrin and claudin-1, yet another TJ protein, in a protein complex, and the loss of claudin-7 disrupts this association.[8] Thus it is tempting to postulate that claudin-7 may serve as a connecting hub between the apical and basolateral domains of the cells, and the loss of its expression may modulate signaling regulated by cell–cell and cell–matrix interaction to induce transformation. Of interest, claudin-7 expression in SW620 cells suppressed ERK and Src activation, which have important roles in EMT and tumorigenesis.[39–41]

Yet another important effect of manipulating claudin-7 expression in colon cancer cells was an inverse effect upon claudin-1 expression. Importantly, claudin-1 expression is highly upregulated in colon cancer, and in our studies, using human patient samples or colon cancer cells, we have demonstrated an inverse correlation between claudin-1 and claudin-7 expression.[13,42] Most notably, in recent studies, we found that intestinal epithelial cell-specific overexpression of clauidn-1 led to the suppression of claudin-7 expression and epithelial differentiation.[42] Here, it also must be noted that manipulation of claudin-1 expression in colon cancer cells also affect E-cadherin expression, however, in an inverse manner and through the regulation of ZEB-1 expression, a transcriptional inhibitor of E-cadherin.[43] We have also reported that claudin-1 expression in colon cancer cells promotes Wnt/β-catenin signaling.[13,44] By contrast, claudin-7 expression induced membrane recruitment of nuclear/cytosolic β-catenin expression while its knockdown in DLD-1 and HT-29 cells resulted in delocalization of the β-catenin to cell cytoplasm. Of note, cytoplasmic/nuclear β-catenin induces Wnt signaling, and abnormal activation of the Wnt/β-catenin signaling pathway occurs in CRCs.[45] These results suggest that claudin-7 modulates canonical Wnt signaling and that it regulates cellular transitions between epithelial and mesenchymal phenotypes and implicates the loss of claudin-7 function as contributing to colon tumorigenesis. The recruitment of β-catenin to cell junctions could also contribute to the reduced invasive ability mediated through claudin-7 expression. Taken together, we hypothesize that a delicate balance between claudin-1 and claudin-7 dictates the epithelial differentiation in colonic epithelial cells, and disruption of this balance imbalances Wnt activation through the modulation of E-cadherin/β-catenin expression/cellular distribution to promote colon tumorigenesis.

We further utilized the comparative functional oncogenomics to connect molecular pathogenic features of human colon cancer to colon cancer cell models with a greater level of confidence to derive a claudin-7-specific gene signature. The resultant clusters discriminated the survival outcomes in human patients with colon cancer with great confidence. We have previously demonstrated that the gene-based metastasis score can identify patients with stage II and III colon cancer at greater risk of colon cancer recurrence and death.[12] Our gene cluster analysis identified a set of 101 genes, which were differentially regulated. Interestingly, the levels of Rab25 were the highest in cluster 2

patients who demonstrated better overall and disease-free survival. In addition, ingenuity pathway analysis also implicated Rab25 in the top network. Notably, Rab25, an epithelial-specific member of the Rab family of small GTPases, has been associated with several epithelial cancers. Rab25 expression is decreased in human colon cancers independent of stage and mouse models of intestinal and colonic neoplasia have demonstrated that Rab25 deficiency markedly promotes the development of neoplasia.[9] In our studies, we show regulation of Rab25 by claudin-7 that suggests an important role of claudin-7 in polarized trafficking to the cell membrane. Most notably, manipulation of Rab25 expression sufficed to overcome the effects of claudin-7 expression on p-ERK1/2 and p-Src signaling, proliferation and cell invasion, suggesting claudin-7 mediates its effect through regulation of Rab25, p-ERK1/2 and/or p-Src signaling. Of note, studies have demonstrated that increase in Rab25 in ovarian cancer cells was associated with aberrations in the cell surface localization of β1-integrin.[46] Another recent study demonstrated that the highest concentration of Rab25 expression in colonic epithelial cells exists in the transition between the crypt and the villus regions of the intestinal mucosa.[9,47] Interestingly, this is also the critical region for the upregulation of β1-integrin expression in the mucosa.[9] Thus it appears that manipulation of Rab25 levels can lead to aberrations in the trafficking of β1-integrin and perhaps other critical regulators of cell adhesion. The effects of Rab25 expression changes on integrins may also lead to alteration in the trafficking of another cargo or changing the effective concentration of direct effectors shared with Rab11a. As described, claudin-7 complexes with integrin α2 and claudin-1 in normal intestine and deletion of claudin-7 gene reduces integrin α2 expression as well as disrupts integrin α2 localization and claudin-7/integrin α2/claudin-1 complex leading to matrix metalloproteases upregulation, intestinal epithelial tissue damage and inflammatory response.[8] Therefore, we propose a possible role of the integrin–Rab25–claudin-1–claudin-7 complex in claudin-7-dependent inhibition of colon carcinogenesis. Such investigation is part of the ongoing study in our laboratory.

Taken together, in current studies, we unravel a previously undescribed role for claudin-7 in the regulation of colonic epithelial cell differentiation. Our data from gene manipulation spanning several colon cancer cell lines and the oncogenomics (combining patient and cell line data) reveal that claudin-7 may serve its anti-tumorigenic function by regulating polarized trafficking of the cell–cell adhesion proteins in a Rab25-dependent manner and/or through the regulation of Wnt-β-catenin signaling in manners dependent upon claudin-1 and E-cadherin expression levels. We further propose that Erk1/2 signaling has important role in claudin-7-dependent regulation of proliferation in colon cancer cells to promote their tumorigenic ability. Future studies in understanding the pathways involved, including Src signaling, and how they are interconnected will help us in developing new strategies for colon cancer prevention.

MATERIALS AND METHODS

Cell culture and transfection

The human colon cancer cell lines DLD-1, HT29, SW480 and SW620 were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 containing 10% fetal bovine

serum. Cells were transfected using the Effectene Transfection Reagent (QIAGEN, Valencia, CA, USA) as described previously.[13] Claudin-7-overexpressing cell population was selected using Zeocin (0.4 mg/ml), and knockdown cells were selected using puromycin (1 mg/ml).

Human tissue, microarray platforms and statistical analysis

The protocols and procedures for the procurement of human tissue samples and details of the microarray platforms and statistical analysis have been described previously.[13,44]

Immunoblot, immunohistochemistry and immunofluorescence analysis

These were performed using the standard protocols as described before.[13,44]

RNA isolation, semiquantitative reverse transcription–PCR and real-time PCR

These analyses were performed using the standard protocols.[13]

Anchorage-independent growth and invasion assay

Assays were done as described previously.[13]

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and apoptosis assays

The CellTiter 96 assay from Promega (Madison, WI, USA) was used to perform the MTT assay using the standard protocol.[13] Apoptosis was determined using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics Corp., Indianapolis, IN, USA) as per the manufacturer's protocol.[13]

Xenograft-tumor studies

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University. The tumorigenicity of cells under study was assessed using subcutaneous flank inoculation of 1×10^6 cells in 6-week-old athymic nude mice. Animals were assessed for 5 weeks after the inoculation for tumor incidence and growth and then killed.

Microarray analysis

Microarray data were normalized using the Robust MultiChip Averaging (RMA) algorithm as implemented in the Bioconductor package *Affy* (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). For pairwise group comparisons, *t*-test in the Bioconductor package *Limma* (Fred Hutchinson Cancer Research Center) was used to identify differentially expressed probe sets between the two groups under comparison (Cldn7 over-expressed versus control). The implementation of *t*-test in *Limma* uses an empirical Bayes method to moderate the s.es. of the estimated log-fold changes; this results in a more stable inference, especially for experiments with a small number of arrays. A false discovery rate of 0.005 was used as a cutoff to have significant gene expression list for the Cldn7 cell line. Cldn7-mediated human tumor profile was generated by fitting linear regression for each of the probes on the Affymetrix array to Cldn7 probe 202790_at based on Vanderbilt_Moffit 250 patient samples. We also used cutoffs of false discovery rate <0.005 to determine

significantly Cldn7-associated gene list. These genes were then overlapped with the Cldn7 expression profile from the cells to generate Cldn7 signature containing 103 probes from 88 genes. We then used the Cldn7 gene signature to execute unsupervised hierarchical cluster analysis and outcome determination. The hierarchical clustering with complete linkage and Euclidean distance was applied to generate heatmaps. The three clusters were discovered by hierarchical clustering, and Kaplan–Meier estimates were performed. The log-rank test was used to determine if there were significant differences across three clusters in terms of survival outcomes. No association was noted with grade or adjuvant treatment; however, a significant association was noted between the clusters and the stage of the patients (P = 0.02).

Methylation-specific PCR

Genomic DNA (200–500 ng) was bisulfite modified using a Zymo EZ Methylation Direct Kit (Zymo Research, Irvine, CA, USA). Modified DNA (40 ng per reaction) was amplified by PCR, using 0.2 μM of each primer, 2 units of hot start *Taq* DNA polymerase and 0.2 mM of each dNTP per reaction. Primers specific for unmethylated DNA were 5′-TGGGGAAA GGGTGGTGTTG-3′ (sense, - 831 to 812) and 5′-TTACCCAATTTTAACC ACCAC-3′ (antisense, - 670 to - 649) yielding a 182-bp product. Primers specific for methylated DNA were 5′-GACGTTAGGTTATTTTCGGTC-3′ (sense, - 550 to 529) and 5′ AAACGCGTTTCTAAACGCCG-3′ (antisense, - 350 to 330) yielding a 220-bp product. The PCR conditions were as follows: one cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °Cfor 45 s; and one cycle of 72 °C for 5 min. PCR samples were resolved by electrophoresis on a 1.5% agarose gel.

Statistical analyses

Results are expressed as mean ± s.e.m. Statistical significance was analyzed by two-tailed *t*-tests and analysis of variance when applicable using Bonferroni's correction for multiple testing. A difference of $P<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2001; 2:285–293. [PubMed: 11283726]
2. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol. 2004; 286:C1213–C1228. [PubMed: 15151915]

3. Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM. Increased tight junctional permeability is associated with the development of colon cancer. Carcinogenesis. 1999; 20:1425–1431. [PubMed: 10426787]

4. Turksen K, Troy TC. Barriers built on claudins. J Cell Sci. 2004; 117:2435–2447. [PubMed: 15159449]

5. Sheehan GM, Kallakury BV, Sheehan CE, Fisher HA, Kaufman RP Jr, Ross JS. Loss of claudins-1 and -7 and expression of claudins-3 and -4 correlate with prognostic variables in prostatic adenocarcinomas. Hum Pathol. 2007; 38:564–5692007. [PubMed: 17306334]

6. De Oliveira SS, De Oliveira IM, De Souza W, Morgado-Diaz JA. Claudins upregulation in human colorectal cancer. FEBS Lett. 2005; 579:6179–6185. [PubMed: 16253248]

7. Swisshelm K, Macek R, Kubbies M. Role of claudins in tumorigenesis. Adv Drug Deliv Rev. 2005; 57:919–928. [PubMed: 15820559]

8. Ding L, Lu Z, Foreman O, Tatum R, Lu Q, Renegar R, Cao J, Chen YH. Inflammation and disruption of the mucosal architecture in claudin-7-deficient mice. Gastroenterology. 2012; 142:305–315. [PubMed: 22044670]

9. Nam KT, Lee HJ, Smith JJ, Lapierre LA, Kamath VP, Chen X, et al. Loss of Rab25 promotes the development of intestinal neoplasia in mice and is associated with human colorectal adenocarcinomas. J Clin Invest. 2010; 120:840–849. [PubMed: 20197623]

10. Cheng JM, Ding M, Aribi A, Shah P, Rao K. Loss of RAB25 expression in breast cancer. Int J Cancer. 2006; 118:2957–2964. [PubMed: 16395697]

11. Cheng JM, Volk L, Janaki DK, Vyakaranam S, Ran S, Rao KA. Tumor suppressor function of Rab25 in triple-negative breast cancer. Int J Cancer. 2010; 126:2799–2812. [PubMed: 19795443]

12. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, et al. Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. Gastroenterology. 2010; 138:958–968. [PubMed: 19914252]

13. Dhawan P, Singh AB, Deane NG, No Y, Shiou SR, Schmidt C, et al. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest. 2005; 115:1765–1776. [PubMed: 15965503]

14. Casanova JE, Wang X, Kumar R, Bhartur SG, Navarre J, Woodrum JE, et al. Rab11a and Rab25 association with the apical recycling system of polarized MDCK cells. Mol Biol Cell. 1999; 10:47–61. [PubMed: 9880326]

15. Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. Traffic. 2006; 7:14–21. [PubMed: 16445683]

16. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res. 2001; 61:225–3229.

17. Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, et al. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. Cancer Res. 2001; 61:2782–2787. [PubMed: 11289162]

18. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, et al. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci USA. 2000; 97:6049–6054. [PubMed: 10811911]

19. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002; 3:415–428. [PubMed: 12042769]

20. Boireau S, Buchert M, Samuel MS, Pannequin J, Ryan JL, Choquet A, et al. DNA-methylation-dependent alterations of claudin-4 expression in human bladder carcinoma. Carcinogenesis. 2007; 28:246–258. [PubMed: 16829686]

21. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. J Clin Invest. 2009; 119:1417–1419. [PubMed: 19487817]

22. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009; 139:871–890. [PubMed: 19945376]

23. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002; 2:563–572. [PubMed: 12154349]

24. Nakayama F, Semba S, Usami Y, Chiba H, Sawada N, Yokozaki H. Hypermethylation-modulated downregulation of claudin-7 expression promotes the progression of colorectal carcinoma. Pathobiology. 2008; 75:177–185. [PubMed: 18550915]
25. Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, et al. Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene. 2003; 22:2021–2033. [PubMed: 12673207]
26. Usami Y, Chiba H, Nakayama F, Ueda J, Matsuda Y, Sawada N, et al. Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. Hum Pathol. 2006; 37:569–577. [PubMed: 16647955]
27. Lourenço SV, Coutinho-Camillo CM, Buim ME, de Carvalho AC, Lessa RC, Pereira CM, et al. Claudin-7 down-regulation is an important feature in oral squamous cell carcinoma. Histopathology. 2010; 57:689–698. [PubMed: 21083599]
28. Melchers LJ, Bruine de Bruin L, Schnell U, Slagter-Menkema L, Mastik MF, de Bock GH, et al. Lack of claudin-7 is a strong predictor of regional recurrence in oral and oropharyngeal squamous cell carcinoma. Oral Oncol. 2013; 49:998–1005. [PubMed: 23953778]
29. Yoshizawa K, Nozaki S, Kato A, Hirai M, Yanase M, Yoshimoto T, et al. Loss of claudin-7 is a negative prognostic factor for invasion and metastasis in oral squamous cell carcinoma. Oncol Rep. 2013; 29:445–450. [PubMed: 23229436]
30. Robertson K. DNA methylation and human disease. Nat Rev Genet. 2005; 6:597–610. [PubMed: 16136652]
31. Laird PW, Jaenisch R. The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet. 1996; 30:441–464. [PubMed: 8982461]
32. Toyota M, Ahuja N, Suzuki H, Ohe-Toyota M, Imai K, Imai K, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res. 1999; 59:5438–5442. [PubMed: 10554013]
33. Bird AP. CpG-rich islands and the function of DNA methylation. Nature. 1986; 321:209–213. [PubMed: 2423876]
34. Issa JP. Cancer prevention: epigenetics steps up to the plate. Cancer Prev Res (Phila Pa). 2008; 1:219–222.
35. Bates RC, Mercurio AM. The epithelial-mesenchymal transition (EMT) and color-ectal cancer progression. Cancer Biol Ther. 2005; 4:365–370. [PubMed: 15846061]
36. Kang Y, Massagué J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell. 2004; 118:277–279. [PubMed: 15294153]
37. Lioni M, Brafford P, Andl C, Rustgi A, El-Deiry W, Herlyn M, et al. Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. Am J Pathol. 2007; 170:709–721. [PubMed: 17255337]
38. Fujita H, Chiba H, Yokozaki H, Sakai N, Sugimoto K, Wada T, et al. Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. J Histochem Cytochem. 2006; 54:933–944. [PubMed: 16651389]
39. Lu Z, Ding L, Hong H, Hoggard J, Lu Q, Chen YH. Claudin-7 inhibits human lung cancer cell migration and invasion through ERK/MAPK signaling pathway. Exp Cell Res. 2011; 317:1935–1946. [PubMed: 21641901]
40. Huang RY, Wong MK, Tan TZ, Kuay KT, Ng AH, Chung VY, et al. An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to e-cadherin restoration by a src-kinase inhibitor, saracatinib (AZD0530). Cell Death Dis. 2013; 4:e915. [PubMed: 24201814]
41. Park GB, Kim D, Kim YS, Kim S, Lee HK, Yang JW, et al. The Epstein-Barr virus causes epithelial-mesenchymal transition in human corneal epithelial cells via Syk/src and Akt/Erk signaling pathways. Invest Ophthalmol Vis Sci. 2014; 55:1770–1779. [PubMed: 24569586]
42. Pope JL, Bhat AA, Sharma A, Ahmad R, Krishnan M, Washington MK, et al. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. Gut. 2014; 63:622–634. [PubMed: 23766441]

43. Singh AB, Sharma A, Smith JJ, Krishnan M, Chen X, Eschrich S, et al. Claudin-1 up-regulates the repressor ZEB-1 to inhibit E-cadherin expression in colon cancer cells. Gastroenterology. 2011; 141:2140–2153. [PubMed: 21878201]

44. Shiou SR, Singh AB, Moorthy K, Datta PK, Washington MK, Beauchamp RD, et al. Smad4 regulates claudin-1 expression in a transforming growth factor-b–independent manner in colon cancer cells. Cancer Res. 2007; 67:1571–1579. [PubMed: 17308096]

45. Miwa N, Furuse M, Tsukita S, Niikawa N, Nakamura Y, Furukawa Y. Involvement of claudin-1 in the βcatenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. Oncol Res. 2001; 12:469–476. [PubMed: 11939410]

46. Caswell PT, Spence HJ, Parsons M, White DP, Clark K, Cheng KW, et al. Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. Dev Cell. 2007; 13:496–510. [PubMed: 17925226]

47. Goldenring JR, Nam KT. Rab25 as a tumour suppressor in colon carcinogenesis. Br J Cancer. 2011; 104:33–36. [PubMed: 21063400]

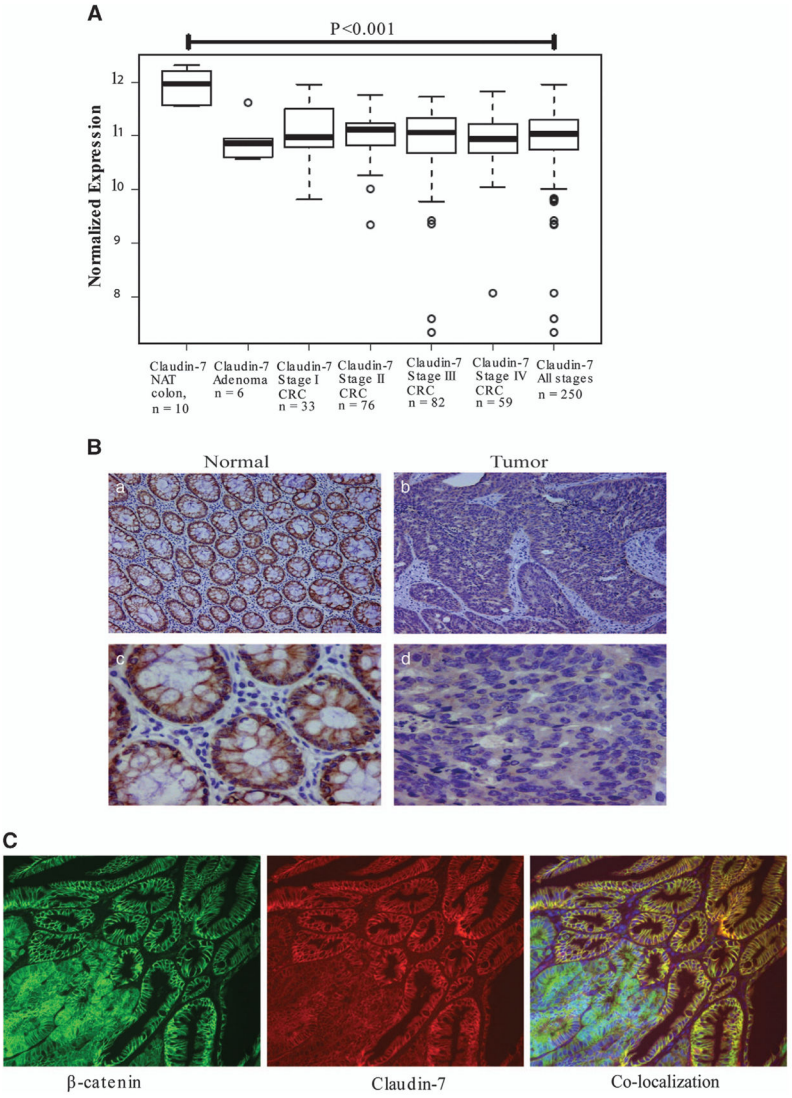

Figure 1.

Claudin-7 expression is reduced in human colon cancer. (**A**) Normalized expression for claudin-7 in 10 normal adjacent specimens versus six adenoma specimens and 250 colon adenocarcinoma samples by microarray analysis. Stage I =33; Stage II =76; Stage III = 82; Stage IV =59; All stages: 250. Claudin-7 expression was significantly downregulated in the adenoma and adenocarcinoma groups compared with the adjacent normals ($P<0.001$). Median and s.d. are shown. (**B**) The expression pattern of claudin-7 was examined by immunohistochemical staining using 11 human colon cancer tissue samples. Decreased claudin-7 expression was observed in paraffin-embedded sections of normal and colonic tumor samples from the same patients. (**C**) Representative immunofluorescence images of tumors from APC^{min} mice immunostained for β-catenin and claudin-7.

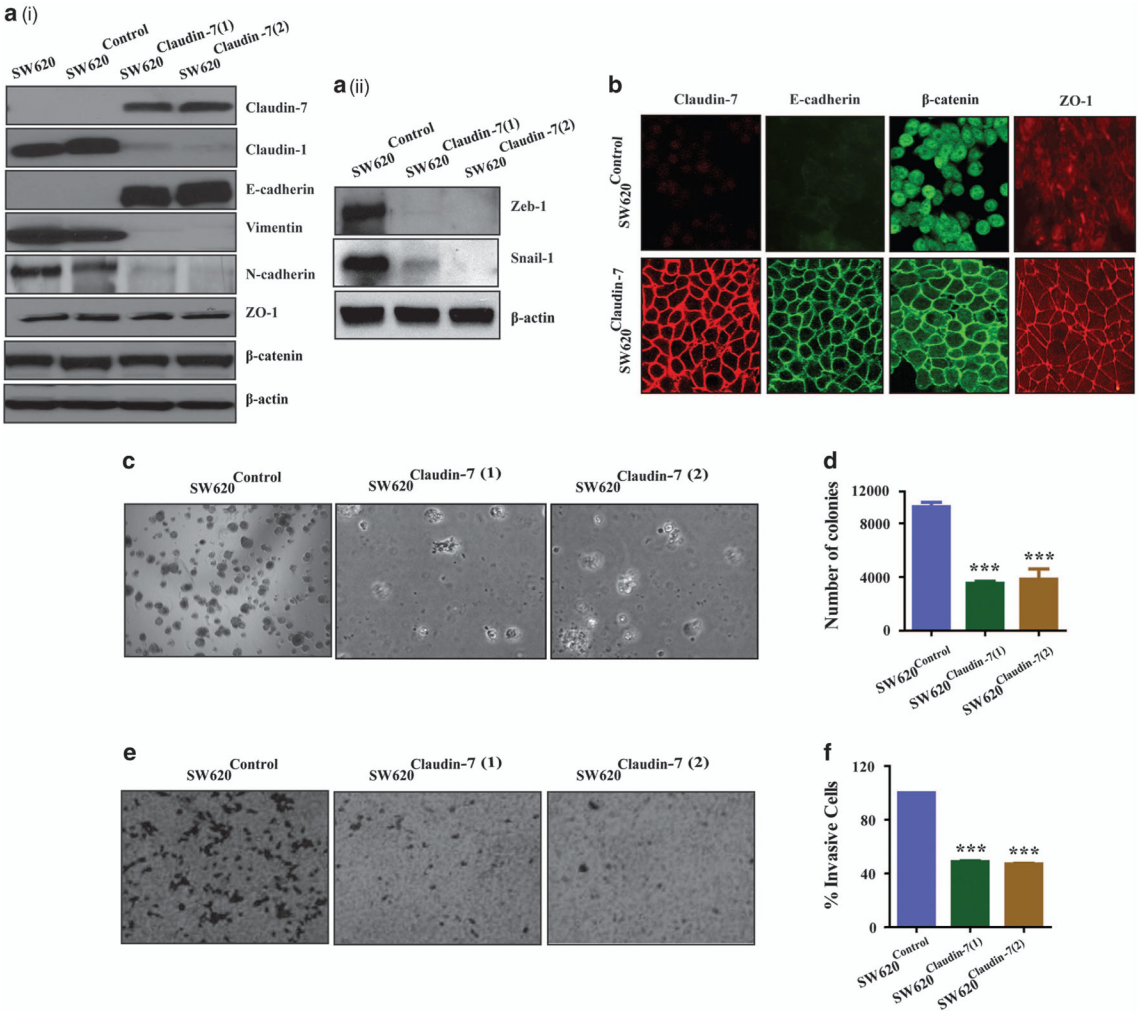

Figure 2.

Manipulation of claudin-7 expression alters EMT and functional characteristics. SW620, $SW620^{control}$, $SW620^{claudin\text{-}7(1)}$ and $SW620^{claudin\text{-}7(2)}$ cells (overexpressing claudin-7) were examined for ((**a**) (i and ii)) expression of EMT markers, including E-cadherin, Vimentin, N-cadherin, ZO-1, β-catenin, Zeb-1 and Snail-1. Actin was used as a loading control. (**b**) Localization of claudin-7, E-cadherin, β-catenin and ZO-1 was examined using immunofluorescence. (**c** and **d**) Anchorage-independent growth assay. Colonies were counted from three individual plates for each sample and were photographed 2 weeks after plating of the cells. The number of soft agar colonies presented is the mean of colony counts from three different experiments. ***P<0.001 compared with $SW620^{control}$. (**e** and **f**) Cell invasion assay. Vector and claudin-7-expressing cells were grown on 24-well transwells coated with collagen type I (100 μg/ml). After 72 h of plating, cells from the top of the filter were removed, and the cells that had invaded the coated membrane were fixed and counted. Data are presented as mean colony counts in six × 20 microscopic fields from triplicate wells. *P<0.01, ***P<0.001, as compared with respective controls.

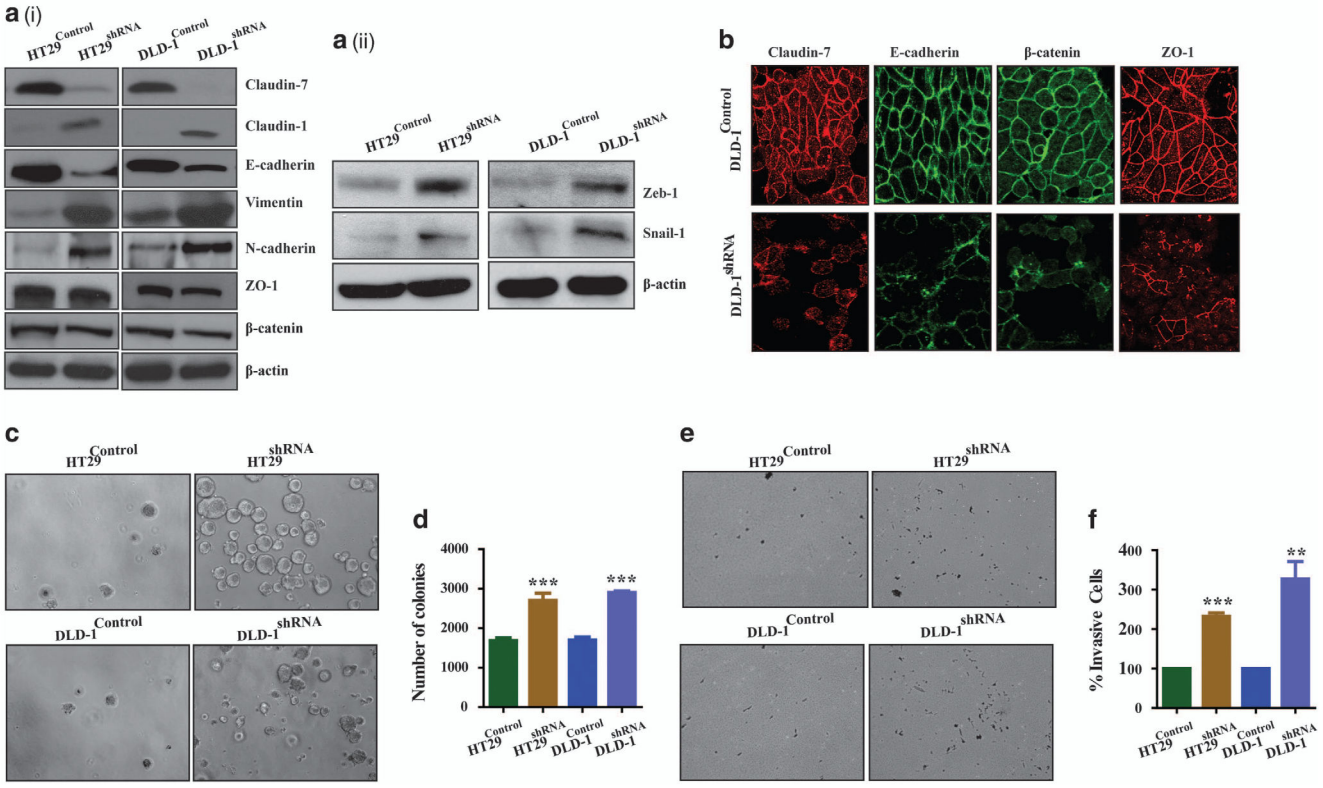

Figure 3.

Loss of claudin-7 induces mesenchymal features in epithelial cells. HT29^{control}, HT29^{shRNA}, DLD-1^{control} and DLD-1^{shRNA} cells (inhibition of claudin-7) were utilized to determine ((**a**) (i and ii)) expression of EMT markers, including E-cadherin, Vimentin, N-cadherin, ZO-1, β-catenin, ZEB-1 and Snail-1, by immunoblotting. Actin was used as a loading control. (**b**) Localization of claudin-7, E-cadherin, β-catenin and ZO-1 was examined using immunofluorescence. (**c** and **d**) Anchorage-independent growth assay. Colonies were counted from three individual plates for each sample and were photographed 2 weeks after plating of the cells. The number of soft agar colonies presented is the mean of colony counts from three different experiments. ***P<0.001 compared with control cells. (**e** and **f**) Cell invasion assay. Vector and claudin-7 knockdown cells were grown on 24-well transwells coated with collagen type I (100 μg/ml). After 72 h of plating, cells from the top of the filter were removed, and the cells that had invaded the coated membrane were fixed and counted. Data are presented as mean colony counts in six × 20 microscopic fields from triplicate wells. **P<0.01. ***P<0.001, as compared with respective controls.

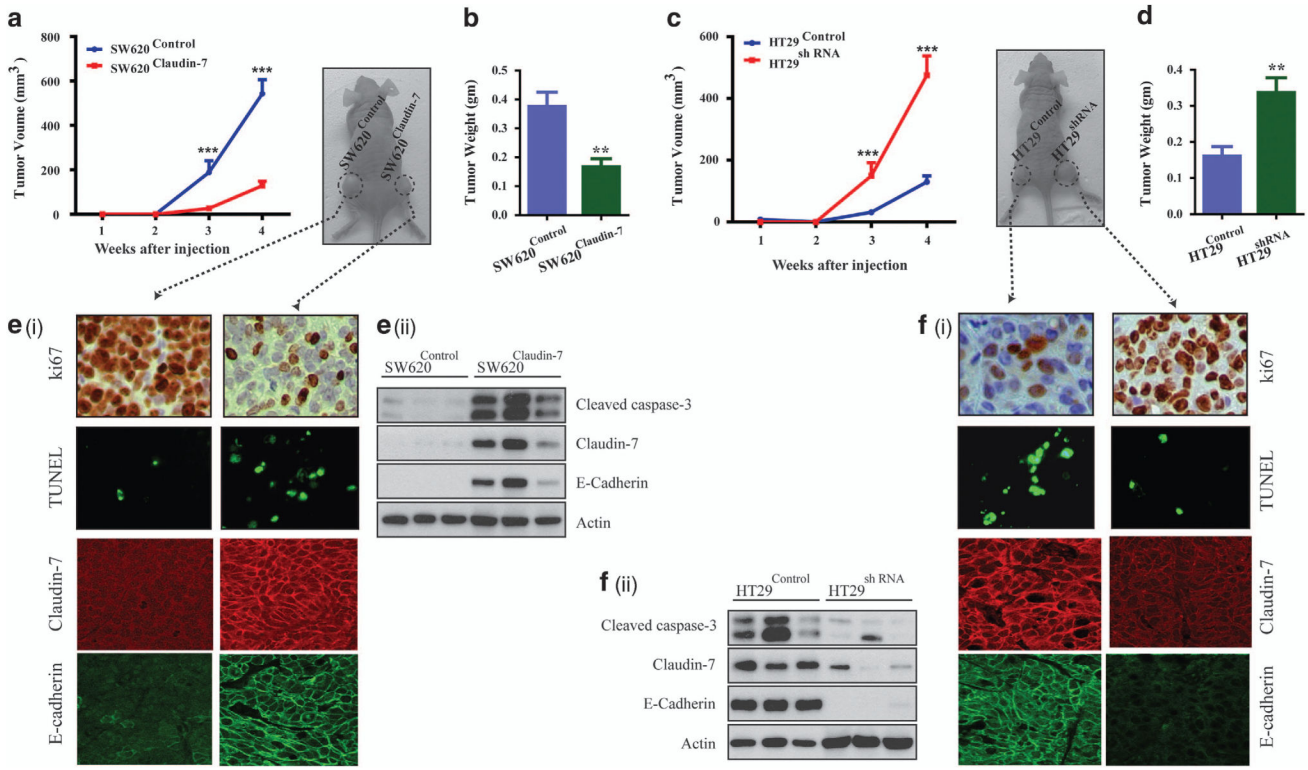

Figure 4.

Effect of modulation of claudin-7 expression on tumor xenograft *in vivo*. SW620^{control} or SW620$^{Claudin\text{-}7}$ cells were subcutaneously injected into the left and right sides of the nude mice (n =7 mice per group). Circles indicate the tumors generated subcutaneously in nude mice. The nude mice were killed 4 weeks after the injection, and the tumors were removed and weighed. Claudin-7 expressing cell-induced tumors in nude mice were smaller in size compared with those of control cells (**a** and **b**). Conversely, HT29^{shRNA} expressing cell-induced tumors in nude mice were bigger in size cells (**c** and **d**). Tumors were evaluated for markers of proliferation (Ki67), apoptosis (TUNEL) as well as claudin-7 and E-cadherin expression by immunostaining (**e** (i) and **f** (i)). Tumors were also immunoblotted for cleaved caspase-3, claudin-7 and E-cadherin (**e** (ii) and **f** (ii)). **P<0.01, ***P<0.001.

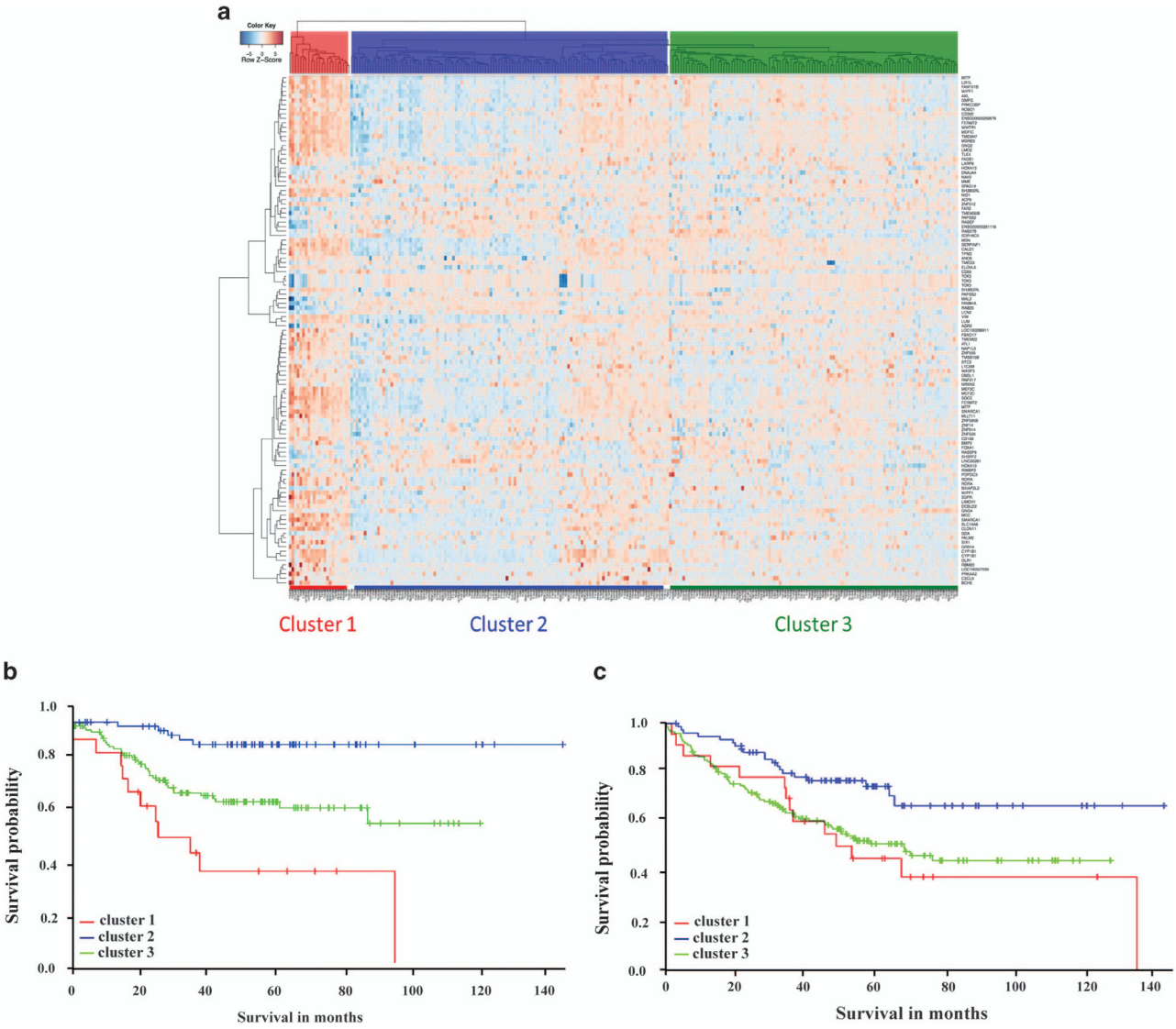

Figure 5.

Claudin-7 expression in human CRC and association with clinical outcomes. (**a**) Unsupervised hierarchical clustering analysis for claudin-7. The claudin-7 gene expression profile (113 probes) separates 250 CRC patients into three clusters. (**b** and **c**) Analysis of clinical outcomes demonstrates that the claudin-7 gene signature is significantly associated with overall and disease-free survival in 250 CRC patients. Cluster 2 (blue) patients demonstrate better overall survival and less recurrent disease than clusters 3 and 1 patients (P =0.005 and <0.001, respectively). No association was noted with grade or adjuvant treatment; however, a significant association was noted between the clusters and the stage of the patients (P =0.02).

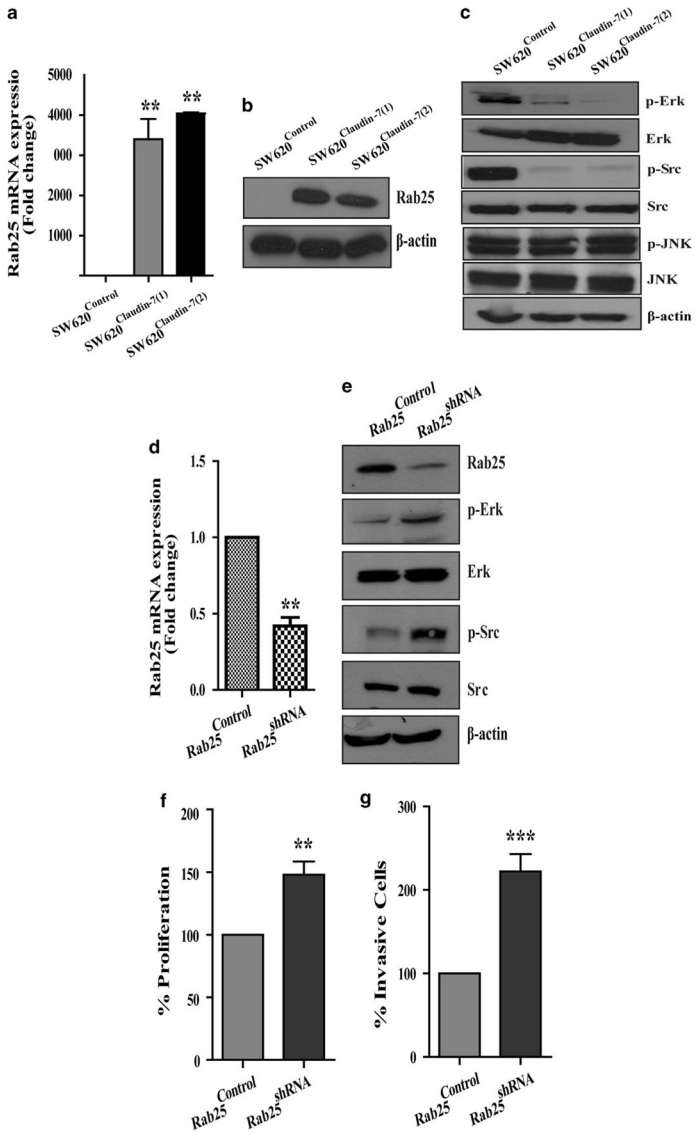

Figure 6.

Claudin-7 effects are mediated through Rab25 signaling. (**a** and **b**) Expression of Rab25 was confirmed at the mRNA and protein levels in claudin-7-manipulated cells using quantitative real-time PCR (qRT–PCR) analysis and immunoblot analysis. Results are plotted as mean ±s.d. from three independent experiments and are presented as fold change. (**c**) Signaling proteins upregulated or downregulated in claudin-7-manipulated cells were determined by immunoblot analysis. (**d**) Silencing of Rab25 expression in SW620$^{\text{claudin-7}}$ cells was confirmed by qRT–pCR. (**e**) Effect of the inhibition of Rab25 expression in SW620$^{\text{claudin-7}}$ cells on ERK/Src signaling, cell proliferation (**f**) and cell invasion (**g**). Cellular proliferation was measured using the MTT assay after 48 h of transfection. Cell invasion was performed as described in Materials and methods. Results were plotted as mean ±s.d. from three independent experiments and presented as fold change. $^{**}P<0.01$, $^{***}P<0.001$ when compared with control.

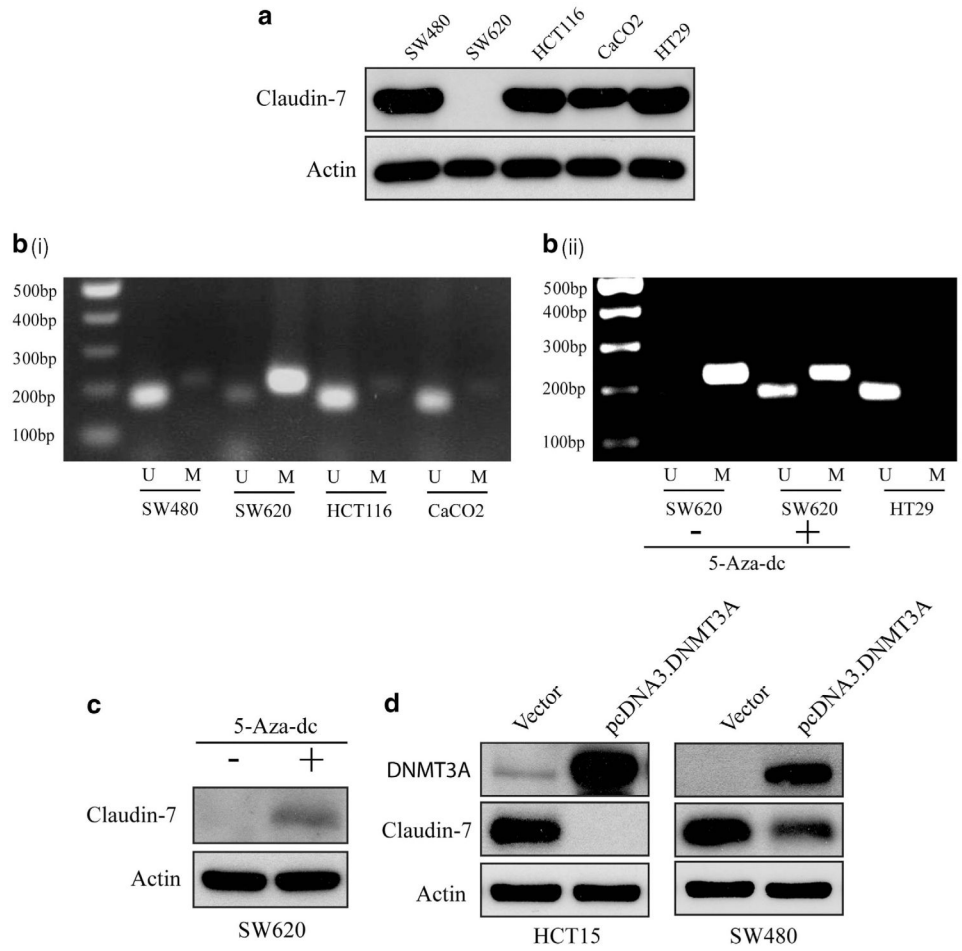

Figure 7.

Suppression of claudin-7 expression is associated with the claudin-7 promoter hypermethylation in SW620 CRC cells. (**a**) Expression of claudin-7 in CRC cell lines determined by western blotting analysis. Actin was used as a loading control. ((**b**) (i and ii)) Methylation status of claudin-7 in colon cancer cell lines. Methylated (M) and unmethylated (U) gene sequences were amplified individually by methylation-specific PCR (MSP) using sodium-bisulfite-treated DNA from the indicated colon cancer cell lines. MSP products were resolved by electrophoresis on a 1.5% agarose gel. ((**b**) (ii)) Re-expression of claudin-7 in SW620 cells following treatment with the demethylating agent 5-aza-dC. Real-time PCR was performed for claudin-7, and PCR products were resolved by electrophoresis on a 1.5% agarose gel. (**c**) Immunoblotting of claudin-7 in SW620 cells following treatment with the demethylating agent 5-aza-dC. (**d**) SW480 and HCT15 cells were transfected with the pcDNA-3 expression plasmid containing the full-length human DNMT3A or vector alone and immunoblotted for DNMT3A and claudin-7 after 48 h of transfection. Actin was used as a loading control.