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ORIGINAL ARTICLE

STIM1, a direct target of microRNA-185, promotes tumor metastasis and is associated with poor prognosis in colorectal cancer

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STIM1 (stromal interaction molecule 1), an endoplasmic reticulum Ca^{2+} sensor that triggers the store-operated Ca^{2+} entry activation, has recently been implicated in cancer progression. However, the role of STIM1 in the progression and metastasis of colorectal cancer (CRC) has not been addressed. In this study, we confirmed increased expression of STIM1 in highly invasive CRC cell lines. Enhanced expression of STIM1 promoted CRC cell metastasis *in vitro* and *in vivo*, whereas silencing of STIM1 with small interfering RNA resulted in reduced metastasis. Ectopic expression of STIM1 in CRC cells induced epithelial-to-mesenchymal transition (EMT), whereas silencing of STIM1 had the opposite effect. Furthermore, STIM1 expression was markedly higher in CRC tissues than in adjacent noncancerous tissues. STIM1 overexpression correlated with poor differentiation and higher tumor node metastasis stage. CRC patients with positive STIM1 expression had poorer prognoses than those with negative STIM1 expression. Moreover, STIM1 was found to be a direct target of miR-185, a microRNA (miRNA) that has not previously been reported to be involved in EMT, in both CRC tissues and cell lines. Taken together, these findings demonstrate for the first time that STIM1 promotes metastasis and is associated with cancer progression and poor prognosis in patients with CRC. In addition, we show that expression of STIM1 is regulated by a posttranscriptional regulatory mechanism mediated by a new EMT-related miRNA. This novel miR-185–STIM1 axis promotes CRC metastasis and may be a candidate biomarker for prognosis and a target for new therapies.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent carcinomas throughout the world, with an estimated one million new cases and half a million mortalities each year.[1,2] Metastasis, one of the six initial cancer hallmarks,[3] is a major cause of CRC-associated mortality.[4] More than one-third of patients with CRC will ultimately develop metastatic disease;[5] however, little is known about the exact molecular mechanisms underlying CRC metastasis.[6] Understanding the key factors in these processes is crucial to the development of novel anticancer therapies.

In most types of cell, the ubiquitous second messenger Ca^{2+} is a critical regulator that mediates various cellular processes.[7] In non-excitable cells, the store-operated calcium influx (SOCE) is the major mechanism of Ca^{2+} entry.[8,9] Recent studies have identified two important molecules responsible for SOCE activation: STIM1 (stromal interaction molecule 1) and Orai1.[8] When Ca^{2+} in the endoplasmic reticulum is depleted, STIM1 serves as a Ca^{2+} sensor and aggregates into multiple punctae that translocate within close proximity of the plasma membranes.[10] Orai1, an essential pore-forming component of the SOCE channel, translocates to these STIM1-containing structures during store depletion and open stomediate Ca^{2+} entry.[11–13] Recently, STIM1 has been reported to have an important role in various pathophysiological conditions, such as the immune response,[11,14] cardiovascular disease,[15,16] sexual dysfunction[17] and pulmonary disease.[18] STIM1 was also found to be involved in cancer and was initially thought to work as a tumor suppressor,[19] although subsequent data indicate an opposite function.[20] More recently, Orai1 and STIM1 were shown to be essential for the migration of breast tumor cells.[21] Another study found that STIM1 has an important role in the growth, migration and angiogenesis of cervical cancer.[22] These studies suggest that STIM1 might have a critical role in cancer.

Epithelial-to-mesenchymal transition (EMT) is a morphogenetic process in which cells lose their epithelial characteristics such as cell polarity and cell–cell contact, and gain mesenchymal properties such as increased motility. EMT also endows cells with invasive properties, induces stem cell properties and prevents apoptosis and senescence. Morphologic evidence of EMT has been found at the invasive front of human tumors.[23] EMT is viewed as an essential early step and a critical process during tumor metastasis.[24–27] Notably, several recent studies suggest that EMT initiated by Oct4 or Snail1 in breast cancer may be associated with STIM1-directed Ca^2 influx.[21,23,28–30] These studies strongly suggest that STIM1 may promote cancer metastasis by regulating EMT.

MicroRNAs (MiRNAs) are noncoding small RNAs that posttranscriptionally regulate protein expression.[31] Several studies have shown that miRNAs have an important role in cancer metastasis.[32] Recently, some studies have highlighted the importance of miRNAs in the regulation of EMT.[33,34] As both miRNAs and STIM1 may have important roles in EMT process, we are interested in whether STIM1 expression is regulated by certain miRNAs in cancer. Notably, a recent study has found that miR-195 directly regulates *STIM1* mRNA decay in normal rat intestinal crypt cells.[35] However,whether posttranscriptional regulation by certain

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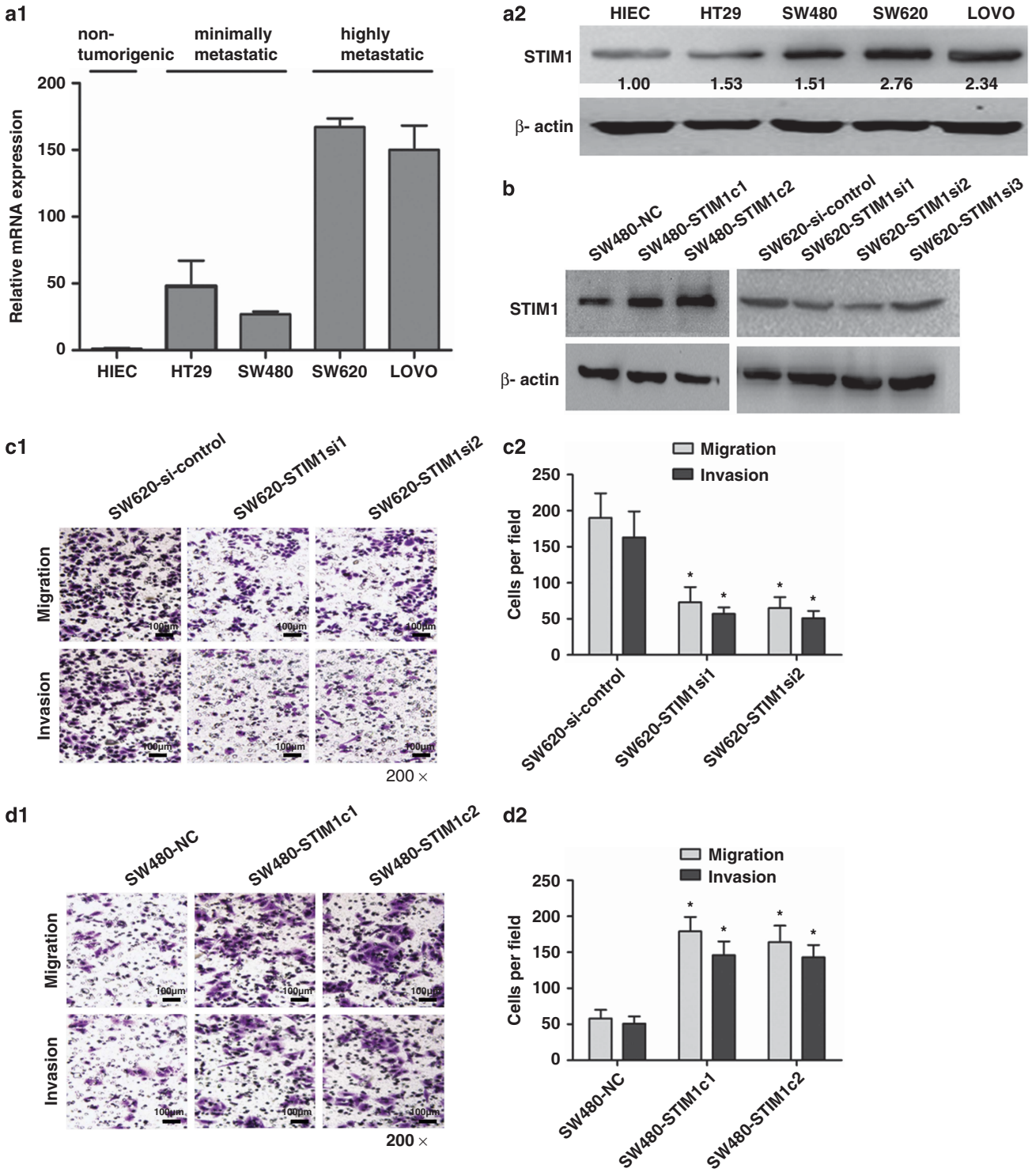


Figure 1. STIM1 promoted CRC cells invasion and metastasis *in vitro*. (**a1**) Expression of STIM1 in four human CRC cell lines relative to the normal human intestinal epithelial cell line (HIEC) was measured by qRT–PCR. (**a2**) Western blotting analysis of STIM1 expression in different CRC cell lines. (**b**) After cells were infected with LV-STIM1 (two clones, **c1** and **c2**) or LV-STIM1si-1, -2 or -3, the level of STIM1 protein expression was detected by western blotting analysis. (**c**) Invasion and migration assay of SW620 cells transfected with LV-STIM1si-1, LV-STIM1si-2 or a negative control. (**c1**) Representative fields of migration (left) or invasive (right) cells on membrane. (**c2**) Average cell number of invasion or migration per field from three independent experiments ± s.e. $*P < 0.05$. (**d1** and **d2**) Invasion or migration assay of SW480 cells transfected with the LV-STIM1 expression vector (two clones, **c1** and **c2**) or the control vector (NC). Scale bar, 100 μm. $*P < 0.05$.

miRNAs may represent one of the upstream regulation mechanisms of STIM1 expression in cancer metastasis remains unknown.

In this study, we provide the first evidence that STIM1 overexpression is associated with progression and poor prognosis in CRC. We show that STIM1 promotes CRC metastasis *in vitro* and *in vivo*. We further demonstrate that STIM1 is a direct functional target of miR-185, a new EMT-related miRNA identified in this study. Our results suggest that the miR-185/STIM1 signaling

pathway might be a potential target for diagnosis and therapy in CRC.

RESULTS

STIM1 promotes migratory and invasive capacities of CRC cells *in vitro* and *in vivo*

To assess whether STIM1 expression correlates with the metastatic potential of CRC cells, we examined the expression of STIM1 in four human CRC cell lines (SW480, SW620, HT29 and LOVO) and in a normal human intestinal epithelial cell line. Basal expression levels of STIM1 were significantly increased in SW620 and LOVO cells, which have highly metastatic propensities, compared with either the poorly metastatic cell lines SW480 and HT29 or the immortalized cell line human intestinal epithelial cell line (Figures 1a1 and a2). The SW480 cell line was obtained from a primary CRC lesion, and the SW620 cell line was established from a lymph node (LN) metastasis in the same patient a year later.[36]

a

Incidence of metastasis in mice

	Lung metastasis	Liver metastasis
SW480-NC	2/10	1/10
SW480-STIM1 c1	6/10	5/10
SW480-STIM1 c2	7/10	5/10
SW620-si-control	8/10	8/10
SW620-STIM1 si1	4/10	3/10
SW620-STIM1 si2	3/10	3/10

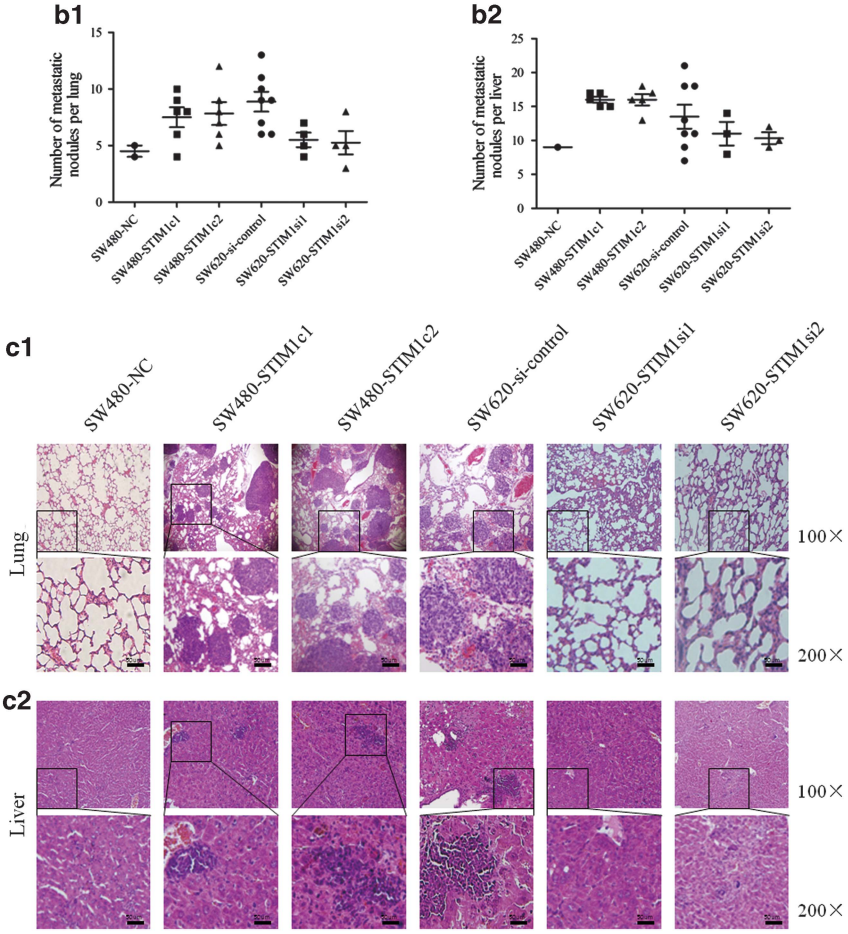


Figure 2. STIM1 promoted CRC cells invasion and metastasis *in vivo*. SW480 cells transfected with the LV-STIM1 expression vector (two clones, **c1** and **c2**) or the control vector and SW620 cells transfected with LV-STIM1si-1, LV-STIM1si-2 or a negative control were injected into nude mice via the tail vein for an *in vivo* metastasis assay. Animals were killed 6 weeks after injection (**a**). Incidence of lung and liver metastasis in each group of nude mice. (**b**) Number of metastatic lung foci observed in each group. (**b2**) Number of metastatic liver foci observed in each group. (**c1**) Images showing representative hematoxylin and eosin staining of lung tissue samples from the different experimental groups. (**c2**) Images showing representative hematoxylin and eosin staining of liver tissue samples from the different experimental groups. Scale bar, 50 μm.

Therefore, these cell lines have the same genetic background but different metastatic potential.[37] To explore whether STIM1 affects the metastatic phenotype of CRC cells, SW620 cells were transfected with STIM1-specific small interfering RNAs (siRNAs) or a control siRNA. Three different siRNAs targeting STIM1 were tested, and the two that most effectively knocked down STIM1

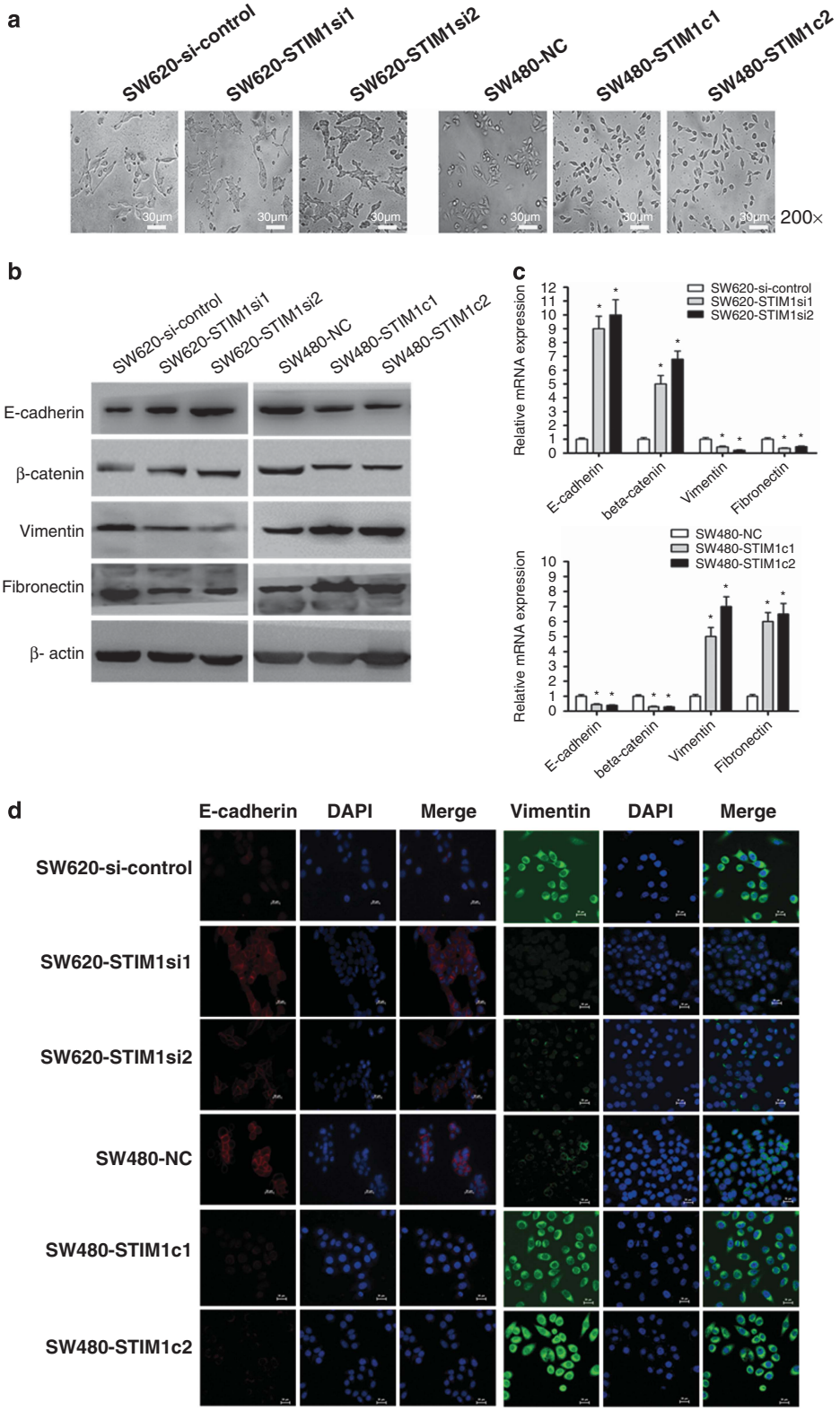
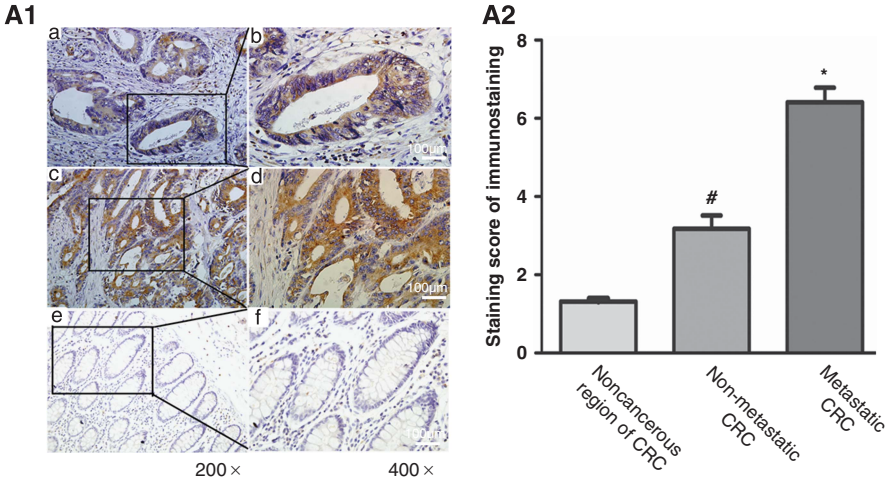


Figure 3. STIM1 regulates EMT in CRC cells. (**a**) Morphological changes by STIM1 in SW620 and SW480 cells. Scale bar, 30 μm. (**b**–**d**) Western blotting, real-time PCR and IF staining show upregulated expression of epithelial markers and downregulated expression of mesenchymal markers in SW620-STIM1si1 and SW620-STIM1si2 cells. In contrast, overexpression of of STIM1 resulted in decreased expression of epithelial markers and increased expression of mesenchymal markers in SW480 cells. Scale bar, 20 μm. *$P < 0.05$.

expression (SW620-STIM1si1 and SW620-STIM1si2) were selected for subsequent studies. SW480 cells were infected with recombinant lentivirus expressing STIM1 (LV-STIM1) or a control lentivirus (LV-NC) and stable clones were established (SW480-STIM1c1, SW480-STIM1c2 and SW480-NC, respectively; Figure 1b). Upregulation and knockdown of STIM1 expression was confirmed by western blot analysis (Figure 1b). Transwell assays showed that downregulation of STIM1 expression in SW620 cells markedly decreased cell migration and invasion (Figures 1c1 and c2). Conversely, upregulation of STIM1 expression in SW480 cells significantly enhanced cell migration and invasion (Figures 1d1 and d2). MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to exclude the contribution of cell proliferation (Supplementary Figures S1A and B).These results suggest that STIM1 promotes metastasis in CRC cells.

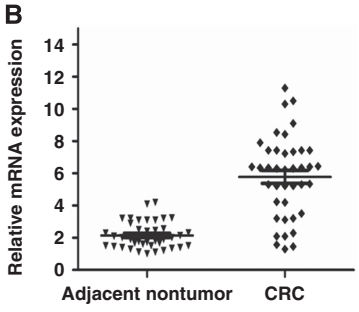
To further explore the role of STIM1, we injected SW480-STIM1c1, SW480-STIM1c2, SW620-STIM1si1 or SW620-STIM1si2 cells into nude mice through the lateral tail vein. Histologic analyses (Figures 2c1 and c2) confirmed that the incidence of lung and liver metastases was markedly increased in mice injected with SW480-STIM1c1 or SW480-STIM1c2 cells and markedly decreased in mice injected with SW620-STIM1si1 or SW620-STIM1si2 cells, compared with the control group. The number of metastatic lung and liver nodules was significantly increased in the SW480-STIM1c1 and SW480-STIM1c2 groups and significantly decreased in the SW620-STIM1si1 and SW620-STIM1si2 groups, compared with the control group (Figures 2a, b1 and b2).



A3

Upregulation of STIM1 in CRC tissues

STIM1 staining	Tumor (90)	Adjacentnontumor (90)	P value
Positive	63	19	P<0.001
Nagetive	27	71	



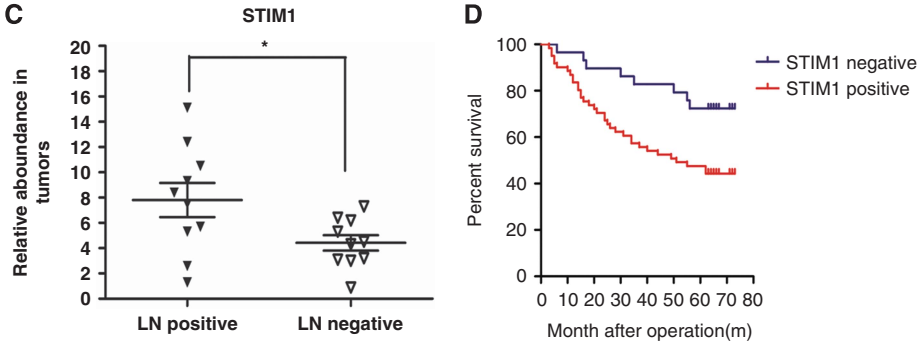


Figure 4. STIM1 is significantly upregulated in CRC tissues. (**A1**) Immunohistochemistry analysis of STIM1 expression in 90 paired CRC tissues. (**a**, **c** and **e**) Magnification, ×200; (**b**, **d** and **f**) magnification, ×400. (**a** and **b**) Primary sites of non-metastatic CRC; (**c** and **d**) primary sites of metastatic CRC; and (**e** and **f**) noncancerous region of CRC. Scale bar, 100 μm. (**A2**) Results of immunohistochemical staining were evaluated by the staining scores. $^{*}P < 0.05$ vs non-metastatic CRC or noncancerous region of CRC. $^{\#}P < 0.05$ vs noncancerous region of CRC. (**A3**) Statistical analysis of STIM1 expression in CRCs. (**B**) Real-time PCR analysis of STIM1 expression in 40 pairs of CRC and adjacent nontumorous tissues. (**C**) Real-time qPCR analysis of STIM1 abundance in human CRC primary tumors, LN-positive ($n = 10$) or -negative ($n = 10$). Matching normal colorectal tissue sample from the same patient was used for normalization. (**D**) Kaplan–Meier's analysis of the correlation between STIM1 expression and overall survival (OS) of CRC patients.

STIM1 regulates EMT in CRC cells

The EMT has a crucial role in cancer metastasis. During the EMT process, differentiated polarized epithelial cells undergo profound morphogenetic changes, typified by the dissolution of cell–cell junctions and loss of apicobasolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties. Moreover, during this process the expression of epithelial markers (such as E-cadherin and β-catenin) is downregulated, whereas the expression of mesenchymal markers (such as vimentin and fibronectin) is upregulated.[38,39]

By comparing the morphology of the cell models described above under a light microscope, we observed that ectopic expression of STIM1 in SW480 cells induced the conversion of polarized epithelial cells to spindle-shaped, fibroblast-like mesenchymal cells with the loss of cell–cell adhesion, whereas knockdown of STIM1 in SW620 cells resulted in a tighter organization of cells in colonies and an epithelioid morphology (Figure 3a).

Next, we assessed the expression of EMT markers in these cell models. Western blotting and quantitative reverse transcription–PCR (qRT–PCR) analysis showed a marked decrease in the expression of vimentin and fibronectin and a significant increase in the expression of E-cadherin and β-catenin in SW620-STIM1si1 and SW620-STIM1si1 cells, compared with control cells. Conversely, a decrease in the expression of E-cadherin and β-catenin and an increase in the expression of vimentin and fibronectin were observed in SW480-STIM1c1 and SW480-STIM1c1 cells, compared with control cells (Figures 3b and c). These changes in E-cadherin and vimentin expression were confirmed by an immunofluorescent assay (Figure 3d). These results suggest that STIM1 is critical for the acquisition of EMT characteristics and may contribute to the EMT-derived invasive phenotype in CRC cells.

STIM1 is significantly upregulated in human CRC tissues and indicates poor prognosis

To explore the potential role of STIM1 in determining the clinical outcome of CRC, we assessed STIM1 expression in a tissue microarray of 90 CRC patients. The results of immunohistochemical staining showed that STIM1 was localized to both the cytoplasm and membranes. Positive STIM1 expression was detected in 63 of 90 (71.1%) primary CRC samples compared with only 19 of 90 (21.1%) adjacent non-tumor tissues. These results were confirmed by scoring the staining intensity across the section (Figures 4A1–3). These findings indicated that STIM1 was significantly overexpressed in human CRC tissues. To investigate the correlation between STIM1 overexpression and CRC metastasis, we compared STIM1 expression in 10 pairs of LN metastasis-positive (LN-positive group) and LN metastasis-negative (LN-negative group) primary CRC specimens. Real-time PCR analysis showed that *STIM1* mRNA levels were much higher in the LN-positive group than in the LN-negative group (Figure 4C). Furthermore, Kaplan–Meier analysis showed that patients with positive STIM1 expression had shorter overall survival than those who were negative for STIM1 expression (Figure 4D). To investigate whether STIM1 expression is associated with the progression of CRC, we investigated the expression of STIM1 in an additional 40 fresh CRC tissues by qRT–PCR (Figure 4B) and compared the results with the clinicopathologic characteristics of the patients. The results showed that overexpression of STIM1 did not correlate with age, gender or tumor size ($P > 0.05$), but was significantly associated with TNM stage and LN status ($P < 0.01$; Table 1). Taken together, these results suggest that the expression level of STIM1 positively correlated with the progression of CRC and that STIM1 overexpression is indicative of poor prognoses of human CRC patients following curative resection.

Table 1. Correlation between STIM1 expression and clinicopathological characteristics of 90 CRC

Variable	*Number of cases*	*STIM1 immunostaining*		P-*value*
		−	+	
Age (years)				
⩾60	66 (73.3%)	18	48	
<60	24 (26.7%)	9	15	0.436
Gender				
Male	47 (52.2%)	15	32	
Female	43 (47.8%)	12	31	0.818
Tumor size (cm)				
⩾5	54 (55.6%)	12	42	
<5	36 (44.4%)	15	21	0.062
TNM stage				
Stage I/II	56 (62.2%)	24	32	
Stage III/IV	34 (27.8%)	3	31	0.001
Lymph node status				
No metastasis	57 (63.3%)	22	35	
Metastasis	33 (36.7%)	5	28	0.031

Abbreviations: CRC, colorectal cancer; STIM1, stromal interaction molecule 1; TNM, tumor node metastasis.

The expression of miR-185 inversely correlates with the expression of STIM1 in CRC cells and is associated with the progression of CRC

The above results indicated that STIM1 overexpression might have a critical role in CRC. However, there have been few studies on the regulation of STIM1 expression in cancer. miRNAs were recently found to have an important role in tumor progression through their function as posttranscriptional regulators. We hypothesized that posttranscriptional regulation by miRNAs may represent one of the upstream regulatory mechanisms of STIM1 expression. To test this idea, we used several web-based target analysis tools (TargetScan, miRanda, RNAhybrid, pictar and DIANAmT) to identify miRNAs that potentially target STIM1. As a result, miR-185 was identified as a potential regulator of STIM1 expression (Figure 5a). This was consistent with the results of our previous miRNA microarray analysis, which identified miR-185 as the most significantly downregulated miRNA in SW620 cells compared with SW480 cells.[36] Therefore, miR-185 was selected for further study.

We examined endogenous miR-185 expression levels in four human CRC cell lines (SW480, SW620, HT29 and LOVO) and in a normal human intestinal epithelial cell line by qRT–PCR. The expression levels of miR-185 were significantly decreased in SW620 and LOVO cell lines (highly metastatic cells) compared with either poorly metastatic or immortalized cells. miR-185 expression negatively correlated with the metastatic potential of the CRC cells (Figure 5b1). Furthermore, the expression of miR-185 and STIM1 was inversely correlated (Figure 5b2). To investigate the correlation of miR-185 with STIM1 expression levels in CRC tissues, we used qRT–PCR to measure the expression levels of miR-185 in the 40 CRC tissues previously described. Our results confirmed that the mRNA level of miR-185 negatively correlated with STIM1 expression (Figure 5c2). The correlation of miR-185 expression with clinicopathologic factors was also examined. In contrast to STIM1 expression, low expression levels of miR-185 were significantly associated with advanced clinical tumor node metastasis stage and LN metastasis ($P < 0.001$). miR-185 expression levels in CRC patients did not correlate with age, gender, tumor size or cell differentiation ($P > 0.05$) (Table 2). These results

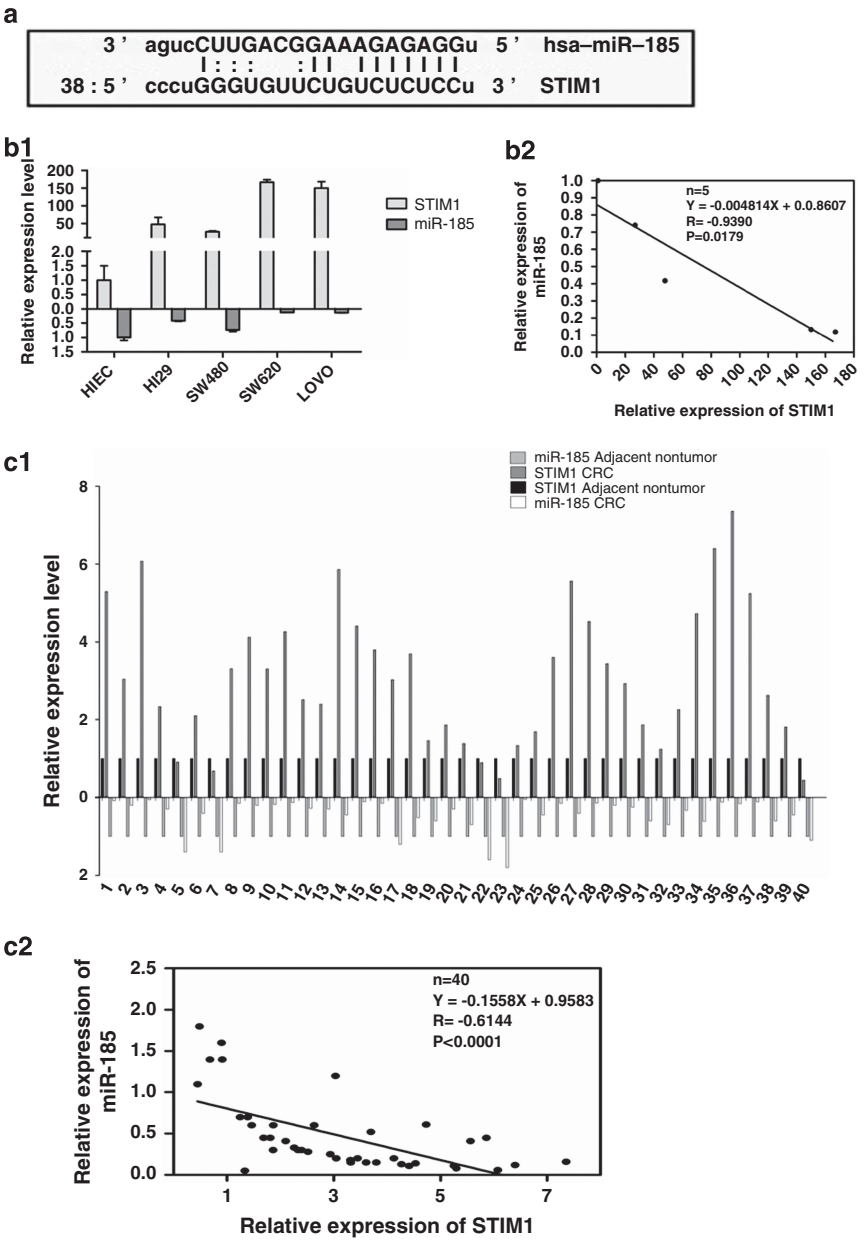


Figure 5. Expression of miR-185 and STM1 negatively correlates in CRC cell lines and clinical samples. (**a**) Sequence alignment of miR-185 with binding site on the STIM1 3′-UTR. (**b1**) Expression of miR-185 and STIM1 in CRC cells was analyzed by qPCR. (**b2**) Scatter plots showing the negative linear correlation between the mRNA expression of miR-185 and that of STIM1 in CRC cells. (**c1**) qPCR analysis of the expression of miR-185 and STIM1 in CRC tissues. (**c2**) Scatter plots showing the negative linear correlation between the mRNA expression of miR-185 and that of STIM1 in CRC tissues.

strongly suggest that miR-185 is one of the upstream molecules regulating STIM1 expression in cancer.

miR-185 represses invasion and regulates EMT in CRC cells

To investigate the role that miR-185 has in the migration and invasion of CRC cells, the SW620 cell line was transiently transfected with miR-185 mimics. Successful upregulation of miR-185 expression was confirmed by qRT–PCR (Figure 6a1). Increased miR-185 expression resulted in significant inhibition of migration and invasion in SW620 cells (Figures 6a2 and a3). To determine whether the loss of miR-185 promotes the migration or invasion of CRC cells, miR-185 was silenced in SW480 cells with an antisense oligonucleotide inhibitor (Figure 6b1). This inhibition of miR-185 significantly promoted cell migration and invasion (Figures 6b2 and b3). These results indicate that miR-185 represses the invasive potential of CRC cells. As STIM1 has been shown to promote EMT in CRC cells and miR-185 has been predicted as a potential regulator of STIM1, we hypothesized that miR-185 may exert an opposite effect on the regulation of EMT in CRC cells. To test this, we compared the morphology of our cell models under a light microscope, and found that upregulating the expression of miR-185 in SW620 cells induced a tighter organization of cells in colonies, whereas downregulating the expression of miR-185 in SW480 cells led to a more fibroblast-like mesenchymal cell morphology (Figure 3c). Western blot and qRT–PCR analyses showed that upregulation of miR-185 in SW620 cells increased the expression of E-cadherin and β-catenin and decreased the

Table 2. Clinicopathological associations of miR-185 expression in primary CRC

Variable	*Number of cases*	*Median expression of miR-185 (range) (2 − ΔΔCT)**	P-*value*
Age (years)			
⩾60	21 (52.5%)	3.7 (1.7–13.1)	0.536
<60	19 (47.5%)	3.6 (1.0–28.2)	
Gender			
Male	23 (57.5%)	3.6 (1.2–28.2)	0.962
Female	17 (42.5%)	3.4 (1.0–13.1)	
Tumor size (cm)			
⩾5	23 (57.5%)	4.2 (1.2–28.2)	0.554
<5	17 (42.5%)	3.4 (1.0–11.4)	
Degree of differentiation			
Well and moderately differentiated	20 (50.0%)	4.2 (1.2–28.2)	0.113
Poorly differentiated	20 (50.0%)	3.1 (1.0–20.4)	
TNM stage			
Stage I/II	25 (62.5%)	4.6 (1.2–28.2)	0.041
Stage III/IV	15 (37.5%)	3.2 (1.0–10.3)	
Lymph node status			
No metastasis	21 (52.5%)	4.7 (1.2–28.2)	0.011
Metastasis	19 (47.5%)	3.2 (1.0–11.4)	

Abbreviations: CRC, colorectal cancer; STIM1, stromal interaction molecule 1; TNM, tumor node metastasis.

expression of vimentin and fibronectin (Figures 6d and e1, 2). Moreover, inhibition of miR-185 in SW480 cells decreased the expression of E-cadherin and β-catenin and increased the expression of vimentin and fibronectin (Figures 6c and d2). Interestingly, these effects of miR-185 on EMT were similar to the effects of STIM1 siRNAs in CRC cells.

STIM1 is a direct functional target of miR-185 in CRC metastasis

As mentioned above, an inverse correlation between miR-185 and STIM1 expression was identified in both CRC cells and tissues. To obtain additional direct evidence that STIM1 expression is regulated by miR-185, we analyzed the binding site of miR-185 in the 3′-untranslated region (UTR) of STIM1 and performed a luciferase reporter assay. An STIM1 3′-UTR reporter (Luc-STIM1) or the corresponding mutant reporter (Luc-STIM1-mu) was co-transfected with miR-185 mimics or mimic controls into SW620 cells. As expected, the luciferase activities of the reporter constructs were reduced significantly only when miR-185 mimics were co-transfected with Luc-STIM1 in SW620 cells. No apparent change in luciferase activities was observed in cells transfected with Luc-STIM1-mu or mimic controls (Figure 7a). To confirm whether targeting of the STIM1 3′-UTR by miR-185 was functional, SW620 cells transfected with miR-185 mimics and SW480 cells transfected with the miR-185 inhibitor were analyzed by qRT–PCR and western blotting. Both mRNA (Figure 7b1) and protein (Figure 7b2) levels of STIM1 were markedly upregulated in SW480 cells transfected with the miR-185 inhibitor, whereas both mRNA (Figure 7c1) and protein (Figure 7c2) levels of STIM1 were effectively downregulated in SW620 cells transfected with the miR-185 mimic. Further experiments confirmed that the altered miR-185 expression does not affect the expression of STIM2, Orai1, Orai2 and Orai3, which may also be involved in SOCE (Supplementary Figures S2A and B). These results suggest that STIM1 is a direct functional target of miR-185. To further corroborate this hypothesis, we performed a 'rescue' experiment by co-transfecting SW620 cells with miR-185 mimics and a pcDNA3.1 vector carrying an STIM1 expression cassette (pcDNA3.1-STIM1) or mutated seed sequences of miR-185 at the STIM1 3′-UTR (STIM1-mu). Western blot analysis demonstrated that co-transfection of SW620 cells with the miR-185 mimic and STIM1-mu restored STIM1 protein expression, whereas co-transfection with pcDNA3.1-STIM1 failed to recover the STIM1 expression that was silenced by the miR-185 mimic. Furthermore, restoration of STIM1 expression by the introduction of STIM1-mu into SW620 cells transfected with the miR-185 mimics inhibited the expression of E-cadherin and β-catenin and increased the expression of vimentin and fibronectin (Figure 7d). In addition, rescuing STIM1 expression in the presence of the miR-185 mimics enhanced the invasion and migration potential of SW620 cells (Figure 7e). Moreover, to determine the effect of the miR-185–STIM1 axis on SOCE, we examined cellular Ca^{2+} distribution by Ca^{2+} imaging in both SW620 and SW480 cells. After ER Ca^{2+} depletion, Ca^{2+} influx through SOCE into SW620 cells was increased compared with SW480 cells (Supplementary Figures S3A and B). We then examined the effect of overexpression of miR-185 and found that SOCE was impaired in SW620–miR-185 mimic cells (Supplementary Figures S3C and D), whereas inhibition of miR-185 in SW480 cells moderately increased SOCE (Supplementary Figures S3E and F). Taken together, these results suggest that STIM1 is a potential functional target of miR-185.

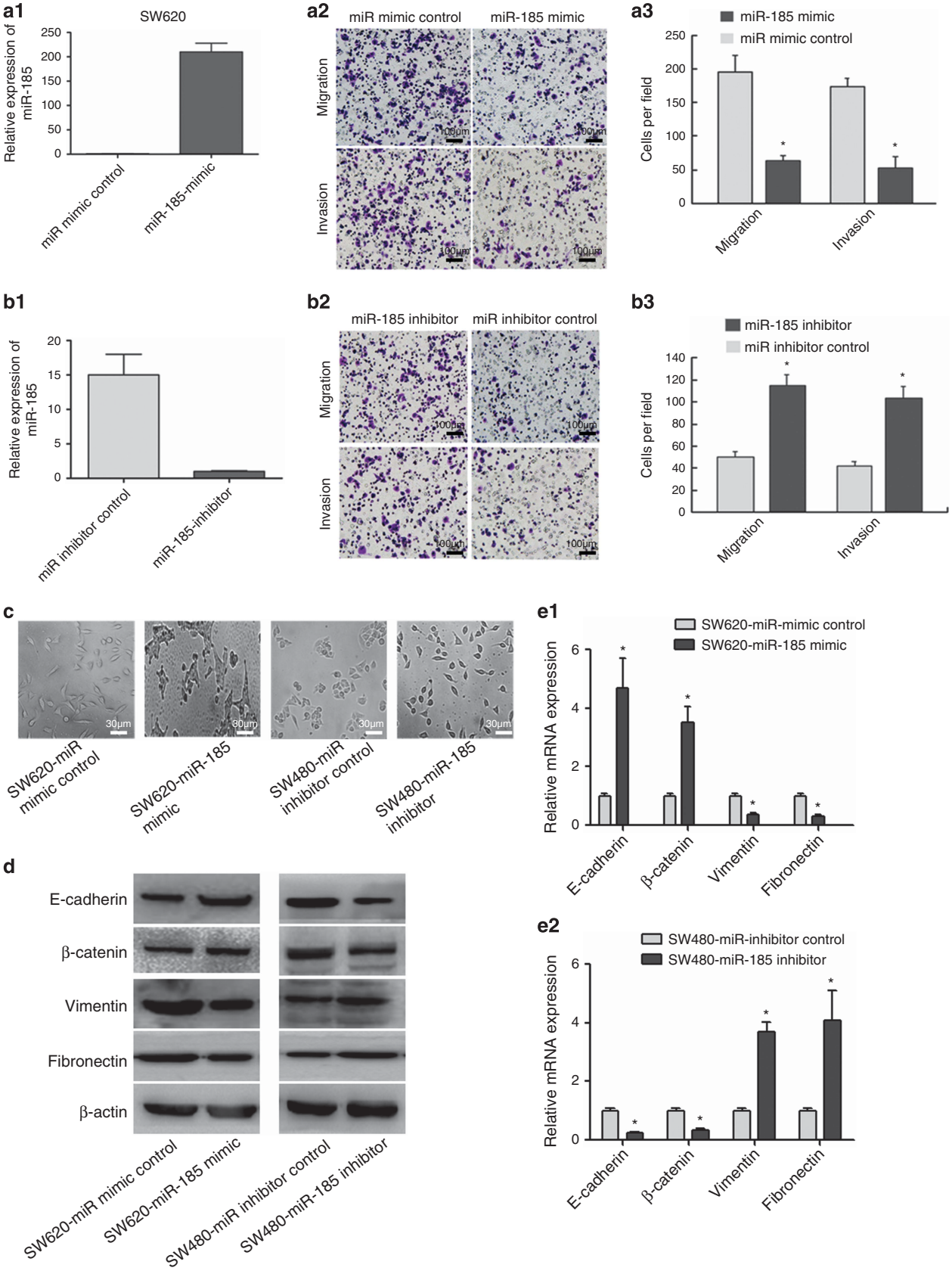
In summary, our results suggest that STIM1 is a direct functional target of miR-185 and that this novel miR-185/STIM1 axis has an important role in the regulation of CRC metastasis.

DISCUSSION

In this study, we demonstrate that STIM1 promotes CRC metastasis. The upregulation of STIM1 in CRC is at least partly attributed to posttranscriptional regulation by miR-185, a novel EMT-regulating miRNA. Additionally, we provide evidence supporting the involvement of STIM1 in the regulation of EMT in cancer.

The influx of Ca^{2+} is essential for the migration of tumor cells.[40,41] STIM1, an endoplasmic reticulum Ca^{2+} sensor, regulates Ca^{2+} influx by activating SOCE.[11,12] STIM1 has recently been proposed to be a critical molecule in the regulation of cancer metastasis,[21,22] although the exact role of STIM1 in metastasis remains controversial. STIM1 was initially implicated as an antimetastasis molecule.[19] However, we found that STIM1 promoted migration and invasive properties in CRC, and that increased expression of STIM1 in CRC was significantly associated with cancer metastasis. In accordance with our study, STIM1 was reported to be one of the molecules essential for breast tumor cell migration *in vitro* and tumor metastasis *in vivo*.[21] Similarly, another study demonstrated that the level of STIM1 was significantly associated with the risk of metastasis in cervical cancer. Moreover, epidermal growth factor-stimulated migration in cervical cancer cells requires STIM1 expression.[22] Our results provide new evidence supporting the involvement of STIM1 in the promotion of cancer metastasis.

It has been reported that expression of constitutively active Ras overcomes the migration-inhibitory function of the Ca^{2+} influx inhibitor and STIM1 siRNA in breast cancer.[21] Interestingly, a previous study has shown that activation of the Ras signaling pathway leads to activation of the transcriptional regulator, Snail1,[28] which appears to have a major role in EMT.[23,29] These studies strongly suggest that STIM1 might promote cancer metastasis by regulating the EMT. In this study, we demonstrated that knockdown of STIM1 induced the conversion of spindle-shaped, fibroblast-like mesenchymal cells to polarized epithelial



cells, enhanced invasion and migration in CRC cells, upregulated epithelial markers and downregulated mesenchymal markers. These findings provided new evidence supporting the involvement of STIM1 in driving metastasis through the regulation of EMT in cancer cells.

We further examined the clinical relevance of STIM1 and found that STIM1 was markedly upregulated in CRC tissues. Moreover, increased levels of STIM1 correlated with a high metastatic potential of CRC cells. In accordance with our findings, a previous study also showed that STIM1 was significantly associated with the risk of metastasis in cervical cancer.[22] Based on these findings, elucidating the mechanism of STIM1 upregulation may contribute to the identification of new cancer therapy targets. A recent study demonstrated that the expression of STIM1 is regulated by Wilms tumor suppressor 1 and early growth response 1 in six specific cancer subtypes.[42] Another study reported that STIM1 is regulated by androgens in prostate stromal cells. However, these observations are not sufficient to explain the mechanism behind the upregulation of STIM1 in cancer. A recent study found that another STIM protein, STIM2, inversely control melanoma growth and invasion,[43] suggesting that STIM proteins, including STIM1 and STIM2, have critical roles in cancer. Interestingly, these STIM proteins may exert different and even opposite functions in different kinds of cancers, which imply complex and important roles in cancer. Further study on STIM family may provide new targets for cancer therapy.

miRNAs have been implicated in the regulation of tumor metastasis[32] and recent studies have highlighted the importance of miRNAs in the regulation of the EMT.[33,34] To explore whether posttranscriptional regulation by certain miRNAs is one of the upstream regulatory mechanisms of STIM1 expression, we used several target prediction algorithms to assay for miRNA binding sites in the STIM1 3′-UTR. miRNA-185 was identified by several algorithms. Surprisingly, our previous miRNA microarray analysis revealed that miR-185 was the most significantly downregulated miRNA in SW620 cells (highly metastatic) compared with SW480 cells (poorly metastatic).[36] In this study, we found that miR-185 expression inversely correlated with the expression of STIM1 in both CRC cells and tissues, and that transfection with miR-185 significantly downregulated the expression of STIM1. These results were further confirmed by luciferase activity assays. miR-185 was recently found to be an independent prognostic factor in gastric cancer[44] and hepatocellular carcinoma.[45] Moreover, miR-185 can induce cell cycle arrest and repress cell proliferation in non-small-cell lung cancer,[46] colorectal cancer,[47] glioma,[48] gastric cancer[49] and breast cancer.[50] miR-185 has also been reported to be involved in metastasis in colorectal cancer[51] and glioma.[48] However, the role of miR-185 in the pathophysiological functions of STIM1 has not previously been reported.

The results presented here show that upregulating the expression of miR-185 inhibited migration and invasion and reversed the EMT in CRC cells. Our data further indicate that STIM1 is a functional target of miR-185 in CRC. Taken together, these findings provide new insight into the mechanism of the regulation of STIM1 expression at the posttranscriptional level, although further studies are required to fully understand the mechanism by which the miR-185-STIM1 pathway regulates EMT in cancer.

In conclusion, we have demonstrated a critical function of STIM1 in promoting CRC metastasis. This study also reveals a new regulatory mechanism of STIM1 expression by miR-185, an miRNA not previously associated with the EMT process. We believe that the miR-185-STIM1 pathway is an attractive target for therapeutic intervention against cancer metastasis.

MATERIALS AND METHODS

Cell culture

The human CRC cell lines were preserved in our institute and were routinely maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin sodium and 100 mg/ml streptomycin sulfate at 37 °C in a humidified air atmosphere containing 5% carbon dioxide. Throughout the experiment, cells were used in the logarithmic phase of growth according to the supplier's instructions.

Clinical samples

Samples from 40 CRC patients (23 males and 17 females) who had undergone proctocolectomy with LN dissection for CRC at Xijing Hospital (Xi'an, China) between November 2008 and April 2009 were included in the study (Supplementary Table S2). None of the patients received preoperative chemotherapy. The resected specimens were histologically examined by hematoxylin and eosin staining. The primary tumor samples and corresponding non-tumor mucosa were collected from each patient immediately after the surgical process and were snap frozen in liquid nitrogen until further use. Total RNA from the frozen tissues was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Oligonucleotide construction, lentivirus production and construct design

An miR-185 mimic, an inhibitor and a negative control oligonucleotides were purchased from RiboBio (Guangzhou, China). Transfection of oligonucleotides was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. On the basis of the STIM1 sequence, three short hairpin RNAs were designed using the siRNA Target Finder (InvivoGene, San Diego, CA, USA): 5′-GGGAAGACCTCAATTACCA-3′, 5′-TCTCCACATTTGGATTCTT-5′ and 5′-TGGATGATGTAGATCATAA-3′. Lentiviral vectors encoding short haipin RNAs were generated using GV248 vector (GeneChem Co., Ltd, Shanghai, China). Stable transfectants overexpressing STIM1 were generated by lentiviral transduction using a GV166 vector (GeneChem Co., Ltd). An empty vector was used as the negative control.

STIM1-expressing vector with miR-185 binding sites or mutated seed sequences of miR-185 at STIM1 3′-UTR (STIM1-mu) was purchased from Open Biosystems (Huntsville, AL, USA) and was subcloned into the eukaryotic expression vector.

Luciferase assay

Luc-STIM1 vector. The STIM1 3′-UTR containing the predicted miR-185 binding site was amplified by RT–PCR from the total RNA of cultured SW620 cells and was cloned into the pCR2.1-TOPO vector (Invitrogen). The

Figure 6. miR-185 represses the migration and invasion potential and reverses the EMT phenotype in CRC cells. (**a1**) qRT–PCR analysis of miR-185 in SW620 cells transfected with an miR-185 mimic or a negative control. (**a2**) Invasion and migration assay of SW620 cells transfected with an miR-185 mimic or a negative control. Representative fields of migration (top) or invasive (bottom) cells on membrane Scale bar, 100 μm. (**a3**) Average number of invasive or migration cells number per field from three independent experiments ± s.e. $*P < 0.05$. (**b1**) qRT–PCR analysis of miR-185 in SW480 cells transfected with an miR-185 inhibitor or a negative control. (**b2**) Invasion or migration assay of SW480 cells transfected with an miR-185 inhibitor or a negative control. Scale bar, 100 μm. (**b3**) Average number of invasive or migration cells number per field from three independent experiments ± s.e. $*P < 0.05$. (**c**) Morphological changes by miR-185 in SW620 and SW480 cells. Scale bar, 30 μm. (**d**) Expression of EMT markers was determined by western blotting in SW620 cells transfected with an miR-185 mimic or a negative control and SW480 cells transfected with an miR-185 inhibitor or a negative control. (**e1** and **e2**) Expression of EMT markers was determined by qRT–PCR in the above four cell lines. $*P < 0.05$.

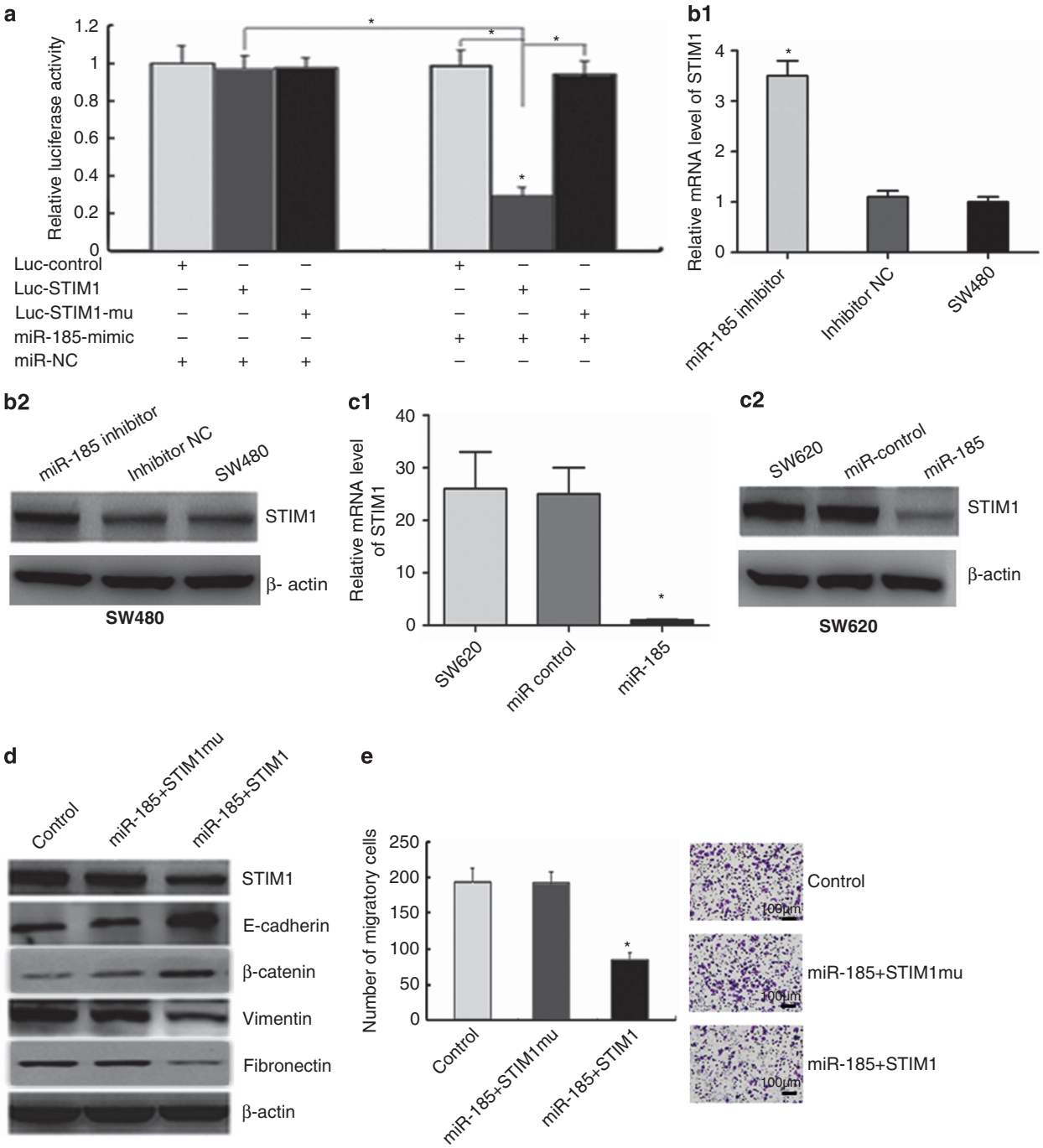


Figure 7. Directly targeting of STIM1 by miR-185 in CRC EMT. (**a**) Luciferase activity assay for targeting of the 3′-UTR of STIM1 by miR-185. The wild-type and mutant miR-185 target sequences of STIM1 were fused with luciferase reporter and transfected into vector control (Luc-STIM1 and Luc-STIM1-mu). Luc-STIM1, Luc-STIM1-mu or the vector control was co-transfected with miR-185 or an miRNA negative control in SW620 cells, and the luciferase activity measured. $P < 0.05$. (**b1** and **c1**) *STIM1* mRNA levels were analyzed by qRT–PCR. (**b2** and **c2**) STIM1 protein levels were analyzed by western blotting. β-Actin was used as an internal control. (**d**) Expression of STIM1 and EMT markers was determined by western blotting in SW620 cells. MiR-185 was co-transfected with an STIM1 wild-type construct (STIM1) or an STIM1 mutant construct in which the target sequence of miR-185 was mutant (STIM1-mu) in SW620 cells. β-Actin was used as an internal control. (**e**) Migration assay of SW620 cells. SW620 cells were treated as in (**d**). Average number of invasive or migration cell number per field from three independent experiments ± s.e. is at the right. *$P < 0.05$ (left). Representative fields of migration (top) or invasive (bottom) cells on the membrane is at the right. Scale bar, 100 μm.

pCR2.1-TOPO-STIM1-3′-UTR construct was digested with *Spe*I and *Hind*III. The resulting fragment was subcloned into the *Spe*I and *Hind*III sites of the pMIR-REPORT miRNA expression reporter vector (Applied Biosystems, Foster City, CA, USA). The first six nucleotides complementary to the miR-185 seed region were deleted from the mutant constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Standard 3′-UTR luciferase assays were performed to validate the *in silico* target predictions. Plasmids containing wild-type and mutant Luc-STIM1 coding sequences were specifically synthesized and used for the luciferase reporter assays. These

plasmids code for firefly luciferase and *Renilla* luciferase, which function as tracking genes. Luciferase activity assays were performed according to the manufacturer's protocols. Briefly, SW620 cells were seeded in six-well plates, co-transfected with the lentiviral constructs containing the miR-185 or vector control and the wild-type or mutated target gene 3′-UTR with Lipofectamine 2000 (Invitrogen). Firefly and *Renilla* luciferase activity levels were measured 48 h after the transfection using a Luc-Pair miR Luciferase Assay Kit (GeneCopoeia Inc., Rockville, MD, USA) according to the manufacturer's recommendations. Activity levels were normalized to *Renilla* luciferase. The results represent three independent experiments, each performed in triplicate.

A detailed description of the Materials and methods used in this study can be found in Supplementary Materials and methods.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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