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TBLR1 is a novel prognostic marker and promotes epithelial–mesenchymal transition in cervical cancer

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Background: Invasion and metastasis remain a critical issue in cervical cancer. However, the underlying mechanism of it in cervical cancer remains unclear. The newly discovered protein, TBLR1, plays a crucial role in regulating various key cellular functions.

Methods: In this study, western blot, real-time RT–PCR, immunohistochemical staining, 3D morphogenesis Matrigel culture, wound healing and Boyden chamber invasion assays, xenografted tumour model, luciferase assays, and chromatin immunoprecipitation assays were used.

Results: The expression of TBLR1 in cervical cancer cell lines and tissues was significantly upregulated at both the RNA and protein levels compared with that in normal cervical cells. Statistical analysis suggested that TBLR1 as an independent prognostic factor was significantly correlated with the clinical stage, survival time and recurrence. Moreover, overexpression of TBLR1 in HeLa and SiHa cell lines promoted invasion *in vitro* and *in vivo* with the increases of the mesenchymal factors vimentin and fibronectin and decreases of the epithelial marker α -catenin. In contrast, RNAi-mediated knockdown of TBLR1 inhibited epithelial–mesenchymal transition *in vitro* and *in vivo*. Further study indicated that this might be mediated via the NF- κ B and Wnt/ β -Catenin signalling pathway, and involve regulation of Snail and Twist.

Conclusions: The TBLR1 protein may be a prognostic marker in cervical cancer and play an important role in the invasion and metastasis of human cervical cancer.

Cervical cancer ranks as the third most frequent female cancer worldwide and the second most frequent in developing countries, where 83% of cases occur (Jemal *et al*, 2011). There are an estimated 529 800 new cases and 275 100 deaths annually (Jemal *et al*, 2011). In spite of its decreasing incidence and mortality in many countries, associated with wide implementation of Pap smear screening programmes in recent years, cervical cancer remains a critical issue (Kodama *et al*, 2010; Jemal *et al*, 2011). The motility and invasiveness of cancer cells play a critical role in the

mortality of cervical cancer patients, and provides important information for determining the treatment approach (Kodama *et al*, 2010; Siegel *et al*, 2011). Previous reports have shown that proteins such as p38 kinase (Kumar *et al*, 2010) and heat shock protein 70-2 (Garg *et al*, 2010) could modulate cancer cell motility and invasiveness, and could serve as prognostic markers for cervical cancer. However, thus far, no sufficiently accurate biomarker for predicting cancer cell aggressiveness preoperatively has been established, and the molecular mechanism underlying

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cervical cancer invasion and metastasis remains unclear (Noordhuis *et al*, 2011). Therefore, it is of great clinical value to identify diagnostic markers as well as novel therapeutic strategies.

The newly discovered protein, transducin β -like protein 1-related protein (TBLR1), was demonstrated to play a key role in mediating the exchange of corepressors, which was also a central aspect in key cellular functions, including growth, anti-apoptosis and inflammation (Hoberg *et al*, 2004; Perissi *et al*, 2004). Interestingly, *TBLR1* gene locates on chromosome 3 at 3q26, and the essential value of detection of genomic amplification of 3q26 in the progression of cervical cancer has been affirmed in several studies (Yang *et al*, 2001; Andersson *et al*, 2006). The TBLR1 is a LisH/WD-40-containing protein, originally associated with an X-linked human disorder in which a microdeletion of the C-terminal part of the *TBLR1* gene was suggested to be responsible for the hearing defect (An *et al*, 2003; Perissi *et al*, 2004). In addition, TBLR1 was initially identified as a component of the SMRT/N-CoR corepressor complexes that regulate multiple signal transduction pathways and gene transcriptions, including the Wnt/ β -catenin, NF- κ B, Notch and nuclear receptor pathways (Zhang *et al*, 2002; Li and Wang, 2008; Perissi *et al*, 2008). In addition, HeLa cells have been used as materials to show that histone deacetylase 3 includes TBLR1 localises to the mitotic spindle and is required for kinetochore-microtubule attachment (Ishii *et al*, 2008). Moreover, TBLR1 expression was upregulated in human primary lung squamous cell carcinoma, breast cancer and colon cancer cells, suggesting that TBLR1 is involved in promoting tumour progression or blocking apoptosis (Hoberg *et al*, 2004; Perissi *et al*, 2008; Choi *et al*, 2011). However, there has been no report on the expression of TBLR1 is correlated with clinical staging or cancer patient survival as a potential oncogene.

In this study, we report that TBLR1 expression was associated with cell aggressiveness, pelvic lymph node metastasis and prognosis in stage Ia2–IIa2 cervical patients, and induced by activating NF- κ B and Wnt/ β -catenin pathways further to regulate the expressions of Snail and Twist in cervical cancer. Thus, TBLR1 is of great clinical value as a potential early biomarker to improve the diagnosis and prognostic assessment of cervical cancer patients.

MATERIALS AND METHODS

Patient information. The current retrospective study enrolled 194 patients diagnosed with cervical cancer who underwent radical hysterectomy and lymphadenectomy in the Department of Gynecology and Obstetrics, the First Affiliated Hospital, Sun Yat-sen University from March 2000 to September 2010. All the enrolled patients were in the Ia2–IIa2 stage. The mean patient age was 42.00 ± 8.94 (range 21–70). Six fresh cervical cancer and paired adjacent noncancerous cervical tissues (ANTs) were collected for quantitative PCR (qPCR) and western blotting analysis. Prior written consent of the patients for the use of these clinical materials for research purposes and approval from the Institutional Ethical Board were obtained. The clinical information is summarised in Supplementary Data and Supplementary Table 1.

Cell lines and plasmids. Primary cultures of normal cervical epithelial cells (NCECs) were established from fresh specimens of patients without cervical lesion as described previously (Song *et al*, 2009). The cervical cancer cell lines HeLa, Siha, ME180 (TBLR1-high), HeLa229, C33A (TBLR1-low), Caski and MS7501 were purchased from the American Type Culture Collection (Manassas, VA, USA) and kept in our laboratory. All cell lines were cultured in RPMI-1640 medium (Gibco BRL, San Francisco, CA, USA). Media were supplemented with 10% fetal bovine serum and 1% antibiotics in a 5% CO₂-humidified atmosphere at 37 °C.

The pMSCV/TBLR1-overexpressing human TBLR1 was generated by subcloning the PCR-amplified human TBLR1 coding sequence into pMSCV vector. To silence endogenous TBLR1, two RNAi oligonucleotides were cloned into the pSuper-retro-puro vector to generate pSuper-retro-TBLR1-RNAi(s), respectively. The cloning primers and RNAi oligonucleotides used are shown in Supplementary Data. The pBabe-Puro-I κ B ζ -mu (plasmid 15,291)-expressing mutant I κ B ζ was from Addgene (Cambridge, MA, USA). Tcf-4 siRNA was synthesised by Shanghai Genepharma (Shanghai, China). Stable cell lines expressing TBLR1 or TBLR1 shRNA were selected for 10 days with $0.5 \mu\text{g ml}^{-1}$ puromycin 48 h after infection. After 10-day selections, the cell lysates prepared from the pooled population of cells in sample buffer were fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the detection of TBLR1 protein level.

The 3D morphogenesis Matrigel culture, wound healing and Boyden chamber invasion assay. The 24-well dishes were coated with growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and covered with growth medium supplemented with 2% Matrigel. Cells were serum starved for 24 h and then seeded at 2×10^4 cells per well, and the medium was replaced with 2% Matrigel for 3–4 days. Then, microscopic images were captured at 2-day intervals for 2 weeks. For the wound-healing assay, the stable HeLa and Siha cell were seeded in six-well plates and grown under permissive conditions until reaching 90% confluence. The cells were then serum starved for 24 h, and a linear wound was created in the confluent monolayer using a pipette tip. Wounds were observed and photographed immediately (time 0) and thereafter at 24 and 48 h. Each experiment was repeated at least three times. In the Boyden chamber invasion assay, 2×10^4 cells were seeded into $8 \mu\text{m}$ pore inserts that were coated with $50 \mu\text{l}$ Matrigel in triplicate wells. After 24 h, the cells that passed through the filter into the lower chamber were fixed with 1% paraformaldehyde, stained with haematoxylin and counted (10 random $100 \times$ fields per well).

Xenografted tumour model. The female BALB/c nude mice (4–5 weeks of age, 18–20 g) were randomly divided into 10 groups ($n = 6/\text{group}$). The stable cells (5×10^6) including TBLR1 vector, TBLR1, RNAi vector and TBLR1 shRNA 1- and 2-transduced HeLa and Siha cells were injected subcutaneously into the dorsal right flank of 10 groups of 4-week-old female Balb/c nude mice, respectively. Tumours were examined twice weekly; length and width measurements were obtained with calipers, and tumour volumes were calculated using the equation $(L \times W^2)/2$. On day 30, animals were killed; tumours were excised, weighed and paraffin embedded. Serial $6.0 \mu\text{m}$ sections were cut and subjected to immunohistochemical staining. All procedures were performed in accordance with ethical committee approval and met the standards required by the United Kingdom Coordinating Committee for Cancer Research (UKCCCR) guidelines. The conduct of all experiments involving mice adhered strictly to the standard as outlined in the guidelines for the welfare and use of animals in cancer research by Workman *et al* (2010).

Xenograft mouse metastatic model. HeLa and Siha stable cells were harvested and washed twice with PBS. The cells ($5 \times 10^6/150 \mu\text{l}$ per mouse) were injected intravenously into the tail vein of female BALB/c-nu mice, respectively. After 6 weeks, the mice were killed and the lungs were removed, fixed with 3.7% formaldehyde and sectioned. The number of mice bearing lung foci was determined. The conduct of all experiments involving mice adhered strictly to the standard as outlined in the guidelines for the welfare and use of animals in the cancer research by Workman *et al* (2010).

Luciferase assay and chromatin immunoprecipitation (ChIP) assays. These were performed as previously described (Song *et al*, 2009), and the detailed information was explicated in Supplementary Data.

Statistical analysis. All statistical analyses were carried out using the SPSS 13.0 statistical software (Chicago, IL, USA) package.

The results were expressed as mean \pm standard error. The χ^2 test and Fisher's exact test were used to analyse the relationship between TBLR1 expression and the clinicopathological characteristics. Survival curves were plotted by the Kaplan–Meier method and compared using the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. In all cases, $P < 0.05$ was considered statistically significant.

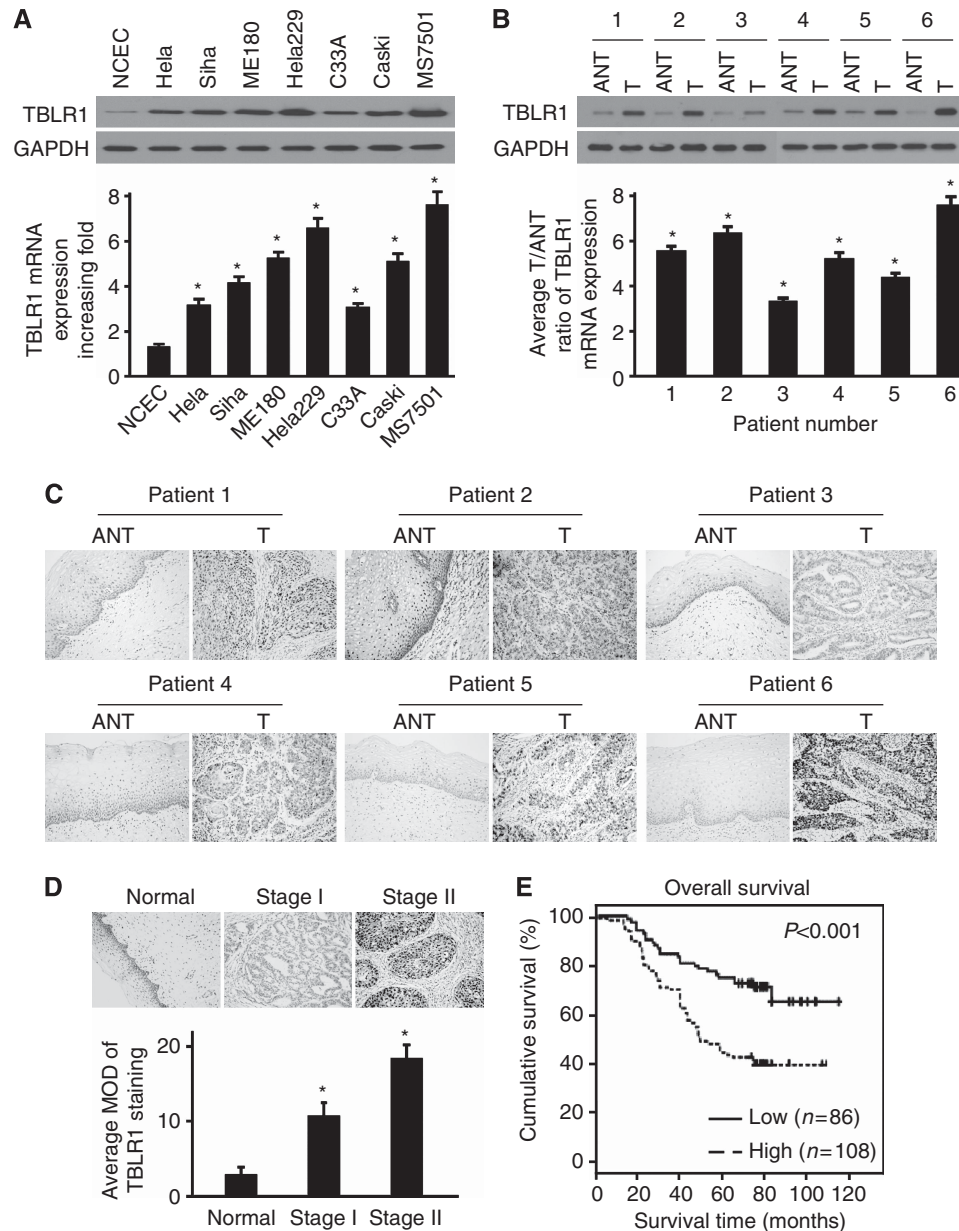


Figure 1. The protein and mRNA expression of TBLR1 determined by western blotting, quantitative PCR (qPCR) and immunohistochemical assay. The expression level was normalised to the expression level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The error bars represent s.d. values calculated from three parallel experiments. The protein expression of TBLR1 was determined by immunohistochemical assay and Kaplan–Meier curves obtained from univariate analyses (log-rank) of cervical cancer patients with low vs high TBLR1 expression. Among 194 patients, 108 showed positive TBLR1 staining, mainly in the cytoplasm, and partly in the nucleus of cancer cells, whereas the others showed marginal or undetectable TBLR1 staining. **(A)** Western blotting and qPCR assay of TBLR1 expression in normal epithelial cells (NCECs) and cervical cancer cell lines. **(B)** Western blotting of TBLR1 protein expression in six pairs of matched cervical cancer (T) and adjacent nontumour cervical tissues (ANTs) with pelvic lymph node metastasis, and T/ANT ratios of TBLR1 mRNA expression quantified by qPCR in six pairs of matched cervical cancer tissues. **(C)** Immunohistochemical assay of TBLR1 protein expression in six pairs of matched cervical cancer tissues with pelvic lymph node metastasis. **(D)** TBLR1 expression gradually increased from NCTs to cervical cancer tissues in stage II. The TBLR1 expression was undetectable in NCTs, marginal or moderate in stage I and strong in stage II of cervical cancer tissues. **(E)** Cumulative survival rate for cases with high vs none or low TBLR1 expression. Survival curves of patients with low vs high expression of TBLR1 ($n = 194$; $P < 0.001$, log-rank test); * $P < 0.05$.

Table 1. Clinicopathological characteristics of patient samples and expression of TBLR1 in cervical cancer and correlation between TBLR1 expression and clinicopathological characteristics of cervical cancer patients (n = 194)

Characteristics	TBLR1		X ² test, P-value	Fisher's exact test, P-value
	Total (n = 194)	No or low expression, 44.3%		
Gender				
Male	0	0 (0.00)	0 (0.00)	1.000
Female	194			
Age (years)				
≥42	91	41 (45.1)	50 (54.9)	0.848
<42	103	45 (43.7)	58 (56.3)	
FIGO stage				
Ia2	18	11 (61.1)	7 (38.9)	0.017
Ib1	81	42 (51.9)	39 (48.1)	
Ib2	29	14 (48.3)	15 (51.7)	
Ila1	23	9 (39.1)	14 (60.9)	
Ila2	43	10 (23.3)	33 (76.7)	
Differentiation grade				
Well	18	9 (50.0)	9 (50.0)	0.324
Moderate	86	35 (40.7)	51 (59.3)	
Poor	66	28 (42.4)	38 (57.6)	
Unclear	24	14 (58.3)	10 (41.7)	
Types of tumour growth				
Ulcerative	39	18 (46.2)	21 (53.8)	0.941
Endophytic	54	23 (42.6)	31 (57.4)	
Exophytic	101	45 (44.6)	56 (55.4)	
Pathologic types				
Squamous	43	17 (39.5)	26 (60.5)	0.764
Adenocarcinoma	132	60 (45.5)	72 (54.5)	
Adenosquamous	19	9 (47.4)	10 (52.6)	
Stromal invasion				
< 1/2	99	54 (54.5)	45 (45.4)	0.003
≥ 1/2	95	32 (33.7)	63 (66.3)	
Lymphovascular space invasion				
Yes	46	11 (23.9)	35 (76.1)	0.001
No	148	75 (50.7)	73 (49.3)	
Pelvic lymph node metastasis				
Yes	67	17 (25.4)	50 (74.6)	< 0.001
No	127	69 (54.3)	58 (45.7)	
Vaginal involvement				
Yes	11	2 (18.2)	9 (81.8)	0.072
No	183	84 (45.9)	99 (54.1)	
Parametrial infiltration				
Yes	24	6 (25.0)	18 (75.0)	0.042
No	170	80 (47.1)	90 (52.9)	
Positive surgical margins				
Yes	22	7 (46.2)	15 (53.8)	0.210
No	172	79 (45.9)	93 (54.1)	

Table 1. (Continued)

Characteristics	TBLR1			χ^2 test, P-value	Fisher's exact test, P-value
	Total (n = 194)	No or low expression, 44.3%	High expression, 55.7%		
High-risk HPV infection					
Yes	118	41 (34.7)	77 (65.3)	0.001	0.001
No	76	31 (40.8)	45 (59.2)		
Postoperative adjuvant therapy					
Chemotherapy	110	40 (36.4)	70 (63.6)	0.011	0.013
Radiation	41	21 (51.2)	20 (48.8)	0.317	0.377
Both	35	16 (45.7)	19 (54.3)	0.855	0.854
Yes (total)	133	57 (42.9)	76 (57.1)	0.542	0.641
No	61	29 (47.5)	32 (52.5)		
Recurrence					
Yes	72	24 (33.3)	48 (66.7)	0.018	0.025
No	122	62 (50.8)	60 (49.2)		
Vital status at follow-up					
Alive	103	60 (58.3)	43 (41.7)	<0.001	<0.001
Death for cervical cancer	91	26 (28.6)	65 (71.4)		

Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus; TBLR1 = transducin β -like protein 1-related protein. P-values <0.05 are indicated in bold.

RESULTS

Elevated expression of TBLR1 in cervical cancer cells at both mRNA and protein levels. The expressions of both the TBLR1 protein and mRNA were markedly upregulated in multiple cervical cancer cell lines, including Hela, Siha, ME180, Hela229, C33A, Caski and MS7501, in comparison with those in NCEC (Figure 1A). Furthermore, comparative analysis showed that TBLR1 mRNA and protein levels were differentially upregulated more than two-fold in all six cervical cancer samples compared with matched normal ANTs (Figure 1B). The upregulation of TBLR1 in these clinical samples was further verified by IHC analysis (Figure 1C). Taken together, our results indicated that TBLR1 was upregulated in cervical cancer.

TBLR1 expression is associated with the clinical characteristics of cervical cancer. To further investigate the clinical relationship between TBLR1 protein expression and cervical cancer progression, the expression of TBLR1 was examined by IHC in paraffin-embedded, archived clinical tumour tissue specimens obtained from 194 cases of cervical cancer, including 18 cases of stage Ia2 (9.3%), 81 cases of stage Ib1 (41.8%), 29 cases of stage Ib2 (14.9%), 23 cases of IIa1 (11.9%), 43 cases of IIa2 (22.1%) and 10 normal cervical tissue (NCT) samples.

The IHC staining showed that TBLR1 expression in cervical cancer increased with advanced clinical stage (Figure 1D). Quantitative analysis of IHC staining showed the level of TBLR1 protein significantly increased in all types of cervical cancer (Supplementary Figure 1), and the percentage of patients expressing TBLR1 increased from stage Ia2 to stage IIa2, but was absent in NCT (Table 1). Statistical analysis revealed that TBLR1 levels were strongly expressed in 55.7% (108 out of 194) of patients with cervical cancer and was strongly associated with FIGO stage ($P=0.017$), stromal invasion ($P=0.003$), lymphovascular space invasion ($P=0.001$), pelvic lymph node metastasis ($P<0.001$), parametrial infiltration ($P=0.042$), high-risk human papillomavirus (HPV) infection ($P=0.001$), postoperative adjuvant

chemotherapy ($P=0.011$), recurrence ($P=0.018$) and patient survival ($P<0.001$) of cervical cancer (Table 1). Significant correlations were identified between TBLR1 expression level and several prognostic risk factors by Spearman's correlation analysis, including FIGO stage ($P=0.001$), stromal invasion ($P=0.003$), lymphovascular space invasion ($P=0.001$), parametrial infiltration ($P=0.042$), pelvic lymph node metastasis ($P<0.001$), high-risk HPV infection ($P=0.001$), postoperative adjuvant chemotherapy ($P=0.01$) and survival time ($P=0.003$) (Supplementary Table 2). Univariate and multivariate Cox regression analyses showed that TBLR1 expression ($P=0.032$), FIGO stage ($P=0.007$), pelvic lymph node metastasis ($P=0.027$) and recurrence ($P<0.001$) were independent prognostic factors that influenced survival (Supplementary Table 3). There was no obvious relationship with age, differentiation grade, types of tumour growth, pathological types, vaginal involvement, positive surgical margins or post-operative adjuvant radiation (Table 1).

Moreover, Kaplan–Meier survival curves and the log-rank test survival analysis showed that the overall survival of patients with high levels of TBLR1 was significantly poorer than patients with low levels of TBLR1 ($P<0.001$; Figure 1E). In addition, similar results were obtained in subgroups of patients with squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma (Supplementary Figure 2A–C). The Cox regression model revealed that TBLR1 expression (relative risk: 1.720, CI 1.061–2.787, $P=0.028$), FIGO stage (relative risk: 1.192, CI 1.061–1.399, $P=0.031$), pelvic lymph node metastasis (relative risk: 1.736, CI 1.064–2.833, $P=0.027$) and recurrence (relative risk: 5.852, CI 3.662–9.350, $P<0.001$) were risk factors (Supplementary Table 3). Collectively, these data demonstrated TBLR1 was linked to the clinical progression of cervical cancer and might represent a valuable prognostic marker for cervical cancer.

TBLR1 overexpression correlates with pelvic lymph node metastasis and clinical progression in cervical cancer. Patient survival analysis showed a clear positive correlation between pelvic lymph node metastasis and the overall survival in patients with cervical cancer ($P<0.001$, Supplementary Figure 2D).

The cumulative 5-year overall survival for patients with pelvic lymph node metastasis was 45.4%, whereas in patients with no pelvic lymph node metastasis the rates were 77.9%. In addition, statistical analyses revealed that TBLR1 expression strongly correlated with pelvic lymph node metastasis ($P < 0.001$; Table 1), suggesting that TBLR1 overexpression is associated with the progression of human cervical cancer.

Upregulation of TBLR1 promotes cervical cancer cell EMT. In light of the obvious connection between TBLR1 expression and pelvic lymph node metastasis, we further investigated the effect of TBLR1 on invasion and metastasis in HeLa and Siha cervical cancer cells. As shown in Figure 2A, HeLa and Siha cells were established to stably overexpress TBLR1. Interestingly, in wound healing assays, HeLa-TBLR1 and Siha-TBLR1 cells migrated and almost filled the gap 48 h after wounding; however, the gap filling was significantly retarded in the control cells, indicating that TBLR1 promoted the migration properties of HeLa and Siha cells (Figure 2D). Moreover, MTT assays of the stable cell lines made it more persuasive by revealing no statistically significant differences in cell proliferation among the stable cell lines ($P > 0.05$, Supplementary Figure 3). Similar results were found in the Boyden chamber assays, another method used to test the migration and metastatic properties of cells (Figure 2C). In addition, 3D spheroid invasion assay revealed that TBLR1-transduced cervical cell lines cultured in Matrigel for 9 days displayed morphologies typical of highly aggressive invasiveness, presenting more outward projections from practically all individual cells, compared with vector control cells (Figure 2B). Moreover, the capability of TBLR1 to promote cervical cancer progression was further verified *in vivo*. The tumours formed by TBLR1-transduced cervical cancer cells were larger and had more weight than vector control tumours (Figure 2E). To further investigate the molecular mechanism mediating the aggressive effect of TBLR1, the levels of epithelial–mesenchymal transition (EMT) markers were examined. Western blotting analysis and immunofluorescence assays revealed that mesenchymal factors vimentin and fibronectin were significantly upregulated and the expression of epithelial marker α -catenin was partially rescued in the TBLR1-transduced HeLa and Siha cells (Figure 3A and B). Nevertheless, HeLa and Siha cells were originally E-cadherin-negative cell lines; therefore, Caski and ME180 cells, as E-cadherin-positive cells, were used in further western blotting analysis to identify whether the expression of the epithelial marker E-cadherin was influenced by TBLR1 in EMT. The TBLR1-transduced Caski and ME180 cervical cancer cells showed visibly downregulated expression of E-cadherin and α -catenin, whereas the expressions of vimentin and fibronectin were upregulated (Supplementary Figure 4). In addition, IHC analysis of the special tumours excised from the four groups of mice, respectively, showed that TBLR1-overexpressing tumours displayed increased expressions of vimentin and fibronectin and lower expressions of α -catenin compared with vector control tumours (Figure 3C). To further quantify metastatic potential *in vivo*, we performed tail vein xenografts and compared the rates of lung colonisation. The upregulation of TBLR1 expression resulted in a significant increase in the number of lung metastatic nodules (Figure 4A and B). In brief, upregulation of TBLR1 promotes cervical cancer cell EMT *in vitro* and *in vivo*.

Downregulation of TBLR1 inhibited cervical cancer cell EMT. To further demonstrate the significance of the pro-EMT function of TBLR1 in cervical cancer cells, the HeLa and Siha cells were infected with empty vector alone and the two shRNAs that suppressed endogenous TBLR1 expression stably. As expected, TBLR1 protein levels were decreased in the TBLR1-silenced cells (Figure 5A). Downregulation of TBLR1 reduced the irregular branched structures of both cells in 3D morphogenesis Matrigel cultures that characterised the invasive phenotype (Figure 5B).

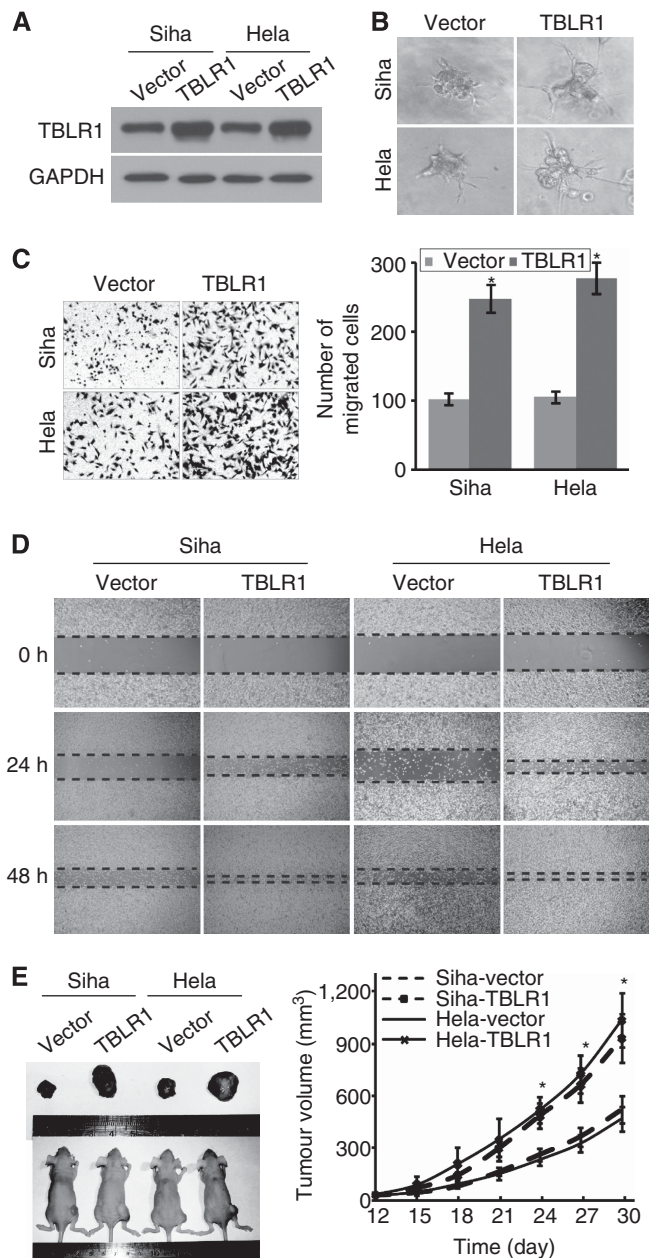


Figure 2. Cell motility and invasion were promoted by TBLR1 overexpression *in vitro* and *in vivo*. (A) Western blotting of TBLR1 expression in vector- and TBLR1-transduced HeLa and Siha cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) The acini formation of cells was tested in 3D morphogenesis Matrigel culture ($\times 200$). (C) The invasive properties induced by fetal bovine serum were analysed by the Boyden chamber invasion assay ($\times 200$, $*P < 0.05$). (D) The mobility of cells was measured by testing the rate of wound closure at 0, 24 and 48 h ($\times 200$). (E) Effect of TBLR1 on tumour formation in nude mouse xenograft model. A representative picture of tumour growth in nude mice subcutaneously inoculated with vector- and TBLR1-transduced HeLa and Siha cells (left). Subcutaneous tumour growth curve of TBLR1-transduced HeLa and Siha cells in nude mice was compared with vector control HeLa and Siha cells in nude mice ($n = 6$) (right); $*P < 0.05$.

Moreover, the wound-healing assay and Boyden chamber invasion assay were dramatically inhibited by the ablation of TBLR1 expression (Figure 5C and D and Supplementary Figure 5). Strikingly, no statistically significant differences were found in MTT assays of the stable cell lines either ($P > 0.05$, Supplementary Figure 3).

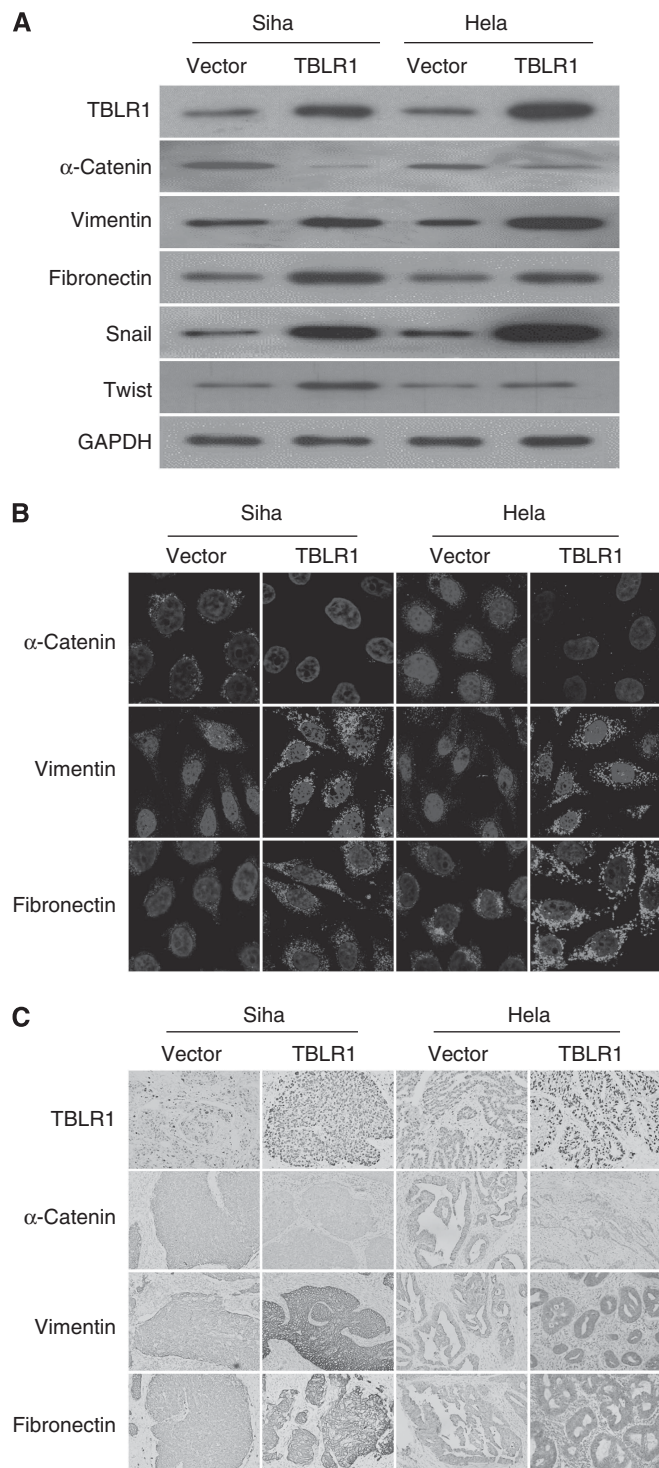


Figure 3. Overexpression of TBLR1 promoted EMT by upregulating the expressions of Snail and Twist in cervical cancer. **(A)** Western blotting of the expressions of TBLR1 and EMT-relevant markers α -Catenin, fibronectin and vimentin, as well as EMT regulators Snail and Twist in the vector- and TBLR1-transduced HeLa and Siha cells, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(B)** Immunofluorescence assay of EMT-relevant markers (α -Catenin, fibronectin and vimentin) ($\times 100$). **(C)** The IHC assay of protein expression of TBLR1 and EMT-relevant markers (α -Catenin, fibronectin and vimentin) in the tumours excised from four groups of mice including the vector- and TBLR1-transduced HeLa and Siha groups, respectively ($\times 200$).

However, tumours formed by TBLR1-silenced cells were smaller, in both size and weight, than the tumours formed by shRNA vector cells (Figure 5E). Besides, the mesenchymal markers vimentin and fibronectin were downregulated, whereas α -Catenin was markedly overexpressed in TBLR1-silenced cells compared with the control cells (Figure 6A and B). Similar results were obtained in TBLR1-silenced Caski and ME180 cells that also showed increased expression of E-cadherin compared with vector control cells (Supplementary Figure 4). Moreover, IHC analysis of mice tumours showed the same trend: decreased expressions of vimentin and fibronectin and increased expressions of α -Catenin in TBLR1-silenced tumours (Figure 6C). *In vivo* metastatic assay, lung metastatic foci were present in the experimental mice, but fewer in the groups of mice injected with TBLR1-silenced cells (Figure 4A). Strikingly, the statistical decreases in the number of lung metastatic nodules resulted from downregulation of TBLR1 (Figure 4B). Consequently, these results indicate that downregulation of TBLR1 inhibited cervical cancer cell EMT *in vitro* and *in vivo*.

TBLR1 induces EMT by regulating the NF- κ B and Wnt/ β -catenin pathways to upregulate the expressions of Snail and Twist. Combining our results with those of previous studies of the biological function and mechanism of TBLR1 (An *et al*, 2003; Perissi *et al*, 2004; Li and Wang, 2008; Choi *et al*, 2011), we decided to test whether the Snail and Twist protein levels were influenced in the stable HeLa and Siha cell lines. Western blotting showed that the expressions of Snail and Twist were significantly higher in TBLR1-overexpressing tumours and lower in TBLR1-silenced tumours than the respective control tumours (Figures 3A and 6A). Further experiments showed that overexpressing TBLR1 significantly enhanced, but silencing TBLR1 reduced, the activities of NF- κ B and Wnt/ β -Catenin luciferase reporters as well as affected the expression levels of their downstream proteins Snail and Twist (Figure 7A and B). To further investigate the mechanism by which TBLR1 promotes cervical cancer pathogenesis, the effects of blocking the NF- κ B and Wnt/ β -Catenin pathway on TBLR1-induced aggressiveness were examined. As shown in Figure 7C and D, either overexpressing an $I\kappa B\alpha$ dominant-negative mutant ($I\kappa B\alpha$ -mu) or silencing TCF4 by RNA interference could significantly decrease the expression levels of EMT regulators, including Snail and Twist, as well as the expression of EMT markers, including vimentin and fibronectin. Intriguingly, the stable HeLa and Siha TBLR1-overexpressing cells treated with both $I\kappa B\alpha$ -mu and TCF4-RNAi showed more significant deregulations of Snail, Twist and the expressions of vimentin and fibronectin (Figure 7C and D). Moreover, by performing a ChIP assay, we found that TBLR1 bound to regions 1 and 5 within the snail promoter but regions 2, 4 and 7 within the Twist promoter (Figure 7E). Therefore, TBLR1 might regulate EMT to induce cervical cancer cell aggressiveness and lymph node metastasis through the NF- κ B and Wnt/ β -catenin pathways by regulating the expressions of Snail and Twist.

DISCUSSION

Our results indicate that TBLR1 has the potential to act as a novel predictor of pelvic lymph node metastasis and recurrence in cervical cancer patients. The standard treatment of early-stage cervical cancer patients is radical hysterectomy plus lymphadenectomy or chemoradiation (Benedet *et al*, 2000). Adjuvant therapies are required for patients with lymph node metastasis that would make the initial surgical procedure unnecessary in retrospect (Benedet *et al*, 2000; Selman *et al*, 2008). Thus, the FIGO guidelines have been used to categorise patients into a high-risk group and an intermediate-risk group according to the pathological risk factors, including positive pelvic lymph nodes, microscopic parametrial invasion, tumour-positive resection margins

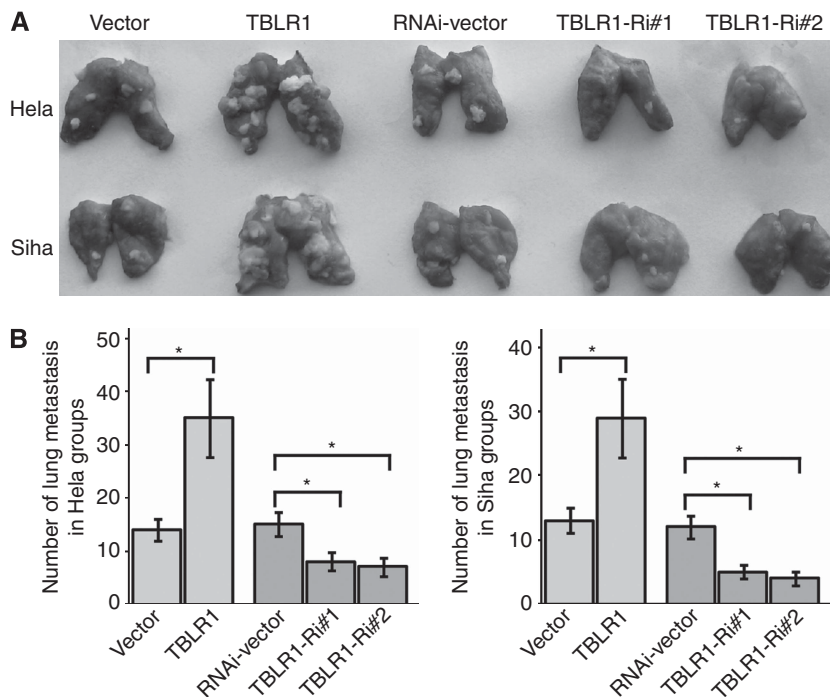


Figure 4. Results of a xenograft mouse metastatic model. The metastatic capacity was increased on TBLR1 overexpression and decreased on TBLR1 knockdown. (A) Lungs removed from mice injected with the indicated cells. (B) The numbers of metastatic tumours were counted. * $P < 0.05$.

and deep stromal invasion (Biewenga *et al*, 2011), that can subsequently affect the decision to perform adjuvant therapy. Among these factors, pelvic lymph node metastases are the most important postoperative risk factor for recurrence or failure to survive, and thus require adjuvant therapy (Kodama *et al*, 2010; Biewenga *et al*, 2011; Chen *et al*, 2013). Unfortunately, gynaecological oncologists could not determine treatment properly because the sentinel lymph node biopsy as a promising technique failed to show sufficiently accuracy to detect micrometastases with a high false negative rate as well as the imaging methods (Altgassen *et al*, 2008; Selman *et al*, 2008; Slama *et al*, 2013).

As treatment strategies are very limited for metastatic and recurrent cervical cancer, the upregulated TBLR1 identified in our study is a potential marker and target for formulating treatment strategies. In this study, the accuracy of predicting pelvic lymph node metastasis by investigating TBLR1 expression level in our cohort was 35.2% (38 out of 108). However, in patients who demonstrated high expression of TBLR1, but were not detected with lymph node metastasis, the recurrence rate reached 31.4%, whereas the rate of recurrence was only 18.5% ($P < 0.05$) in the group of low expression of TBLR1. Given that the reported pelvic lymph node micrometastasis rate in early-stage cervical cancer patients was 15% (Wangsa *et al*, 2009), it is highly likely that the 'real' accuracy of predicting pelvic lymph node metastasis by investigating TBLR1 expression level (high vs low) could be as high as 50.2% (35.2% plus 15%).

Human papillomavirus (HPV) infection is a necessary causal factor in cervical cancer development (Muñoz *et al*, 2003). The average HPV infection rate among different cervical cancer subgroups reached 99.7% in squamous cell carcinoma, whereas in adenocarcinoma and adenosquamous carcinoma it ranged from 94% to 100% (de Sanjose *et al*, 2010). Moreover, the E8^AE2C protein has been studied in high-risk HPV, where it has shown to regulate viral genome levels and to repress transcription from the viral promoter (Laudadio, 2013). Interestingly, using an unbiased proteomic analysis, Powell *et al* (2010) identified six high-confidence candidate interacting proteins for E8^AE2C: the top two were NCoR1 and TBLR1. Consistent with previous study, we

found TBLR1 was associated with HPV as well as progression of cervical cancer ($P < 0.05$).

Previously, it was observed that overexpression of TBLR1 could act as a novel diagnostic or therapeutic target in lung squamous cell carcinomas by immortalising a bronchial epithelial cell line that was considered 'precancerous' (An *et al*, 2003; Huang *et al*, 2009). Interestingly, TBLR1 was also amplified in breast cancer, where it might play an important part in progression (Perissi *et al*, 2008). Furthermore, knockdown of TBL1 and TBLR1 significantly suppressed the invasive growth of head and neck squamous cell carcinoma cells (Li and Wang, 2008), suggesting that TBLR1 may be required for tumourigenesis and metastasis. In the present study, the oncogenic potential of TBLR1 suggested by its ability to promote cervical cancer EMT *in vitro* and *in vivo*. Epithelial-mesenchymal transition plays key roles in determining cervical cancer risk factors, including invasion, metastatic dissemination and acquisition of therapeutic resistance of cancer cells (Polyak and Weinberg, 2009). Multiple different signalling pathways, including NF- κ B, Wnt and JAK/STAT3, have been reported to regulate EMT in cervical cancer (Hsu *et al*, 2007; Mayrand *et al*, 2007; Li *et al*, 2009; Wu *et al*, 2013). Among them, NF- κ B has been reported to be constitutively activated in cervical cancer to promote EMT (Huang *et al*, 2009). Epigenetic silencing of SFRP genes contributed to cervical cancer progression through EMT by Wnt/ β -Catenin pathway (Chung *et al*, 2009). Notably, both NF- κ B and Wnt/ β -Catenin pathways play vital roles in regulating EMT in cervical cancer. Interestingly, TBLR1 was reported to be essential for transcriptional activation of NF- κ B (Perissi *et al*, 2004, 2008). In addition, Perissi *et al* (2008) provided evidence that dedicated strategies based on the indispensable actions of TBLR1 were adopted by NF- κ B-regulated genes in HeLa cells. Depletion of TBLR1 by specific siRNAs in TNF- α -stimulated endothelial cells result in significant inhibition of the NF- κ B-dependent gene activation programme (Perissi *et al*, 2004). A prerequisite for NF- κ B transcription and survival is that SMRT is derepressed by I κ B kinase α with a loss of HDAC3 and with the recruitment of TBLR1 exchanged for co-activators (Grandori *et al*, 2000; Hoberg *et al*, 2004). On the other hand, mammalian TBLR1 was also shown to collaborate with Siah1 in a novel pathway for

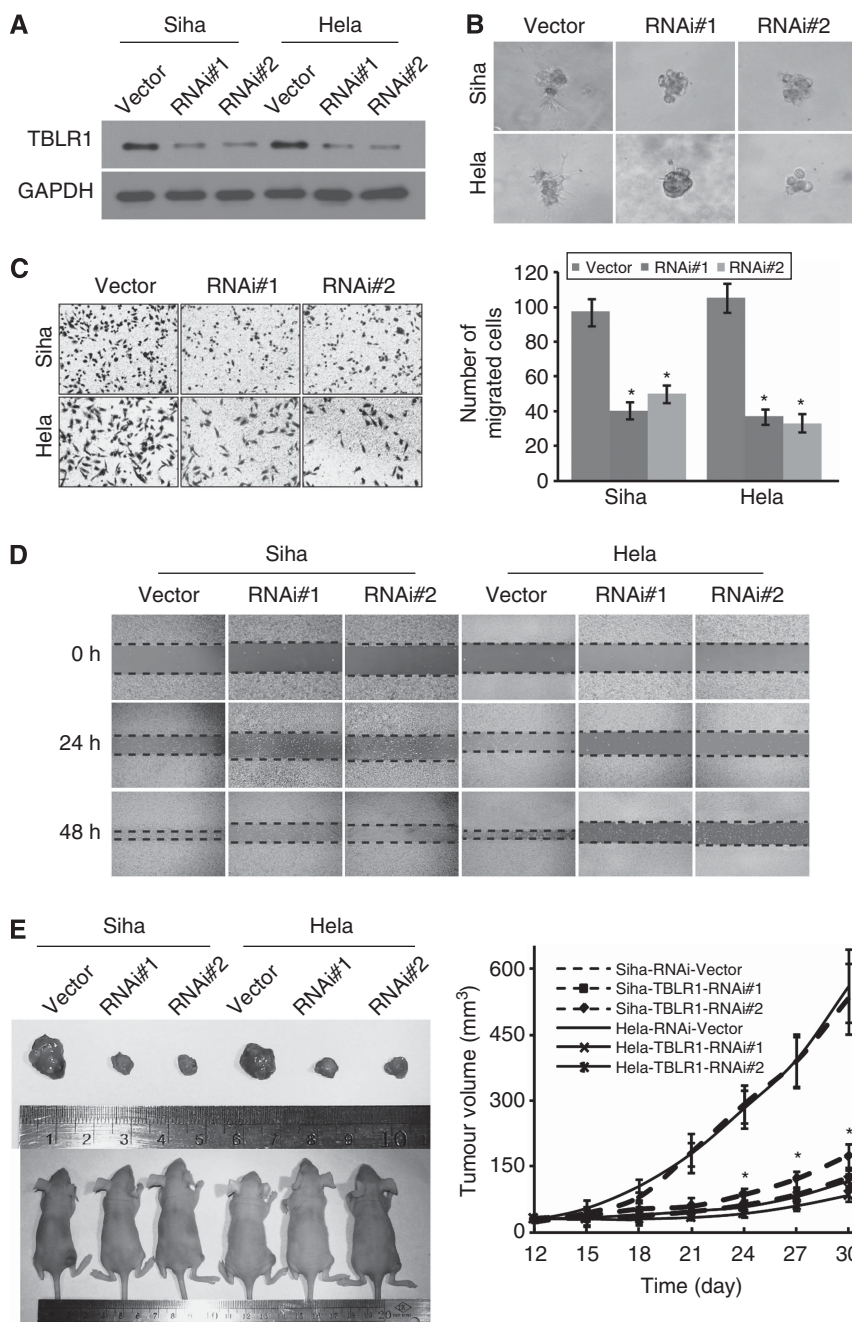


Figure 5. Downregulation of TBLR1 repressed cellular motility and invasion *in vitro* and *in vivo*. **(A)** Western blotting of the expression of TBLR1 in RNAi vector and TBLR1 shRNA 1- and 2-transduced HeLa and Siha cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(B)** The acini formation of cells was tested in 3D morphogenesis Matrigel culture ($\times 200$). **(C)** The invasive properties induced by fetal bovine serum were analysed by the Boyden chamber invasion assay ($\times 200$, $*P < 0.05$). **(D)** Cell mobility was measured by testing the rate of wound closure at 0, 24 and 48 h ($\times 200$). **(E)** Effect of TBLR1 on tumour formation in nude mouse xenograft model. A representative picture of tumour growth in nude mice subcutaneously inoculated with RNAi vector and TBLR1 shRNA 1- and 2-transduced HeLa and Siha cells (left). Subcutaneous tumour growth curve of TBLR1 shRNA 1- and 2-transduced HeLa and Siha cells in nude mice was compared with RNAi vector HeLa and Siha cells in nude mice ($n = 6$) (right). $*P < 0.05$.

β -catenin degradation. Furthermore, the role of TBLR1 in the promotion of Wnt/ β -catenin-mediated oncogenesis has been emphasised recently in a Wnt signalling-dependent manner (Yoon *et al*, 2005). In addition, TBLR1 may promote cervical cancer EMT as an indispensable factor for β -catenin-mediated invasive growth that plays a critical role in p53-induced β -catenin degradation, because knockdown of TBLR1 strongly inhibited tumour cell invasion through the Matrigel-coated membrane in head and neck squamous cell carcinoma (Li and Wang, 2008). Interestingly, previous studies show that TCF-4 mediates a

nuclear response to Wnt signals by interacting with β -catenin, and TCF4-RNAi could then directly downregulate the expression of TCF-4 as an effective inhibitor of Wnt/ β -Catenin pathway (Ravindranath *et al*, 2011; Zhang *et al*, 2011). Moreover, I κ B α -mu effectively inactivated the I κ B α protein (Cabannes *et al*, 1999), thus causing NF- κ B to be significantly inactive in the tumour cells and this activity contributes to the different malignant state of these tumour cells such as the inhibiting the progression of oesophageal squamous cell carcinoma (Song *et al*, 2012). In light of these findings, we further validated that both NF- κ B and

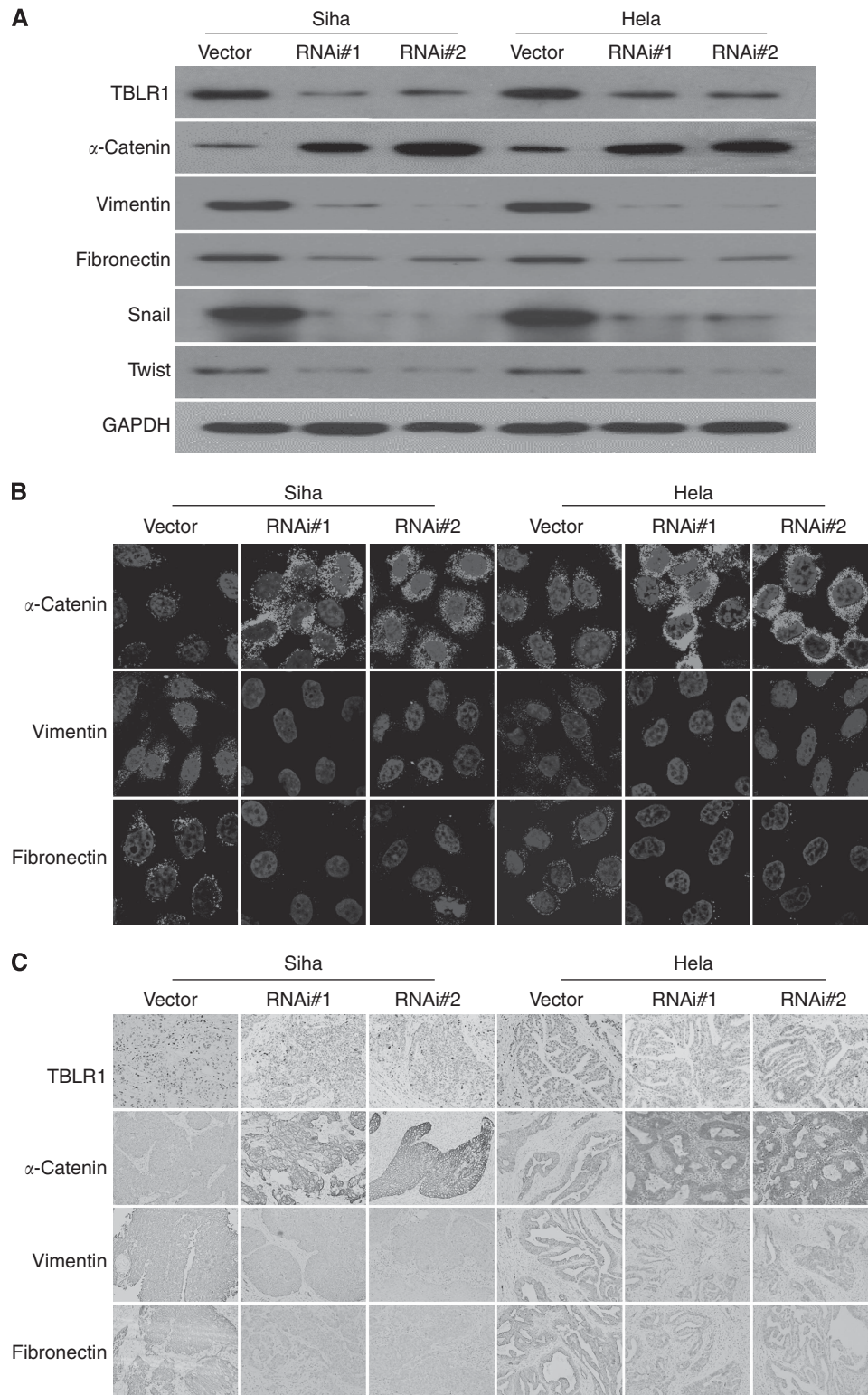


Figure 6. Knockdown of TBLR1 inhibited EMT by downregulating the expressions of Snail and Twist in cervical cancer. **(A)** Western blotting of the expressions of TBLR1 and EMT-relevant markers α -Catenin, fibronectin and vimentin, as well as EMT regulators Snail and Twist in RNAi vector and TBLR1 shRNA 1- and 2-transduced HeLa and Siha cells. Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(B)** Immunofluorescence assay of EMT-relevant markers (α -Catenin, fibronectin, vimentin) ($\times 100$). **(C)** The IHC assay of protein expression of TBLR1 and EMT-relevant markers (α -Catenin, fibronectin and vimentin) in the tumours excised from six groups of mice including the RNAi vector and TBLR1 shRNA 1- and 2-transduced HeLa and Siha groups, respectively ($\times 200$).

Wnt/ β -Catenin pathways, under the sophisticated regulation of TBLR1, play the essential roles in promoting EMT in cervical cancer by increasing the expression levels of EMT markers, including vimentin and fibronectin. Consistent with previous

reports, we demonstrated that TBLR1 might contribute to invasion, metastasis and progression of cervical cancer, and silencing TBLR1 inhibited the EMT of human cervical cancer cells through NF- κ B and Wnt/ β -Catenin pathways.

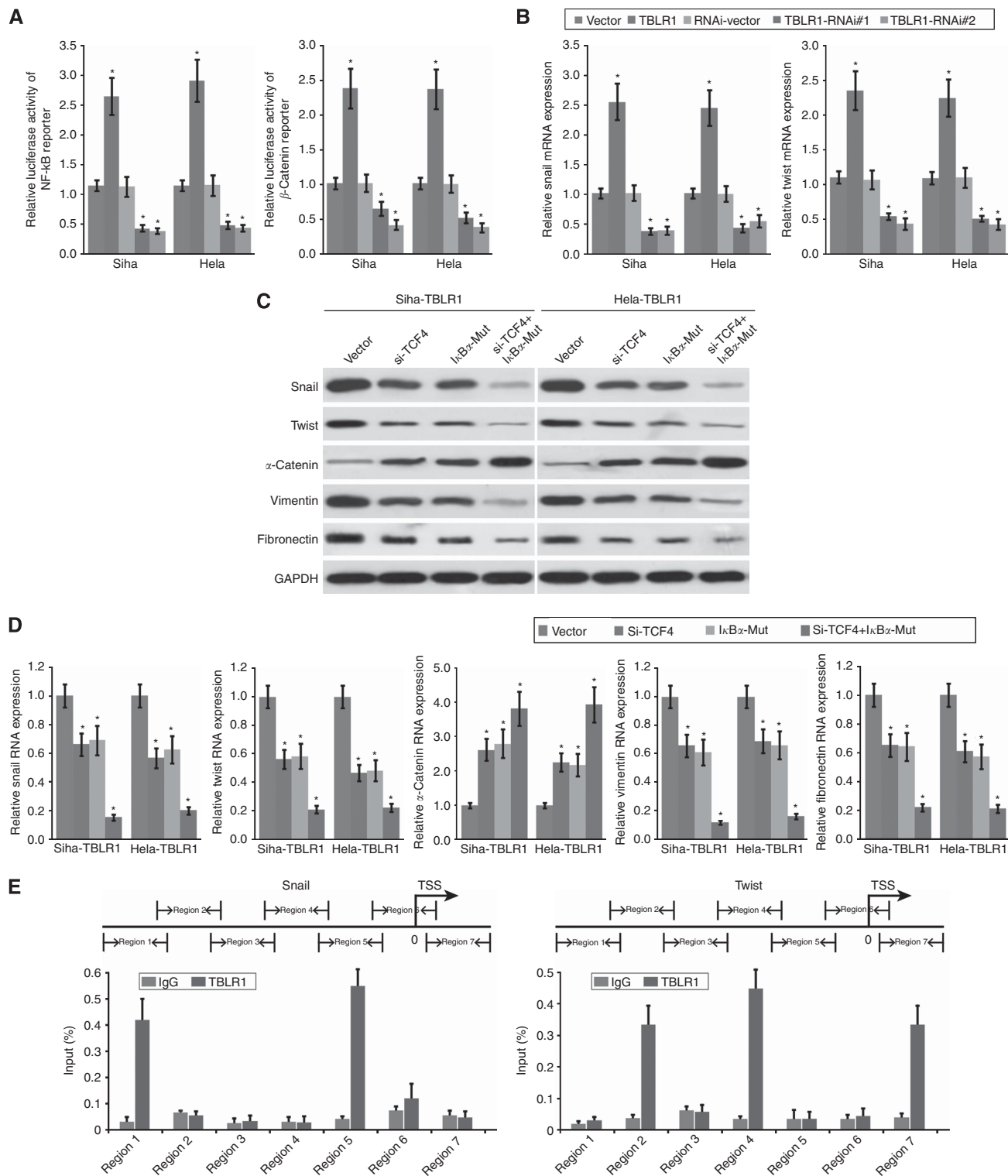


Figure 7. (A) Relative luciferase activities of NF-κB and β-Catenin reporters, respectively, in the stable cell lines. Error bars represent the mean ± s.d. from three independent experiments. **(B)** Real-time PCR analysis indicating an apparent overlap between TBLR1 and Snail mRNA expression, and between TBLR1 and Twist mRNA expression, respectively, in the stable HeLa and Siha cells. Bars represent the mean ± s.d. values. Experiments were performed in triplicate. **(C)** Western blotting of the expressions of EMT-relevant markers including α-Catenin, fibronectin and vimentin and EMT regulators, Snail and Twist, in the stable TBLR1-overexpressing cells, and the stable TBLR1-overexpressing cells transfected with TCF4-RNAi or (and) IκBζ-mu, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(D)** Real-time PCR analysis of the expressions of EMT-relevant markers including α-Catenin, fibronectin, vimentin, Snail and Twist in the above cells, respectively. Bars represent the mean ± s.d. values. Experiments were performed in triplicate. **(E)** Binding of TBLR1 to the Snail and Twist promoters was analysed by ChIP, respectively. The results display the means ± s.e.m. performed in triplicate. *P<0.05.

The molecular mechanisms underlying the role of TBLR1 in cervical cancer EMT in NF- κ B and Wnt/ β -Catenin pathways, however, remain largely unknown. A preliminary study on the molecular mechanisms demonstrated the indispensable roles of Snail and Twist that were regulated by the NF- κ B and Wnt/ β -Catenin pathways. In cervical cancer, gene expression arrays have identified an increase in the expression of the EMT-related genes (Snail, Twist and E-cadherin) in lymph node micrometastases compared with the primary tumours (Julien *et al*, 2007; Hsu *et al*, 2007; Chung *et al*, 2009). Among the various signals associated with the EMT programme, Snail was a central transcription factor (Shagieva *et al*, 2012). Lee *et al* (2008) showed that EMT could be modulated by the signal from extracellular matrix fibronectin and $\alpha 5 \beta 1$ integrin in cervical cancer cells via upregulating Snail expression. Recently, Baritaki *et al* (2010) proposed that activation of the NF- κ B pathway regulated the expression of the EMT phenotype by inducing the Snail family of transcription factors. Snail was regulated as a direct target of NF- κ B to promote EMT via the amyl hydrocarbon receptor (Barberà *et al*, 2004; Min *et al*, 2008). Twist2 overexpression was also significantly linked to cervical cancer progression, in combination with aberrant E-cadherin expression in primary cervical cancer tissues, predicting the malignant transformation and distal metastasis (Shibata *et al*, 2008; Li *et al*, 2012). Moreover, SFRP1 and SFRP2 have been demonstrated to suppress EMT in cervical cancer by enhancing E-cadherin through inhibition of Snail and Twist that were regulated by Wnt/ β -Catenin pathway (Chung *et al*, 2009). Consistent with previous studies, our findings provided further insights into the contribution of Snail and Twist as downstream signal molecules of NF- κ B and Wnt/ β -Catenin signalling pathways in promoting EMT, further enhancing the invasion and metastasis of cervical cancer.

In conclusion, we have demonstrated an important role for TBLR1 in cervical cancer, where it is remarkably associated with poor prognosis and enhanced progression of cervical cancer. Future studies, which are already underway in our laboratory, should address the detailed molecular mechanisms underlying the role of TBLR1 in the carcinogenesis and the exact relationship between TBLR1 and HPV infection of cervical cancer.

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