

## ORIGINAL ARTICLE

Transducin ( $\beta$ )-like 1 X-linked receptor 1 promotes gastric cancer progression via the ERK1/2 pathwayQ Zhou<sup>1</sup>, X Wang<sup>1</sup>, Z Yu, X Wu, X Chen, J Li, Z Zhu, B Liu and L Su

Gastric cancer (GC) is one of the most common types of cancer worldwide, and it involves extensive local tumour invasion, metastasis and poor prognosis. Understanding the mechanisms regulating the progression of GC is necessary for the development of effective therapeutic strategies. Transducin ( $\beta$ )-like 1 X-linked receptor 1 (TBL1XR1) is an important regulator controlling gene activation and repression, which has been thought to be involved in tumorigenesis. However, the role of TBL1XR1 in human GC remains largely unknown. Here, we find that TBL1XR1 is aberrantly expressed in human GC tissues, and TBL1XR1 levels are highly correlated with local tumour invasion, late tumor, lymph node, metastasis (TNM) stage and poor prognosis. Knockdown of TBL1XR1 by shRNA inhibits GC cell proliferation, migration, invasion, epithelial-mesenchymal transition (EMT) *in vitro*, as well as tumorigenesis and peritoneal metastasis *in vivo*, whereas overexpression of TBL1XR1 produces the opposite effects. These effects are mediated by activation of the ERK1/2 signalling pathway, and inhibition of this pathway with a specific ERK1/2 inhibitor (U0126) significantly impairs the tumour-promoting effects induced by TBL1XR1. Moreover, TBL1XR1 mediated ERK1/2 activation is dependent on the  $\beta$ -catenin/MMP7/EGFR signalling pathway. In conclusion, TBL1XR1 contributes to GC tumorigenesis and progression through the activation of the  $\beta$ -catenin/MMP7/EGFR/ERK signalling pathway and may act as a new therapeutic target for GC.

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## INTRODUCTION

Gastric cancer (GC) poses a tremendous health burden on communities worldwide, with 723 100 deaths reported in 2012.<sup>1–3</sup> Despite the declining incidence of GC, patients have poor prognosis due to the high rate of metastasis and postsurgical recurrence. Surgical resection and adjuvant chemotherapy are the main methods used for the treatment of GC, the overall 5-year survival rate remains grim, with less than 30% survival worldwide.<sup>4,5</sup> Therefore, it is vital for scientists and physicians to explore the molecular mechanisms underlying the development and progression of GC and identify new therapeutic targets.

Transducin ( $\beta$ )-like 1 X-linked receptor 1 (TBL1XR1 or TBLR1) is a newly discovered F-box/WD-40-containing protein that is mainly expressed in CD34<sup>+</sup>CD38<sup>-</sup> cells and plays a crucial role in regulating the activation of corepressors.<sup>6–8</sup> TBL1XR1 is an essential component of the SMRT (silencing mediator of retinoic acid and thyroid hormone receptors)/N-CoR (nuclear receptor corepressor)/HDAC3 (histone deacetylase 3)/TBL1 (transducin ( $\beta$ )-like 1)/GPS2 (G-protein pathway suppressor 2) nuclear receptor corepressor complex,<sup>9–12</sup> and overexpression of TBL1XR1 can induce gene repression by targeting gene promoters through transcription factors (TFs) and nuclear receptors (NRs).<sup>12–14</sup> TBL1XR1 also acts as a special adaptor molecule for the localization of the ubiquitin-19S/proteasome complex, which modulates the efficient exchange of corepressors for coactivators.<sup>11,15</sup> Many recurrent mutations and *de novo* deletions have been found in the *TBL1XR1* gene, which are associated with intellectual disability.<sup>16–20</sup> Studies have shown that TBL1XR1 can

mediate the activation of many different transduction pathways, such as NF- $\kappa$ B, NRs, Wnt/ $\beta$ -catenin and Notch pathways.<sup>9–12,15,21,22</sup>

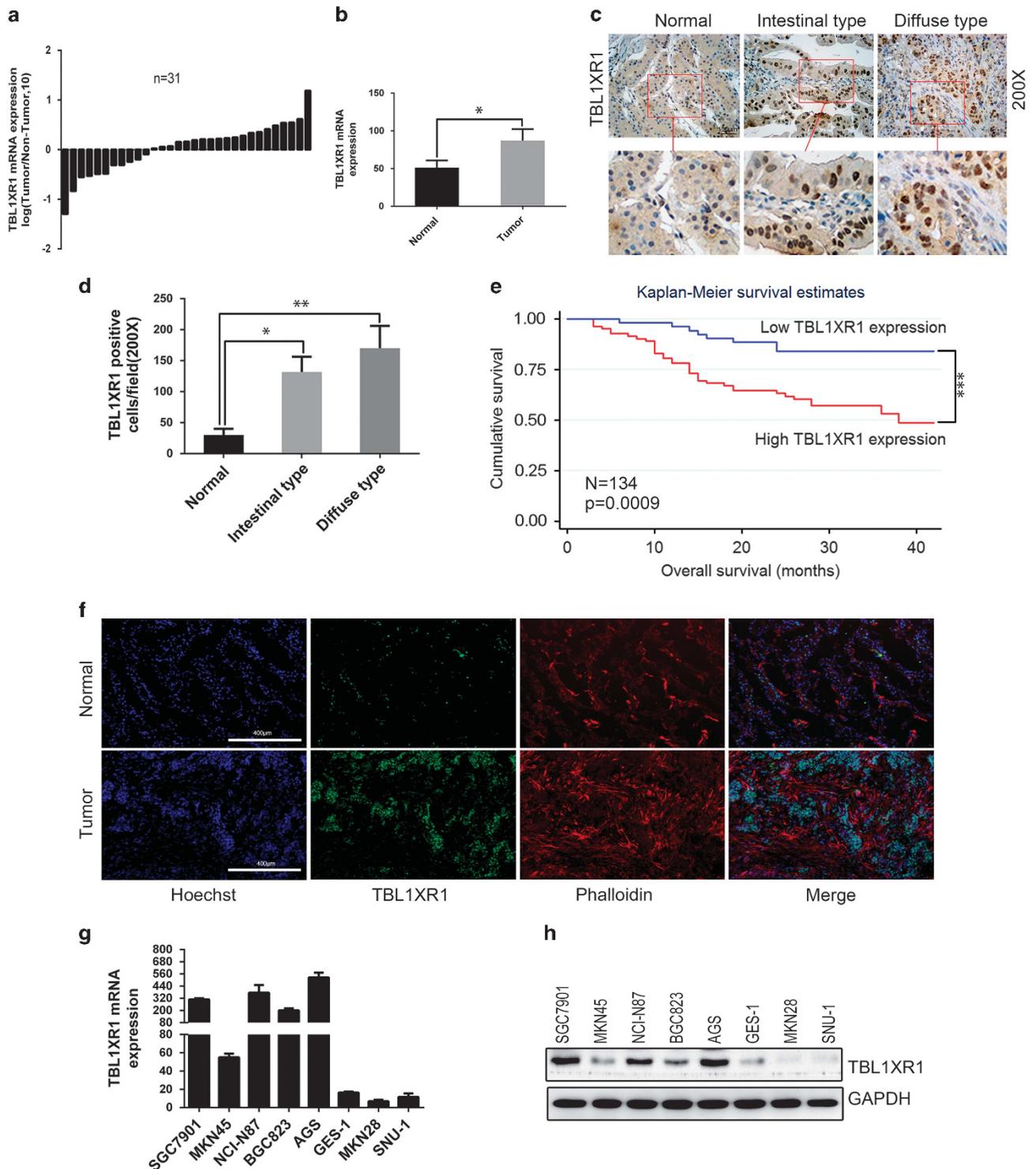
Recently, accumulating evidences have indicated that TBL1XR1 plays a crucial role in tumorigenesis, tumour progression and metastasis.<sup>23–29</sup> Liu *et al* indicated that TBL1XR1 is highly expressed in human primary lung squamous cell carcinoma tissues.<sup>20</sup> Li *et al* showed that TBL1XR1 is amplified in breast cancer and plays an oncogenic role in breast cancer progression through activation of the Cyclin D1 and  $\beta$ -catenin signalling pathway.<sup>25</sup> Liu *et al* demonstrated that TBL1XR1 mediates VEGF-C expression by binding to the promoter region of the VEGF-C gene, which induces lymphangiogenesis and lymphatic metastasis in esophageal squamous cell carcinoma.<sup>28</sup> A recent study showed that TBL1XR1 can also serve as an androgen receptor coactivator and suppress prostate cancer growth by directly activating androgen receptor target genes involved in cell differentiation and growth suppression, instead of cell proliferation.<sup>24</sup> Although previous findings strongly suggest the critical role of TBL1XR1 in tumorigenesis and progression of many types of cancer, the biological functions and clinical significance of TBL1XR1 in GC are not clear.

In this study, we show that TBL1XR1 is significantly elevated in GC and its overexpression is closely associated with local invasion, late tumor, lymph node, metastasis (TNM) stage and short survival of patients with GC. Studies of TBL1XR1 with gain- and loss-of-function approaches in GC cells reveal that TBL1XR1 regulates cell growth, migration, invasion and epithelial-mesenchymal transition (EMT) *in vitro*, as well as tumorigenesis and peritoneal metastasis *in vivo* through the activation of the  $\beta$ -catenin/MMP7/EGFR/ERK pathway in

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**Figure 1.** Expression of TBL1XR1 in GC tissues and cell lines. **(a)** QRT-PCR analyses of *TBL1XR1* mRNA expression in GC and patient-matched normal tissues ( $n = 31$ ). **(b)** Histogram displaying the relative mRNA expression of *TBL1XR1* in 31 GC tissues. **(c)** Immunohistochemical staining of TBL1XR1 in GC tissues. Representative images of TBL1XR1 staining in surgical specimens from normal tissues, intestinal GC tissues and diffuse GC tissues (magnification:  $\times 200$ ). **(d)** Histogram shows the number of TBL1XR1 positive cells in gastric tumour tissues. **(e)** Cumulative survival curve of GC patients. **(f)** Immunofluorescence shows the nuclear localization of TBL1XR1 in GC tissues and paired normal tissues (magnification:  $\times 200$ ). **(g)** Histogram displaying the relative mRNA expression of *TBL1XR1* in seven gastric tumour cell lines (SGC7901, MKN45, NCI-N87, BGC823, AGS, MKN28 and SNU-1) and one immortalized normal gastric epithelial cell line (GES-1). **(h)** The expression of TBL1XR1 protein in gastric tumour cell lines and immortalized normal gastric epithelial cell line (GES-1) was examined by western blot analysis. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

GC cells. These findings indicate that TBL1XR1 plays a pivotal role in the progression of GC and it may be of clinical value as a therapeutic target to improve the treatment of GC.

## RESULTS

TBL1XR1 is highly expressed in human GC and correlated with poor prognosis

To determine the biological role of TBL1XR1 in human GC progression, TBL1XR1 expression was first examined in human GC tissue samples. The mRNA expression of *TBL1XR1* was measured in 31 pairs of GC and patients-matched normal tissues by quantitative RT-PCR (qRT-PCR). As shown in Figures 1a and b, the *TBL1XR1* mRNA level was significantly higher in GC tissues than in normal tissues. TBL1XR1 protein expression was then examined in 134 pairs of GC and corresponding normal tissues by immunohistochemical staining (IHC). Here, 82 of 134 GC tissues (the positive rate: 61.2%) and 40 of 134 adjacent normal tissues (the positive rate: 30.7%) were found positive for TBL1XR1 expression ( $P < 0.05$ ). Typical immunostaining of TBL1XR1 in normal and GC tissues was shown in Figure 1c and positive staining of TBL1XR1 protein was mainly identified in the nuclei of GC cells. The number of TBL1XR1 positive cells per field (200×) in tumour tissues was significantly higher than in normal tissues (Figures 1c and d). Moreover, the Pearson  $\chi^2$  test showed the expression of TBL1XR1 protein was significantly associated with local invasion ( $P = 0.040$ ) and late TNM stage ( $P < 0.001$ ), but not with other clinicopathological parameters including age, tumour differentiation or tumour size (Table 1). Furthermore, Kaplan–Meier survival analysis showed that patients with high TBL1XR1 expression had a shorter overall survival (Figure 1e). In addition, immunofluorescence also proved that TBL1XR1 was highly expressed in the nuclei of GC cells (Figure 1f). TBL1XR1 expression was also evaluated in seven GC cell lines and one normal gastric epithelial cell line (GES-1). As shown in Figures 1g and h, both mRNA and protein levels of TBL1XR1 were higher than GES-1 in seven GC cell lines. Thus, these results indicate that TBL1XR1 is overexpressed in GC and may play an important role in GC progression.

### TBL1XR1 promotes GC cell growth *in vitro*

Given a potential link between TBL1XR1 overexpression and GC progression, we determined whether knockdown of TBL1XR1 expression could affect cell growth in GC. To further elucidate the biological functions of TBL1XR1 in GC progression, TBL1XR1 expression in GC cells was silenced by transfecting TBL1XR1-shRNA (sh#1, sh#2, sh#3 and sh#4) into NCI-N87 and SGC7901 cells expressing high levels of TBL1XR1 (Figures 2a–c). Sh#3 showed the most efficiency in silencing TBL1XR1 expression in GC cells, therefore, it was selected to establish NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells stably expressing low levels of TBL1XR1 (Figures 2a and b). Meanwhile, TBL1XR1 expression in GC cells was upregulated by transfecting TBL1XR1-expressing vector into BGC823 and MKN45 cells with low levels of TBL1XR1 (Figure 2c). As shown in Figure 2d, knockdown of TBL1XR1 (NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA) significantly attenuated GC cell proliferation compared with negative controls (NCI-N87/nc-shRNA and SGC7901/nc-shRNA). However, BGC823/TBL1XR1 and MKN45/TBL1XR1 cells proliferated faster than the empty vector groups (BGC823/vector and MKN45/vector) (Figure 2e). Cell apoptosis and cell cycle were then examined by flow cytometry and results showed that the percentage of apoptotic cells was significantly higher in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA groups than in NCI-N87/nc-shRNA and SGC7901/nc-shRNA groups (negative control) (Figures 2f and g), while knockdown of TBL1XR1 expression did not affect cell cycle of GC cells (data not shown). A colony

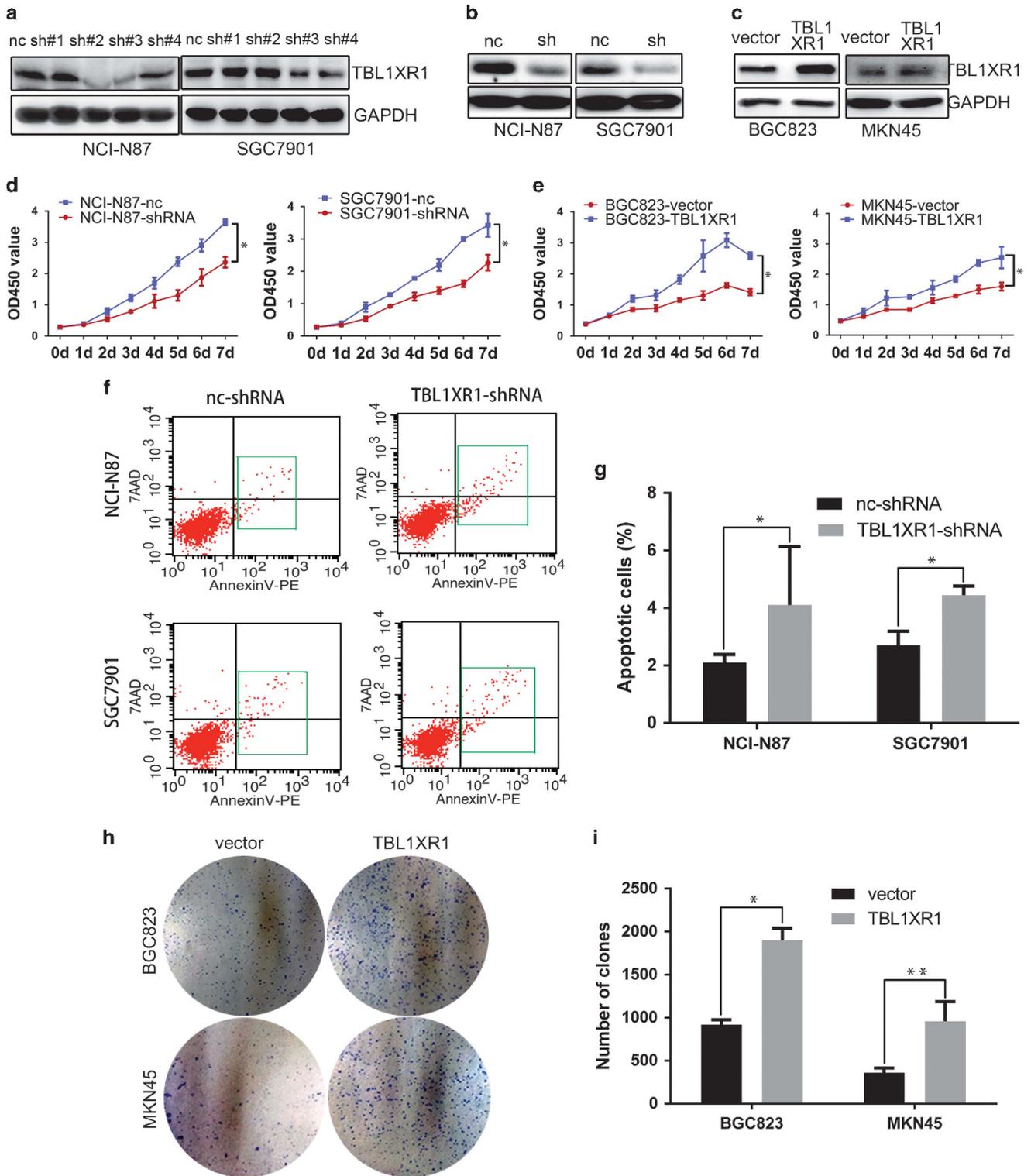
**Table 1.** Relationship between TBL1XR1 expression level and clinicopathologic variables in 134 GC tissues

Clinicopathologic parameters	Number of cases	TBL1XR1 immunostaining		P
		Weak positive (n = 52)	Strong positive (n = 82)	
<i>Age (years)</i>				
≥ 60	64	23	41	0.595
> 60	70	29	41	
<i>Gender</i>				
Male	95	31	64	0.019
Female	39	21	18	
<i>Tumour size (cm)</i>				
≥ 5	69	28	41	0.724
> 5	65	24	41	
<i>Lauren classification</i>				
Intestinal	74	25	49	0.126
Diffuse	60	27	33	
<i>Differentiation</i>				
Poorly, undifferentiated	76	27	49	0.379
Well, moderately	58	25	33	
<i>Local invasion</i>				
T1,T2	33	18	15	0.040
T3,T4	101	34	67	
<i>Lymph node metastasis</i>				
No	57	23	34	0.445
Yes	77	29	48	
<i>TNM stage</i>				
I,II	38	24	14	< 0.001
III,IV	96	28	68	

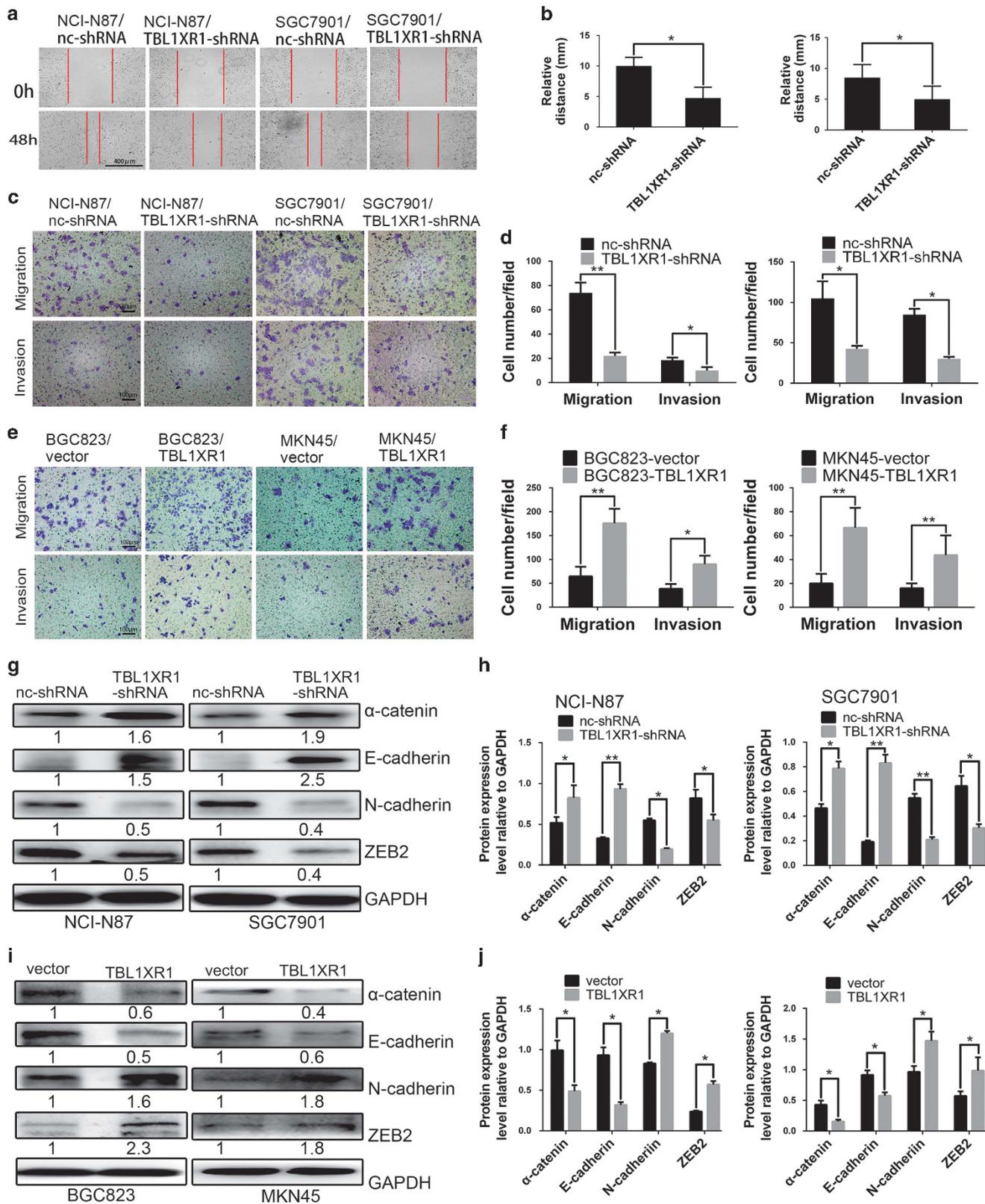
formation assay was performed to further validate the proliferative effect of TBL1XR1 on the growth of GC cells. As shown in Figures 2h and i, the number of colonies from BGC823 and MKN45 cells transfected with TBL1XR1-expressing vector was more than those with control vectors. Together, these results indicate that TBL1XR1 promotes GC cell growth *in vitro*.

### TBL1XR1 promotes the migration, invasion and EMT of GC cells

We further assessed the tumour cell migration and invasion ability using wound healing assays, transwell migration and invasion assays. The wounds healed better and faster in NCI-N87/nc-shRNA and SGC7901/nc-shRNA cells than in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells (Figures 3a and b). We further tested GC cell migration and metastatic properties using transwell migration and invasion assays. Knockdown of TBL1XR1 significantly decreased the migration and invasion ability of NCI-N87 and SGC7901 cells (Figures 3c and d), while overexpression of TBL1XR1 significantly increased the migration and invasion ability of BGC823 and MKN45 cells (Figures 3e and f). EMT plays a crucial role in tumour cell migration and invasion. To further investigate the molecular mechanism mediating the aggressive effects of TBL1XR1 on GC cells, we next explored whether TBL1XR1 status regulates EMT in GC cells. As shown in Figures 3g and h, TBL1XR1 knockdown significantly increased the expression levels of



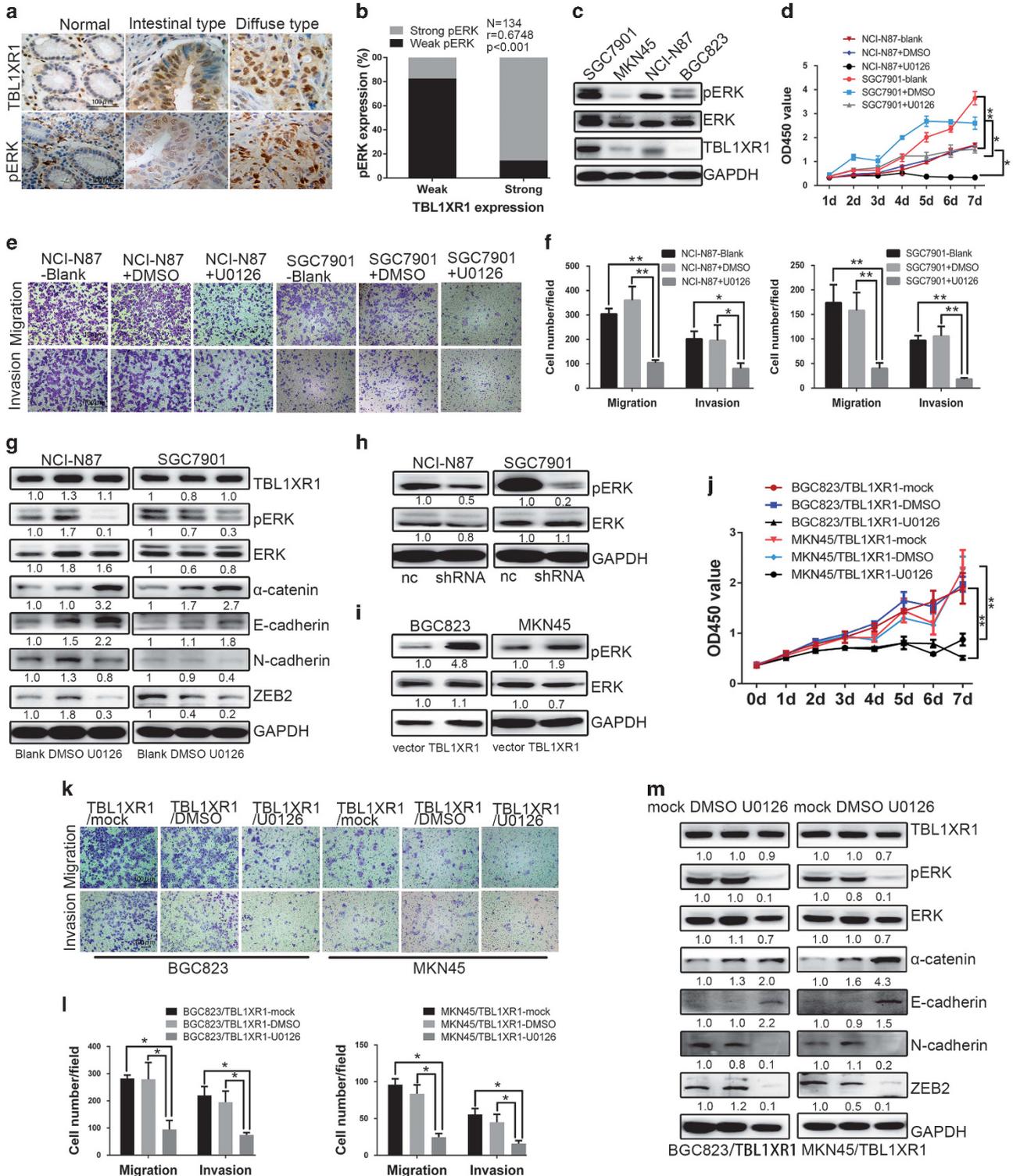
**Figure 2.** The effects of TBL1XR1 on tumour cell proliferation and apoptosis. **(a)** Western blot analyses of TBL1XR1 expression in NCI-N87 and SGC7901 cells underwent transient transfection of TBL1XR1 shRNA (sh#1, sh#2, sh#3 and sh#4). **(b)** TBL1XR1 protein expression in NCI-N87 and SGC7901 cells transfected with TBL1XR1-shRNA (sh#3) was confirmed by western blot analysis. **(c)** TBL1XR1 protein expression in BGC823 and MKN45 cells transfected with TBL1XR1-constructed plasmid. **(d)** The effect of TBL1XR1 knockdown on NCI-N87 and SGC7901 cell proliferation was analysed by CCK8 assay. **(e)** The effect of TBL1XR1 overexpression on BGC823 and MKN45 cell proliferation was analysed by CCK8 assay. **(f)** and **(g)** Knockdown of TBL1XR1 induced the apoptosis of NCI-N87 cells. **(h)** and **(i)** Overexpression of TBL1XR1 promoted the clone formation of BGC823 cells. Data are representative of three independent experiments (mean  $\pm$  s.d.). \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 3.** TBL1XR1 enhances the migratory and invasive ability of GC cells. (a), (c) and (e) Effects of knockdown and overexpression of TBL1XR1 on GC cell wound healing, migration and invasion was measured and representative images of distance (mm) of wound healing, migrated and invaded cells (magnification:  $\times 100$ ) are shown. (b), (d) and (f) Histogram shows the relative distance (mm) (magnification:  $\times 100$ ) of wound healing and the number of migrated and invaded cells. Five random fields were selected for statistical analysis. (g) and (i) The effects of knockdown and overexpression of TBL1XR1 on EMT markers was determined by western blot analysis. Densitometry shows relative protein expression normalized for GAPDH. (h) and (j) Densitometric analysis shows the effects of knockdown and overexpression of TBL1XR1 on EMT markers of GC cells. Data are shown as mean  $\pm$  s.d. of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

epithelial cell markers ( $\alpha$ -catenin and E-cadherin), and decreased the expression levels of mesenchymal cell markers (N-cadherin and ZEB2), which suggests that TBL1XR1 silencing could reverse the EMT phenotypes of GC cells. However, overexpression of TBL1XR1 significantly promotes the EMT phenotypes of BGC823 and MKN45 cells (Figures 3i and j). Taken together, these data suggest that TBL1XR1 may promote GC cell migration and invasion via the induction of EMT.

The tumour-promoting effects induced by TBL1XR1 in GC are mediated through the activation of ERK1/2 pathway  
The ERK1/2 signalling pathway plays a central role in the process of tumour cell migration and invasion induced by EMT.<sup>30–33</sup> To better understand the signalling pathways involved in the tumour-promoting properties of TBL1XR1 in GC, we investigated the possible involvement of ERK1/2. We first examined the TBL1XR1 and pERK1/2 (Thr202/Tyr204 phosphorylation of ERK)



protein expression levels in GC and patient-matched normal gastric tissues by IHC and immunofluorescence staining, and found that the pERK1/2 expression level was positively correlated with TBL1XR1 (Figures 4a and b, Supplementary Figure S1). We next investigated the protein expression levels of pERK1/2 and TBL1XR1 in four GC cell lines, and found that both proteins were highly expressed in SGC7901 and NCI-N87 cells, while expression levels were significantly lower in MKN45 and BGC823 cells (Figure 4c), suggesting that the ERK1/2 signalling pathway may be involved in tumour-promotion effects induced by TBL1XR1.

To explore the involvement and functional effect of ERK1/2 activation in tumour promotion induced by TBL1XR1 in GC, we first treated SGC7901 cells and NCI-N87 cells expressing high levels of TBL1XR1 with a specific ERK1/2 inhibitor U0126. We demonstrated that U0126 (20  $\mu$ M) significantly suppressed GC cell proliferation, migration and invasion (Figures 4d–f). Additionally, U0126 also reversed the EMT of GC cells (Figure 4g). We then determined whether knockdown of TBL1XR1 expression could inhibit the phosphorylation of ERK1/2 in GC cells. As shown in Figures 4h and i, TBL1XR1 knockdown significantly reduced the phosphorylation of ERK1/2 in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells, while TBL1XR1 overexpression significantly increased the phosphorylation of ERK1/2 in BGC823/TBL1XR1 and MKN45/TBL1XR1 cells. We then explored whether TBL1XR1 could enhance GC cell proliferation, migration and invasion through the activation of the ERK1/2 signalling pathway. After incubation with U0126, the proliferation, migration and invasion ability of BGC823/TBL1XR1 and MKN45/TBL1XR1 cells was significantly reduced in comparison with the control groups (Figures 4j–l). Western blot analysis demonstrated that EMT induced by overexpression of TBL1XR1 was also reversed after the treatment of BGC823/TBL1XR1 and MKN45/TBL1XR1 cells with U0126 (Figure 4m). However, U0126 had no effects on the expression of TBL1XR1 in GC cells (Figures 4g and m). Thus, these data suggest that TBL1XR1 regulates GC cell proliferation, migration, invasion and EMT by activating the ERK1/2 signalling pathway.

The activation of ERK1/2 induced by TBL1XR1 is mediated via the  $\beta$ -catenin/MMP7/EGFR signalling pathway

Aberrant activation of  $\beta$ -catenin signalling plays an important role in various cancers such as gastrointestinal and colorectal cancer.<sup>34–36</sup> Recent findings indicated that TBL1XR1 and  $\beta$ -catenin can recruit each other to Wnt target gene promoters to facilitate transcriptional activation and oncogenesis.<sup>10</sup> TBL1XR1 can promote the EMT of tumour cells via activating  $\beta$ -catenin signalling.<sup>19,25,37</sup> To determine whether  $\beta$ -catenin signalling is involved in the activation of ERK1/2 induced by TBL1XR1, we first detected the phosphorylation levels of  $\beta$ -catenin (S675) and ERK1/2 in GC cells with different status of TBL1XR1 expression. We found that knockdown of TBL1XR1 expression significantly

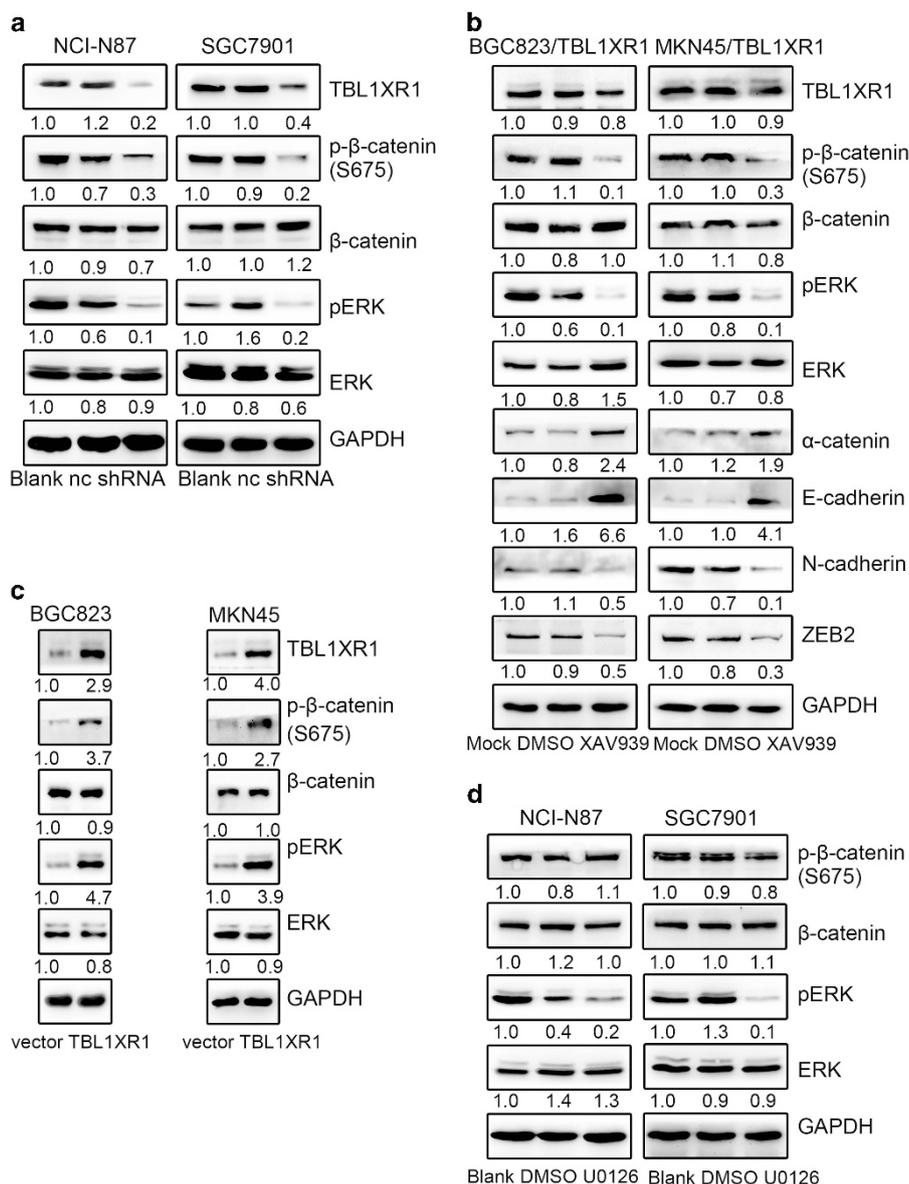
inhibited the phosphorylation of  $\beta$ -catenin (p- $\beta$ -catenin) and ERK1/2 in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells (Figure 5a). In contrast, TBL1XR1 overexpression significantly increased the phosphorylation of  $\beta$ -catenin and ERK1/2 in BGC823/TBL1XR1 and MKN45/TBL1XR1 cells (Figure 5b). We then explored whether TBL1XR1 could promote the phosphorylation of ERK1/2 and EMT in GC cells through the activation of  $\beta$ -catenin. As shown in Figure 5c, after treatment with  $\beta$ -catenin inhibitor XAV939 (10  $\mu$ M), the phosphorylation of ERK1/2 induced by overexpression of TBL1XR1 in BGC823/TBL1XR1 and MKN45/TBL1XR1 cells was significantly inhibited. Moreover, the EMT phenotypes induced by overexpression of TBL1XR1 were also reversed after the treatment with XAV939 (Figure 5c). Furthermore, the phosphorylation of  $\beta$ -catenin induced by overexpression of TBL1XR1 was not affected by U0126 (Figure 5d). Thus, these data suggest that TBL1XR1 promotes the activation of ERK1/2 and EMT in GC cells by activating the  $\beta$ -catenin signalling.

To further determine how TBL1XR1 activates ERK1/2 by  $\beta$ -catenin signalling, we detected the expression of MMP7 (Matrix metalloproteinase-7), a target gene of  $\beta$ -catenin, in GC cells. We showed that MMP7 was elevated or downregulated in TBL1XR1-overexpressing or TBL1XR1-silencing GC cells (Figures 6a and b). It has been reported that MMP7 is responsible for EGF receptor (EGFR) transactivation, downstream ERK activation and increased cell proliferation.<sup>38</sup> This pathway is also confirmed in our study using  $\beta$ -catenin, MMP7, EGFR and ERK inhibitors (Figure 6). We showed that TBL1XR1 knockdown significantly decreased the expression of p- $\beta$ -catenin, MMP7, pEGFR and pERK1/2 in NCI-N87 cells (Figure 6a). However, TBL1XR1 overexpression showed the opposite results in BGC823 cells (Figure 6b). Moreover, Batimastat (MMP7 inhibitor) inhibited the expression of MMP7, pEGFR and pERK, but had no effects on the expression of TBL1XR1 and p- $\beta$ -catenin in both NCI-N87 and BGC823/TBL1XR1 cells (Figures 6c and d). Furthermore, Afatinib (EGFR inhibitor) attenuated the activation of EGFR and ERK, but had no effects on the expression of TBL1XR1, p- $\beta$ -catenin and MMP7 (Figure 6d). Thus, these results suggest that TBL1XR1 indirectly promotes the activation of ERK1/2 via the  $\beta$ -catenin/MMP7/EGFR signalling pathway.

TBL1XR1 facilitates GC cell growth and peritoneal dissemination *in vivo*

We further evaluated whether TBL1XR1 knockdown or overexpression could regulate gastric tumour growth and metastatic potential *in vivo*. NCI-N87/TBL1XR1-sh cells, NCI-N87/nc-shRNA cells, BGC823/TBL1XR1 and BGC823/vector cells were subcutaneously transplanted into the nude mice. As shown in Figures 7a–d, NCI-N87/nc-shRNA and BGC823/TBL1XR1 cells showed progressive growth, whereas the growth of NCI-N87/TBL1XR1-sh and BGC823/vector cells was significantly slower compared with NCI-N87/nc-shRNA and BGC823/TBL1XR1 cells. Additionally, the average weight of

**Figure 4.** TBL1XR1 enhances GC cell proliferation, migration and invasion via the ERK1/2 signalling pathway. **(a)** The expression of TBL1XR1 and pERK1/2 in 134 pairs human GC tissues were assessed by IHC. The representative images show the expression of TBL1XR1 and pERK1/2 in intestinal and diffuse type of GC (scale bar = 100  $\mu$ m). **(b)** Spearman correlation was used to analyse the correlation between TBL1XR1 and pERK1/2 expression in 134 pairs of GC tissues. **(c)** The expression of pERK1/2, ERK1/2 and TBL1XR1 in four GC cell lines were determined by western blot analysis. **(d)** ERK1/2 specific inhibitor U0126 (20  $\mu$ M) suppressed the proliferation of NCI-N87 and SGC7901 cells. **(e)** and **(f)** U0126 (20  $\mu$ M) inhibited the migratory and invasive ability of NCI-N87 and SGC7901 cells (scale bar = 100  $\mu$ m). **(g)** U0126 (20  $\mu$ M) inhibited the phosphorylation of ERK1/2 and EMT changes in NCI-N87 cells and SGC7901 cells. **(h)** The pERK1/2 level in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells was determined by western blot analysis. **(i)** The effect of TBL1XR1 overexpression on pERK1/2 level in BGC823 and MKN45 cells. **(j)** BGC823/TBL1XR1 and MKN45/TBL1XR1 cell proliferation were measured in the presence of U026 (20  $\mu$ M). **(k)** and **(l)** The effect of U0126 on the migratory and invasive ability of BGC823/TBL1XR1 and MKN45/TBL1XR1 cells was analysed and representative images are shown (magnification:  $\times$  100, scale bar = 100  $\mu$ m). **(m)** The effect of U0126 (20  $\mu$ M) on the EMT changes of BGC823/TBL1XR1 and MKN45/TBL1XR1 cells was analysed by western blot analysis. Densitometry shows relative protein expression normalized for GAPDH. Data are representative of three independent experiments (mean  $\pm$  s.d.). \* $P$  < 0.05, \*\* $P$  < 0.01.

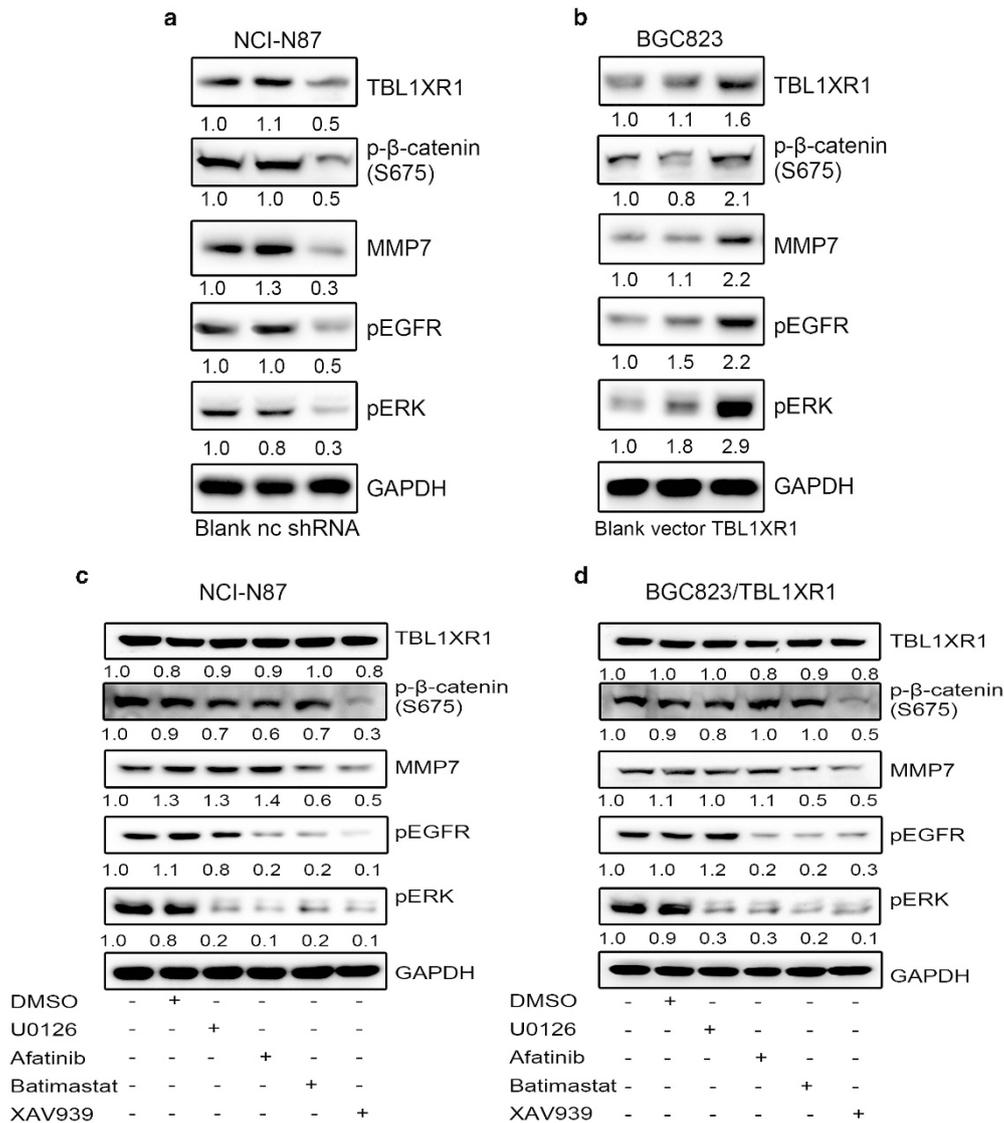


**Figure 5.** The activation of ERK1/2 induced by TBL1XR1 is mediated via the  $\beta$ -catenin signalling pathway. **(a)** The phosphorylation levels of  $\beta$ -catenin (p- $\beta$ -catenin) and ERK1/2 in NCI-N87/TBL1XR1-shRNA and SGC7901/TBL1XR1-shRNA cells were detected by western blot analysis. **(b)** The phosphorylation levels of  $\beta$ -catenin and ERK1/2 in BGC823/TBL1XR1 and MKN45/TBL1XR1 cells were detected by western blot analysis. **(c)** The phosphorylation level of ERK1/2 and EMT in BGC823/TBL1XR1 and MKN45/TBL1XR1 cells treated with XAV939 were detected by western blot analysis. **(d)** The effect of U0126 (20  $\mu$ M) on phosphorylation of  $\beta$ -catenin in NCI-N87 and SGC7901 cells was analysed by western blot analysis. Densitometry shows relative protein expression.

tumours generated from NCI-N87/TBL1XR1-sh and BGC823/vector cells was significantly reduced in comparison with tumours from the NCI-N87/nc-shRNA and BGC823/TBL1XR1 cells ( $0.989 \pm 0.094$  g vs  $4.290 \pm 0.224$  g,  $P < 0.001$ ,  $1.581 \pm 0.116$  g vs  $3.679 \pm 0.210$  g,  $P < 0.001$ , Figures 7e and f). To further assess whether the regulation of cell proliferation, EMT and the ERK1/2 pathway by TBL1XR1 could be recapitulated *in vivo*, we analysed the expression of Ki-67 antigen (a cellular marker for proliferation), pERK1/2, E-cadherin and ZEB2 protein expression by IHC. As shown in Figure 7g, there were fewer Ki-67 antigen-positive cells in tumours derived from NCI-N87/TBL1XR1-sh cells than in tumours derived from NCI-N87/nc-shRNA cells. Consistent with the *in vitro* data shown above, the expression of pERK1/2 and ZEB2 in NCI-N87/TBL1XR1-sh cells was also significantly lower than

in NCI-N87/nc-shRNA cells, while E-cadherin showed increased expression in NCI-N87/TBL1XR1-sh cells. This further indicated that the knockdown of TBL1XR1 expression could inhibit cell growth and EMT of GC cells via the ERK1/2 pathway.

To further quantify metastatic and dissemination potential *in vivo*, NCI-N87/TBL1XR1-sh and NCI-N87/nc-shRNA cells were intraperitoneally transplanted into nude mice. The average volume of ascites NCI-N87/TBL1XR1-sh group was smaller than NCI-N87/nc-shRNA group (Figure 7h). Moreover, there were significantly fewer visible peritoneal nodules in the NCI-N87/TBL1XR1-sh group compared with controls ( $7.667 \pm 1.202$  vs  $28.670 \pm 3.180$ ,  $P < 0.01$ , Figures 7i and k). In contrast, treatment of nude mice with BGC823/TBL1XR1 cells increased the formation of peritoneal metastatic nodules compared with BGC823/vector



**Figure 6.** The activation of ERK1/2 induced by TBL1XR1 is mediated via the  $\beta$ -catenin/MMP7/EGFR signalling pathway. **(a)** and **(b)** The expression levels of TBL1XR1, p- $\beta$ -catenin, MMP7, pEGFR and pERK1/2 in NCI-N87/TBL1XR1-shRNA cells **(a)** and BGC823/TBL1XR1 cells **(b)** were detected by western blot analysis. **(c)** and **(d)** The expression levels of TBL1XR1, p- $\beta$ -catenin, MMP7, pEGFR and pERK1/2 in NCI-N87 cells **(c)** and BGC823/TBL1XR1 cells **(d)** in the presence of XAV939 (10  $\mu$ M), Batimastat (10  $\mu$ M), Afatinib (5  $\mu$ M) and U0126 (20  $\mu$ M) were detected by western blot analysis. Densitometry shows relative protein expression.

cell treatment (Figures 7j and l). These results indicate that TBL1XR1 expression promotes gastric tumour growth, peritoneal dissemination and metastasis potential *in vivo*.

## DISCUSSION

This study investigated the biological role of TBL1XR1 and its precise mechanisms in GC. The data show that TBL1XR1 expression is elevated in GC cells and closely correlated with poor prognosis of GC patients. Moreover, the data further reveal a potential molecular mechanism by which TBL1XR1 promotes tumorigenesis and progression of GC via activation of the ERK1/2 pathway. These findings indicate the key role of TBL1XR1 as an oncogenic protein and uncover a novel molecular mechanism underlying the development and progression of GC.

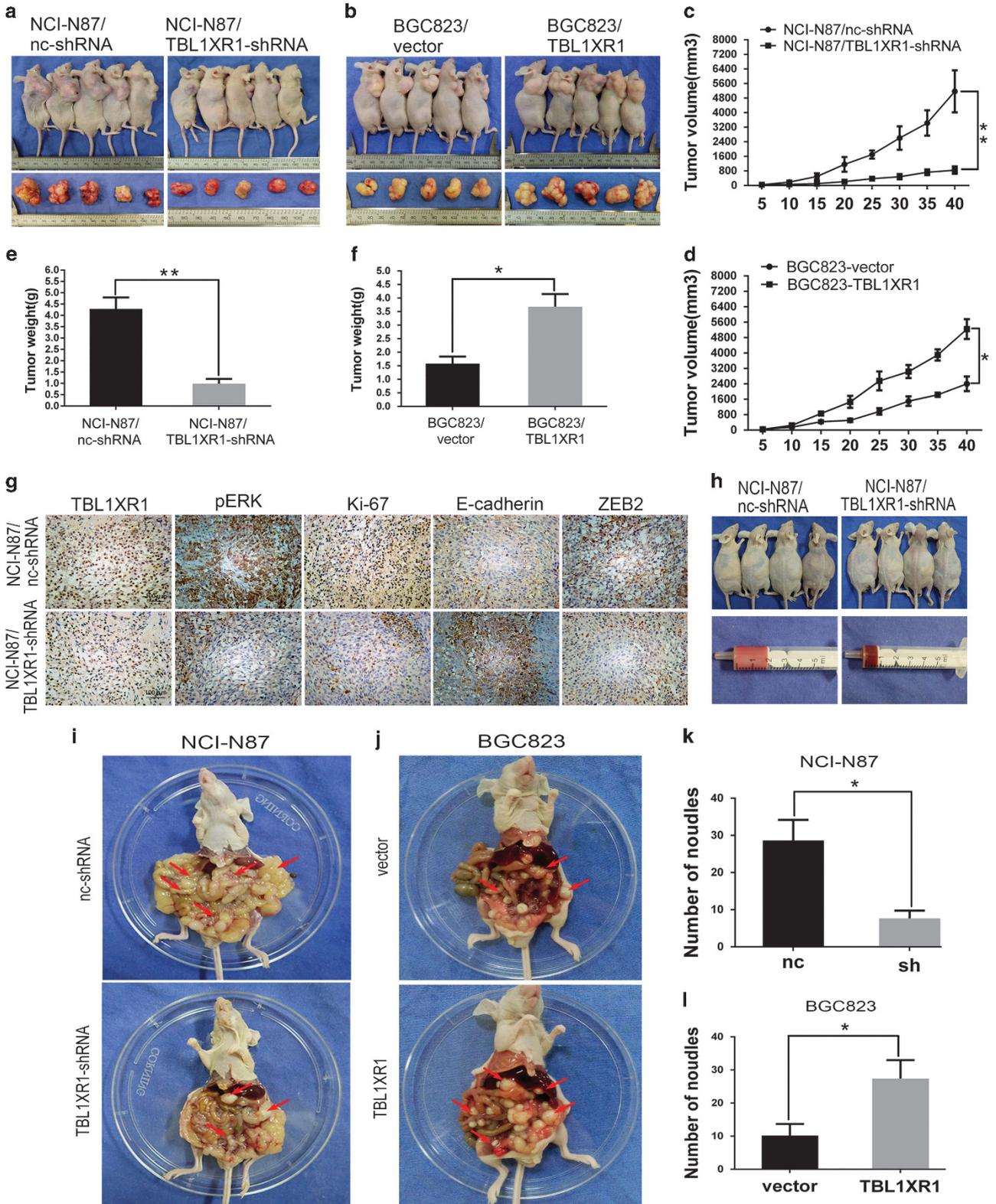
Overexpression of TBL1XR1 has been confirmed in several types of cancer, including esophageal squamous cell carcinoma, squamous cell lung carcinoma, cervical cancer and breast cancer.

Liu *et al* found that overexpression of TBL1XR1 enhanced lymphangiogenesis and lymphatic metastasis in esophageal squamous cell carcinoma and was positively correlated with tumour stage and overall survival,<sup>28</sup> which suggest that TBL1XR1 might serve as an independent unfavourable prognostic factor for outcome of esophageal squamous cell carcinoma patients. TBL1XR1 amplification has also been shown in breast cancer, and inactivation of TBL1XR1 attenuated tumour cell migration and invasion.<sup>23</sup> Here, we find that TBL1XR1 is upregulated in GC cells, and correlates with clinicopathological characteristics such as local invasion and late TNM stage. Furthermore, Kaplan–Meier survival analysis shows that patients with high TBL1XR1 expression have a shorter overall survival. Thus, these results indicate that TBL1XR1 can act as a novel predictor of poor prognosis in human GC patients.

Accumulating evidences indicate that TBL1XR1 has an important role in cell cycle regulation, anti-apoptosis, inflammatory responses, resistance to therapies and EMT.<sup>22,37</sup> In accordance

with previous studies, we find that TBL1XR1 acts as a multi-functional protein that affects tumour cell proliferation, migration and invasion, and knockdown of TBL1XR1 significantly inhibited gastric tumour growth and metastatic potential both *in vitro* and *in vivo*. Notably, TBL1XR1 inactivation attenuates GC cell

proliferation by inducing apoptosis. Aberrant apoptotic events are closely involved in oncogenesis and tumour regression in numerous cancers, including GC. Thus, elevated TBL1XR1 conferred growth advantages to GC cells, which may be linked to the chemoresistance of GC. However, further studies are needed to



support this hypothesis. EMT, a well-characterized embryological process, contributes to the enhanced motility and invasiveness of cancer cells, which is critical to tumour metastasis. Present data demonstrate that TBL1XR1 status regulates the EMT phenotype, cell migration, invasion and the formation of experimental peritoneal metastasis. Thus, TBL1XR1 overexpression in GC promoting tumour aggressiveness suggests that TBL1XR1 could be a feasible target in cancer therapy.

Multiple different signalling pathways, including NF- $\kappa$ B, Wnt/ $\beta$ -catenin, ERK1/2 and JAK/STAT3, have been reported to regulate EMT in tumour progression.<sup>39,40</sup> ERK1/2 is one of the most important pathways contributing to cell proliferation, EMT and metastasis during tumorigenesis, and the expression of E-cadherin, N-cadherin and ZEB1 mediated by the ERK1/2 pathway during EMT is regulated by the corepressor CtBP.<sup>30</sup> Researchers have shown that gene activation in cancer requires the dismissal of both N-CoR/SMRT- and CtBP1/2-dependent repression, via the interaction of TBL1XR1 and TBL1, which allow the ubiquitination and degradation of N-CoR/SMRT- and CtBP1/2, respectively.<sup>11</sup> In the present study, we show that the tumour-promoting effects induced by TBL1XR1 in GC are mediated through the activation of ERK1/2 pathway. TBL1XR1 knockdown attenuated the phosphorylation of ERK1/2 and consequently led to decreased cell growth, migration, invasion and EMT in GC cells *in vitro* and *in vivo*. In contrast, TBL1XR1 overexpression exhibited the opposite results, which supports that TBL1XR1 is an upstream activator of ERK1/2 and serves as an important regulator of cell growth and EMT. Additionally, we find that the inhibitory effects of ERK1/2 inhibitor U0126 on GC cells are much more significant than those of silencing TBL1XR1 alone. We consider that there are several reasons resulting in such phenotypes. First, ERK1/2 is known as a convergence point of multiple tumour-promoting cascades, and TBL1XR1 is one of these receptors that can activate the ERK1/2 signalling pathway in tumour microenvironment. Second, RNAi usually does not completely shut off the gene, the knockdown efficiency of RNAi was 70–80% in silencing TBL1XR1 expression in GC cells, and the rest unchanged TBL1XR1 might still promote GC progression. Third, we cannot exclude the possibility of the dose effect of MEK inhibitor U0126 on GC cells.

Recent findings have shown that TBL1XR1 and  $\beta$ -catenin can recruit each other to Wnt target gene promoters to facilitate transcription activation and oncogenesis.<sup>10</sup> Beta-catenin activation could promote the phosphorylation of ERK1/2, leading to the increased expression of Bcl-2.<sup>40</sup> The activation of ERK1/2 mediated by TBL1XR1 was dependent on the phosphorylation of  $\beta$ -catenin. Wang *et al* have indicated that TBL1XR1 could promote EMT via the  $\beta$ -catenin signalling pathway in cervical cancer.<sup>37</sup> MMP7 (Matrix metalloproteinase-7) serves as a target gene of  $\beta$ -catenin.<sup>41</sup> Beta-catenin activation promotes prostate tumour progression by upregulating MMP7.<sup>42</sup> Further study showed that MMP7 is responsible for EGFR transactivation, downstream ERK1/2 activation and increased cell proliferation.<sup>38</sup> This signalling pathway is confirmed in our study using  $\beta$ -catenin, MMP7, EGFR and ERK1/2 inhibitors. Here, we find that  $\beta$ -catenin, MMP7 and

EGFR are involved in the activation of ERK1/2 induced by TBL1XR1, and treatment of GC cells with  $\beta$ -catenin inhibitor XAV939, MMP inhibitor Batimastat or EGFR inhibitor Afatinib leads to the inhibition of ERK1/2 phosphorylation and reversion of EMT induced by overexpression of TBL1XR1. Thus, these data indicate that the activation of ERK1/2 induced by TBL1XR1 is dependent on the  $\beta$ -catenin/MMP7/EGFR signalling.

In conclusion, we demonstrated that TBL1XR1 plays a crucial role in tumour growth, metastasis and prognosis via activation of the  $\beta$ -catenin/MMP7/EGFR/ERK pathway (Figure 8). Thus, this study links TBL1XR1/ $\beta$ -catenin/MMP7/EGFR/ERK signalling to the control of the progression of GC and establishes TBL1XR1 as a potential biomarker for clinical prognosis and a therapeutic target in GC.

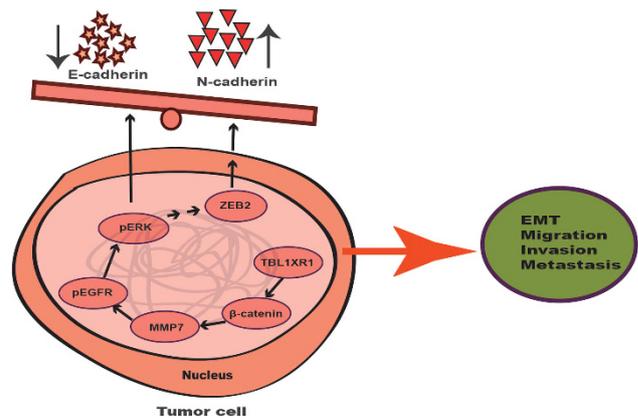
## MATERIALS AND METHODS

### Ethical statement

An institutional review board from the local Human Research Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, China approved the study. All human participants provided informed consent. All animal experiments were also approved by the local Laboratory Animal Ethics Committee of Ruijin Hospital and conducted based on the Guide for the Care and Use Laboratory Animals of Ruijin Hospital. All samples tested for GC were anonymous in accordance with legal and ethical standards.

### Reagents and cell cultures

Seven GC cell lines including SGC7901, MKN45, NCI-N87, BGC823, AGS, MKN28, SNU-1 and one immortalized normal gastric epithelial cell line GES-1 were cultured and passaged according to the manufacturer's instructions. All cell lines were cultured in RPMI-1640 medium (Gibco BRL, San Francisco, CA, USA) containing 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine



**Figure 8.** Schematic model of TBL1XR1-promoting ERK activation and gastric cancer progression. GC cell-derived TBL1XR1 promotes the phosphorylation of ERK1/2 via activating  $\beta$ -catenin/MMP7/EGFR, which facilitates the EMT, migration, invasion and metastatic potential of GC cells.

**Figure 7.** TBL1XR1 promotes subcutaneous tumour growth and peritoneal dissemination in nude mice. NCI-N87/nc-shRNA, NCI-N87/TBL1XR1-shRNA, BGC823/vector and BGC823/TBL1XR1 cells were subcutaneously or intraperitoneally injected into nude mice. Tumour volume was monitored twice a week and all mice were sacrificed under general anesthesia 6 weeks after injection. (a) and (b) Representative images of tumour-bearing mice and tumour mass. (c) and (d) Tumour growth curves. (e) and (f) Average tumour weight of each group ( $N=5$ ). (g) Immunohistochemistry analyses of TBL1XR1, pERK1/2, Ki67, E-cadherin and ZEB2 protein expression in xenograft tumours. (h–j) NCI-N87/TBL1XR1-shRNA, NCI-N87/nc-shRNA, BGC823/TBL1XR1 and BGC823/vector cells were intraperitoneally transplanted into the nude mice, the volume of ascites and the number of peritoneal nodules from each mouse were measured. Macroscopic images of the peritoneal disseminations are shown and red arrows in the images indicate tumours developing peritoneal invasion and dissemination. (k) and (l) Average of the peritoneal tumour nodules developed by NCI-N87/TBL1XR1-shRNA, NCI-N87/nc-shRNA, BGC823/TBL1XR1 and BGC823/vector cells was quantified. Data are representative of three independent experiments (mean  $\pm$  s.d.). \* $P < 0.05$ , \*\* $P < 0.01$ .

serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO<sub>2</sub>-humidified incubator. ERK1/2 specific inhibitor U0126 was purchased from Cell Signaling Technology (Danvers, MA, USA; no. 99035). Beta-catenin specific inhibitor XAV939, MMP7 inhibitor Batimastat and EGFR inhibitor Afatinib were purchased from Selleck (Houston, TX, USA; no. S1180, no.S7155, no.S1011).

#### Tissue specimens and histological examination

GC tissues and corresponding normal tissues were collected from 134 patients who underwent gastrectomy between 1 January 2011 and 30 December 2013 in the Department of Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. None of these patients had received chemotherapy or radiation therapy before. All samples from GC patients were confirmed by two experienced pathologists according to the seventh edition of the American Joint Committee on Cancer staging system. After being fixed with formalin and embedded in paraffin, all samples were made into tissue microarrays (Outdo Biotech Co. Ltd., Shanghai, China). Thirty pairs of tissues were immediately embedded in Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, CA, USA) with Tissue-Tek Cryomold (Sakura Finetek) and stored at -80 °C. Frozen sections were prepared with a cryostat. Forty pairs of tissues were stored at -80 °C for protein and RNA extraction.

#### Immunofluorescence

The frozen sections of tissue samples were fixed in 4% paraformaldehyde at room temperature for 10 min. Sections were gently washed three times in 1 × phosphate buffered saline (PBS) and then permeabilized with 0.5% NP-40 (Sigma, St Louis, MO, USA) in 1 × PBS for 10 min. After being blocked with 10% normal goat serum for 45 min, the sections were incubated with anti-TBL1XR1 (Abcam, Cambridge, MA, USA; no. ab117761), anti-pERK1/2 (Cell Signaling Technology; no. 91005) primary antibodies for 2.5 h at room temperature and gently washed three times in PBS. Sample sections were then incubated with Alexa Fluor 488 dye-conjugated secondary antibody, Alexa Fluor 568 dye-conjugated phalloidin, and Hoechst 33342 (Molecular Probes, Waltham, MA, USA). Pictures were taken using an Olympus BX50 microscope (Olympus, Tokyo, Japan).

#### Immunohistochemical staining (IHC)

Immunohistochemical staining was performed on the tissue microarray according to a previously reported standard protocol used in Shanghai Institute of Digestive Surgery.<sup>43</sup> Two independent board-certified pathologists evaluated the protein expression levels in an unbiased fashion. The staining intensity and the proportion of cell staining were used to score the overall tissue sections. The staining intensity was graded in four segments on a 3-point scale (staining scores): no staining (0 points), light brown staining (1 point), brown staining (2 points) and dark brown staining (3 points). The number of positive cells was divided into four grades (percentage scores): < 5% (0), 5–30% (1), 31–70% (2) and 71–100% (3). TBL1XR1 staining was calculated by the following formula: overall staining score = intensity score × percentage score. A final score ≤ 3 was defined as negative staining, and > 3 as positive staining.

#### Western blot analysis

Cell sample extracts were prepared in RIPA cell lysis buffer (Kangwei, Beijing, China) supplemented with phosphatase inhibitor Cocktail III (Cell Signaling Technology) and the protease inhibitor phenylmethane-sulfonyl fluoride. The concentration of protein was quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) against a bovine serum albumin standard curve. A total of 40 µg of protein was loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel and then transferred onto 0.22 µm PVDF membranes (Millipore, MA, USA). The membranes were blocked with 1 × TBST buffer containing 5% bovine serum albumin and incubated with the corresponding primary antibodies at 4 °C overnight. Anti-GAPDH (no. HRP-60004), anti- $\alpha$ -catenin (no. 12831-1-AP), anti-E-cadherin (no. 20874-1-AP), anti-N-cadherin (no. 22018-1-AP), anti- $\beta$ -catenin (no. 51067-2-AP) and anti-MMP7 (10374-2-AP) antibodies were purchased from Proteintech (Rosemont, IL, USA). Anti-TBL1XR1 (no. ab117761) and anti-ZEB2 (no. ab138222) antibodies were purchased from Abcam. Anti-ERK1/2 (no. 91005), anti-pERK1/2 (no. 91005), anti-phospho- $\beta$ -catenin (Ser675) (no. 95675), anti-pEGFR (Tyr1068) (no. 37775) and HRP-conjugated secondary (1:5000) antibodies were purchased from Cell Signaling Technology. After the addition of secondary antibodies, membranes were visualized with Thermo Pierce

chemiluminescent (ECL) Western Blotting Substrate (Thermo, Waltham, MA, USA) using a Tanon 5200 system (Tanon, Shanghai, China).

#### Cell proliferation assay

GC cells were cultured in 96-well plates at a density of  $2 \times 10^3$ /well (200 µl/well). A cell proliferation assay was conducted using a CCK-8 (Cell Counting Kit-8) (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. OD 450 was measured by spectrophotometry (BioTek, Vermont, USA) 2 h after being incubated with 20 µl of CCK-8 reagent.

#### Quantitative real-time PCR (QRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA synthesis was performed using a reverse transcription kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The mRNA level of *TBL1XR1* was measured using the SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and the Applied Biosystems 7900HT sequence detection system (Applied Biosystems). *TBL1XR1* mRNA relative expression level was evaluated using the  $2^{-\Delta\Delta Ct}$  method and normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

#### Plasmids construction and transfection

TBL1XR1 shRNA sequences and negative control sequences were designed and synthesized by Genechem (Genechem Co. Ltd., Shanghai, China). The corresponding vector was hU6-MCS-Ubiquitin-EGFP-IRES-puromycin. SGC7901 and NCI-N87 cells were cultured in six-well plates and transfected with 4 µg shRNA plasmids using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Cells were treated with puromycin (1 µg/ml) (InvivoGen, San Diego, CA, USA) to produce stably transfected cells (NCI-N87/nc-shRNA, NCI-N87/TBL1XR1-shRNA, SGC7901/nc-shRNA and SGC7901/TBL1XR1-shRNA) for further experiments. The full length TBL1XR1 cDNA was synthesized by RT-PCR from GC tissues. And then TBL1XR1 cDNA was sub-cloned into the CN550-pLOV-EF1a-PuroR-CMV-eGFP-2A-3FLAG plasmid (Obio Technology Co. Ltd., Shanghai, China). The TBL1XR1 plasmid and corresponding empty vector were transfected into GC cells (BGC823 and MKN45) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Stably transfected cells (BGC823/vector, BGC823/TBL1XR1, MKN45/vector and MKN45/TBL1XR1) were selected by using puromycin (1 µg/ml) (InvivoGen). Knockdown and overexpression of TBL1XR1 were confirmed by western blot analysis.

#### Flow cytometry

For cell cycle analysis, tumour cells were collected and fixed in 70% ice-cold ethanol at 4 °C overnight. After washing with PBS, cells were incubated with 100 µg/ml RNase A at 37 °C for 20 min. Cells were subsequently stained with propidium iodide (50 µg/mL) and cell cycle analysis was performed by flow cytometry on a FACScan (Beckman Instruments, Fullerton, CA, USA) instruments. To assess apoptosis, unfixed tumour cells were washed with PBS and incubated with Annexin V and propidium iodide (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction.

#### Cell migration, invasion and wound healing assays

Cell migration and invasion assays were conducted using 24-well plates and 8 µm transwell inserts (Corning Life Science, Acton, MA, USA). For migration assays, tumour cells were suspended in 200 µl serum-free RPMI 1640 medium ( $4 \times 10^4$  cells) and cultured in the upper chamber. Fetal bovine serum-conditioned medium (10%) (800 µl) was added to the lower 24-well plates. For invasion assays, the inserts were coated with Matrigel (50 µl/well) (BD Biosciences) before adding the cells. After 24 h of culture, tumour cells remaining in the upper side of the inserts were removed with cotton swabs. Tumour cells that migrated to the lower side of the inserts were fixed in methanol and stained with 0.5% crystal violet for 20 min. Migrated cells were photographed using Nikon Digital Sight DS-U2 (Nikon, Tokyo, Japan) and Olympus BX50 microscopes (Olympus Optical Co. Ltd., Tokyo, Japan). Six visual fields were randomly chosen to calculate the number of migrated cells. For the wound healing assay, tumour cells were cultured in six-well plates until confluent, and scratched with a 20 µl pipette tip. The previous medium was replaced with fresh medium containing 1% fetal bovine serum. Cells were photographed at 0, 12, 24 and 48 h after the scratches were made.

### In vivo tumorigenesis and dissemination

Male BALB/c nude mice (4-weeks-old) (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) were housed in a specific pathogen-free room in the Animal Experimental Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, China. Animal experiments were performed in accordance with the Institution's guidelines and animal research principles. Twenty mice were randomly divided into four groups (five mice per group). The single blind method was used in our experiments. Mice were subcutaneously injected with  $1 \times 10^6$  tumour cells (NCI-N87/TBL1XR1-shRNA, NCI-N87/nc-shRNA, BGC823/vector and BGC823/TBL1XR1) suspended in 100  $\mu$ l PBS (five mice per group). Tumour length (L) and width (W) were measured every 5 days using digital Vernier caliper. Tumour volume was determined using the following formula: volume =  $(W+L)/2 \times W \times L \times 0.5236$ .<sup>44</sup> Mice were intraperitoneally transplanted with  $4 \times 10^5$  tumour cells (NCI-N87/TBL1XR1-shRNA, NCI-N87/nc-shRNA, BGC823/vector and BGC823/TBL1XR1) suspended in 200  $\mu$ l PBS (five mice per group). All mice were sacrificed under general anesthesia 6 weeks after injection. Tumour grafts and peritoneal nodules were weighed and observed systematically.

### Statistical analysis

All experimental results were repeated at least three times and are shown as mean  $\pm$  standard deviation (s.d.). The association between TBL1XR1 expression and clinicopathological characteristics was statistically determined using the Pearson  $\chi^2$  test. Differences between treated and control groups were analysed using the Student's *t*-test and one-way ANOVA. A two-tailed value of  $P < 0.05$  was considered statistically significant. All statistical analyses were performed with the Stata software 12.0 (Stata Corporation, College Station, TX, USA).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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