



<b>Title</b>	<b>Wnt2 secreted by tumour fibroblasts promotes tumour progression in oesophageal cancer by activation of the Wnt/ - catenin signalling pathway</b>
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# Wnt2 secreted by tumour fibroblasts promotes tumour progression in oesophageal cancer by activation of the Wnt/ $\beta$ -catenin signalling pathway

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## ABSTRACT

**Objectives** Interaction between neoplastic and stromal cells plays an important role in tumour progression. It was recently found that *WNT2* was frequently overexpressed in fibroblasts isolated from tumour tissue compared with fibroblasts from non-tumour tissue normal fibroblasts in oesophageal squamous cell carcinoma (OSCC). This study aimed to investigate the effect of TF-secreted Wnt2 in OSCC development via the tumour–stroma interaction.

**Methods** Quantitative PCR, western blotting, immunohistochemistry and immunofluorescence were used to study the expression pattern of Wnt2 and its effect on the Wnt/ $\beta$ -catenin pathway. A Wnt2-secreting system was established in Chinese hamster ovary cells and its conditioned medium was used to study the role of Wnt2 in cell proliferation and invasion.

**Results** Expression of Wnt2 could only be detected in TF but not in OSCC cancer cell lines. In OSCC tissues, Wnt2 (+) cells were mainly detected in the boundary between stroma and tumour tissue or scattered within tumour tissue. In this study, Wnt2-positive OSCC was defined when five or more Wnt2(+) cells were observed in 200 $\times$  microscopy field. Interestingly, Wnt2-positive OSCC (22/51 cases) was significantly associated with lymph node metastases ( $p=0.001$ ), advanced TNM stage ( $p=0.001$ ) and disease-specific survival ( $p<0.0001$ ). Functional study demonstrated that secreted Wnt2 could promote oesophageal cancer cell growth by activating the Wnt/ $\beta$ -catenin signalling pathway and subsequently upregulated cyclin D1 and c-myc expression. Further study found that Wnt2 could enhance cell motility and invasiveness by inducing epithelial–mesenchymal transition.

**Conclusions** TF-secreted Wnt2 acts as a growth and invasion-promoting factor through activating the canonical Wnt/ $\beta$ -catenin signalling pathway in oesophageal cancer cells.

Mounting evidence suggests that the tumour microenvironment, composed of non-cancer cells and their stroma, plays an important role in cancer development and progression.<sup>1,2</sup> Our previous study has described a broad range of deregulated genes between fibroblasts isolated from oesophageal squamous cell carcinoma (OSCC) and their adjacent non-tumorous oesophageal tissues. Approximately 43% (126/292) of known deregulated genes in tumour fibroblasts (TF) were associated with cell proliferation, extracellular matrix remodelling and immune response.<sup>3</sup> Notably, several members of the

## Significance of this study

### What is already known about this subject?

- Tumour fibroblasts (TF) play an essential role in the complex process of tumour–stroma interactions and tumorigenesis.
- The *WNT2* gene is frequently overexpressed in fibroblasts isolated from tumour tissue (TF) compared with fibroblasts from non-tumour tissue normal fibroblast in oesophageal squamous cell carcinoma (OSCC).
- The specific roles of TF-secreted Wnt2 in the oesophageal cancer microenvironment are still unknown.

### What are the new findings?

- Wnt2 displays unique expression patterns in OSCC and correlates with the unfavourable clinical features and survival of patients with OSCC.
- TF-secreted Wnt2 can promote oesophageal cancer cell growth by activating Wnt/ $\beta$ -catenin signalling and its downstream targets cyclin D1 and c-myc.
- TF-secreted Wnt2 can enhance oesophageal cancer cell motility and invasiveness by inducing epithelial–mesenchymal transition.

### How might it impact on clinical practice in the foreseeable future?

- Based on our findings, future therapeutic targets for interfering with Wnt signalling include cell surface receptors and secreted signalling molecules, which mediate signalling between cancer cells and the stromal environment, and may serve as effective treatment approaches to patients with OSCC.

Wnt pathway, including *WNT2*, *WNT5A*, *LEF1* and *WISP1*, were upregulated in TF, suggesting that Wnt signalling might be involved in the tumour–stromal interactions in oesophageal cancer.<sup>3</sup>

The Wnt proteins are secreted ligand proteins (approximately 40 kDa in size) that act in a paracrine fashion by activating diverse signalling cascades inside their target cells. The Wnt signalling pathway diversifies into three main branches.<sup>4</sup> The best understood is the so-called canonical pathway, which activates target genes through stabilisation of  $\beta$ -catenin in the nucleus. Wnt proteins can also signal by activating calmodulin kinase II and protein

kinase C (known as the Wnt/Ca<sup>2+</sup> pathway), which involves an increase in intracellular calcium (Ca<sup>2+</sup>), or Jun N-terminal kinase (known as the planar cell polarity pathway), which controls cytoskeletal rearrangements and cell polarity. The canonical Wnt pathway is the most prevalent Wnt signalling pathway in the development of cancer. The Wnt2 gene, located on chromosome 7q31.3, is highly expressed in fetal lungs and weakly expressed in the placenta.<sup>5</sup> Blasband *et al*<sup>6</sup> has reported that overexpression of Wnt2, with signalling through  $\beta$ -catenin, promotes transformation of mammary epithelial cells in the mouse cell line C57MG, demonstrating the oncogenic activity of Wnt2.

As some Wnt ligands are stromally secreted with cognate receptors expressed by the epithelia, Wnt proteins are good candidates for mediating tumour–stromal interactions.<sup>7 8</sup> Our previous study has identified WNT2 as one of the upregulated WNT genes in TF isolated from primary OSCC. In the present study, we further investigated the role of Wnt2 secreted by TF in OSCC tumorigenesis. Our results showed that Wnt2-positive expression was significantly correlated with poor clinical outcomes of OSCC patients. We also demonstrated that secreted Wnt2 acts as a growth and invasion-promoting factor through activating the canonical Wnt/ $\beta$ -catenin signalling pathway in oesophageal cancer cells.

## MATERIALS AND METHODS

### Cell lines and primary tumour specimens

Two Chinese OSCC cell lines (EC18 and EC109) were kindly provided by Professor Tsao GS (Department of Anatomy, The University of Hong Kong). Five Japanese OSCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410 and KYSE510) were obtained from DSMZ (Braunschweig, Germany), the German Resource Centre for Biological Material.<sup>9</sup> The Chinese hamster ovary cell line (CHO-K1) was purchased from the American Type Culture Collection (Manassas, Virginia, USA). Primary OSCC tissues and their adjacent normal oesophageal tissues were collected immediately after surgical resection in the Cancer Center of Sun Yat-sen University (Guangzhou, China). A total of 51 formalin-fixed and paraffin-embedded OSCC were also kindly provided by the Cancer Center of Sun Yat-sen University. The clinical data of patients included in this study are detailed in supplementary table S1 (available online only). All patients did not receive preoperative treatment. Clinical samples used in this study were approved by the Committee for Ethical Review of Research at Sun Yat-sen University.

### Isolation of fibroblasts

TF and their matched normal fibroblasts were isolated from primary OSCC as described previously.<sup>3</sup> Briefly, freshly collected OSCC tumour tissue and its paired non-tumorous oesophageal tissue were cut into as small pieces as possible in sterile phosphate-buffered saline (PBS) solution, followed by collagenase digestion. The suspension was filtered through 20  $\mu$ m stainless steel wire mesh to collect a single cell suspension. The filtrate was centrifuged and washed before being finally plated on 6 cm tissue culture dishes in 5 ml DMEM medium supplemented with 20% fetal bovine serum (FBS). After culturing for 30 min at 37°C, unadherent cells (mainly tumour cells) were removed to obtain pure fibroblasts. The adherent fibroblasts were subcultured for further study.

### Plasmid construction and transfection

The WNT2 gene was PCR amplified, cloned into pcDNA3.1/V5-His TOPO TA vector (Invitrogen, Carlsbad, California, USA), and transfected into CHO cells using Lipofectamine 2000

(Invitrogen), following the manufacturer's instructions. Stably Wnt2-expressing clones (CHO-Wnt2) were screened. CHO cells transfected with the empty pcDNA3.1 vector (CHO-Vec) were used as control.

### Conditioned medium

Stably Wnt2-expressing cells (CHO-Wnt2) and empty vector transfected cells (CHO-Vec) were cultured in DMEM medium with 10% FBS until 70% confluence. After complete removal of the normal culture medium, CHO-Wnt2 and CHO-Vec cells were continually cultured in DMEM medium with 3% FBS for 24 h before medium collection. Culture medium was then centrifuged at 1000 g for 30 min and the supernatant was collected as conditioned medium for further study.

### Quantitative and semiquantitative RT–PCR

Total RNA was extracted by Trizol (Invitrogen). Two micrograms of total RNA was used to synthesise complimentary DNA with the Advantage RT-for-PCR Kit (Clontech Laboratories, Inc, Mountain View, California, USA), following the standard protocols provided by the manufacturer. Semiquantitative reverse transcriptase (RT)–PCR was performed by using AmpliTaq (Applied Biosystems, Foster City, California, USA). GAPDH was used as a control. For quantitative real-time PCR (qPCR), cDNA products were amplified using a SYBR Green PCR Kit (Applied Biosystems). The amplification protocol consisted of incubations at 95°C for 15 s, 60°C for 1 min and 72°C for 1 min for 40 cycles. Quantification was done using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). All gene expression values were normalised using the housekeeping genes GAPDH and calculated using the comparative C<sub>T</sub> method ( $\Delta\Delta$  C<sub>T</sub> method).<sup>3</sup>

### Immunohistochemical staining

Immunohistochemical staining was performed using a standard streptavidin–biotin–peroxidase complex method. In brief, paraffin block sections of oesophageal cancers were deparaffinised, blocked with 10% normal rabbit serum for 10 min, and incubated with rabbit anti-human Wnt2 polyclonal antibody (MBL, 1:150 dilution) overnight at 4°C. The slides were then incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:100 at 37°C for 30 min. The status of the cytoplasmic expression of Wnt2 was assessed by three independent investigators without previous knowledge of clinicopathological data. Positive expression of Wnt2, primarily a cytoplasmic pattern, was mostly detected in the stromal compartment within tumour tissue. In the present study, a positive result was defined when five or more Wnt2-positive cells were detected per microscopy field (20 $\times$  in magnification).

### Immunofluorescence

Cells cultured with conditioned medium from Wnt2-expressing CHO cells and control cells were grown on gelatin-coated coverslips for the indicated time points and then fixed in 4% *p*-formaldehyde at room temperature for 20 min. Cells were incubated with PBS-B solution at 37°C for 30 min to block non-specific interactions and stained with anti- $\beta$ -catenin mouse monoclonal antibody (Immunofluorescence (IF) Preferred; Cell Signalling Technology, Danvers, Massachusetts, USA) at 4°C overnight. After several washes in PBS, cells were incubated with optimal concentrations of FITC-labelled goat anti-mouse secondary antibody (eBioscience, San Diego, California, USA) at room temperature for 1 h. Anti-fade DAPI solution was added and images were obtained. For immunofluorescence

double-labelling, paraffin block sections of oesophageal cancers were incubated with a mouse antibody against vimentin and a rabbit antibody against Wnt2 simultaneously at 37°C overnight. After brief washing, slides were incubated with FITC goat anti-mouse (eBioscience) and Texas-red goat anti-rabbit (eBioscience) secondary antibodies at room temperature for 1 h. After brief washing, slides were counterstained with DAPI in anti-fade solution.

### Cell proliferation assay

To test the effect of Wnt2 secretion on cell growth rate, KYSE30 and EC109 cells were seeded onto 96-well plates at a density of  $2 \times 10^3$  per well. After 24-h incubation, normal growth media was replaced by conditioned medium from Wnt2-expressing clones and control cells. The cell growth rate was detected using the cell proliferation XTT kit (Sigma, St Louis, Missouri, USA) according to the manufacturer's instruction.

### Migration and invasion assays

For cell migration assay, KYSE30 and EC109 cells were grown to confluence and then mechanically scratched with a sterile pipette tip. Cells were rinsed with PBS and grown in CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium for an additional 72 h. The cell motility in terms of wound closure was measured by photographing at three random fields at time points 0 and 72 h. For invasion assay, KYSE30 and EC109 cells were starved with serum-free medium for 24 h before the assay. Cells ( $5 \times 10^4$ ) were suspended in 0.5 ml serum-free medium and loaded on the upper compartment of the invasion chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower compartment was filled with CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium as chemoattractant. After 48 h, invasive cells were fixed, stained and counted under a microscope. Three independent experiments were done with triplicates each time for both migration and invasion assays.

### Western blotting analysis

Western blotting was done according to the standard protocol with antibodies for rabbit anti-Wnt2 (R&D Systems, Minneapolis, Minnesota, USA; 1:400), rabbit anti-cyclin D1, -c-myc,  $\alpha$ -catenin, and  $\beta$ -catenin (Cell Signalling Technology; 1:1000), and mouse anti-E-cadherin,  $\alpha$ -SMA, -Vimentin and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2000).

### Statistical analysis

Statistical analysis was performed using SPSS standard V.13.0 software. Data are expressed as mean  $\pm$  SD from at least three independent determinations. Significance of difference was analysed using Student's *t* tests. The correlation between Wnt2 expression and clinicopathological characteristics was analysed using Fisher's exact test. Disease-specific survival was calculated from the date of diagnosis to the date of cancer-related death or last follow-up. Survival curves was assessed by the Kaplan–Meier method and compared by the log-rank test. Differences were considered significant for  $p < 0.05$ .

## RESULTS

### Wnt2 is overexpressed mainly in OSCC TF

qPCR was used to compare *WNT2* expression status between TF and normal fibroblasts isolated from 10 primary OSCC. Consistent with our previous finding,<sup>3</sup> *WNT2* expression was approximately fourfold higher in the TF than the normal fibroblasts (figure 1A). Statistical analysis showed that the difference in *WNT2* expression was significant ( $p < 0.05$ ,

independent Student's *t* test) between normal fibroblasts and TF. Western blot analysis confirmed that the expression of Wnt2 protein was also higher in TF than that in normal fibroblasts (figure 1B). Interestingly, no visible protein expression of Wnt2 was detected in all tested OSCC cancer cell lines (figure 1C).

### Distribution of Wnt2-positive cells in primary OSCC

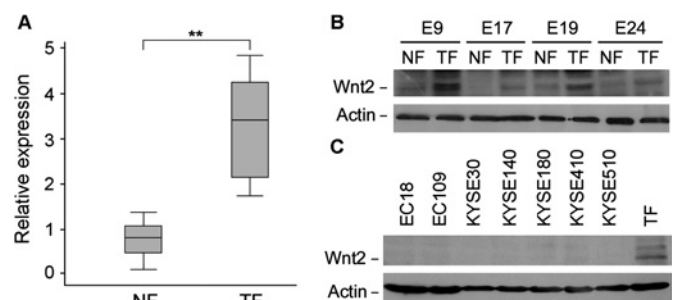
To study further the distribution of Wnt2(+) cells in OSCC, immunohistochemical staining was used to detect Wnt2 expression cells in 51 primary OSCC cases. Wnt2(+) cells were almost undetectable in adjacent non-tumorous oesophageal tissue (figure 2A). In OSCC tissues, Wnt2(+) cells could be detected in 42/51 (82.4%) cases. Wnt2(+) cells were mainly detected in the boundary between stroma and tumour tissue, stroma between tumour nests and scattered within tumour tissue (figure 2A). Two-colour (green vimentin; red Wnt2) staining immunofluorescence confirmed that Wnt2 and vimentin were co-expressed in the same cells, suggesting that Wnt2(+) cells were fibroblasts (figure 2B,C). Wnt2-positive OSCC, which was defined when five or more Wnt2(+) cells were observed in 200 $\times$  microscopy field, was detected in 22/51 (43.1%) of OSCC in the present study.

### The correlation of Wnt2-positive expression with OSCC prognosis

Correlation of the Wnt2-positive expression with clinicopathological features was studied in 51 OSCC. The results found that Wnt2(+) OSCC was significantly associated with TNM stage ( $p = 0.001$ , Fisher's exact test) and lymph node metastasis ( $p = 0.001$ , Fisher's exact test; table 1). Furthermore, the log rank test showed that the disease-specific survival of OSCC with Wnt2(+) OSCC (median survival time, 16 months) was significantly shorter than patients with Wnt2(-) OSCC (median survival time, 51 months;  $p < 0.0001$ ; figure 3).

### Establishment of secreted Wnt2 conditioned medium

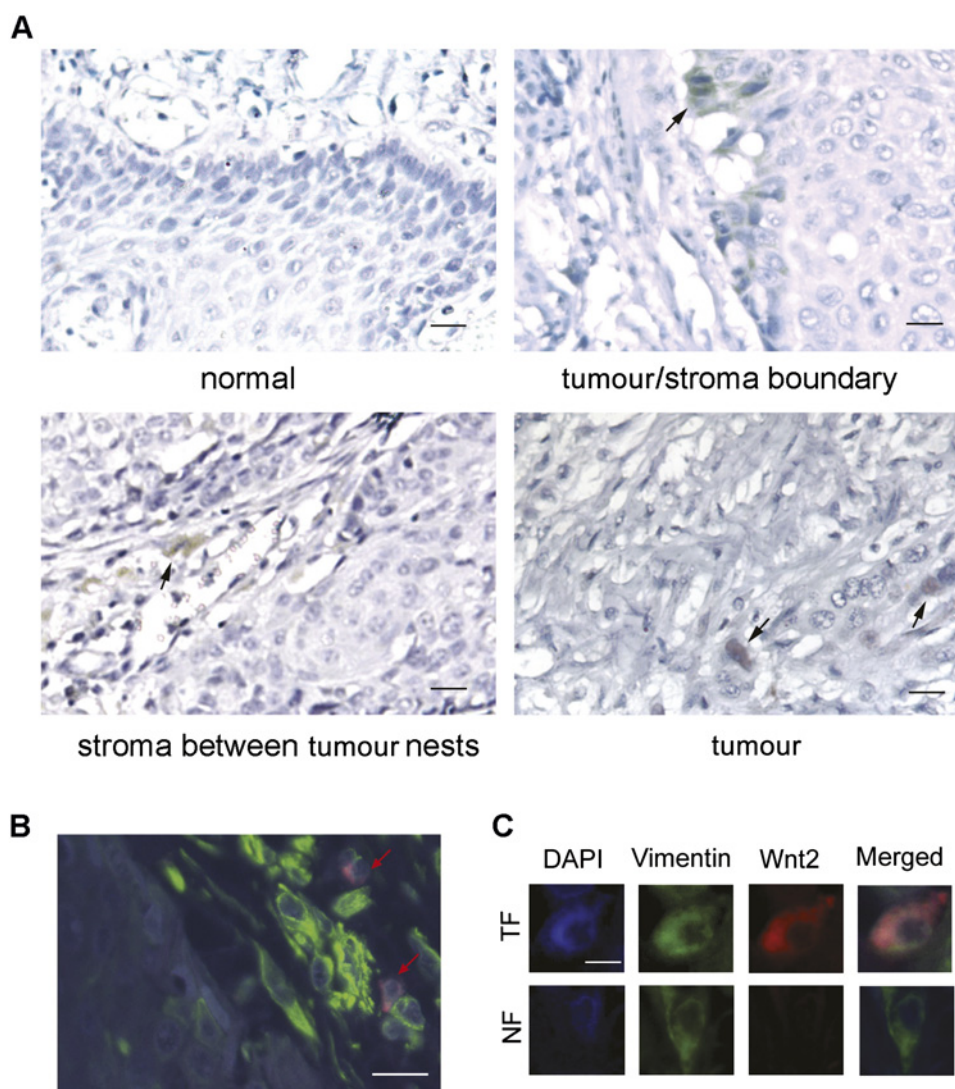
To investigate further the effect of secreted Wnt2 on OSCC cells, an in-vitro Wnt2 secreting system was established using CHO cells. Briefly, Wnt2 expression plasmid was stably transfected into CHO cells (CHO-Wnt2), and empty vector-transfected CHO cells (CHO-Vec) were used as controls. After a short



**Figure 1** Detection of Wnt2 expression in oesophageal squamous cell carcinoma (OSCC). (A) Relative expression level of *WNT2* was analysed by quantitative real-time PCR in tumour fibroblasts (TF) and their paired normal fibroblasts (NF) in 10 OSCC cases. *GAPDH* was used as endogenous control. Data represent the mean  $\pm$  SD derived from three independent experiments (\*\* $p < 0.01$ , independent Student's *t* test). (B) Representative of Wnt2 expression in protein level detected by western blot analysis.  $\beta$ -actin was used as loading control. (C) No expression of Wnt2 was detected in OSCC cancer cell lines by western blot analysis.  $\beta$ -Actin was used as an internal control.

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**Figure 2** Distribution of Wnt2(+) cells in oesophageal squamous cell carcinoma (OSCC). (A) Immunostaining with anti-Wnt2 antibody showed that Wnt2(+) cell was seldom observed in normal esophageal tissue. In OSCC, Wnt2(+) cell (indicated by arrows) was frequently observed in tumour/stroma boundary, stroma between tumour nests and scattered within tumour tissue.  $\times 200$  in magnification. Scale bars 20  $\mu\text{m}$ . (B) Representative of Wnt2(+) cells (indicated by arrows) detected in tumour/stroma boundary by two-colour immunofluorescent staining with antibodies against vimentin (green colour) and Wnt2 (red colour). Nuclei were counterstained with DAPI (blue colour). Scale bar 20  $\mu\text{m}$ . (C) Representative images of tumour fibroblasts (TF) and normal fibroblasts (NF) labelled by antibodies against vimentin (green colour) and Wnt2 (red colour). Nuclei were counterstained with DAPI (blue colour). Scale bar 10  $\mu\text{m}$ .



culture (24 h) in 3% FBS, culture medium was collected as conditioned medium for further study (figure 4A). The ectopic expression of Wnt2 in CHO cells was confirmed at both messenger RNA and protein (figure 4B) levels. Secreted Wnt2

was also detected in the conditioned medium collected from CHO-Wnt2 cells (figure 4B).

**Table 1** Association of positive Wnt2 expression with clinicopathological features in 51 OSCC

Clinicopathological characteristics	N	Wnt2 expression no (%)		p Value
		Wnt2(+)	Wnt2(-)	
Age (years)				
$\leq 57$	25	11 (44.0)	14 (56.0)	0.903
$> 57$	26	11 (42.3)	15 (57.7)	
Sex				
Male	39	19 (48.7)	20 (51.3)	0.192
Female	12	3 (25.0)	9 (75.0)	
TNM stage				
I	2	0 (0)	2 (100)	0.001*
II	31	8 (25.8)	23 (74.2)	
III	18	14 (77.8)	4 (22.2)	
Lymph node metastasis				
N <sub>0</sub>	23	4 (17.4)	19 (82.6)	0.001*
N <sub>1</sub>	28	18 (64.3)	10 (35.7)	

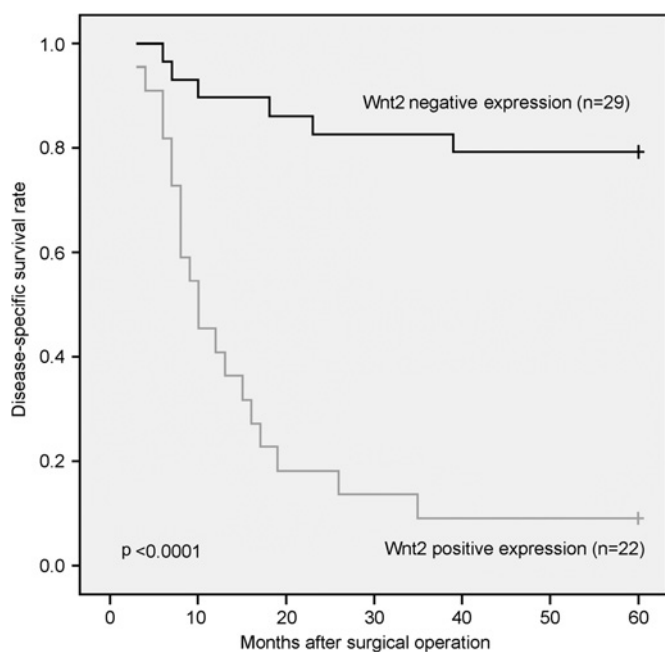
\* $p < 0.05$  (Fisher's exact test).  
OSCC, oesophageal squamous cell carcinoma.

### Secreted Wnt2 promotes cell proliferation in OSCC cells

To analyse the effect of Wnt2 on cell proliferation, OSCC cell lines (KYSE30 or EC109) were incubated in DMEM medium with 10% FBS, conditioned medium from CHO-Wnt2 or CHO-Vec cells, respectively, for 4 days. Cell proliferation (XTT) assay showed that the cell growth rate was significantly higher ( $p < 0.001$ ) in cells cultured with conditioned medium from CHO-Wnt2 than that of cells cultured with conditioned medium from CHO-Vec (figure 4C,D).

### Wnt2 promotes cell proliferation via nuclear translocation of $\beta$ -catenin

In an attempt to explore the potential molecular mechanism of the proliferating promotion effect of secreted Wnt2 on OSCC, the Wnt/ $\beta$ -catenin signalling pathway in KYSE30 and EC109 cells was characterised. Immunofluorescence was used to visualise the localisation of  $\beta$ -catenin at different time points (0, 1, 2 and 6 h) after Wnt2-conditioned medium incubation. As shown in figure 5A,  $\beta$ -catenin was mainly localised on the membrane of KYSE30 cells before incubation with the CHO-Wnt2 conditioned medium (CHO-Wnt2 conditioned medium). After incubation with CHO-Wnt2 conditioned medium for 1 h,

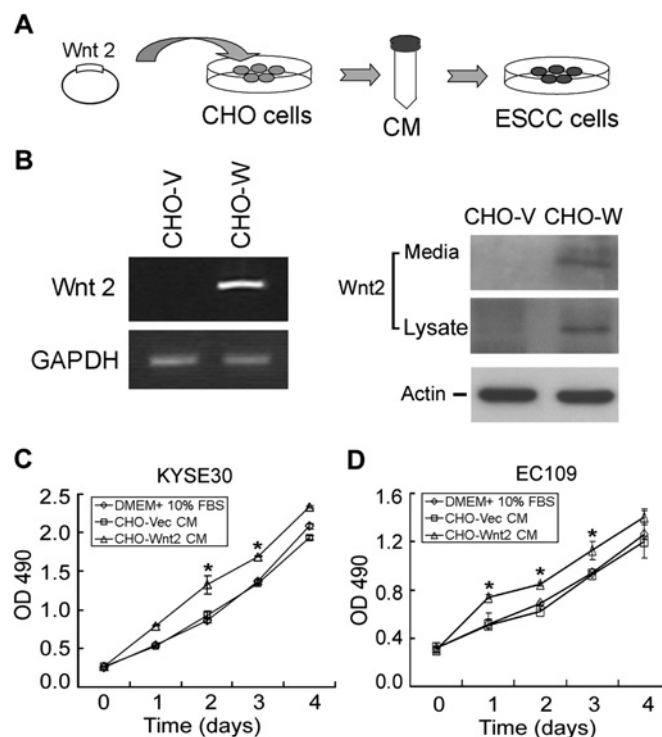


**Figure 3** Overexpression of Wnt2 was associated with poor prognosis of oesophageal squamous cell carcinoma determined by Kaplan–Meier analysis. Black, patients with negative Wnt2 expression (n=29, median survival 51 months); grey, patients with positive expression of Wnt2 (n=22, median survival 16 months; \*\* $p < 0.0001$ , log-rank test).

membranous  $\beta$ -catenin started to enter the cytoplasm of KYSE30 cells. At 2 h,  $\beta$ -catenin was translocated into the nucleus. Interestingly,  $\beta$ -catenin appeared in the membrane of KYSE30 cells again after 6 h incubation with CHO-Wnt2 conditioned medium (figure 5A). As expected, nuclear translocation of  $\beta$ -catenin could not be observed when KYSE30 cells were incubated with CHO-Vec conditioned medium (figure 5A).

To investigate whether the nuclear translocation of  $\beta$ -catenin could upregulate the expression of its downstream targets, expressions of cyclin D1 and c-myc at both mRNA and protein levels were compared between KYSE30 cells cultured with CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium by qPCR and western blot analysis. qPCR results showed that expressions of *CCND1* and *MYC* in KYSE30 cells were increased 1 h after culture with CHO-Wnt2 conditioned medium compared with cells cultured with CHO-Vec conditioned medium (figure 5B). At 2 h, expressions of *CCND1* and *MYC* were significantly increased in KYSE30 cells incubated with CHO-Wnt2 conditioned medium compared with cells incubated with CHO-Vec conditioned medium. At 4 h, expressions of *CCND1* and *MYC* were decreased to the levels before CHO-Wnt2 conditioned medium treatment (figure 5B). No increased *CCND1* and *MYC* expression was observed in KYSE30 cells treated with CHO-Vec conditioned medium.

Similarly, elevated protein levels of cyclin D1 and c-myc in response to the nuclear translocation of  $\beta$ -catenin were also detected by western blot analysis (figure 5C). Increased cyclin D1 and c-myc expression was detected at 2 h and 4 h after CHO-Wnt2 conditioned medium treatment (figure 5C). No increased cyclin D1 and c-myc expression was observed in KYSE30 cells treated with CHO-Vec conditioned medium. To confirm further the effect of Wnt2 on  $\beta$ -catenin translocation and the subsequent cyclin D1 and c-myc upregulation, OSCC cell line EC109 cells were also treated with CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium. Similar



**Figure 4** Secreted Wnt2 promotes cell proliferation in oesophageal squamous cell carcinoma (OSCC) cells. (A) Schematic diagram of the Wnt2-secretion system established in Chinese hamster ovary (CHO) cells. Plasmid containing *WNT2* was stably transfected into CHO cells and conditioned medium (CM) containing secreted Wnt2 was collected for further study. (B) Expression of *WNT2* in Wnt2-transfected CHO cells (CHO-W) was confirmed by reverse transcriptase (RT)–PCR (left). Western blot analysis showed that Wnt2 protein could be detected in both CHO-W cells and conditioned medium of CHO-W (right). CHO-V, empty vector-transfected CHO cells. (C and D) The effect of normal growth media (DMEM + 10% fetal bovine serum), control-conditioned media (CHO-Vec conditioned medium), or Wnt2-conditioned media (CHO-Wnt2 conditioned medium) on the growth of OSCC tumour cells (KYSE30 and EC109) was compared by XTT assay. The results are expressed as mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ , independent Student's t test.

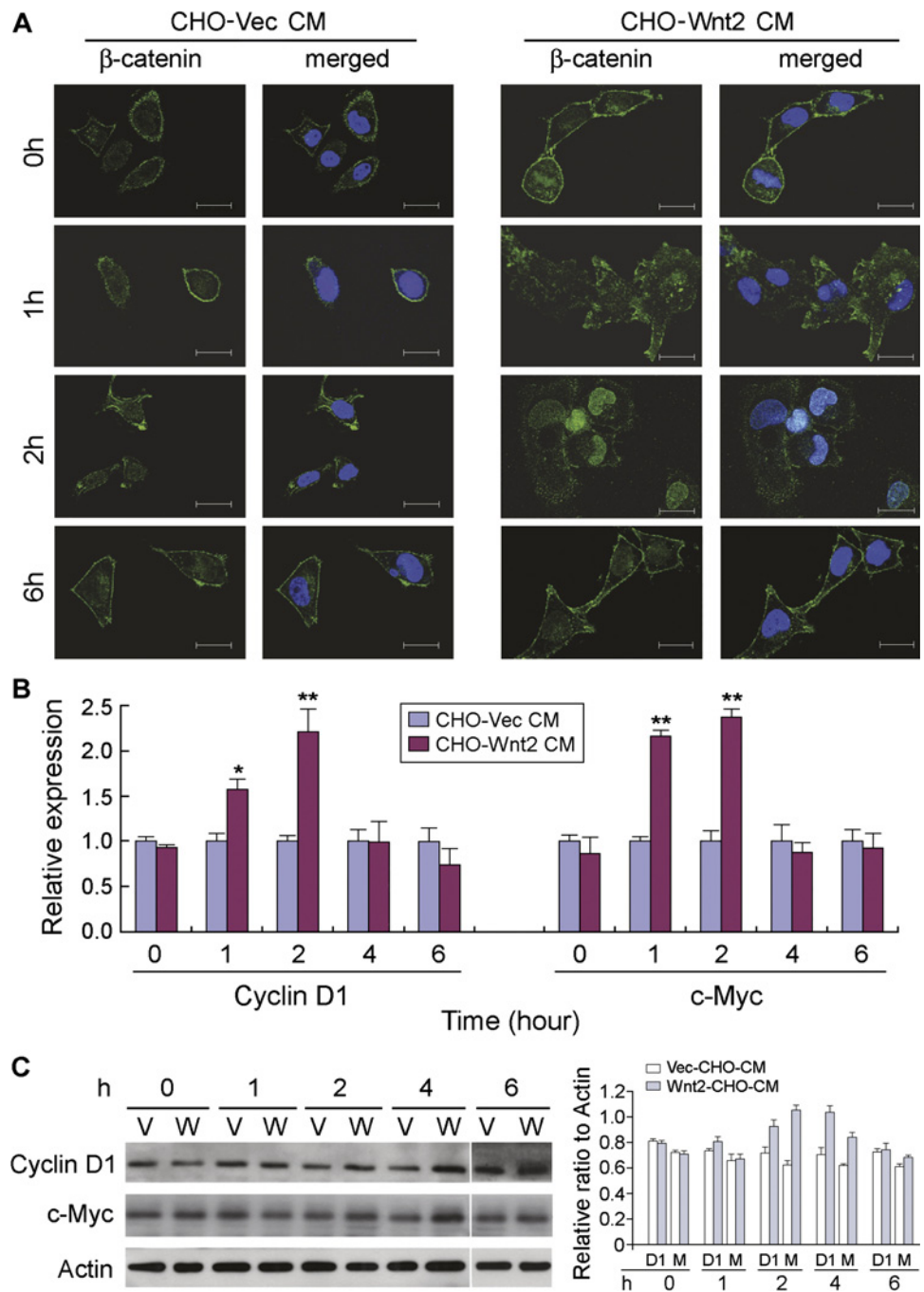
effects of Wnt2 on the nuclear translocation of  $\beta$ -catenin, as well as the upregulation of cyclin D1 and c-myc, were observed when cells were incubated with CHO-Wnt2 conditioned medium (figure 6).

#### Wnt2 enhances cell motility and invasiveness via epithelial–mesenchymal transition

As our correlation study showed that the positive expression of Wnt2 was closely associated with OSCC metastasis, the metastatic role of Wnt2 was studied by wound-healing and invasion assays. In the wound-healing assay, no significant difference was observed between CHO-Vec and CHO-Wnt2 conditioned medium cultured KYSE30 or EC109 cells over a 24-h incubation. As shown in figure 7A, CHO-Wnt2 conditioned medium could dramatically enhance cell migration ability in OSCC cells at 72 h after incubation, compared with control conditioned medium ( $p < 0.05$ ). Subsequently, we measured the capacity of OSCC cells to invade through matrigel, an artificial extracellular matrix, after incubation with CHO-Wnt2 conditioned medium or control conditioned medium. KYSE30 and EC109 cells cultured with CHO-Wnt2 conditioned medium showed an increased invasion by 53% and 75%, respectively ( $p < 0.01$ ; figure 7B).

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**Figure 5** Secreted Wnt2 induces nuclear translocation of  $\beta$ -catenin and activates Wnt/ $\beta$ -catenin signalling pathway in KYSE30 cells. (A) Representative immunofluorescence images showing that the translocation of  $\beta$ -catenin from the membrane to nucleus was only detected in KYSE30 cells treated with CHO-Wnt2 conditioned medium (CM) but not with CHO-Vec conditioned medium at the indicated time points. Antibody against  $\beta$ -catenin was labelled with green colour and nuclei were counterstained with DAPI (blue colour).  $\times 400$  in magnification. Scale bars 20  $\mu\text{m}$ . (B) Expression of  $\beta$ -catenin regulated targets *CCND1* and *MYC* was compared by quantitative real-time PCR between KYSE30 cells treated with CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium at the indicated time points. *GAPDH* was used as endogenous control. Data represent the mean  $\pm$  SD derived from three independent experiments (\* $p < 0.05$ ; \*\* $p < 0.01$ , independent Student's t test). (C) Expression of cyclin D1 and c-myc was also compared by western blot analysis between KYSE30 cells treated with CHO-Vec conditioned medium (V) and CHO-Wnt2 conditioned medium (W) at the indicated time points.  $\beta$ -Actin was used as a loading control. The relative expression ratio to  $\beta$ -actin was summarised in the right panel.

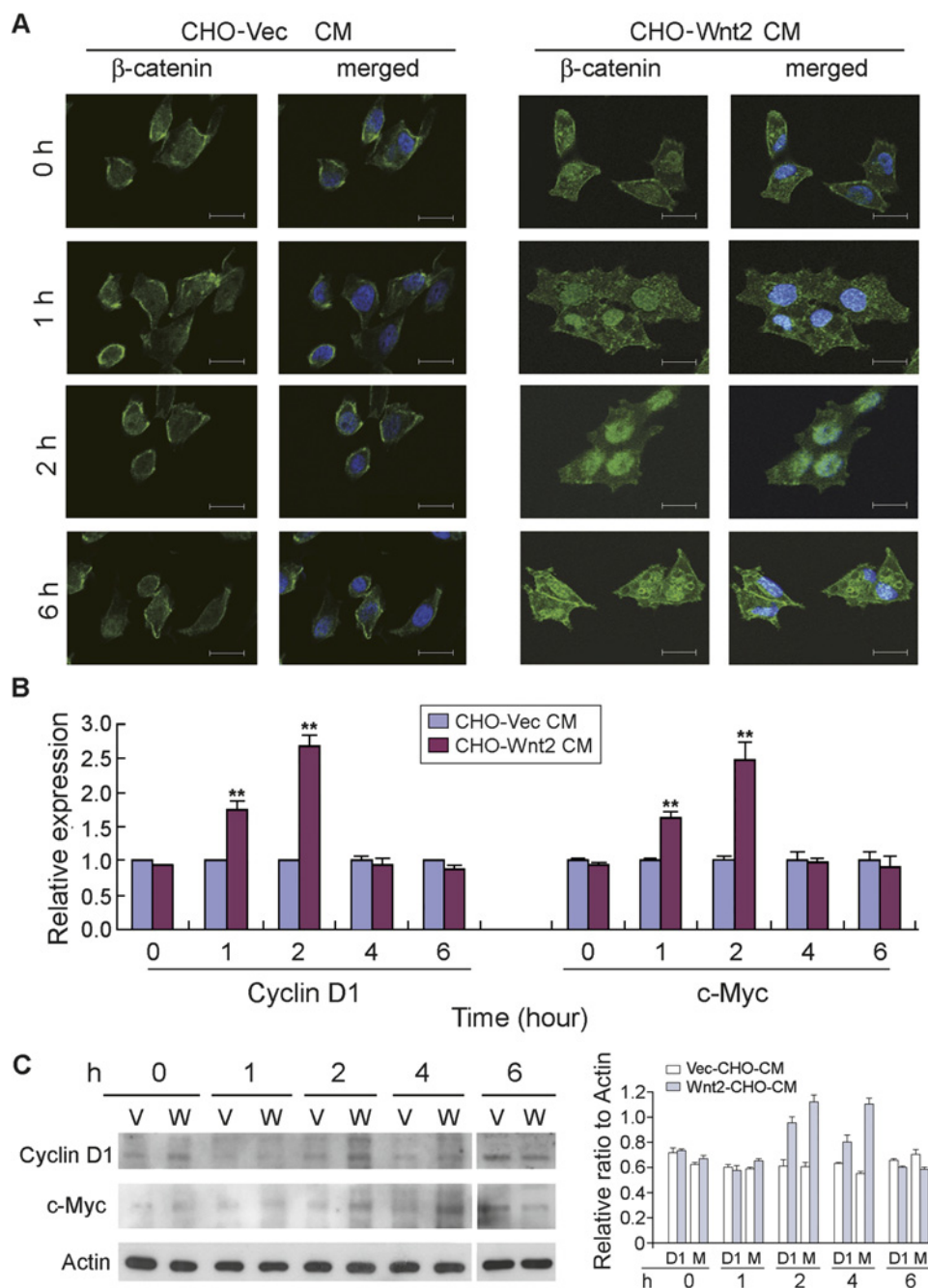


To determine whether the effect of Wnt2 on cell migration and invasion was associated with epithelial–mesenchymal transition, expressions of several epithelial markers ( $\alpha$ -catenin,  $\beta$ -catenin and E-cadherin) and mesenchymal markers ( $\alpha$ -SMA and vimentin) were compared between CHO-Wnt2 conditioned medium and control conditioned medium cultured OSCC cells at the 6-h time point by western blot analysis. Except for the absent expression of E-cadherin in EC109 cells, the results showed that the expression of all the tested epithelial markers was significantly downregulated, whereas the mesenchymal markers were upregulated in CHO-Wnt2 conditioned medium cultured KYSE30 and EC109 cells (figure 7C). These findings indicated that Wnt2 increased cell migration and invasion was through epithelial–mesenchymal transition.

## DISCUSSION

The role of the Wnt signalling pathway in normal development and organogenesis has been widely investigated in recent years.<sup>10–13</sup> An aberrantly activated Wnt signalling pathway may lead to the development of cancer.<sup>14 15</sup> Among members of the human *WNT* gene family, *WNT2* has been reported as a proto-oncogene with the potential to activate the WNT/ $\beta$ -catenin signalling pathway. Upregulation of Wnt2 has been detected in colorectal cancer,<sup>16</sup> gastric cancer<sup>17</sup> and oesophageal adenocarcinoma.<sup>18</sup> In the present study, we found that Wnt2 was seldom expressed in OSCC tumour cells. However, Wnt2(+) cells could be detected in 82.4% of primary OSCC cases, mainly distributed in the boundary between tumour and its surrounding stroma tissues, or in the stroma tissue between tumour nests, or scattered within tumour tissue. This distribution pattern is

**Figure 6** Secreted Wnt2 activates Wnt/ $\beta$ -catenin signaling pathway in EC109 cells. (A) Representative immunofluorescence images of  $\beta$ -catenin (green) subcellular location in EC109 cells treated with CHO-Wnt2 conditioned medium (CM) at the indicated time points. Nuclei were counterstained with DAPI (blue).  $\times 400$  in magnification. Scale bars 20  $\mu$ m. (B) Expression of  $\beta$ -catenin regulated targets *CCND1* and *MYC* was compared by quantitative real-time PCR between EC109 cells treated with CHO-Wnt2 conditioned medium and CHO-Vec conditioned medium at the indicated time points. *GAPDH* was used as endogenous control. Data represent the mean  $\pm$  SD derived from three independent experiments (\*\* $p < 0.01$ , independent Student's *t* test). (C) Expression of cyclin D1 and c-myc was detected by western blot analysis in EC109 cells treated with CHO-Vec conditioned medium (V) and CHO-Wnt2 conditioned medium (W) at the indicated time points.  $\beta$ -Actin was used as a loading control. The relative expression ratio to  $\beta$ -actin was summarised in the right panel.



similar to that of FGFR2(+) cells, which have been shown to be TF in our previous study.<sup>3</sup> An immunofluorescence study with antibodies to Wnt2 and the fibroblast marker vimentin showed that Wnt2 and vimentin were co-expressed in the same cells, suggesting that Wnt2(+) cells were TF. In addition, OSCC patients with more Wnt2(+) cells in OSCC tissue had shorter survival times, and the association was significant ( $p < 0.0001$ ). These data suggest that Wnt2 might play an important role in the development and progression of OSCC.

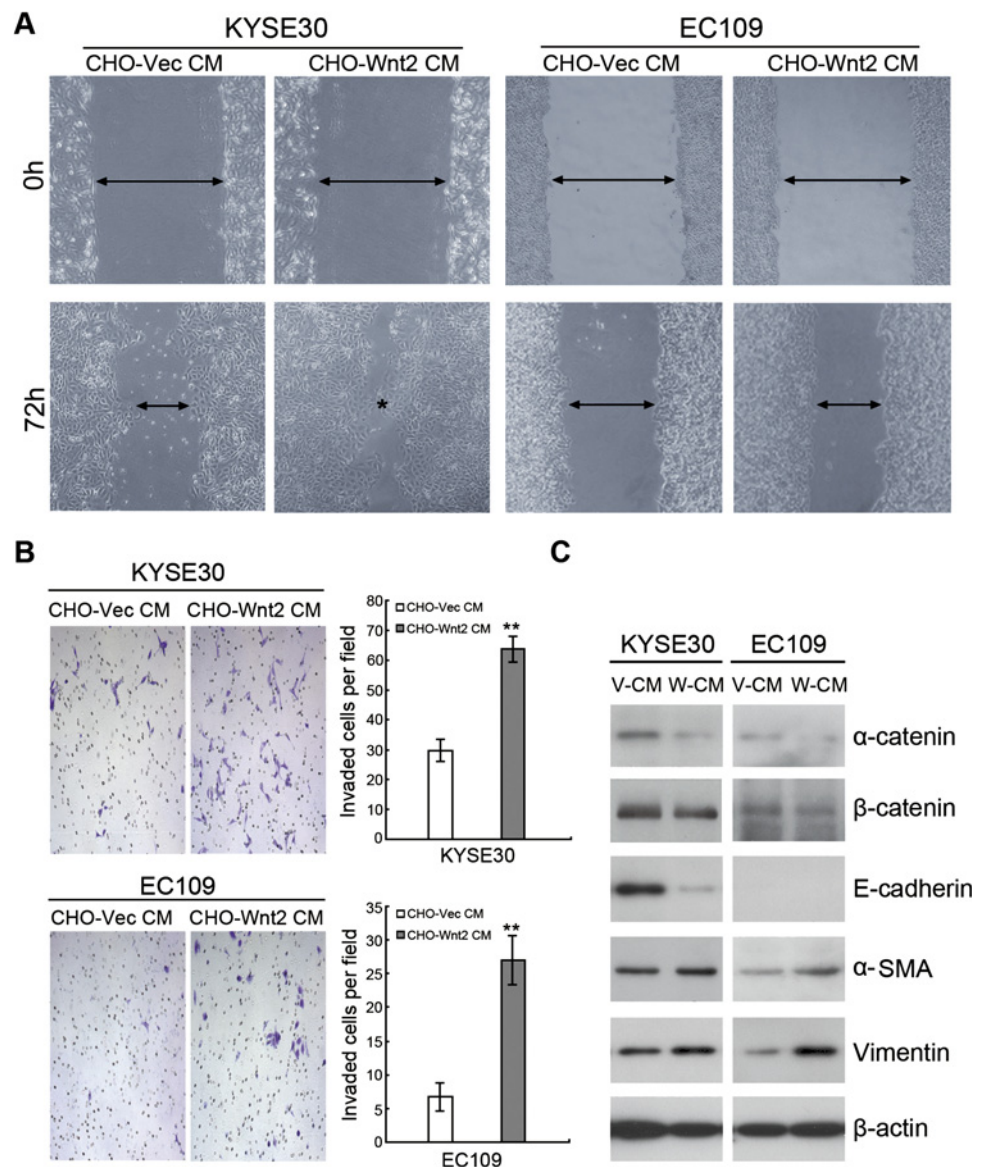
To investigate the effect of TF-secreted Wnt2 on oesophageal cancer cell growth, we established an in-vitro Wnt2 secretion system. The conditional medium collected from cultured CHO-Wnt2 cells, which contains secreted human Wnt2, could promote cell growth. This finding suggests that Wnt2 secreted by TF can modulate the growth of cancer cells during tumour development. Secreted WNT proteins act on target cells by binding to two

distinct families of cell surface receptors: the Frizzled receptors and the single-pass transmembrane low-density lipoprotein receptor-related proteins 5 and 6.<sup>19</sup> These receptor complexes transduce a signal to  $\beta$ -catenin via dishevelled proteins. In normal cells, most of the  $\beta$ -catenin molecules are localised at the plasma membrane where they link E-cadherin to the actin cytoskeleton via  $\alpha$ -catenin. In addition,  $\beta$ -catenin is inactivated by a multi-molecular complex containing the adenomatous polyposis coli protein, axin, and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). Phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$  results in its ubiquitination and proteolysis. Canonical Wnt signalling inhibits  $\beta$ -catenin degradation by inactivation of GSK-3 $\beta$ . Hypophosphorylated  $\beta$ -catenin then translocates to the nucleus, where it binds to transcription factors of the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family and initiates transcription of target genes, such as *MYC*, *CCND1* and *MMP7*.<sup>20</sup> Noteworthy, the



## Oesophagus

**Figure 7** Secreted Wnt2 promotes cell mobility and invasion by inducing epithelial–mesenchymal transition. (A) The effect of Wnt2 on cell migration was determined by wound-healing assay. During a period of 72 h, the spreading speed of CHO-Wnt2 conditioned medium (CM)-treated cells along the wound edge was faster than that in CHO-Vec conditioned medium-treated cells. (B) Representative images showed the CHO-Wnt2 and CHO-Vec conditioned medium-treated cells that invaded through the matrigel. The number of invaded tumour cells was quantified in the right panel. Columns, mean of triplicate experiments; \*\* $p < 0.01$ . (C) Expressions of epithelial markers  $\alpha$ -catenin,  $\beta$ -catenin and E-cadherin and mesenchymal markers  $\alpha$ -SMA and vimentin were compared by western blotting analysis between CHO-Wnt2 and CHO-Vec-treated KYSE30 and EC109 cells.  $\beta$ -actin was used as a loading control.



downstream effect of  $\beta$ -catenin on promoting cell proliferation might be a feedback response of Wnt2 on oesophageal cancer cells. Therefore, it would be interesting to study further the influence of  $\beta$ -catenin knockdown on cell proliferation, migration and invasion, which could exclude the effects that are not caused by  $\beta$ -catenin signalling.

In the present study, we found that secreted Wnt2 could activate the canonical Wnt signalling pathway, which was characterised by  $\beta$ -catenin accumulation in the cytoplasm, translocation into the nucleus and activation of transcription of Wnt target genes including *MYC* and *CCND1*. Our results clearly showed that  $\beta$ -catenin was accumulated in the cytoplasm at 1 h and entered into the nucleus at 2 h after oesophageal cancer cells treated with CHO-Wnt2 conditioned media, suggesting that the presence of Wnt2 in the conditioned media successfully activated the canonical Wnt signalling pathway.

Interestingly, nuclear  $\beta$ -catenin was declined while membranous  $\beta$ -catenin was increased after 6 h of incubation with CHO-Wnt2 conditioned medium, resulting in a decrease of c-myc and cyclin D1 compared with those seen at 2 h and 4 h. It has also been reported that Wnt-1 conditioned media led to an accumulation of cytoplasmic  $\beta$ -catenin in OSCC cells at 1.5 h and

sustained until 4 h after incubation.<sup>21</sup> Taken together, these findings suggest that secreted Wnt2 may act as an initiating factor during the stromal–epithelial interaction. However, the subsequent crosstalk between the Wnt2-mediated signalling pathway and other signalling networks, which contribute to cancer cell proliferation, need to be investigated further.

As a key component of the Wnt signalling pathway,  $\beta$ -catenin has been implicated in many human cancers,<sup>22</sup> and its oncogenic potential has been extensively studied in in-vitro tissue culture models<sup>23–24</sup> and in-vivo animal models.<sup>25–28</sup> The stabilisation of  $\beta$ -catenin, lack of degradation and ultimately nuclear accumulation is used as evidence of an activated Wnt/ $\beta$ -catenin pathway. Mutations, including large interstitial deletions involving exon 3 of the  $\beta$ -catenin gene, have been found to lead to the accumulation of  $\beta$ -catenin in the cytoplasm and nucleus of cancer cells in several types of cancers.<sup>29–31</sup> However, mutations in  $\beta$ -catenin are not responsible for the abnormal localisation of  $\beta$ -catenin in OSCC.<sup>32–33</sup> Nuclear accumulation of  $\beta$ -catenin has also been observed in Barrett's oesophagus, a precursor of adenocarcinoma of the distal oesophagus,<sup>34–36</sup> suggesting an involvement of Wnt signalling in the pathogenesis of both adenocarcinomas and squamous cell carcinomas of the

oesophagus. Further analysis showing a fraction of OSCC tumour cells have abnormal nuclear accumulation of  $\beta$ -catenin accompanied by increased stromal Wnt2 expression would improve our understanding of the growth-promoting effect of secreted Wnt2 in vivo.

Another interesting finding of this study is the promoting effect of Wnt2 on tumour metastasis in OSCC. Our data showed that exogenous Wnt2 could promote cell motility and invasiveness of OSCC cells in vitro. This mirrored the findings of Wnt2(+) OSCC was significantly associated with advanced clinical stage and lymph node metastasis. Increasing attention towards the role of epithelial–mesenchymal transition as a critical component of metastasis<sup>37</sup> has been thought to underlie the spread of malignant cells from a primary carcinoma to distant sites.<sup>38</sup> Several studies suggest that the Wnt/ $\beta$ -catenin signal pathway may play an important role in epithelial–mesenchymal transition. Indeed, our results showed that  $\beta$ -catenin entered into the nucleus at 2 h and removed from the nucleus to membrane at 6 h with the presence of Wnt2. Furthermore, we detected the decreased total protein level of  $\beta$ -catenin at 6 h after incubation with Wnt2, accompanied by the decreased expression of  $\alpha$ -catenin and E-cadherin and increased expression of  $\alpha$ -SMA and vimentin. These results strongly suggested that the Wnt2– $\beta$ -catenin–epithelial–mesenchymal transition pathway may play a critical role in OSCC progression.

In summary, we report that TF-secreted Wnt2 acts in a paracrine manner in oesophageal cancer initiation and progression by activating the canonical Wnt/ $\beta$ -catenin signalling pathway. Despite the importance of tumour–stromal interactions, our knowledge of the genes that mediate changes in the tumour microenvironment and tumour–stromal interactions in oesophageal cancer is still limited. Based on our findings, future therapeutic targets for interfering with Wnt signalling include cell surface receptors and secreted signalling molecules, which mediate signalling between cancer cells and the stromal environment, and may serve as effective treatment approaches to OSCC patients.

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**Contributors** The first two authors contributed equally to this paper. LF and CZ: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript and obtaining funding; LYZ, SSD, LHL, JC, KLK and YL: acquisition of data, technical support; YD and DLK: material support; XYG: study concept and design, critical revision of the manuscript for important intellectual content and obtaining funding.

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## Wnt2 secreted by tumour fibroblasts promotes tumour progression in oesophageal cancer by activation of the Wnt/ $\beta$ -catenin signalling pathway

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