

# Endostatin is a potential inhibitor of Wnt signaling

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Endostatin (ES) is a fragment of collagen XVIII that possesses antiangiogenic activity. To gain insight into ES-mediated signaling, we studied the effects of ES RNA on *Xenopus* embryogenesis and observed developmental abnormalities consistent with impaired Wnt signaling. ES RNA blocked the axis duplication induced by  $\beta$ -catenin, partially suppressed Wnt-dependent transcription, and stimulated degradation of both wild-type and “stabilized” forms of  $\beta$ -catenin, the latter suggesting that ES signaling does not involve glycogen synthase kinase 3. Moreover, ES uses a pathway independent of the Siah1 protein in targeting  $\beta$ -catenin for proteasome-mediated degradation. ES failed

to suppress the effects of T cell-specific factor (TCF)-VP16 (TVP), a constitutive downstream transcriptional activator that acts independently of  $\beta$ -catenin. Importantly, these data were replicated in endothelial cells and also in the DLD-1 colon carcinoma cells with the mutated adenomatous polyposis coli protein. Finally, suppression of endothelial cell migration and inhibition of cell cycle by ES were reversed by TVP. Though high levels of ES were used in both the *Xenopus* and endothelial cell studies and the effects on  $\beta$ -catenin signaling were modest, these data argue that at pharmacological concentrations ES may impinge on Wnt signaling and promote  $\beta$ -catenin degradation.

## Introduction

Angiogenesis, the formation of new capillaries through sprouting, is the primary process responsible for tumor neovascularization (Hanahan and Folkman, 1996; Carmeliet, 2000). This process depends on the balance between the effects of pro- and antiangiogenic molecules. Endostatin (ES),\* a COOH-terminal fragment of collagen XVIII, is a recently described antiangiogenic factor (O’Reilly et al., 1997). Exogenous recombinant ES inhibits tumor growth in several animal models (O’Reilly et al., 1997; Blezinger et al., 1999; Dhanabal et al., 1999a; Yoon et al., 1999; Sauter et al., 2000; Yokoyama et al., 2000; Feldman et al., 2001), and its usefulness for human cancer treatment is being assessed in clinical trials (Herbst et al., 2001). In vitro, ES blocks endothelial cell migration, promotes apoptosis,

and induces cell cycle arrest (Dhanabal et al., 1999a,b,c). Currently, which of these effects are most relevant in vivo when ES is administered for tumor therapy is unclear.

Both collagen XVIII and ES are involved in many aspects of embryonic development. Collagen XVIII is present in the tubular kidney basement membrane and is necessary for ureter morphogenesis (Lin et al., 2001). ES has been purified from the cultured supernatant of a kidney ureteric bud cell line and shown to modulate the mesenchymal–epithelial transition (Yung, J., T. Novak, M. Dhanabal, V. Sukhatme, and J. Barasch. 2000. Endostatin stimulates growth of epithelial precursors. American Society of Nephrology Annual Meeting, Toronto, Ontario, Canada. A2187. [Abstr.]). Moreover, we have shown recently that ES blocks renal branching morphogenesis and tubulogenesis (Karihaloo et al., 2001; Karumanchi et al., 2001). A splice mutation in human collagen XVIII that leads to a truncated protein lacking the ES fragment occasionally causes abnormalities in the renal collecting duct (Knobloch syndrome) (Sertie et al., 2000). The major phenotype in this disease is a failure of neural tube closure and vitreal and retinal degradation. At present, the physiological role of collagen type XVIII in control of vasculogenesis remains unclear. Nevertheless, mice lacking collagen type XVIII gene display retinal vessel abnormality (Fukai et al., 2002). In *Caenorhabditis elegans*, loss of the ES domain in the *cle-1* gene, a collagen XVIII homologue, results in multiple

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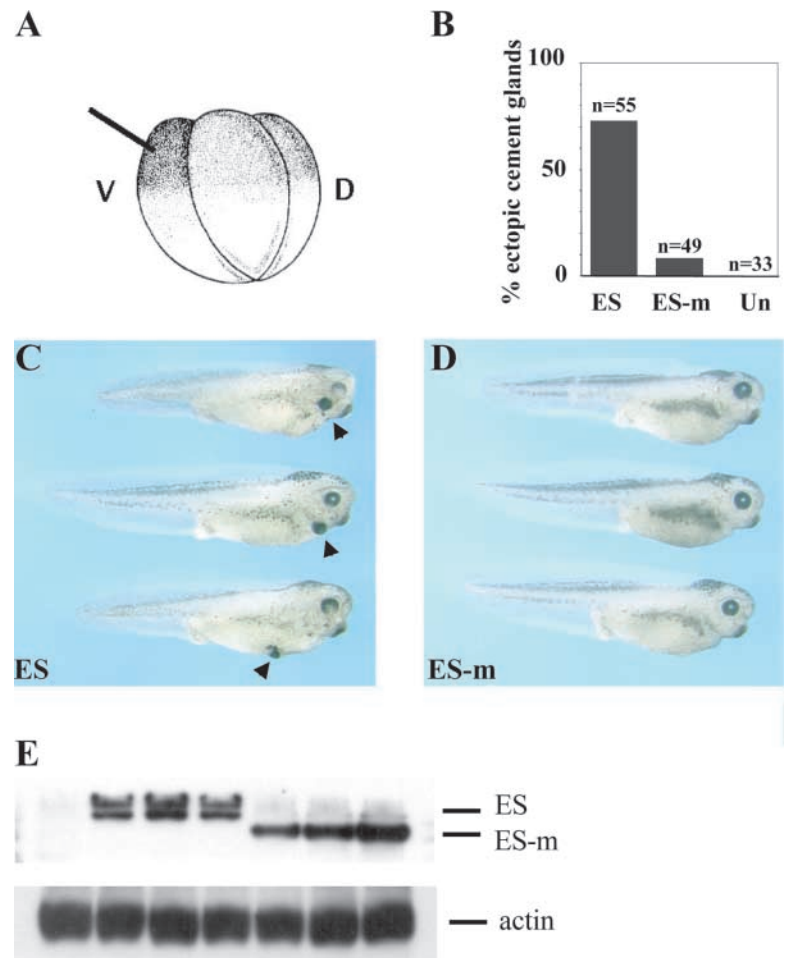
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\*Abbreviations used in this paper: APC, adenomatous polyposis coli; bFGF, basic FGF; CPAE, calf pulmonary artery endothelial; Dsh, Dishevelled; ES, endostatin; Frz, frizzled; GSK, glycogen synthase kinase; HUVECs, human umbilical vein endothelial cells; TCF, T cell-specific factor; TVP, TCF-VP16.

Key words: endostatin;  $\beta$ -catenin; migration; angiogenesis; *Xenopus*

**Figure 1. Developmental effects of ES in *Xenopus* embryos.** Four to eight cell embryos were injected into the animal side of one ventral (V) blastomere (A) with wild-type or mutated ES RNAs (2 ng each) and cultured for 48 h at room temperature to assess cement gland development. (B) Summary of the data. (C) Embryos injected with ES RNA develop ectopic cement glands (arrowheads). (D) Embryos injected with RNA encoding an NH<sub>2</sub>-terminal deletion construct (ES-m) are indistinguishable from uninjected embryos. (E) Protein expression levels in embryos injected with ES and ES-m RNAs assessed by anti-ES antibody.



cell migration and axon guidance defects (Ackley et al., 2001). Besides ES, the collagen XVIII gene contains an alternatively spliced fragment encoding a domain similar to the extracellular region of the frizzled (Frz) family members (Zatterstrom et al., 2000). Since Frz proteins function as cell surface receptors for secreted Wnt ligands (Bhanot et al., 1996; Wang et al., 1996; Zatterstrom et al., 2000), the structure of collagen XVIII suggests its possible involvement in Wnt signaling.

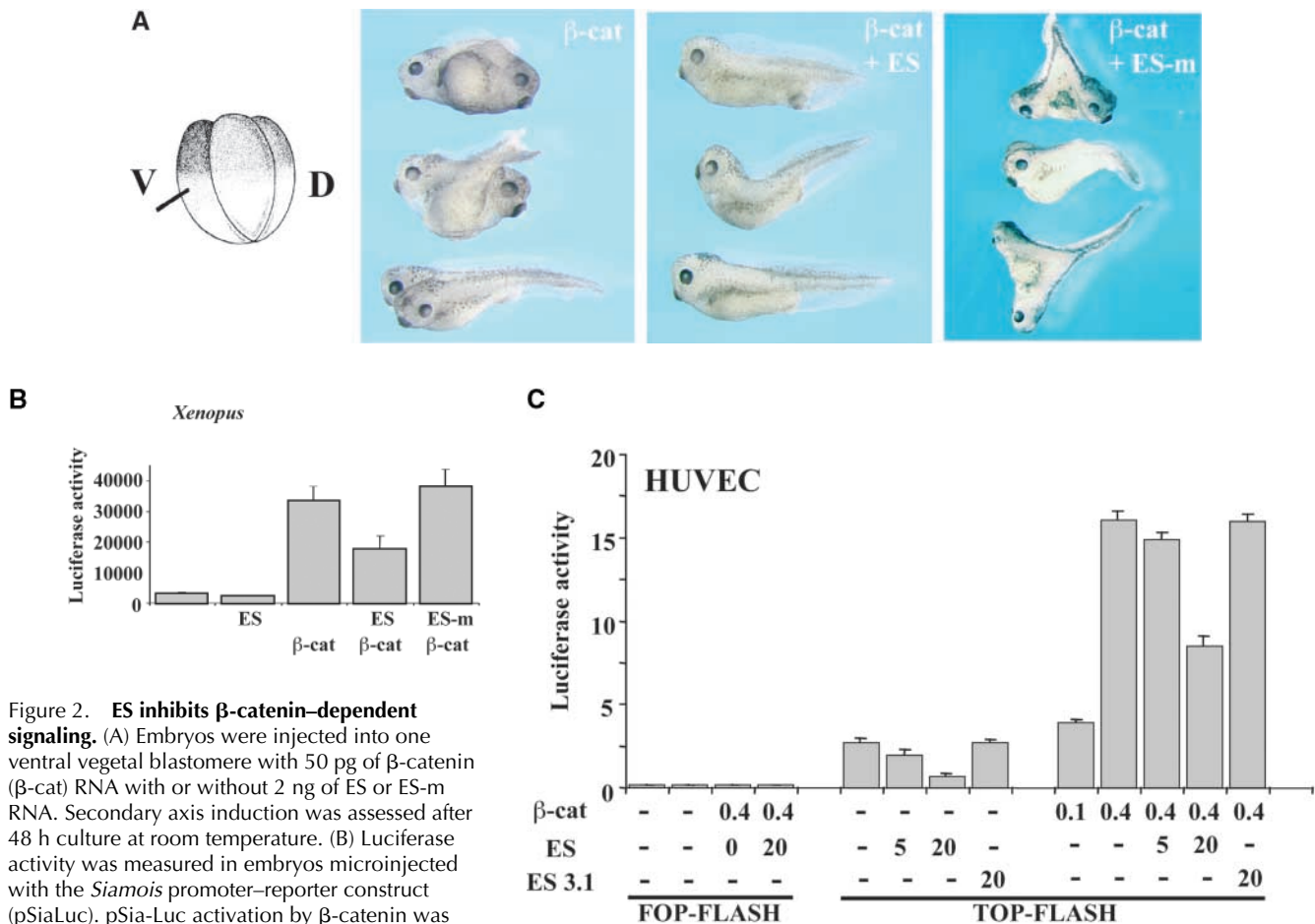
Wnt signaling pathways play important roles in the regulation of cellular proliferation, differentiation, motility, and morphogenesis (Wodarz and Nusse, 1998; Akiyama, 2000; Bienz and Clevers, 2000; Polakis, 2000). Signaling by the Frz receptors results in activation of the cytoplasmic Dishevelled (Dsh) proteins. Dsh antagonizes the effects of glycogen synthase kinase (GSK3), thus leading to  $\beta$ -catenin stabilization (Cadigan and Nusse, 1997; Gumbiner, 1997; Sokol, 1999). Stability of  $\beta$ -catenin is a critical point in Wnt signaling that is regulated by many cytoplasmic proteins including Axin, Frat/GBP, protein phosphatase 2A, adenomatous polyposis coli (APC), and GSK3 (Yost et al., 1996, 1998; Zeng et al., 1997; Kishida et al., 1999; Smalley et al., 1999; Ikeda et al., 2000; Itoh et al., 2000; Polakis, 2000). Stabilized  $\beta$ -catenin translocates to the nucleus where it binds to members of the T cell-specific factor (TCF)/lymphoid enhancer binding factor 1 transcription factor family and stimulates transcription of the target genes including c-Myc, cyclin D, and Siamese (Peifer and Polakis, 2000; Polakis, 2000).

Little is known about ES signal transduction. We have reported recently that glypicans are low affinity ES receptors critically important in mediating ES activities, such as the inhibitory effects on both endothelial cell and renal tubular branching morphogenesis (Karihaloo et al., 2001; Karumanchi et al., 2001). Although ES has been reported to activate tyrosine kinase signaling through the Shb adaptor protein (Dixelius et al., 2000), it remains to be defined how ES signals are transduced inside the cell. To gain insight into signal transduction pathways driven by ES, we used *Xenopus* embryogenesis as a model system that is well characterized with respect to several signaling pathways (Harland and Gerhart, 1997; Kimelman and Griffin, 2000). Here, we show that at high concentrations ES can antagonize the Wnt pathway in *Xenopus* embryos and in mammalian cells. Furthermore, our results suggest that ES may inhibit endothelial cell migration and arrest the cell cycle by inhibiting TCF-dependent transcription.

## Results

### ES causes specific developmental defects in *Xenopus* embryos

To gain insight into signal transduction pathways that involve ES, we used overexpression analysis in early *Xenopus* embryos. This *in vivo* system is well suited for the analysis of several pathways, including FGF, transforming growth



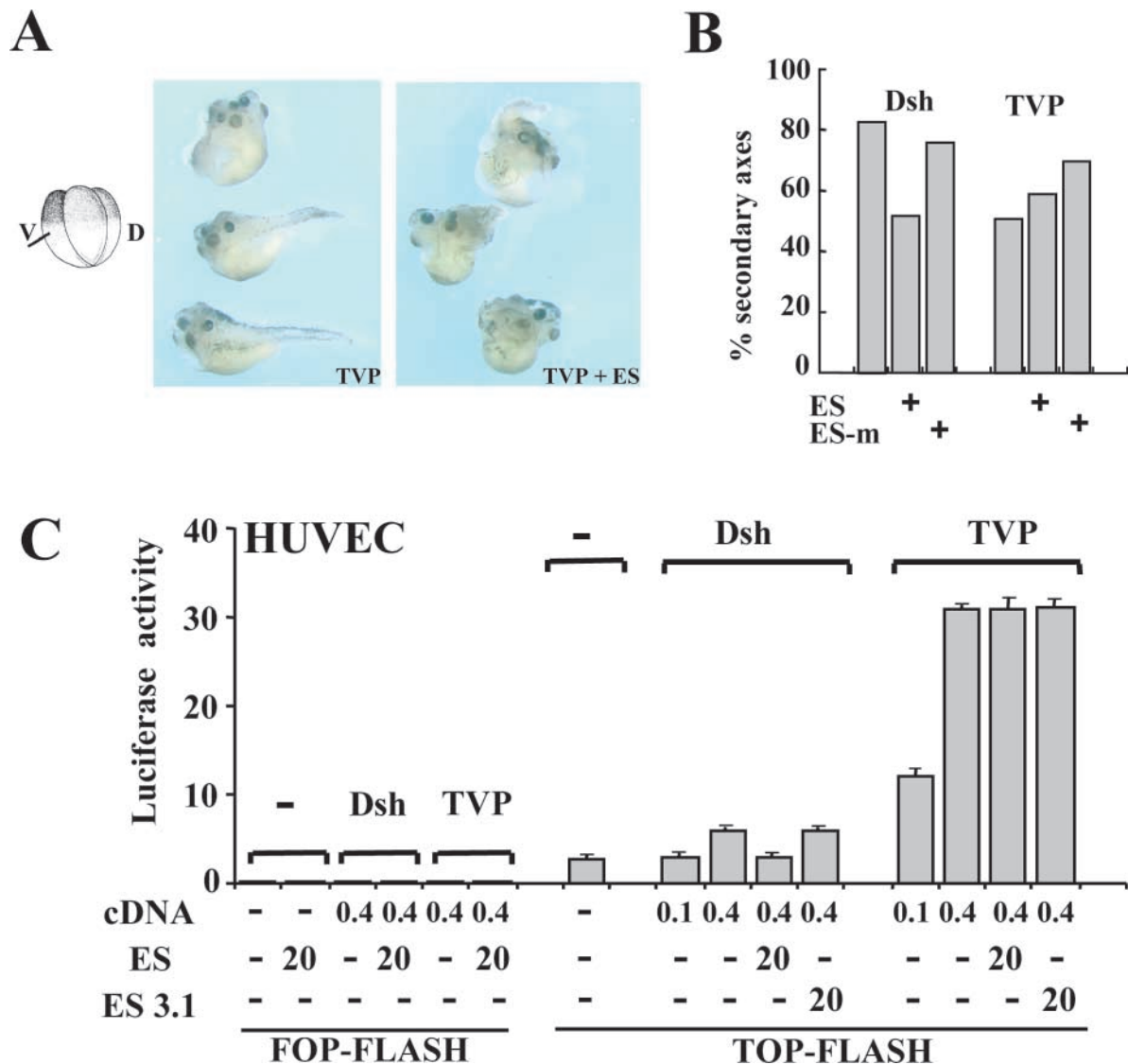
**Figure 2. ES inhibits  $\beta$ -catenin-dependent signaling.** (A) Embryos were injected into one ventral vegetal blastomere with 50 pg of  $\beta$ -catenin ( $\beta$ -cat) RNA with or without 2 ng of ES or ES-m RNA. Secondary axis induction was assessed after 48 h culture at room temperature. (B) Luciferase activity was measured in embryos microinjected with the *Siamois* promoter-reporter construct (pSiaLuc). pSia-Luc activation by  $\beta$ -catenin was reduced by ES but not by ES-m. (C) The effect of ES on  $\beta$ -catenin signaling in endothelial cells. Transcriptional activation of the TOP-FLASH and FOP-FLASH reporters was measured in HUVECs transfected with indicated plasmids in the presence or absence ES or ES mutant (ES 3.1). Numbers show the amount of ES in  $\mu$ g/ml and the amount of transfected  $\beta$ -catenin plasmid per well of a 6-well plate. Cells were transfected with 0.8  $\mu$ g of each reporter plasmid.

factor- $\beta$ , and Wnt signaling, since activation of these pathways results in developmental changes that are easily detectable at the morphological level. To analyze the effects of ES in frog embryos, we constructed a recombinant form of ES, which contains a signal peptide allowing secretion and does not require proteolytic processing (Karumanchi et al., 2001). In vitro synthesized mRNAs encoding wild-type and mutated forms of ES were microinjected into a single animal ventral or dorsal blastomere of *Xenopus* embryos at the 4–8-cell stage. Injected embryos were cultured, and their development was closely monitored. Starting at neurula stages, 72.7% of embryos injected ventrally with 2 ng ES RNA developed an ectopic cement gland, an anterior ectodermal organ that forms near the future mouth of the tadpole ( $n = 55$ ) (Fig. 1), whereas dorsal injection resulted in eye defects (data not shown). In contrast, mutated ES with an NH<sub>2</sub>-terminal deletion (ES-m) failed to interfere with normal development (Fig. 1, B and D). Both ES and ES-m proteins were expressed at comparable levels (Fig. 1 E). These defects are similar to those obtained by overexpression of GSK3 $\beta$ , an inhibitor of Wnt signaling (Itoh et al., 1995), or partial depletion of  $\beta$ -catenin, a key mediator of Wnt signal transduction (Heasman et al., 2000). Thus, the phenotype induced by ES in *Xenopus* embryos suggests that ES might antagonize Wnt signaling.

Since several inhibitors of  $\beta$ -catenin signaling, including Axin and a dominant negative TCF, are known to inhibit dorsal development (Gumbiner, 1997; Sokol, 1999), we assessed the effect of ES after injection of RNA in dorsovegetal blastomeres. No significant suppression of dorsal structures was observed (unpublished data), suggesting that the signaling pathway activated by ES differs from the canonical Wnt-dependent mechanisms for  $\beta$ -catenin degradation.

### ES impinges on Wnt signaling

To evaluate whether ES can modulate the Wnt pathway, we assessed its effect on  $\beta$ -catenin activity.  $\beta$ -catenin is an essential mediator of Wnt signaling and is known to induce a secondary axis when overexpressed in *Xenopus* embryos (Guger and Gumbiner, 1995). Injection of  $\beta$ -catenin RNA into a ventral blastomere of 4-cell embryos resulted in a significant percentage of embryos with duplicated head-containing body axes (53%, total number = 45) (Fig. 2). In contrast, most embryos coinjected with ES RNA (86%,  $n = 29$ ) developed only a single axis or a partial secondary axis with trunk and tail structures but without head, thus demonstrating the inhibitory activity of ES. An ES deletion mutant (ES-m) did not have this activity (81% of embryos had secondary axes;  $n = 25$ ). Similarly, axis-inducing activity of Wnt8 was also suppressed



**Figure 3. Effects of ES in response to increasing Dsh and TVP activity.** (A and B) Embryos were injected into one ventral vegetal blastomere as indicated with the following RNAs: TVP (0.7  $\mu$ g), Dsh (0.7 ng); ES and ES-m (2 ng). (A) ES failed to inhibit TVP induced secondary axes. (B) The effect of ES constructs on secondary axes induced by Dsh and TVP. Total number of injected embryos varied from 14 to 70 per group. (C) The effect of ES on transcriptional activation of TOP-FLASH in response to Dsh and TVP. HUVECs were transfected with 0.4  $\mu$ g of TVP or empty vector in the presence (5 or 20  $\mu$ g/ml) or absence of ES. ES inhibited Dsh-mediated but not TVP-mediated activation of the reporter.

by ES (unpublished data). These findings are consistent with the hypothesis that ES functions as an inhibitor of the Wnt pathway.

Next, we wanted to determine whether the suppression of  $\beta$ -catenin-mediated axis induction by ES results from direct inhibition of early Wnt signal transduction or represents an effect of ES on non-Wnt signaling pathways involved in axis determination later in development. Therefore, we studied whether ES inhibits Wnt/ $\beta$ -catenin-dependent transcriptional targets and analyzed the effect of ES and ES-m on *Siamois* promoter activation by  $\beta$ -catenin (Fig. 2 B) or Wnt8 (data not shown). ES but not ES-m reduced *Siamois* promoter activation by  $\beta$ -catenin (Fig. 2 B) or Wnt8 (data not shown). Because *Siamois* is an immediate target for Wnt signaling (Brannon et al., 1997; Fan et al., 1998), this result suggests that ES inhibits the Wnt/ $\beta$ -catenin pathway in a direct manner.

We also examined whether the effects of ES in *Xenopus* embryos could be reproduced in endothelial cells in which ES activity was initially demonstrated (O'Reilly et al., 1997). TOP-FLASH, a  $\beta$ -catenin responsive reporter with multimerized TCF sites, but not the control FOP-FLASH construct with mutated TCF sites (van de Wetering et al., 1991, 1996), was activated in human umbilical vein endothelial cells (HUVECs), transiently transfected with a  $\beta$ -catenin plasmid (Fig. 2 C). At 5–20  $\mu$ g/ml doses, ES repressed TOP-FLASH activation in HUVECs by 30–60%. Lower ES doses (100 ng/ml) produced similar inhibition in calf pulmonary arterial endothelial (CPAE) cells (unpublished data). On the other hand, ES with double point mutations, which lacks activity in endothelial migration assays (ES3.1) (Karumanchi et al., 2001), had no effect on promoter activation (Fig. 2 C). Together, these experiments suggest that ES can inhibit the Wnt pathway both in *Xenopus* embryos and endothelial cells.

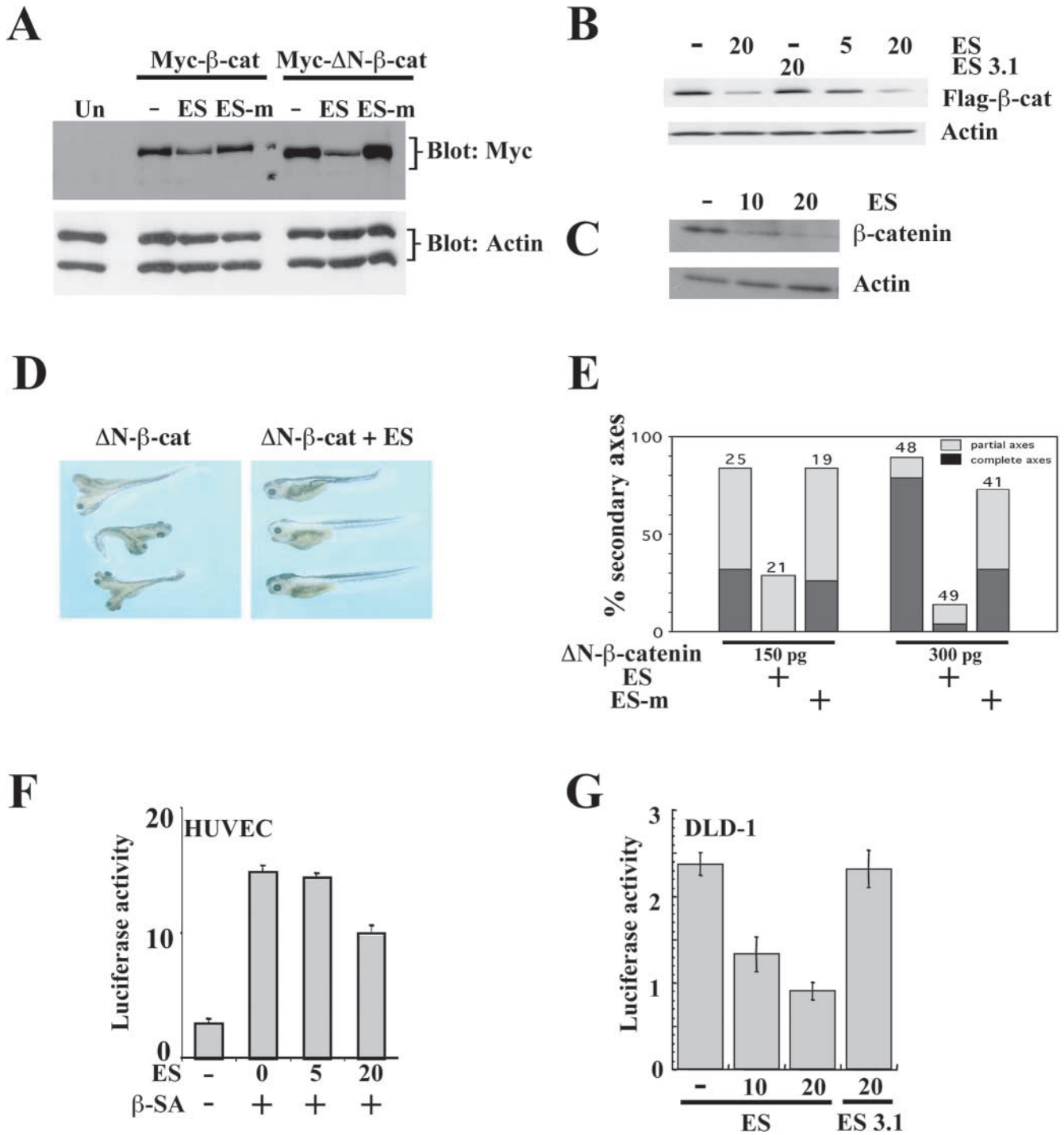


Figure 4. **ES down-regulates β-catenin levels.** (A) ES decreases steady state β-catenin levels in embryo lysates. Embryos were coinjected with Myc-tagged β-catenin (β-cat) or ΔN-β-catenin (ΔN-β-cat) RNAs (300 and 100 pg, respectively) and either wild-type or mutated ES RNA. At embryonic stages 8–8.5, β-catenin levels were analyzed by Western blotting with anti-Myc antibodies. ES but not ES-m reduced both β-catenin and ΔN-β-catenin protein levels. (B) Effect of ES on β-catenin levels in HUVECs. ES (5 or 20 μg/ml) but not mutant ES 3.1 (20 μg/ml) reduced Flag-β-catenin levels in transfected HUVECs. (C) ES down-regulates endogenous β-catenin in CPAE cells. Cells were incubated with indicated amounts of ES (μg/ml) for 24 h, and cell lysate were collected for western analysis. (D and E) ES blocks the signaling activity of stabilized β-catenin in *Xenopus*. Embryos were injected with 150–300 pg of ΔN-β-catenin RNA and 2 ng of ES or ES-m RNA as described in the Fig. 2 legend. (D) Suppression of secondary axes induced by ΔN-β-catenin. (E) Summary of the data. (F) ES blocks the signaling activity of stabilized β-catenin in HUVECs. Transcriptional activation of TOP-FLASH in HUVECs transfected with 0.4 μg of β-catenin S>A mutant (β-SA) or empty vector in the presence or absence of 5–20 μg/ml of ES. (G) ES down-regulates TOP-FLASH in the colon cancer DLD-1 cells with constitutively high levels of β-catenin signaling. DLD-1 cells were transfected with 0.9 μg of TOP-FLASH reporter plasmid and incubated in the presence (10 or 20 μg/ml) or absence of ES. Cell lysates were collected 24 h after transfection for luciferase activity measurement.

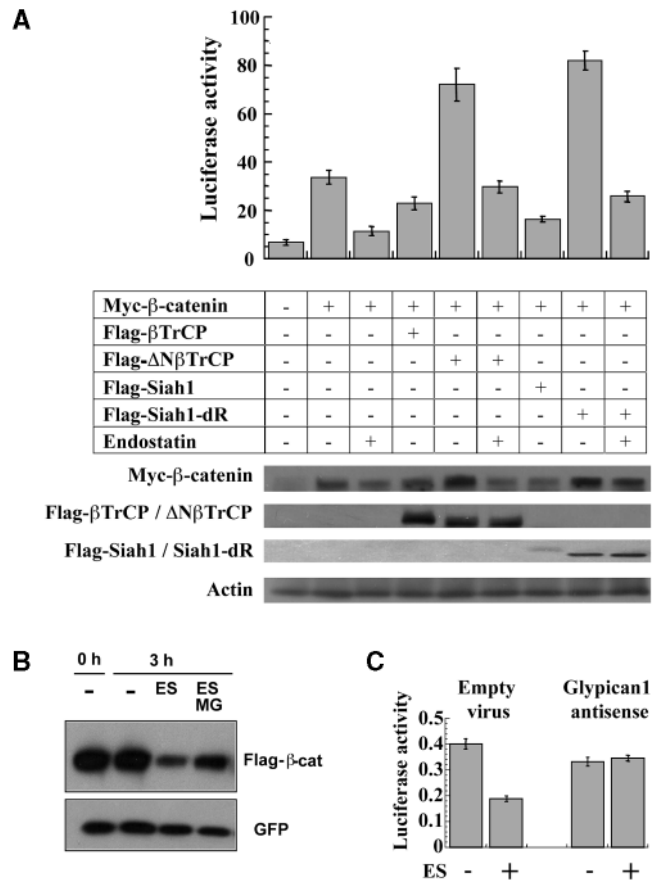
### $\beta$ -catenin as a target for ES

The canonical Wnt pathway has been shown to involve several molecular components, which function sequentially (Cadigan and Nusse, 1997; Sokol, 1999). Therefore, we tested the ability of ES to block signal transduction at different levels using both *Xenopus* embryos and endothelial cells. Injection of TCF-VP16 (TVP) RNA, encoding NH<sub>2</sub>-terminally deleted Xtc3 fused to the VP16 transcriptional activator (Vonica et al., 2000), resulted in axis duplication (Fig. 3). TVP lacks the  $\beta$ -catenin binding site and is a constitutive transcriptional activator that is independent of  $\beta$ -catenin (Vonica et al., 2000). Importantly, ES failed to inhibit TVP-mediated axis duplication (Fig. 3, A–B). In contrast, secondary axes induced by Dsh, an upstream component of the pathway, were inhibited by ES (Fig. 3 B). Similar data were obtained by measuring TOP-FLASH reporter activation in HUVECs (Fig. 3 C). These findings show that ES operates downstream of or parallel to Dsh, possibly at the level of  $\beta$ -catenin.

Since ES strongly inhibited the function of  $\beta$ -catenin but failed to modulate activity of TVP, we evaluated whether  $\beta$ -catenin stability was affected by ES. Treatment with ES but not with the mutated forms of ES decreased steady-state levels of tagged  $\beta$ -catenin in both *Xenopus* embryos and endothelial cells (Fig. 4, A and B). Moreover, endogenous  $\beta$ -catenin was also down-regulated in endothelial cells by ES (Fig. 4 C). These findings suggest that  $\beta$ -catenin may be a target for ES.

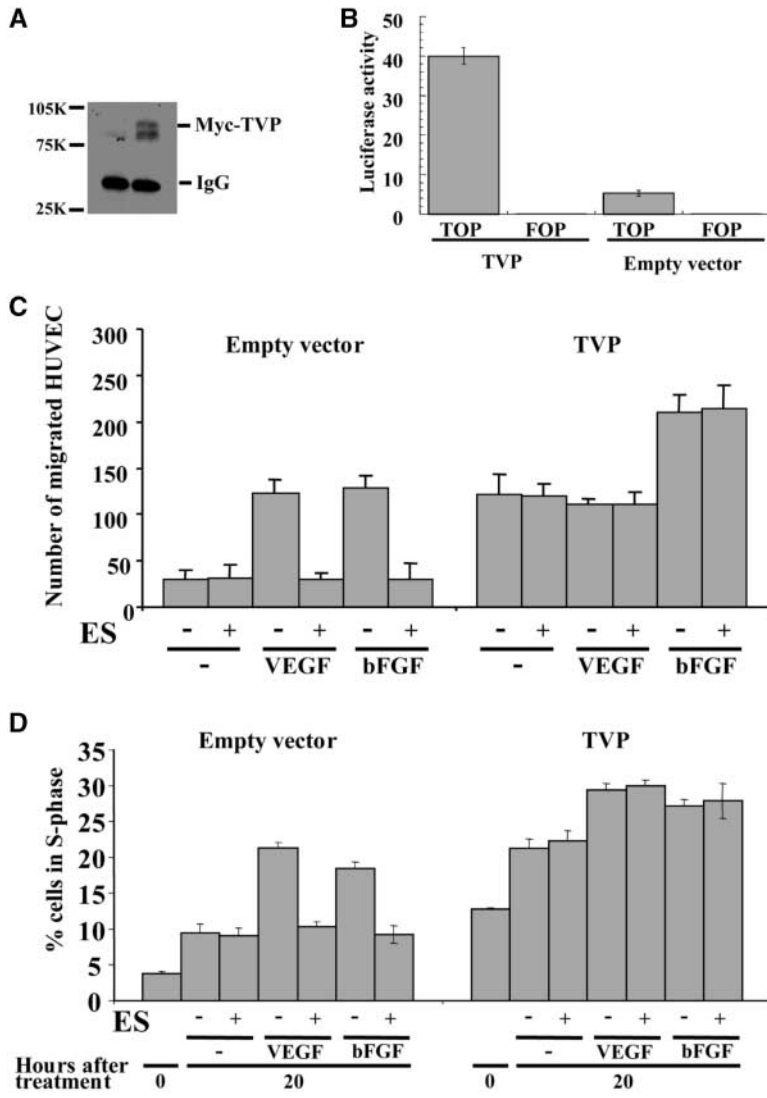
Next, we asked whether the stable form of  $\beta$ -catenin, lacking NH<sub>2</sub>-terminal phosphorylation sites ( $\Delta$ N- $\beta$ -catenin [Yost et al., 1996]), is affected by ES. NH<sub>2</sub>-terminal phosphorylation has been shown to be required for  $\beta$ -catenin degradation (Yost et al., 1996), and  $\Delta$ N- $\beta$ -catenin is insensitive to upstream antagonists of Wnt signaling, such as GSK3 $\beta$ , APC, and axin (Peifer and Polakis, 2000). We found that ES but not ES-m decreased expression of  $\Delta$ N- $\beta$ -catenin (Fig. 4 A) and suppressed the ability of  $\Delta$ N- $\beta$ -catenin to induce secondary axes in frog embryos (Fig. 4, D and E). Consistent with these *Xenopus* data, ES reduced the TOP-FLASH activation induced by the stabilized S>A  $\beta$ -catenin mutant ( $\beta$ -SA) (Liu et al., 1999) in endothelial cells (Fig. 4 F). These data indicate that ES modulates the Wnt pathway by regulating  $\beta$ -catenin stability via a novel GSK3-independent mechanism. Moreover, ES but not the ES mutant ES3.1 down-regulated TOP-FLASH activity in DLD-1, colon cancer cells carrying an APC mutation that results in constitutively high  $\beta$ -catenin signaling (Morin et al., 1997) (Fig. 4 G). Together, these observations suggest that ES works in a GSK3- and an APC-independent manner.

The  $\beta$ TrCP protein associates with  $\beta$ -catenin through recognition of specific NH<sub>2</sub>-terminal sites for GSK3 phosphorylation, thereby targeting it for ubiquitin-proteasome-dependent degradation (Jiang and Struhl, 1998; Liu et al., 1999; Maniatis, 1999). In CPAE cells,  $\beta$ -catenin levels were decreased by  $\beta$ TrCP, whereas a dominant negative  $\beta$ TrCP ( $\Delta$ N $\beta$ TrCP) interfered with this degradation pathway resulting in upregulation of  $\beta$ -catenin (Fig. 5 A). ES decreased  $\beta$ -catenin levels and TOP-FLASH activity in the presence of  $\Delta$ N $\beta$ TrCP, indicating that it can promote  $\beta$ -catenin degradation even when the  $\beta$ TrCP function is blocked, lending additional support for GSK3-independent action of ES.



**Figure 5. ES effects on  $\beta$ -catenin are not mediated through  $\beta$ TrCP or Siah1.** (A) CPAE cells were transfected with 0.4  $\mu$ g of myc- $\beta$ -catenin and 0.8  $\mu$ g of each Flag- $\beta$ TrCP, Flag- $\Delta$ N $\beta$ TrCP, Flag-Siah1, or Flag-Siah1-dR, as indicated, in the presence (20  $\mu$ g/ml) or absence of ES. ES reduced transcriptional activation of TOP-FLASH by  $\beta$ -catenin in the presence of dominant negative forms of  $\beta$ TrCP or Siah1.  $\beta$ -catenin levels were measured in the same experiment in the presence (20  $\mu$ g/ml) or absence of ES. (B) Proteasome inhibitor treatment reverses the effect of ES on  $\beta$ -catenin. CPAE cells were transfected with the Flag- $\beta$ -catenin and GFP plasmids. After 24 h, cells were incubated with ES (10  $\mu$ g/ml) and/or the proteasome inhibitor MG132 (MG) for 3 h as indicated. Cell lysates were collected for Western blotting with anti-Flag and anti-GFP antibodies. (C) ES effect on TOP-FLASH requires glypican 1. Transcriptional activity of TOP-FLASH was measured in glypican 1 antisense virus- or control vector-infected HUVECs.

A novel pathway for  $\beta$ -catenin degradation has been described that involves Siah1, a human homologue of *seven in absentia* (Della et al., 1993; Liu et al., 2001; Matsuzawa and Reed, 2001). Since this degradation route is GSK3 and TrCP independent, we explored a possible role for Siah1 in ES signaling. In CPAE cells,  $\beta$ -catenin levels were down-regulated by wild-type Siah1 and up-regulated by the dominant negative Siah1 (Siah1-dR [Hu and Fearon, 1999]) consistent with previous reports (Fig. 5 A). Importantly, ES still decreased  $\beta$ -catenin levels and TOP-FLASH activity in cells transfected with Siah1-dR (Fig. 5 A), demonstrating that ES-mediated degradation of  $\beta$ -catenin does not depend on Siah1. Nevertheless, MG132, a proteasome inhibitor (Jensen et al., 1995), significantly decreased ES activity and up-regulated  $\beta$ -catenin levels (Fig. 5 B), indicating that ubiquitin-dependent proteolysis is involved.



**Figure 6. Activated TCF (TVP) reverses the inhibitory effects of ES on endothelial cell migration and cell cycle.** For migration assays and cell cycle analysis, TVP-infected HUVECs (TVP-HUVEC) or empty virus-infected HUVECs were used. TVP-HUVECs expressed Myc-TVP as detected by anti-Myc antibodies (A) and had elevated TOP-FLASH reporter activity (B). (C) Migration assays were performed in Boyden chambers with VEGF (20 ng/ml) or bFGF (20 ng/ml) as stimulants. ES (1  $\mu$ g/ml) had no effect on TVP-HUVECs. (D) FACS<sup>®</sup> analysis of the cell cycle stage was performed with VEGF (20 ng/ml) or bFGF (20 ng/ml) as stimulants in the presence or absence of ES (20  $\mu$ g/ml). ES reduced the number of cells entering the S phase in control HUVECs but not in TVP-HUVECs.

### ES effects on $\beta$ -catenin require glypican 1

Previously, we showed that glypicans function as low affinity receptors for ES (Karumanchi et al., 2001). To test whether glypicans are involved in the effect of ES on  $\beta$ -catenin signaling (Fig. 5 C), we used the antisense approach. In HUVECs stably infected with a glypican 1 antisense virus, surface expression of glypican 1 is reduced by  $\sim$ 80% (Karumanchi et al., 2001). ES inhibited TOP-FLASH activation in control HUVECs but not in these HUVECs carrying the antisense retrovirus. These data point to the importance of glypican 1 in ES signaling.

### ES inhibits endothelial cell migration and entry into the S phase of the cell cycle by targeting LEF/TCF sites

The antiangiogenic activity of ES has been connected with its ability to modulate endothelial cell migration and cause  $G_1$  arrest of the cell cycle (Dhanabal et al., 1999c). Whereas our findings show that ES can decrease  $\beta$ -catenin stability and inhibit Wnt-dependent transcriptional targets, it is unclear whether the effects of ES on endothelial cell migration or the cell cycle are due to the modulation of the Wnt pathway. Since TVP, a constitutive active transcriptional activator of TCF-dependent transcription, acts in a  $\beta$ -catenin-indepen-

dent manner and ES down-regulates  $\beta$ -catenin, we asked whether this reagent can rescue the inhibitory effects of ES on endothelial cell migration and cell cycle progression.

HUVECs infected with a TVP-containing retrovirus (TVP-HUVEC) express TVP in cell lysates (Fig. 6 A) and reveal elevated TOP-FLASH reporter activity (Fig. 6 B). We subsequently studied the effects of ES and TVP on endothelial cell migration in the Boyden chamber assay in response to the angiogenic factors VEGF and basic FGF (bFGF) (Yamaguchi et al., 1999; Boilly et al., 2000). Interestingly, in TVP-HUVECs, bFGF but not VEGF augmented the migratory response compared with control vector-infected HUVECs, suggesting that the effects of the two angiogenic factors involve different signaling pathways. ES inhibited both bFGF- and VEGF-induced migration of control HUVECs but had no effect on TVP-HUVECs (Fig. 6 C). This observation shows that TVP overcomes the block in cell migration imposed by ES.

To evaluate whether TVP can reconstitute cell cycle progression blocked by ES, we performed cell cycle analysis using control HUVECs and TVP-HUVECs treated with the combination of VEGF, bFGF, and ES. ES inhibited S phase entry of control HUVECs stimulated by VEGF or bFGF

(Fig. 6 D). However, no inhibitory effect of ES was detected in TVP-HUVECs. Together, these results suggest that ES targets transcription through LEF/TCF binding sites.

## Discussion

Antiangiogenic properties of ES have been attributed to a block in endothelial cell migration and cell cycle arrest (Dhanabal et al., 1999a; Shichiri and Hirata, 2001), yet underlying signal transduction pathways remain elusive. Using both *Xenopus* embryos and mammalian cultured cells, this study demonstrates that ES triggers GSK3-independent degradation of  $\beta$ -catenin, a key mediator of Wnt signaling. Both activities of ES, the block of  $\beta$ -catenin function and inhibition of endothelial cell migration and S phase entry, can be rescued by the downstream transcriptional activator TVP, which acts independently of  $\beta$ -catenin. These findings suggest that one effect of ES arises from its ability to down-regulate Wnt signaling. Additionally, our results implicate Wnt/ $\beta$ -catenin signaling in the regulation of endothelial cell migration and cell cycle progression.

The major limitation of our gain of function experiments is that they employ fairly high concentrations of ES and that only modest effects on  $\beta$ -catenin are observed. However, our experiments are controlled by ES mutants that have no effect at comparable doses. Therefore, the findings may be most relevant to antiangiogenic effects seen in vivo, when pharmacological doses of ES are administered. Moreover, it is possible that ES may interfere with extracellular matrix assembly and thereby elicit antiangiogenic effects through this mechanism. The recent description of collagen XVIII expression in the early frog embryo (Elamaa et al., 2002) is also consistent with a biological role for ES in normal development.

### $\beta$ -catenin as a target of ES

To investigate the signaling events triggered by ES, we took advantage of *Xenopus* embryogenesis, which involves a cascade of inductive events with only a small number of signal transduction pathways (Harland and Gerhart, 1997). Microinjection of ES RNA into animal blastomeres of early embryos resulted in specific developmental abnormalities: ectopic cement gland formation and suppression of eye development. These phenotypes are characteristic of embryos in which Wnt signaling has been inhibited by overexpression of GSK3 or partial depletion of  $\beta$ -catenin (Itoh et al., 1995; Heasman et al., 2000). The hypothesis that ES functions by impinging on Wnt signaling has been supported using several experimental approaches in both frog embryos and human cells. The doses of injected ES RNA (2 ng) are relatively high compared with those typically used for other signaling proteins in the *Xenopus* embryos. It is possible that the requirement for high levels of ES arises from the fact that mammalian ES is labile in frog cells or that binding of human ES to its frog receptor is suboptimal.

Several inhibitors of  $\beta$ -catenin signaling are known to inhibit dorsal development; however, animal ventral injections of RNA (Fig. 1) do not allow one to assess dorsal development. Therefore, we tested the effect of ES on embryos injected into dorsal vegetal blastomeres that are known to contribute to dorsal development. ES had very little effect on

endogenous axis formation even when injected into dorso-vegetal blastomeres (data not shown). Thus, ES appears to affect only the secondary but not the primary body axis. We note that overexpression of GSK3, a well-characterized inhibitor of  $\beta$ -catenin, also has only mild effects on endogenous axis development (He et al., 1995; Pierce and Kimelman, 1995; unpublished data). Although the reasons for this observation are currently unclear, the lack of the effect may be due to targeting a subcellular pool of  $\beta$ -catenin that is not directly involved in axis specification (Guger and Gumbiner, 2000). ES would also fail to cause an effect in dorsovegetal cells if they lack the ES receptor or contain an endogenous inhibitor. Alternatively, overexpressed ES may function at a later stage than needed for severe axis disruption or may have a partial effect on  $\beta$ -catenin.

### ES activates a novel molecular pathway leading to $\beta$ -catenin degradation

To define at which level ES impinges on Wnt signal transduction, we have shown that ES suppressed axis-inducing activities of Dsh and  $\beta$ -catenin, yet failed to block the activity of TVP, a downstream component of the pathway. These results, obtained in *Xenopus* embryos and extended to mammalian endothelial cells, suggest that ES modulates the Wnt pathway at the level of  $\beta$ -catenin. Consistent with this hypothesis,  $\beta$ -catenin levels were diminished in the presence of ES. Interestingly, stabilized  $\beta$ -catenin, which is insensitive to GSK3-mediated phosphorylation and degradation, was also affected by ES, indicating that the underlying pathway is GSK3 independent. Also, ES suppressed  $\beta$ -catenin signaling in the DLD-1 carcinoma cell line with constitutively high  $\beta$ -catenin signaling (Morin et al., 1997). This observation is consistent with our conclusion that ES acts via a novel pathway and raises an intriguing possibility that ES may have direct antitumor effects.

Among mechanisms regulating  $\beta$ -catenin are stabilization by the upstream components of the Wnt pathway Dsh, axin, and GSK3 (Gumbiner, 1997; Salic et al., 2000), or association of  $\beta$ -catenin and TCF (Lee et al., 2001), and p53-dependent destruction by Siah1, a mammalian homologue of Sina (Matsuzawa and Reed, 2001). Our data suggest that ES targets  $\beta$ -catenin for proteasome-mediated degradation using pathways independent of  $\beta$ TrCP and Siah1. We also show that the ES ability to inhibit Wnt signaling depends on glypican 1, since cells infected with a glypican 1 antisense virus fail to down-regulate TCF reporter activity in response to ES (Fig. 5 C). This result is supported by our recent study showing that glypicans function as low affinity ES receptors (Karumanchi et al., 2001). At present, it remains unclear whether the requirement of glypicans for ES binding and function is related to the involvement of glypicans in Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001).

Our data argue that a function of ES is to inhibit Wnt signaling. Interestingly, a list of genes rapidly down-regulated by ES (Shichiri and Hirata, 2001) includes *c-myc*, a known direct target of Wnt signaling (He et al., 1998). Also, we have shown recently that cyclin D1, another direct target of Wnt signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999), is repressed by ES, leading to G<sub>1</sub> arrest (Hanai et al., 2002). Moreover, critical cyclin D1 promoter se-



quences responsible for ES effects have been mapped to the LEF/TCF recognition site (Hanai et al., 2002). Together, these findings support our hypothesis that ES might act by blocking Wnt/ $\beta$ -catenin signaling.

### A role for Wnt signaling in endothelial cells

The Wnt signaling pathway is involved in the control of multiple cellular processes (Cadigan and Nusse, 1997; Sokol, 1999; Peifer and Polakis, 2000). In endothelial cells, Wnt signaling has been reported to increase cell proliferation (Wright et al., 1999), whereas the secreted Frz-related protein FrzA has the opposite effect (Dennis et al., 1999; Duplaa et al., 1999). Moreover, defects of yolk sac and placental angiogenesis in mice lacking the Frz5 gene directly demonstrate the involvement of Wnt signaling in vascular development (Ishikawa et al., 2001). Our results provide further insight into a possible function of the Wnt/ $\beta$ -catenin pathway in angiogenesis. In particular, our data reveal that endothelial cell migration and cell cycle progression depend on activation of TCF-responsive target genes. Identification and characterization of these targets will be an important focus of future studies.

## Materials and methods

### Reagents and plasmid constructs

The mouse ES protein (ES) and ES mutant (ES3.1) were produced and purified from yeast as described previously (Dhanabal et al., 1999a; Karumanchi et al., 2001). ES cDNA with K-cadherin signal peptide sequence in the NH<sub>2</sub> terminus was amplified by PCR and cloned into pCS2+ (Turner and Weintraub, 1994). An NH<sub>2</sub>-terminal deletion of ES (ES-m) was generated by removing aa 22–84 using the primer 5'-AACACCCCCTGTCTGGATC-CCTGTTTTCTGGCTC-3' as described (Makarova et al., 2000). TOP/FOP-FLASH (a gift of H. Clevers, University Medical Center Utrecht, Utrecht, Netherlands) (van de Wetering et al., 1991, 1996) and pSia-Luc (Fan et al., 1998) luciferase reporters and expression plasmids for Myc- and Flag-tagged  $\beta$ -catenin (Yost et al., 1996; Liu et al., 1999), pCS2-Xdsh (Sokol, 1996), and pCS2-TVP (Vonica et al., 2000) have been described previously. The TVP sequence was subcloned from pCS2-TVP into EcoRI and NotI-digested retroviral vector pLXSHD<sup>2</sup> (a gift from D. Miller and A. Kazlauskas, Harvard Medical School, Boston, MA) (Karumanchi et al., 2001), confirmed by sequencing, and referred to as the TVP retroviral vector. Two different stabilized constructs of  $\beta$ -catenin were used in this study, the NH<sub>2</sub>-terminal deletion construct  $\Delta$ N- $\beta$ -catenin (Yost et al., 1996) and the S>A  $\beta$ -catenin mutant (Liu et al., 1999). Flag-Siah1 and Flag-Siah1-dR (gifts of E. Fearon, University of Michigan Medical School, Ann Arbor, MI) have been described previously (Hu and Fearon, 1999). Flag- $\beta$ TrCP and Flag- $\Delta$ N $\beta$ TrCP (gifts of N. Matsunami, University of Utah, Salt Lake City, UT) have also been described (Liu et al., 2001).

### Cell culture and DNA transfection

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics. CPAE cells and DLD-1 cells were obtained from American Type Culture Collection. HUVECs and CPAE cells were used between passages 2 and 3. HUVECs were maintained in EGM2-MV medium (Clonetics) containing endothelial basal medium (EBM-2), supplemented with 5% FBS, gentamicin, amphotericin B, hydrocortisone, ascorbic acid, and the following growth factors: VEGF, bFGF, hEGF, and IGF-1. CPAE cells were maintained in DME supplemented with 10% FCS and penicillin/streptomycin (P/S). DLD-1 cells were maintained in RPMI 1640 supplemented with 10% FCS and P/S. All cell lines were grown at 37°C in a 100% humidified incubator with 5% CO<sub>2</sub>. Cells were grown to 80–90% confluency, harvested with trypsin, and resuspended to the cell density required for each assay. For transient transfections, 60–70% confluent cells in 6-well plates were transfected using Lipofectamine 2000 (Life Technologies) for HUVECs and CPAE cells and Lipofectamine Plus (Life Technologies) for DLD-1 cells. Total amount of transfected DNA in each experimental group was adjusted to the same value by adding vector DNA. MG132 was purchased from Sigma-Aldrich.

### Retrovirus production

Retrovirus production was performed as described before (Ory et al., 1996). Briefly, 20  $\mu$ g of retroviral plasmids were transfected into the 293 GPG packing cell line using the Calphos transfection kit (CLONTECH Laboratories, Inc.). 48 h after transfection, the packing cell line supernatant was collected and used to infect target cells (HUVECs) in complete medium (EGM-2MV).

### Xenopus eggs and embryos

Eggs were obtained from *Xenopus* females injected with 600 U of human chorionic gonadotropin, fertilized in vitro, and cultured in 0.1 $\times$  Marc's modified Ringer's medium as described previously (Newport and Kirschner, 1982). Embryonic stages were determined according to Nieuwkoop and Faber (1967).

### RNA microinjections

Capped synthetic RNAs were generated with SP6 or T7 RNA (Krieg and Melton, 1984) by in vitro transcription of plasmids using mMessage mMachine kits (Ambion). RNA microinjections were performed as described (Itoh et al., 1995).

### Luciferase assay

After transient transfection of the plasmids, cells were incubated for 20 h in 10% FCS (for the TOP-FLASH and FOP-FLASH promoters), and luciferase activity in the cell lysates was determined using a luminometer normalized using sea-pansy luciferase activity under the control of the thymidine kinase promoter. The Dual-Luciferase Reporter Assay System was purchased from Promega. For luciferase assays of embryo cell lysate, embryos were injected in the animal pole with 30  $\mu$ g of the *Siamois*-luciferase reporter (pSia-Luc) DNA (Fan et al., 1998) and mRNAs for ES and ES mutant, and the luciferase activity was measured in light units as described (Fan and Sokol, 1997).

### Immunoprecipitation and immunoblotting

Collected cell lysates or cell lysates immunoprecipitated as described (Kawabata et al., 1998) were separated by PAGE (precast gels; Bio-Rad Laboratories). This was followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking with 2% BSA in Tris buffered saline/Tween-20 (TBS-T) for 1 h, the polyvinylidene difluoride membrane was incubated overnight with each primary antibody. After washing with TBS-T, the membrane was incubated with the secondary anti-mouse Ig at a 1:5,000 dilution for 0.5 h. Protein bands were detected using SuperSignal<sup>®</sup> West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Primary antiserum against ES was used as described (Dhanabal et al., 1999a). Injected embryos were lysed in 500  $\mu$ l of lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>) when sibling embryos had developed to late blastula stages (stage 8+). Western blot analysis was performed as described (Itoh et al., 1998).

### Migration assays

Migration assays were performed using 12-well Boyden chemotaxis chambers (Neuro Probe, Inc.) with a polycarbonate membrane (25  $\times$  80-mm, PVD free, 8  $\mu$ m pore size; Osmonics) as described previously (Dhanabal et al., 1999a). Briefly, polycarbonate membrane coated with Vitrogen 100 (Collagen Biomaterials, Inc.) collagen solution (0.3 mg/ml) on both sides was placed over a 0.1% gelatin-coated bottom chamber. The lower chamber was filled with DME containing 0.1% BSA and 20 ng/ml bFGF or 20 ng/ml VEGF (R & D Systems) or nothing. The upper chamber was seeded with 60,000 cells/well with different concentrations of recombinant ES in triplicate. Endothelial cells were labeled with Dil and allowed to migrate for 5 h at 37°C as described (Dhanabal et al., 1999a).

### Cell cycle analysis

HUVECs were growth arrested by contact inhibition for 48 h. The 0 h value refers to the percentage of cells in S phase at this time point. Cells (0.1  $\times$  10<sup>6</sup> cells/well) were harvested and plated into a 6-well plate in 1% FCS/EGM2-MV with recombinant VEGF or bFGF with or without ES. The cells were harvested at various time points and then fixed in ice-cold ethanol. Fixed cells were dehydrated at 4°C for 0.5 h in PBS containing 2% FCS and 0.1% Tween-20 and then centrifuged and resuspended in 0.5 ml of the same buffer. RNase digestion (5  $\mu$ g/ml) was performed at 37°C for 1 h followed by staining with propidium iodide (5  $\mu$ g/ml). The cells were analyzed using a FACScan Becton Dickinson flow cytometer.

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