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ORIGINAL ARTICLE

Critical role of endoglin in tumor cell plasticity of Ewing sarcoma and melanoma

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Tumor cell plasticity enables certain types of highly malignant tumor cells to dedifferentiate and engage a plastic multipotent embryonic-like phenotype, which enables them to 'adapt' during tumor progression and escape conventional therapeutic strategies. This plastic phenotype of aggressive cancer cells enables them to express endothelial cell-specific markers and form tube-like structures, a phenotype that has been linked to aggressive behavior and poor prognosis. We demonstrate here that the transforming growth factor (TGF)-β co-receptor endoglin, an endothelial cell marker, is expressed by tumor cells and its expression correlates with tumor cell plasticity in two types of human cancer, Ewing sarcoma and melanoma. Moreover, endoglin expression was significantly associated with worse survival of Ewing sarcoma patients. Endoglin knockdown in tumor cells interferes with tumor cell plasticity and reduces invasiveness and anchorage-independent growth *in vitro*. Ewing sarcoma and melanoma cells with reduced endoglin levels showed reduced tumor growth *in vivo*. Mechanistically, we provide evidence that endoglin, while interfering with TGF-β signaling, is required for efficient bone morphogenetic protein, integrin, focal adhesion kinase and phosphoinositide-3-kinase signaling in order to maintain tumor cell plasticity. The present study delineates an important role of endoglin in tumor cell plasticity and progression of aggressive tumors.
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Keywords: endoglin; tumor cell plasticity; transforming growth factor-β; bone morphogenetic proteins; Ewing sarcoma; melanoma

Introduction

Several studies have suggested that aggressive tumor cells can embrace the characteristics of stem cells and acquire a pluripotent, embryonic-like phenotype, which enables them to adapt to the microenvironment and transdifferentiate into other cell types (Hendrix *et al*., 2003, 2007; Staege *et al*., 2004). In this way high-grade tumors can mimic an endothelial-like phenotype and participate in the formation of vascular-like structures, which could contribute to tumor perfusion and eventually tumor metastasis (Maniotis *et al*., 1999). This form of tumor cell plasticity was termed vasculogenic mimicry and it has been described in several tumor types, such as melanoma, Ewing sarcoma, ovarian and breast cancer, and multiple myeloma (Maniotis *et al*., 1999; Sharma *et al*., 2002; Shirakawa *et al*., 2002; van der Schaft *et al*., 2005; Scavelli *et al*., 2008). Melanoma cells were shown to participate in neovascularization in a circulation-deficient muscle microenvironment *in vivo* (Hendrix *et al*., 2002). Additionally, it was demonstrated that EW7 Ewing sarcoma tumors growing in athymic mice can form vasculogenic-like structures that contribute to circulation (van der Schaft *et al*., 2005; Hillen *et al*., 2008). Similar channels were visualized in choroidal melanoma patients (Mueller *et al*., 1998). In this way, tumor cell growth may not only be dependent on angiogenesis for supply of oxygen and nutrients and thus escape conventional antiangiogenic therapies. Thus, characterization of the molecular mechanisms underlying tumor cell plasticity may provide new putative targets for therapeutic interventions against cancer.

Endoglin is an accessory receptor of the transforming growth factor-β (TGF-β) family of proteins, which includes TGF-βs, bone morphogenetic proteins (BMPs) and activins (ten Dijke *et al*., 2008). TGF-β family members are pleiotropic cytokines that signal through heteromeric complexes of transmembrane serine/threonine type I (also known as activin receptor-like kinases (ALKs)) and type II kinase receptors (ten Dijke and Arthur, 2007). On ligand binding, the type II receptors phosphorylate the type I receptors, which in turn phosphorylate specific receptor-regulated Smads, TGF-β induces Smad2/3 phosphorylation and BMPs Smad1/5/8

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phosphorylation. Phosphorylated receptor-regulated Smads form heteromeric complexes with the common mediator Smad4 and regulate expression of target genes (ten Dijke and Arthur, 2007). Endoglin can modulate TGF-β signaling by regulating access of ligands to signaling receptors and has an important role in angiogenesis. Endoglin knockout mice die *in utero* owing to defects in vasculogenesis, and mutations in endoglin result in the human vascular disease, hereditary hemorrhagic telangiectasia (ten Dijke *et al*., 2008). A soluble form of endoglin was shown to be expressed and to have a role in the pathogenesis of pre-eclampsia by inducing endothelial cell dysfunction. Additionally, endoglin is upregulated in tumor-associated endothelium and correlates with poor prognosis, and tumor vascularization and growth are decreased in endoglin heterozygous mice (ten Dijke *et al*., 2008).

In the present study we demonstrate that endoglin is expressed on Ewing sarcoma and melanoma tumor cells. We show that depletion of endoglin results in reduced plasticity, invasiveness and anchorage-independent growth and thereby in decreased tumor growth *in vivo*. These properties of endoglin are related to its role in the regulation of TGF-β, BMP, integrin, FAK and PI3K signaling pathways in tumor cells.

Results

Endoglin expression correlates with the plastic tumor cell phenotype of Ewing sarcoma and melanoma cells

Several studies have focused on the characterization of the key molecular determinants of tumor cell plasticity. Gene-expression profiling suggested that expression of endothelial cell markers, such as vascular endothelial-Cadherin (Hendrix *et al*., 2001) and the Ephrin receptor tyrosine kinase A2 (Hess *et al*., 2001), is upregulated in aggressive human melanoma cells compared with less aggressive melanoma cells.

Endoglin is an endothelial cell marker, highly expressed on activated endothelial cells (ten Dijke *et al*., 2008). We sought to investigate whether endoglin expression correlates with tumor cell plasticity as measured by the ability of tumor cells to form a tube-like network on matrigel (VM+). Using quantitative real-time PCR and fluorescence-activated cell sorting analysis, we found that the Ewing sarcoma cell line EW7 expressed higher levels of endoglin compared with the A673, RD-ES and SIM/EW27 cell lines, which form hardly any structures on matrigel (van der Schaft *et al*., 2005). Endoglin was also found to be highly expressed on MUM-2B and C8161 melanoma cell lines when compared with the less aggressive, isogenically matched MUM-2C and C81-61 cell lines (Figure 1a and Supplementary Figure 1a). These results suggest that endoglin expression correlates with the plastic phenotype of melanoma and Ewing sarcoma.

To determine whether endoglin is expressed in primary Ewing sarcomas and cutaneous melanoma, we examined histological sections of tissue biopsy samples from patients. We found that endoglin is expressed on tumor cells lining the blood lakes in Ewing sarcoma and in small clusters of tumor cells in primary cutaneous melanoma (Figures 1b and c). Additionally, endoglin expression was confined on endothelial cells in the tumor (Figures 1b and c). It has been suggested that the blood lakes seen in Ewing sarcoma are hypoxic vasculogenic structures (van der Schaft *et al*., 2005). Accordingly, hypoxia was shown to induce vasculogenic mimicry channel formation and tumor invasion in mouse melanoma B16 cells (Sun *et al*., 2007). To test the effect of hypoxia on endoglin expression, we analyzed the expression of endoglin in EW7 and MUM-2B cultured under hypoxic conditions. We found that hypoxia induced endoglin expression in both EW7 and MUM-2B (Supplementary Figure 1b).

To further characterize the role of endoglin in tumor progression, we performed tissue microarrays (TMAs) containing tissues of primary Ewing sarcoma. Endoglin was expressed on endothelial cells in all the tumor samples (Supplementary Figure 2). Analysis of endoglin expression revealed that 45% of the samples showed expression of endoglin on tumor cells. Evaluation of endoglin expression on tumor cells revealed that 55% of the tumors were negative (scale 0), 17% showed low endoglin expression and 27% high endoglin expression (scale 2) (Figure 1d). The intensity of endoglin staining was scored on a 0–3 scale. Intensity of staining was higher in the vessels (scale 3) than in the tumor cells (scale 0–2). The percentage of tumor cells expressing endoglin varied between the positive samples (Figure 1e). To address the significance of endoglin expression on tumor cells, we examined whether clinical outcomes correlated with endoglin expression. Kaplan–Meier survival curves revealed that endoglin expression on tumor cells significantly (log-rank 7.161, $P=0.007$) correlated with decreased survival of Ewing sarcoma patients (Figure 1f).

Endoglin has a critical role in Ewing sarcoma and melanoma tumor cell plasticity

We next sought to assess the role of endoglin in tumor cell plasticity. For this, we established EW7 and MUM-2B short hairpin RNA (shRNA) cell lines, attaining significant silencing of endoglin expression (by >90%) (Figure 2a). Knockdown of endoglin resulted in significant reduction of network formation on matrigel in EW7 and MUM-2B, by 80 and 60%, respectively; this effect could be rescued by ectopic expression of endoglin (Figures 2b–d). In addition, endoglin overexpression in control shRNA cells significantly increased network formation of EW7 and MUM-2B on matrigel. Similar results were obtained with independent EW7 and MUM-2B clones from different shRNAs (Supplementary Figures 3b–d). Likewise, endoglin downregulation prevented the formation of vasculogenic-like network when cells were grown on three-dimensional collagen matrices (Figure 2e). However, ectopic expression of endoglin in the poorly aggressive melanoma cell line MUM-2C failed to induce formation of networks on

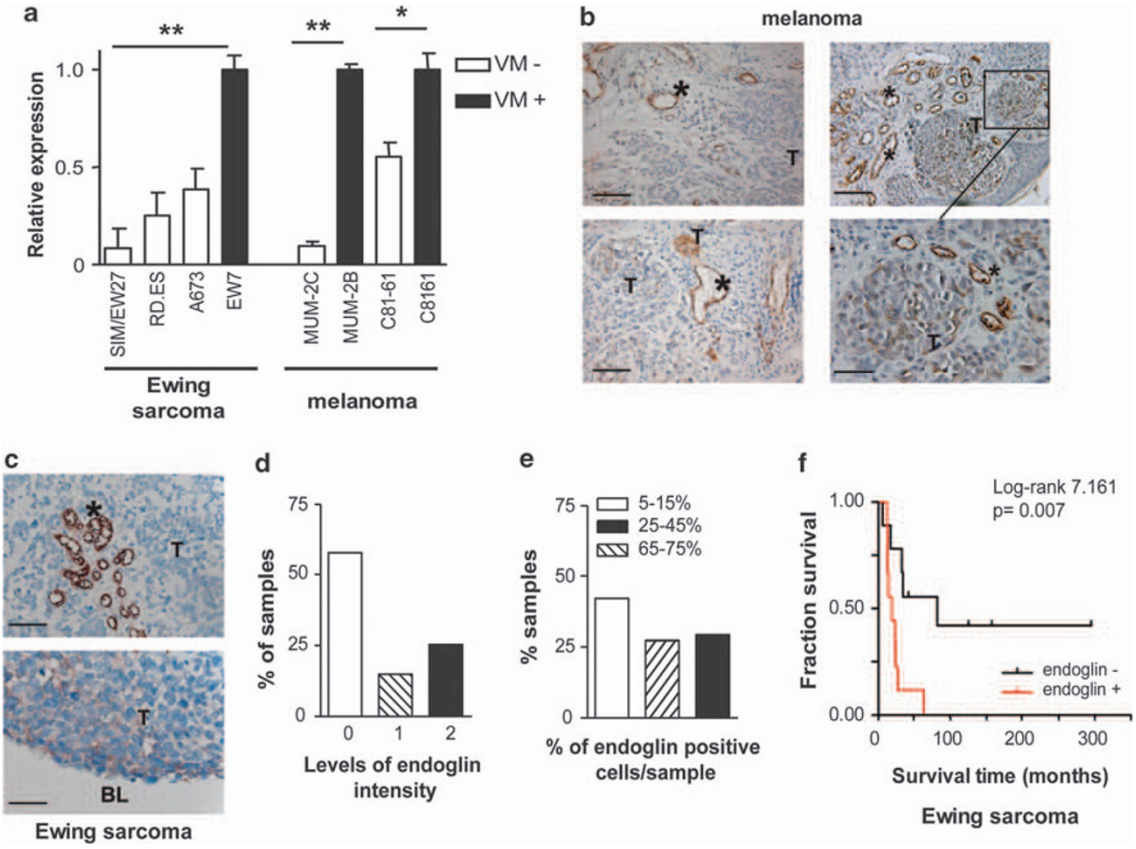

Figure 1 Endoglin is expressed in Ewing sarcoma and melanoma cell lines and lesions. (**a**) Endoglin expression in Ewing sarcoma and melanoma cell lines correlates with their plastic phenotype. Endoglin mRNA levels measured by quantitative real-time RT–PCR in the highly aggressive, vasculogenic mimicry positive (VM +) Ewing sarcoma (EW7) and melanoma (MUM-2B and C8161) compared with the less aggressive VM negative (VM-), Ewing sarcoma (A673, RD-ES and SIM/EW27) and melanoma (MUM-2C and C81-61) cell lines ($n = 3$). (**b**) Immunohistochemical analysis of endoglin expression on primary cutaneous melanoma lesions shows expression of endoglin on blood vessels and on the cell membrane of tumor cells. The images on the left panel represent areas of the same tumor. The images on the right panel represent different magnification of the same tumor. (**c**) Immunohistochemical analysis of endoglin expression on Ewing sarcoma lesions shows expression of endoglin on blood vessels and on the tumor cells around the blood lakes (the images represent different areas of the same tumor). (**d**–**f**) Histochemical analysis of endoglin expression on an Ewing sarcomaTMA. (**d**) Analysis of endoglin expression levels on tumor cells of Ewing sarcoma (TMA) (0, negative; 1, low expression; 2, high expression). (**e**) Percentage of endoglin-expressing tumor cells/sample varies between the endoglin-positive Ewing sarcoma samples (TMA). (**f**) Kaplan–Meier survival curve showing that Ewing sarcoma patients with endoglin expression on tumor cells (endoglin +) had significant worse survival than patients with no endoglin expression on tumor cells (endoglin −) (Log-rank 7.161, $P = 0.007$). *, blood vessel; BL, blood lake; T, tumor (*$P \leqslant 0.05$, **$P \leqslant 0.01$).

matrigel or collagen (data not shown), suggesting that endoglin is required, but not sufficient, for inducing their pluripotent phenotype.

Recent studies have suggested that soluble endoglin can interfere with endoglin function on endothelial cells (Venkatesha *et al*., 2006). Therefore, we examined the effect of soluble endoglin on the ability of Ewing sarcoma and melanoma to form networks. Treatment of EW7 and MUM-2B with endoglin-Fc significantly reduced tube network formation by 80 and 40%, respectively (Figure 2f), whereas a control Fc protein had no effect on network formation. Similar results were obtained with the cells infected with adenovirus expressing soluble endoglin (Venkatesha *et al*., 2006) (data not shown). Interestingly, soluble Flt1 (VEGF receptor I) had no effect on network formation (data not shown). Together, gain- and loss-of-function studies suggest that endoglin expression and function is essential for tumor cell plasticity in Ewing sarcoma and melanoma.

Interplay between endoglin, TGF-β and BMP signaling pathways is crucial for Ewing sarcoma and melanoma tumor cell plasticity

Endoglin has been reported to have an inhibitory effect on the TGF-β/ALK5 pathway and to potentiate the BMP pathway (ten Dijke *et al*., 2008). To characterize the expression of TGF-β receptors, we performed affinity-labeling experiments. We found that TGF-β strongly binds to TβRII and ALK5 on EW7, MUM-2B and MUM-2C cell lines (Supplementary Figure 4). Interestingly, although TGF-β treatment had no effect on network formation (data not shown), overexpression of a constitutively active form of TβRI/ALK5 (caALK5) inhibited tube formation by 40 or 50% in MUM-2B and EW7, and this effect could be rescued by addition of the ALK4/5/7 kinase inhibitor SB-431542 (Figure 3a). These results suggest that EW7 and MUM-2B cells have lost their responsiveness to TGF-β owing to increased endoglin expression. We therefore hypothesized

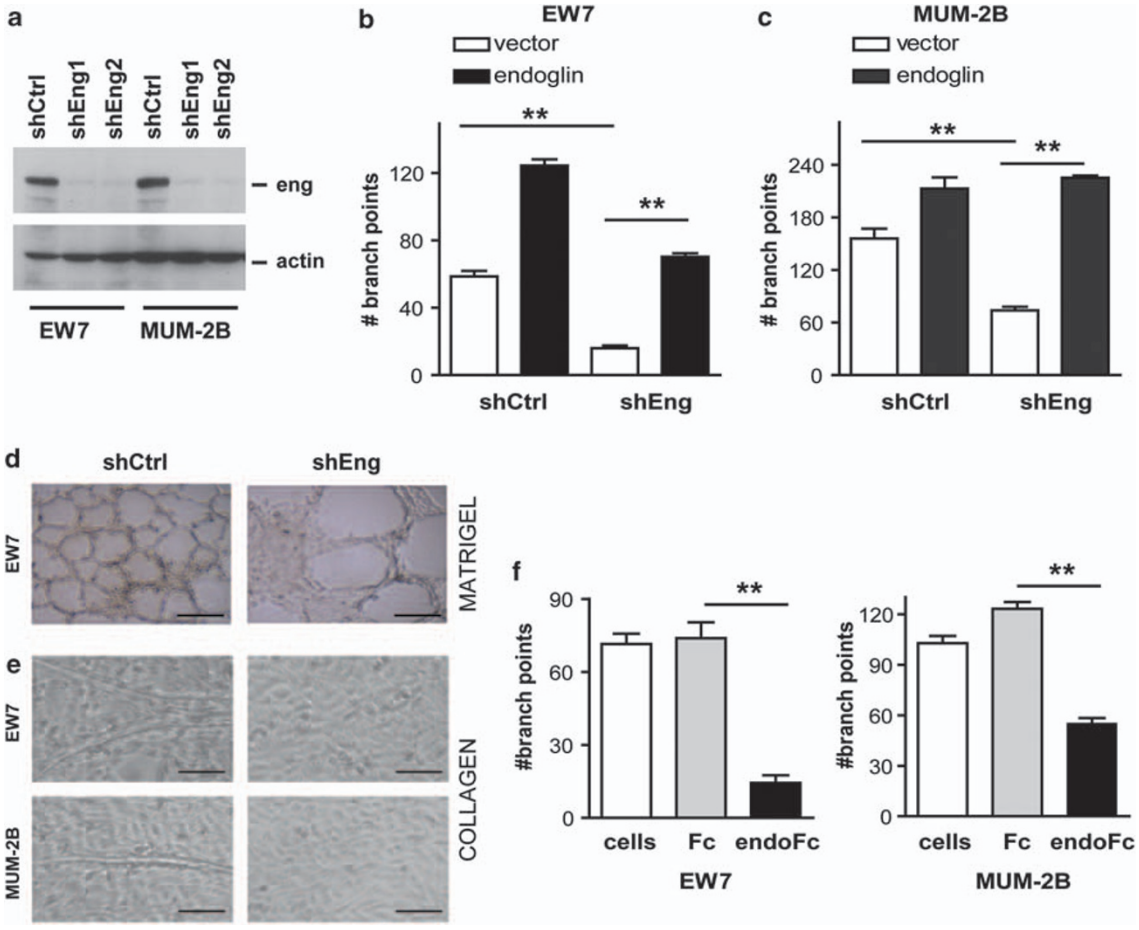

Figure 2 Endoglin downregulation interferes with tumor cell plasticity. (**a**) Protein analysis of the effect of shRNA-mediated silencing of endoglin in EW7 and MUM-2B cells. (**b**, **c**) Control shRNA (shCtrl) or endoglin shRNA (shEng1) EW7 (**b**) and MUM-2B (**c**) stable clones infected with empty vector (white bars) or endoglin (black bars) overexpressing lentivirus were plated on matrigel to analyze their ability for network formation. Data represent the number (mean ± s.d.) of junctions formed during the assay ($n = 3$). (**d**) Bright-field images of shCtrl or shEng1 EW7 cultured on 2-D matrigel. (**e**) Bright-field images of shCtrl or shEng1 EW7 and MUM-2B cells cultured on 3-D collagen matrices. (**f**) EW7 and MUM-2B cells were treated with Fc or endoglin-Fc (Endo-Fc) protein for 24 h. Cells were harvested and seeded on matrigel with Fc or endoglin-Fc to analyze network formation. The number (mean ± s.d.) of junctions formed during the assay was counted ($n = 3$) (**$P \leqslant 0.01$).

that increased TGF-β/ALK5 signaling, due to endoglin downregulation, resulted in reduced tube formation. Endoglin downregulation resulted in increased TGF-β-induced Smad2 and Smad3 phosphorylation in EW7 (Figure 3b) and increased TGF-β/Smad3-induced transcriptional activity in EW7 (Figure 3c) and MUM-2B (Supplementary Figures 5a and b). Moreover, expression of the TGF-β/ALK5 target gene *PAI-1* was significantly increased in the endoglin knockdown EW7 cells (Figure 3d). Next we investigated whether inhibition of the enhanced TGF-β/ALK5 pathway could reverse the inhibitory effect of endoglin downregulation on network formation. However, inhibition of TGF-β signaling with the ALK4/5/7 kinase inhibitor did not rescue the effect of endoglin downregulation on vasculogenic network formation (Figure 3e). These results suggest that the defects seen in the endoglin knockdown cells not only result from enhanced TGF-β signaling and imply an additional, not only TGF-β-dependent, function of endoglin in network formation and tumor cell plasticity.

Addition of the BMP2/4/7 inhibitor noggin resulted in 50 and 20% reduction in tube formation in EW7 and MUM-2B, respectively (Figure 4a). It has been described that endoglin promotes BMP signaling, raising the possibility that the effects seen in the endoglin knockdown cells are due to attenuated BMP signaling. Indeed, endoglin downregulation resulted in reduced BMP-induced Smad1 phosphorylation (Figure 4b) and BMP transcriptional responses in EW7 and MUM-2B (Figure 4c and Supplementary Figure 5c). In addition, noggin did not affect network formation in EW7 endoglin knockdown cells, suggesting a deregulated endogenous BMP pathway (Figure 4d). As noggin is a natural antagonist of BMP2/4/7, we sought to investigate which of these BMPs are expressed in EW7 and MUM-2B cells. Quantitative real-time PCR analysis showed that both BMP2 and BMP4 are expressed in EW7 and MUM-2B cells, although BMP6 and BMP7 are not expressed at detectable levels (Figure 4e and Supplementary Figure 5d). These data suggest an important stimulatory role of endoglin in promoting

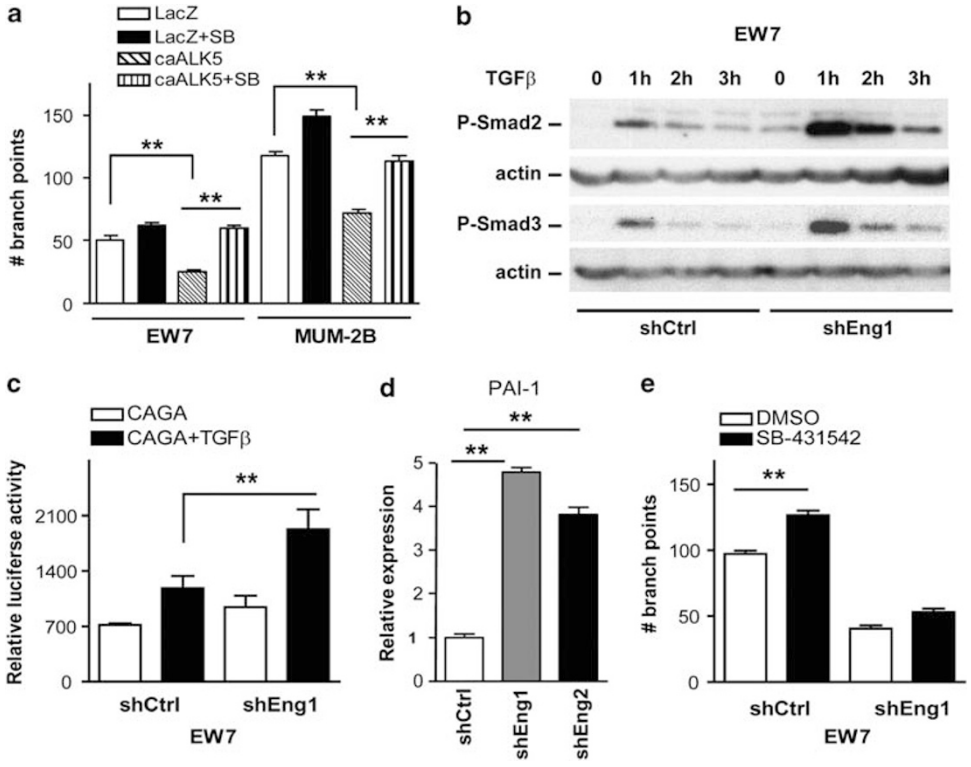

Figure 3 TGF-β signaling inhibits tumor cell plasticity of Ewing sarcoma and melanoma. (**a**) EW7 and MUM-2B cells infected with adenoviruses expressing caALK5 or LacZ were plated on matrigel and allowed to form networks in the presence or absence of the ALK4/5/7 inhibitor SB-431 542. The number (mean ± s.d.) of junctions formed is shown ($n=3$). (**b**) shCtrl and shEng1 EW7 cells were exposed to TGF-β3 (5 ng/ml) for the indicated time periods. Phosphorylation of Smad2 and Smad3 was determined by western blotting. Actin served as a loading control. (**c**) TGF-β-induced transcription was determined by transient transfection of (CAGA) 12-luciferase in shCtrl and shEng1 EW7 cells. Cells were stimulated without (white bars) or with TGF-β3 (5 ng/ml) (black bars) for 16 h and relative luciferase activity was measured ($n=3$). (**d**) Quantitative real-time PCR analysis of PAI-1 in shCtrl or shEng1 and shEng2 EW7 cells. Data are presented as the relative gene expression compared with ARP as a mean of three measurements ± s.d ($n=3$). (**e**) shCtrl and shEng1 cells were cultured for 48 h in the presence or absence of SB-431 542 (10 μM). Cells were plated on matrigel in the presence or absence of SB-431 542 to analyze network formation. The number (mean ± s.d.) of junctions is shown ($n=3$) (**$P\leqslant 0.01$).

tumor cell plasticity by facilitating more effective BMP signaling.

Endoglin downregulation interferes with tumor cell plasticity by interfering with FAK, PI3K and integrin pathways

To further explore the molecular mechanisms by which endoglin regulates tumor cell plasticity, we surveyed the potential links between endoglin and signaling pathways of known relevance to tumor cell plasticity. Focal adhesion kinase (FAK) was shown to have an important role in tumor cell plasticity of melanoma cells (Hess *et al*., 2005). Therefore, we assessed the effect of endoglin downregulation on the FAK pathway. We found that endoglin downregulation resulted in reduced FAK Y397-phosphorylation in both EW7 and MUM-2B (Figure 5a). Moreover, phospho-paxillin stainings revealed that organization of focal adhesions was altered in the EW7 endoglin shRNA compared with control shRNA (Supplementary Figure 6). In addition, endoglin shRNA cells displayed increased actin stress fibers as shown by phalloidin staining (Supplementary Figure 6). These results suggest that endoglin knockdown interferes with the FAK pathway in Ewing sarcoma and melanoma.

The phosphoinositide-3-kinase (PI3K) pathway was previously shown to have an important role in tumor cell plasticity of melanomas (Hess *et al*., 2003). Endoglin downregulation in the EW7 cells resulted in decreased basal PI3K activity, which was shown by performing the lipid PI3K kinase assay (Figure 5b). Treatment of control shRNA cells with the PI3K inhibitor LY294002 resulted in inhibition of tube formation; however, the PI3K inhibitor had no effects on tube formation of endoglin knockdown cells (Figure 5c), suggesting that endoglin downregulation results in attenuated PI3K signaling.

Previous studies have suggested that increased serum osteopontin levels, one of the ligands for integrin αvβ3, correlates with melanoma metastasis and that integrin signaling has an important role in tumor progression (Dome *et al*., 2005; Zhou *et al*., 2005; Kadkol *et al*., 2006). Quantitative PCR analysis revealed that expression of integrin β3 and osteopontin was downregulated in the endoglin shRNA cells (Figures 5d and e). Moreover, overexpression of integrin β3 could rescue the effect of endoglin knockdown on tube formation of EW7 cells (Figure 5f). Collectively, these data suggest

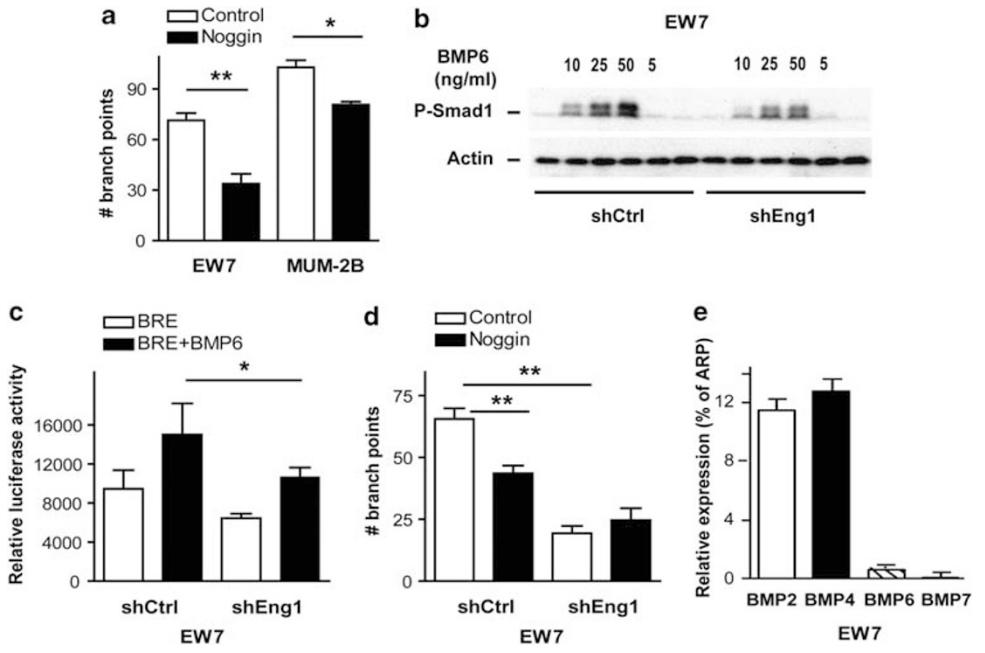

Figure 4 Endoglin and BMP signaling have an important role in tumor cell plasticity of Ewing sarcoma and melanoma. (**a**) EW7 and MUM-2B were plated on matrigel and allowed to form networks in the absence (white bars) or presence (black bars) of the BMP inhibitor noggin (300 ng/ml). The number (mean ± s.d.) of junctions formed during the assay is shown ($n=3$). (**b**) shCtrl and shEng1 EW7 were exposed to indicated concentrations of BMP6 for 45 min. Phosphorylation of Smad1 was determined by western blotting. Actin served as loading control. (**c**) BMP-induced transcription was determined by transient transfection of BRE-luciferase in shCtrl or shEng1 EW7 cells. Cells were starved and then incubated without (white bars) or with BMP6 (50 ng/ml) (black bars) for 16 h and relative luciferase activity was measured. (**d**) shCtrl and shEng1 cells were plated on matrigel in the absence (white bars) or presence (black bars) of noggin and the number (mean ± s.d.) of junctions was quantified ($n=3$). (**e**) Quantitative real-time PCR analysis of BMP2, 4, 6 and 7 in EW7 cells. Data are presented as the % of relative gene expression compared with ARP ± s.d ($n=3$) (*$P\leqslant 0.05$, **$P\leqslant 0.01$).

that endoglin downregulation, by interfering with the expression of integrin β3, affects tumor cell plasticity.

As endoglin downregulation results in reduced BMP signaling, we wondered whether decreased integrin β3 and osteopontin expression is due to decreased BMP signaling. Therefore, we analyzed the effect of BMP and noggin treatment on integrin β3 and osteopontin expression. BMP treatment resulted in small but significant increase in integrin β3 and osteopontin mRNA expression in EW7 and MUM-2B (Supplementary Figures 7a and c), but not in endoglin knockdown EW7 cells (Supplementary Figures 7b and d). However, noggin had no effects on integrin β3 and osteopontin expression. These results suggest that endoglin may also regulate expression of integrin β3 and osteopontin via other signaling pathways besides BMP signaling.

Endoglin downregulation attenuates the aggressive phenotype of Ewing sarcoma and melanoma

As endoglin expression correlates with tumor cell plasticity, we wondered whether it associates with more aggressive behavior. To address this possibility, we examined the effect of endoglin downregulation on anchorage-independent growth and invasiveness. Endoglin knockdown resulted in decreased colony formation of EW7 in soft agar (Figure 6a). Moreover, invasiveness of EW7 endoglin shRNA cells in a three-dimensional collagen spheroid assay was reduced by 40% (Supplementary Figure 8a). These results suggest that endoglin expression is important for the aggressive phenotype of Ewing sarcoma *in vitro*.

To determine the role of endoglin on tumor progression of Ewing sarcoma and melanoma *in vivo*, we transplanted control and endoglin shRNA clones into the flank of Swiss nude mice. Endoglin knockdown resulted in a 50–70% decrease in tumor volume of both tumor types (Figures 6b and c), despite the fact that some of the clones regained endoglin expression after *in vivo* passaging (Supplementary Figures 8b and c). Immunohistochemical analysis of phosphorylated histone H3 levels in tumors revealed that endoglin downregulation significantly reduced proliferation of EW7 and MUM-2B *in vivo* (Figures 6d and e). The observed effects were not due to proliferation defects *in vitro* as there were no significant differences in their proliferative capacity (Supplementary Figures 8d and e) or the levels of phosphorylated histone H3 (Supplementary Figures 9a and b). In addition, an increased rate of necrosis was evident in the tumors from endoglin knockdown cells (Figure 6f). Decreased tumor growth and increased level of necrosis might be the result of reduced angiogenesis. However, histological staining for endothelial cell-specific markers revealed that endoglin downregulation in the tumor cells did not affect microvascular density (Supplementary Figures 9c and d). These results show that endoglin downregulation attenuates tumor growth of Ewing sarcoma and melanoma *in vivo*.

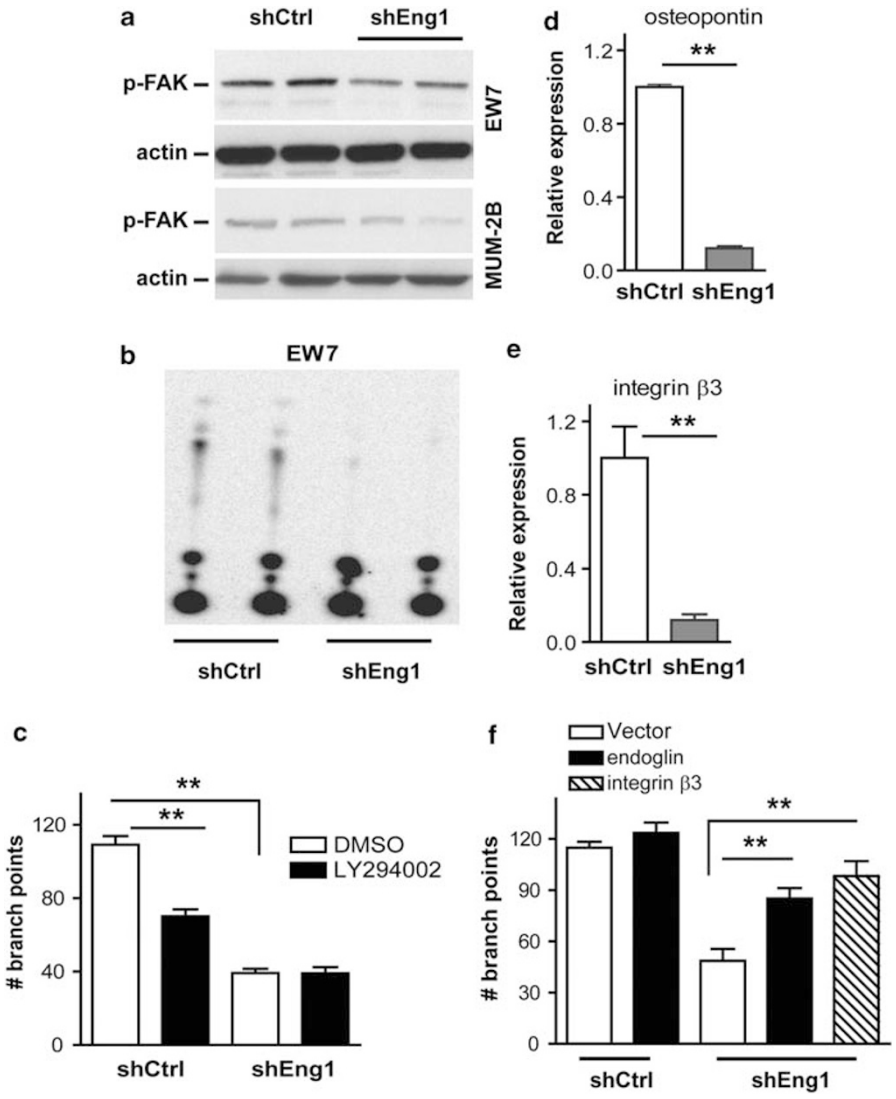

Figure 5 Endoglin knockdown interferes with tumor cell plasticity of Ewing sarcoma and melanoma by deregulating FAK, PI3K and integrin signaling. (**a**) Western blot analysis of protein lysates from shCtrl control and shEng1 endoglin knockdown EW7 and MUM-2B cells for pY397-FAK protein levels. Actin served as loading control. (**b**) shCtrl and shEng EW7 cells were harvested and lysates subjected to immunoprecipitation with phosphotyrosine antibody. *In vitro* kinase activity of PI3K was examined using L-α-phosphatidylinositol as a substrate. Lipids were extracted and resolved on oxalate-coated TLC plates. (**c**) shCtrl and shEng1 EW7 cells were plated on matrigel in the presence or absence of the PI3K inhibitor LY29400 and the number (mean ± s.d.) of junctions was quantified ($n = 3$). (**d**, **e**) Quantitative PCR analysis of osteopontin (**d**) and integrin β3 (**e**) in shCtrl and shEng1 cells. Data are presented as the relative gene expression compared with ARP as a mean of three measurements ± s.d. (**f**) shCtrl and shEng1 EW7 cells infected with control empty virus (vector), or virus overexpressing endoglin or integrin β3, plated on matrigel and the number (mean ± s.d.) of junctions was quantified ($n = 3$) (**$P \leqslant 0.01$).

Discussion

Ewing sarcoma and melanoma are aggressive tumor types with high incidence of metastasis and most importantly poor prognosis. An evolving concept in cancer is that of tumor cell plasticity. Aggressive tumor cells have the plasticity to transdifferentiate and to express genes usually expressed in various normal tissues and they are resistant to current cancer therapeutics (Hendrix *et al*., 2003, 2007). Thus, delineation of their molecular characteristics is essential for the development of new therapeutic strategies.

Experimental data have provided evidence that tumor cell plasticity of both Ewing sarcoma and melanoma enables them to embrace endothelial-like characteristics (Hendrix *et al*., 2003). Here we present data suggesting that endoglin expression correlates with the plastic phenotype of two types of aggressive tumor cells, Ewing sarcoma and melanoma. Immunohistochemical analysis revealed that endoglin is expressed on the vasculature as well as on the tumor cells of Ewing sarcoma and primary melanoma samples. Analysis of a TMA containing Ewing sarcoma tissues revealed endoglin expression on tumor cells in 45% of the samples.

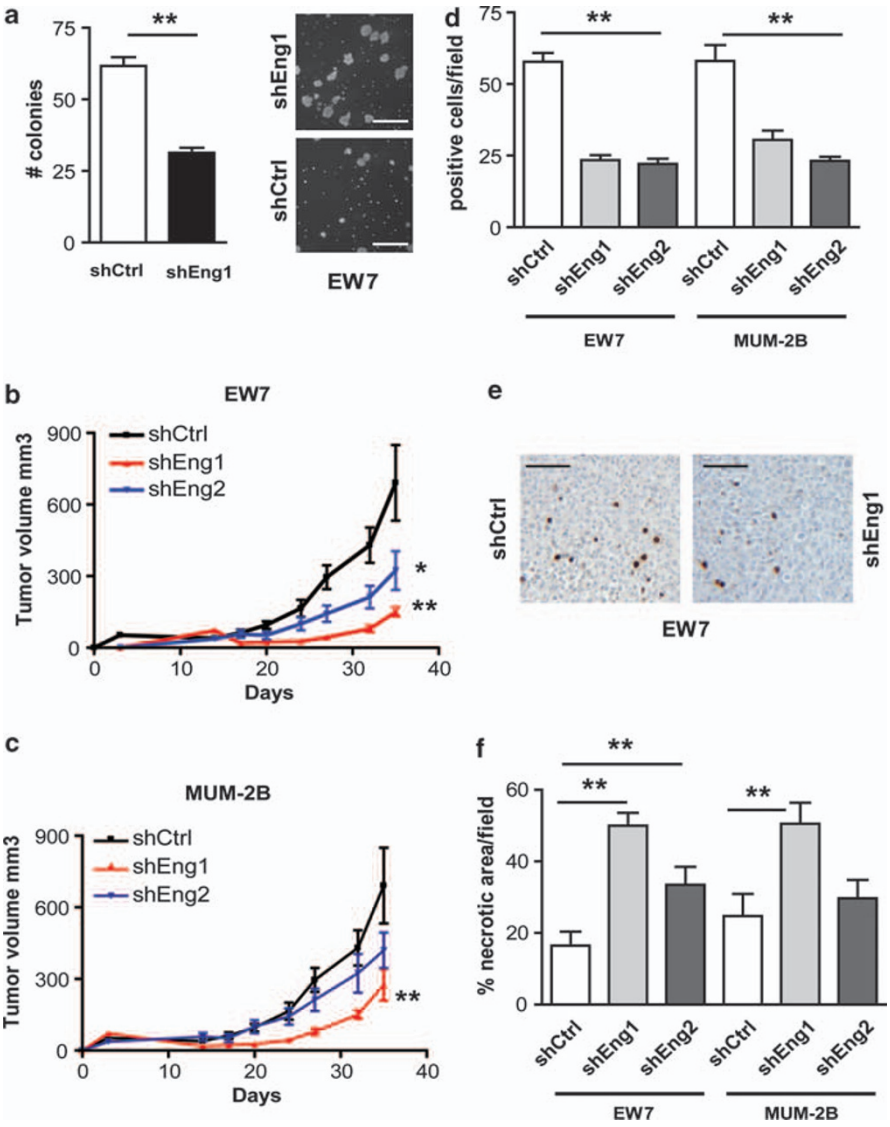

Figure 6 Endoglin downregulation abrogates Ewing sarcoma and melanoma tumorigenicity. (**a**) shCtrl control and shEng1 endoglin knockdown EW7 cells were cultured for 14 days in a soft agar assay. Colonies were stained and quantified. Phase-contrast microscopy of colonies in soft agar. (**b**, **c**) 1×10^6 of shCtrl, and two different shEng clones (different shRNAs) of Ew7 (**b**) and MUM-2B (**c**) cells, were inoculated into the flank of Swiss nude mice ($n = 8$) and tumor volumes were measured at the indicated time points (mean ± s.d.). (**d**) *In vivo* proliferation was assessed by immunohistochemical detection of phospho-histone-H3 on tumor sections obtained from the inoculated control or knockdown cell lines. Relative number (mean ± s.d.) of phospho-histone-H3 positive cells was quantified using Image J software. (**e**) Representative images of immunohistochemical analysis of phospho-histone-H3 in tumor sections from shCtrl control and shEng1 endoglin knockdown EW7 cells. (**f**) Tumors derived from the shCtrl and shEng inoculated cells were stained with H&E. Necrotic area (mean ± s.d.) was quantified using Image J software (*$P \leqslant 0.05$, **$P \leqslant 0.01$).

Endoglin expression on tumor cells was associated with decreased survival of Ewing sarcoma patients. These results suggest that endoglin may be used as a prognostic marker for Ewing sarcomas or other types of aggressive tumors. Further studies in a larger group of Ewing sarcoma patients as well as other types of aggressive tumors will validate the potential of endoglin as a prognostic marker for highly aggressive tumors and its association with poor prognosis.

Downregulation of endoglin interferes with tumor cell plasticity as endoglin knockdown cells have decreased ability to form vasculogenic-like networks on 2-D and 3-D matrices *in vitro*. *In vitro* studies have suggested that formation of tumor-lined vascular channels will probably escape conventional antiangiogenic therapies (van der Schaft *et al*., 2004). Several studies have considered endoglin as a therapeutic target in antiangiogenic therapies. Endoglin-neutralizing antibodies can target tumor vasculature and inhibit tumor growth in mouse tumor models (Seon *et al*., 1997) and soluble endoglin can interfere with endoglin function on endothelial cells and inhibit angiogenesis (Venkatesha *et al*., 2006; Hawinkels *et al*., 2010). Endoglin-Fc and soluble endoglin, but not soluble Flt1, could inhibit network formation of Ewing sarcoma and melanoma. Furthermore, endoglin knockdown resulted in reduced

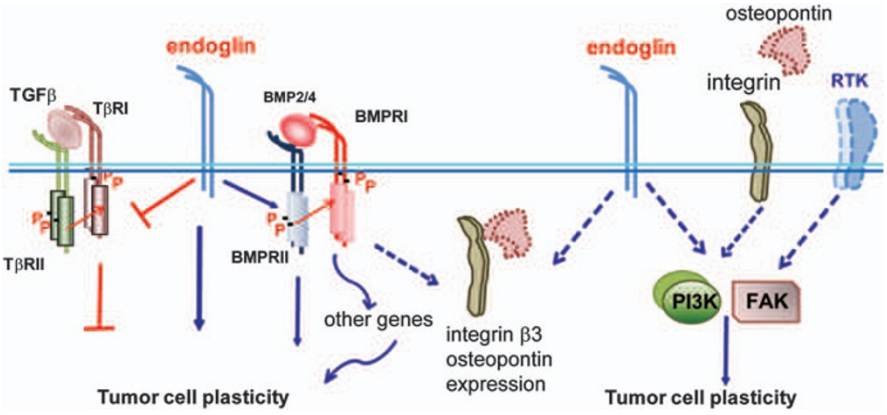

Figure 7 Schematic model outlining the role of endoglin in tumor cell plasticity. Endoglin acts in concert with several signaling molecules to maintain tumor cell plasticity. Endoglin by promoting BMP signaling and inhibiting TGF-β signaling potentiates the plastic phenotype of tumor cells. Involvement of endoglin, either directly or indirectly, in the activity of FAK and PI3K signaling and the regulation of integrin β3 and osteopontin expression further contributes to the plastic phenotype of aggressive tumor cells. BMPRI, BMP receptor I; BMPRII, BMP receptor II; RTK, receptor tyrosine kinase; TβRI, TGF-β receptor I (ALK5); TβRII, TGF-β receptor II.

invasiveness and anchorage-independent growth of EW7 cells. Finally, downregulation of endoglin expression significantly reduced proliferation and tumor growth of both tumor types *in vivo*, despite the fact that some of the clones regained endoglin expression. Additionally, tumors from endoglin knockdown cells showed large areas of necrosis. This was not due to decreased angiogenesis, suggesting that endoglin downregulation affects solely the tumor cells and their growth *in vivo*.

In several systems endoglin expression inhibits the TGF-β/ALK5 (Guo *et al*., 2004; Lebrin *et al*., 2004; Bernabeu *et al*., 2009) while promoting the BMP signaling pathway (David *et al*., 2007). TGF-β had no effect on the ability of Ewing sarcoma and melanoma lines to form networks, due to increased endoglin expression. However, overexpression of a constitutively active form of the ALK5 receptor resulted in decreased network formation, suggesting an inhibitory role of TGF-β signaling in tumor cell plasticity (Figure 7). Although endoglin downregulation resulted in enhanced TGF-β/ALK5 signaling, inhibition of the enhanced TGF-β pathway could not reverse the effects seen in the endoglin knockdown cells, suggesting the involvement of other pathways. Previous studies suggested that another member of the TGF-β superfamily, Nodal, has an important role in tumor cell plasticity and tumor growth of melanomas by activating the Smad2/3 pathway (Topczewska *et al*., 2006). However, our results demonstrate that activation of the ALK5/Smad2/3 pathway has inhibitory effects on tumor cell plasticity of aggressive Ewing sarcoma and melanoma. TGF-β signaling can be modulated not only on the level of Smad phosphorylation. For example, upregulation of inhibitors like Ski and Sno in melanoma can inhibit TGF-β signaling downstream of Smad2/3 phosphorylation (Medrano 2003). In addition, it has been shown that during mouse development, Nodal activates BMP4 signaling and together they regulate mesoderm and mesendoderm formation (Ben-Haim *et al*., 2006). Although there are no studies on the role of endoglin in Nodal signaling, it will be interesting to investigate the interplay of endoglin and Nodal signaling in tumor cell plasticity.

Our results suggest that endoglin downregulation resulted in decreased BMP signaling and that autocrine BMP signaling (presumably BMP2 or BMP4) has an important role in tumor cell plasticity of EW7 and MUM-2B cells (Figure 7). In line with our results, previous studies suggested that BMPs promote melanoma tumor growth and invasion. It was shown that BMP2 and BMP4 have an important role in melanoma plasticity and that melanoma cells with reduced BMP signaling showed reduced tumor growth and large necrotic areas (Rothhammer *et al*., 2005, 2007). However, thus far, there are no studies on the role of BMPs on the aggressiveness of Ewing sarcoma. Future studies will shed more light on the role of BMPs in Ewing sarcoma development.

It was shown that the FAK and PI3K pathways have an important role in melanoma tumor cell plasticity (Hess *et al*., 2003, 2005) and that integrin signaling regulates anchorage-independent growth, invasiveness and tumor growth. In addition, expression of integrin αvβ3 and osteopontin is increased in aggressive, highly metastatic cancer cells (Dome *et al*., 2005; Kadkol *et al*., 2006). Our results suggest that depletion of endoglin interferes with the activity of the FAK and PI3K pathways. Additionally, endoglin downregulation resulted in decreased expression of integrin β3 and osteopontin. Interestingly, integrin β3 overexpression could rescue the effect of endoglin knockdown on network formation. These results suggest a crosstalk between endoglin and the integrin/FAK/PI3K pathways. Future studies will shed more light on the molecular mechanisms by which endoglin associates with these pathways. Taking together, our results suggest that endoglin is required for efficient signaling

of multiple pathways in order to maintain tumor cell plasticity and tumor growth, and that there is an active crosstalk and tight balance between TGF-β/BMP and non-TGF-β/BMP pathways wherein endoglin has an important role (Figure 7).

Despite the fact that Ewing sarcoma and melanoma are tumors of different origin, endoglin downregulation inhibited tumor cell plasticity and tumor growth of both tumor types. In addition, endoglin expression is higher in the more aggressive MUM-2B melanoma cell line compared with the MUM-2C cell line derived from the same explants of metastatic uveal melanoma, further exemplifying the positive correlation between endoglin expression and tumor progression. In line with our observations, endoglin RNA expression was shown to be upregulated in aggressive ovarian and prostate cancer cell lines that can form tube-like networks *in vitro* (Sood *et al*., 2001; Sharma *et al*., 2002). Endoglin was also found to be expressed on glioblastoma stem-like cells that can form vascular-like structures (Hallani *et al*., 2010). It was also shown that endoglin expression on breast cancer cells enhances their invasive phenotype by inducing invadopodia formation (Oxmann *et al*., 2008). Interestingly, in this system endoglin potentiated the chemotactic effect of TGF-β on the breast cancer cells. These studies suggest that endoglin may act as a tumor promoter. However, studies in prostate carcinoma and esophageal squamous cell carcinoma lines suggested that endoglin interferes with their invasive phenotype and tumor growth *in vivo* by inhibiting TGF-β signaling (Liu *et al*., 2002; Wong *et al*., 2008). Other studies have also shown that endoglin acts as a tumor suppressor during late stages of skin carcinogenesis by inhibiting TGF-β signaling (Perez-Gomez *et al*., 2007; Bernabeu *et al*., 2009). Endoglin downregulation enhances TGF-β/Smad2,3 signaling and its antiproliferative effects, resulting in delayed tumor latencies of squamous cell carcinoma and an acelerated transition to spindle cell carcinoma. On the contrary, studies in melanoma suggested that endoglin contributes to the antiproliferative effect of TGF-β on these cells (Altomonte *et al*., 1996). These results suggest that endoglin function is context-dependent and demonstrate once again the complexity of the TGF-β superfamily signaling. The controversial results of different studies, wherein endoglin exhibits distinct effects on tumorigenesis, are probably because of the type (origin) of tumor cells, which may represent different phases of the multistep carcinogenesis process, the relative expression levels of endoglin and other signaling molecules, and their synergistic and/or antagonistic interactions with TGF-β and non-TGF-β signaling pathways.

In summary, we have shown that endoglin expression correlates with tumor cell plasticity and that it is expressed in aggressive tumors of different origin at varying levels. Endoglin expression is associated with shortened survival time of Ewing sarcoma patients, indicating that endoglin may serve as a molecular diagnostic and prognostic marker. Moreover, endoglin downregulation results in reduced tumor growth in xenograft mouse models. Our results suggest a novel role of endoglin in cancer progression providing a target for new therapeutic strategies against tumor angiogenesis, tumor cell plasticity and tumor progression.

Materials and methods

Cell culture and reagents
Ewing sarcoma cell lines EW-7, A673, RD-ES and SIM/EW27 (Szuhai *et al*., 2006; Ottaviano *et al*., 2010) and melanoma cell lines MUM-2B, MUM-2C, C8161 and C81-61 were previously characterized and cultured as described (Maniotis *et al*., 1999; Seftor *et al*., 2002; van der Schaft *et al*., 2005).

Generation of constructs and knockdown cells
Knockdown endoglin was achieved using pRetroSuper technology targeting the following 19-nucleotide sequences: 5′-AG AAAGAGCTTGTTGCGCA-3′ (shEng1), 5′-TGTCCTTGA TCCAGACAAA-3′ (shEng2) and 5′-TGGTACATCTACTC GCACA-3′ (shEng3). The non-targeting sequence was 5′-GGT ACGAATGTTAGCGAAC-3′ (shCtrl). Single-cell clones were selected with puromycin (1 μg/ml) for generation of endoglin knockdown cell lines. To generate knockdown-rescue cell lines, we generated lentiviral constructs encoding human and mouse endoglin. The integrin β3 retroviral construct was a kind gift of Dr E Danen (LACDR, Leiden, The Netherlands). Knockdown cell lines were infected with viruses; cells were allowed to recover and used in the experiments.

Patient material and immunohistochemistry
We obtained formalin-fixed, paraffin-embedded archival tissue from individuals with primary cutaneous melanoma (Maastricht University, Maastricht, The Netherlands) or Ewing sarcoma (Leiden University Medical Center, Amsterdam, The Netherlands). All patient materials were handled in a coded fashion according to the protocols as detailed by the Dutch Association of Medical Scientific Associations.

Xenograft assays
All animal experiments were approved by the local animal ethics committee. 1×10^6 cells were injected subcutaneously into the flank of 6-week-old female Swiss/nude mice. Primary tumor growth rates were analyzed by measuring tumor length (L) and width (W) and calculating tumor volume based on the formula $V = (W^2 \times L)/2$.

Statistical analysis
All results are expressed as the mean ± s.d. Statistical differences were examined by performing two-tailed Student's t-test. Survival curves were constructed using the method of Kaplan and Meier including log-rank tests. Differences were considered significant when $P \leqslant 0.05$ ($^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$).

Additional procedures
Descriptions of additional experimental procedures used are given in Supplementary Methods.

Conflict of interest

The authors declare no conflict of interest.

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