Snail is required for TGFβ-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells

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Summary

Epithelial-mesenchymal transition (EMT) plays important roles in various physiological and pathological processes, and is regulated by signaling pathways mediated by cytokines, including transforming growth factor β (TGFβ). Embryonic endothelial cells also undergo differentiation into mesenchymal cells during heart valve formation and aortic maturation. However, the molecular mechanisms that regulate such endothelial-mesenchymal transition (EndMT) remain to be elucidated. Here we show that TGFβ plays important roles during mural differentiation of mouse embryonic stem cell-derived endothelial cells (MESECs). TGFβ2 induced the differentiation of MESECs into mural cells, with a decrease in the expression of the endothelial marker claudin 5, and an increase in expression of the mural markers smooth muscle α-actin, SM22α and calponin, whereas a TGFβ type I receptor kinase inhibitor inhibited EndMT. Among the transcription factors involved in EMT, Snail was induced by TGFβ2 in MESECs. Tetracycline-regulated expression of Snail induced the differentiation of MESECs into mural cells, whereas knockdown of *Snail* expression abrogated TGFβ2-induced mural differentiation of MESECs. These results indicate that Snail mediates the actions of endogenous TGFβ signals that induce EndMT.

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Key words: TGFβ2, TβR-I inhibitor, Snail, EMT, EndMT, Embryonic stem cell, Claudin 5, Smooth muscle α-actin

Introduction

Blood vessels are lined by endothelial cells and, with the exception of capillaries, are surrounded by mural cells (pericytes or smooth muscle cells). Embryonic endothelial cells arise from mesodermal cells that express vascular endothelial growth factor receptor 2 (VEGFR2; also known as KDR) (Carmeliet 2005; Coultas et al., 2005). Previous studies have described embryonic mural cells as originating from neural crest and locally differentiating mesenchymal cells (Hirschi and Majesky, 2004). Although embryonic stem cell (ESC)-derived vascular progenitor cells have been shown to differentiate into both endothelial and mural cells (Yamashita et al., 2000; Marchetti et al., 2002; Ema et al., 2003), there is no direct evidence to show that mural cells differentiate from common vascular progenitor cells in vivo.

Differentiated endothelial cells have been shown to differentiate into mesenchymal cells in vivo. During heart development, cardiogenic mesodermal cells give rise to two types of heart cells: myocardial and endocardial cells. Endocardial cells acquire endothelial markers, such as VE-cadherin (cadherin 5) and platelet-endothelial cell adhesion molecule 1 (PECAM1). A population of endocardial/endothelial cells in the atrioventricular (AV) canal gives rise to the mesenchymal heart cushion cells, which form the mesenchymal region of cardiac septa and valves (Markwald et al., 1977; Potts et al., 1991). Furthermore, during maturation of dorsal aorta in quail, endothelial cells experimentally labeled with a wheat germ agglutinin-colloidal gold marker were shown to differentiate into subendothelial mesenchymal cells that were positive for both endothelial and mural markers in the aortic wall (DeRuiter et al., 1997; Arcinegas et al., 2000).

During these processes, endothelial cells undergo a process similar to epithelial-mesenchymal transition (EMT). EMT converts polarized epithelial cells into motile mesenchymal cells (Lee et al., 2006). EMT plays important roles in gastrulation and cancer cell invasion (Huber et al., 2005). During EMT, the epithelial markers E-cadherin (cadherin 1) and zona occludens 1 (ZO1; also known as TJP1) are downregulated, whereas the mesenchymal markers smooth muscle α-actin (SMA) and vimentin are upregulated. During endothelial-mesenchymal transition (EndMT) in heart development, expression of VE-cadherin is downregulated, whereas that of SMA is upregulated. EndMT in heart valve formation is regulated by signaling pathways mediated by multiple cytokines, including Wnt, Notch and transforming growth factor β (TGFβ).

Members of the TGFβ family bind to two different types of serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor, which, in turn, activates the downstream signal transduction cascades, including Smad pathways. Activins and TGFβs signal through the type I receptors known as activin receptor type B (ACVR1B, hereafter referred to as ALK4) and transforming growth factor beta receptor 1 (TGFβR1; hereafter referred to as ALK5), respectively. The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and 3 transduce signals for TGFβs and activins, whereas Smad1, 5 and 8 (also known as Smad9) are specific for signaling of bone

morphogenetic proteins (BMPs) (Feng and Derynck, 2005). As an exception, ALK1 (ACVRL1), which is preferentially expressed in endothelial cells, binds TGFβ and activates Smad1/5 pathways (Oh et al., 2000). Recently, BMP9 and BMP10 were reported to bind to ALK1 (David et al., 2007; Scharpfenecker et al., 2007). Once activated, R-Smads form a complex with the common mediator Smad4 (co-Smad) and translocate to the nucleus, where Smad complexes regulate transcription of target genes through their interaction with various transcription factors. In addition, TGFβ has been shown to activate a diversity of non-Smad parallel downstream pathways, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 MAP kinase (Derynck and Zhang, 2003).

Knockout mice deficient in various TGFβ family signaling components exhibit defects in cardiovascular tissues, implicating a role for TGFβ family proteins in cardiovascular development (Goumans and Mummery, 2000; Mercado-Pimentel and Runyan, 2007). In particular, TGFβ2-deficient mice have multiple defects in AV cushion formation, suggesting a role in EndMT of endocardiac tissues (Sanford et al., 1997; Bartram et al., 2001). Furthermore, various in vitro studies have shown that TGFβs induce the differentiation of vascular endothelial cells into mural cells (Arciniegas et al., 1992; Frid et al., 2002; Ishisaki et al., 2003). However, the molecular mechanisms that govern TGFβ-induced EndMT remain largely unknown.

Several transcription factors, including Snail (SNAI1), Slug (SNAI2), δEF1 (ZEB1), SIP1 and Twist, have been implicated in EMT (Peinado et al., 2007). Snail, a zinc-finger-containing transcription factor, represses E-cadherin expression and induces EMT when overexpressed in epithelial cells (Cano et al., 2000; Batlle et al., 2000). Knockout mice deficient for *Snail* die at gastrulation because they fail to undergo a complete EMT process, forming an abnormal mesodermal layer that maintains E-cadherin expression (Carver et al., 2001). Although TGFβ signals have been shown to induce the expression of Snail, SIP1 and δEF1 during EMT of mammary epithelial cells (Peinado et al., 2003; Shirakihara et al., 2007), the causal relationship between TGFβ-induced Snail expression and EMT has not yet been fully elucidated.

In order to elucidate the roles of TGFβ signaling in the differentiation of embryonic endothelial cells into mural cells, we used endothelial cells derived from mouse ESCs. TGFβ2 decreased the expression of an endothelial marker, claudin 5, and increased that of mural markers, SMA, SM22α (transgelin) and calponin. We also found that Snail is necessary for the TGFβ2-induced mural differentiation of endothelial cells. These results reveal that Snail plays important roles in the TGFβ-induced EndMT.

Results

TGFβ and activin induce differentiation of ESC-derived endothelial cells into SMA-expressing cells

When VEGFR2-expressing vascular progenitor cells derived from mouse ESCs were cultured for 3 days with VEGF (VEGFA), we obtained cells positive for mural cell marker SMA, which surround endothelial cells positive for PECAM1 and CD34 (Yamashita et al., 2000). These mixed vascular cell populations were sorted using anti-CD34 antibodies in order to purify endothelial cells. The proportion of PECAM1$^+$ cells in the sorted population was nearly 100% (data not shown). We used these mouse ESC-derived endothelial cells (MESECs) in the present study (supplementary material Fig. S1).

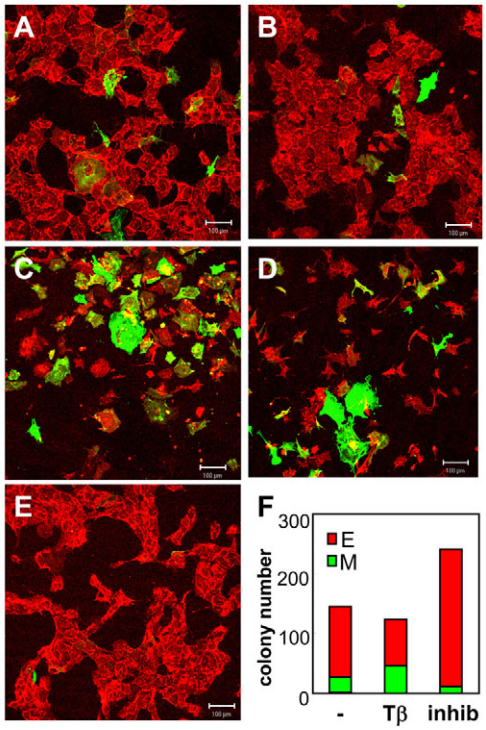

Fig. 1. Effects of TGFβ family members on mouse ESC-derived endothelial cells (MESECs). (A-E) MESECs were obtained by CD34-sorting of vascular (endothelial and mural) cells derived from mouse ESCs, and cultured in the presence of 10% fetal calf serum (FCS) and VEGF (A), followed by immunofluorescence staining for PECAM1 (red) and SMA (green). MESECs were also treated with BMP4 (B), TGFβ2 (C), activin (D) and TβR-I inhibitor (E). Scale bars: 100 μm. (F) Quantitative analysis of the effects of TGFβ signals on colony formation from single MESECs. MESECs were cultured at low density with 10% FCS and VEGF in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) for 4 days, and then stained for PECAM1 and SMA. The number of colonies per well was counted to assess the effect of TGFβ signals on colony formation of MESECs. Two colony types were observed: those consisting of pure endothelial cells (E) and those also containing mural cells (M). Experiments were repeated at least three times with essentially the same results.

After MESECs were cultured for 4 days in the presence of VEGF, the proportion of endothelial cells remained higher than 95% (Fig. 1A). Previous reports have shown that cultured endothelial cells differentiate into mesenchymal cells expressing SMA under long-term stimulation by TGFβ (Paranya et al., 2001; Frid et al., 2002). In order to study the effects of TGFβ on MESECs, we used TGFβ2, which seems to be the physiologically most relevant TGFβ isoform for EndMT during heart cushion development (Camenisch et al., 2002), as well as activin and BMP4, other members of the TGFβ family. Whereas BMP4 did not exhibit significant effects (Fig. 1B), TGFβ2 and activin led to a decrease in the number of PECAM1$^+$ sheets of endothelial cells and to an increase in SMA$^+$ cells (Fig. 1C,D). We also compared the effects of different isoforms of TGFβ on MESECs and found that TGFβ1 and 3 induced the EndMT in a similar manner to TGFβ2 (supplementary material Fig. S2).

Furthermore, inhibition of endogenously activated TGFβ/activin signals in MESECs (Watabe et al., 2003) by a small-molecule that inhibits kinases of receptors for TGFβ, actin and nodal (TβR-I inhibitor, also known as LY364947) (Sawyer et al., 2003), led to a decrease in SMA$^+$ cells (Fig. 1E). These results

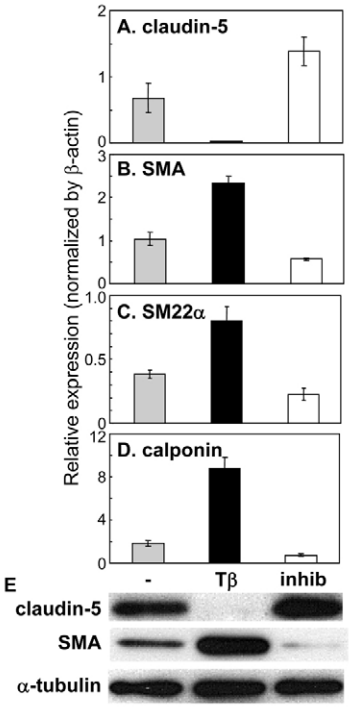

Fig. 2. Effect of TGFβ signals on expression of endothelial and mural markers in MESECs. (A-D) Levels of mRNA expression for claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs cultured in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) as analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

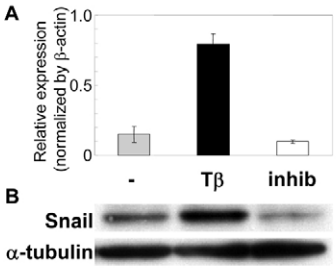

Fig. 3. Effect of TGFβ signals on Snail expression in MESECs. (A) Levels of expression of Snail in MESECs cultured in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) as analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (B) Protein levels of Snail (top) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the HUVECs cultured in the absence or presence of TGFβ2 or TβR-I inhibitor.

suggest that activation of ALK5-mediated signaling pathways induces the differentiation of MESECs into SMA-expressing cells.

Quantitative analysis of the effects of TGFβ signals on the differentiation of MESECs into SMA-expressing cells

In order to further dissect the roles of TGFβ in the differentiation of MESECs into SMA^+ cells, we performed quantitative analysis using a limiting-dilution assay. When MESECs were plated at a low density, single MESECs formed individual colonies, and the total number of colonies varied depending on the culture conditions. TGFβ2 decreased the number of colonies slightly, whereas the TβR-I inhibitor strongly increased it (Fig. 1F).

We next evaluated the phenotypes of the colonies by immunohistochemical analysis. Culturing MESECs with VEGF induced two types of colonies emerging from single MESECs: $PECAM1^+$ pure endothelial cells (E), and mixtures of endothelial and SMA^+ mural cells (M) (Fig. 1F). Whereas the frequency of mural colonies was 19% when cultured in the presence of VEGF, addition of TGFβ2 or TβR-I inhibitor reproducibly increased (to 37%) or decreased (to 5%) this frequency, respectively, further suggesting that TGFβ signals induce the differentiation of MESECs into SMA^+ cells.

TGFβ regulates the expression of various endothelial and mural cell markers

Vascular endothelial cells express various markers, including PECAM1, VE-cadherin and claudin 5, whereas mural cells express markers such as SMA, SM22α and calponin. During the EndMT in heart cushion development, expression of VE-cadherin is downregulated with concomitant upregulation of SMA expression. We studied the effects of TGFβ signals on the expression of various markers for endothelial and mural cells in MESECs using quantitative RT-PCR and immunoblot analyses. Whereas the expression of VE-cadherin was unaffected by TGFβ2 (data not shown), TGFβ2 treatment resulted in a decrease in claudin 5 expression (Fig. 2A) with a concomitant increase in SMA expression at both the mRNA (Fig. 2B) and protein (Fig. 2E) levels. By contrast, addition of TβR-I inhibitor increased the expression of claudin 5 and decreased SMA expression (Fig. 2A,B,E).

We also studied the expression of other mural cell markers. As shown in Fig. 2C,D, SM22α and calponin were both upregulated by TGFβ2 and downregulated by TβR-I inhibitor. These results suggest that TGFβ2 induces the EndMT of MESECs.

TGFβ induces the expression of Snail during mural cell differentiation of MESECs

Recent studies have revealed that several transcription factors are involved in EMT (Peinado et al., 2007). In order to elucidate the molecular mechanisms that govern TGFβ-induced EndMT of MESECs, we studied the expression of various EMT-related transcription factors after treatment of MESECs with TGFβ2 or TβR-I inhibitor. As shown in Fig. 3A, expression of Snail was significantly increased by TGFβ2 treatment, whereas it was slightly decreased by the addition of TβR-I inhibitor. By contrast, expression of Slug, SIP1, δEF1 or Twist, was unaffected by TGFβ2 treatment in MESECs (data not shown).

We further studied the effect of TGFβ signals on the expression of Snail protein in human umbilical vein endothelial cells (HUVECs). In accordance with the results in MESECs, expression of Snail was induced by TGFβ2 and decreased by TβR-I inhibitor (Fig. 3B). These results suggest Snail as a possible regulator of EndMT in response to TGFβ2 treatment.

Snail expression in MESECs induces EndMT

Since Snail expression was induced by TGFβ, we examined the effects of Snail expression on MESECs. Because we wished to induce the expression of Snail in differentiated endothelial cells instead of undifferentiated ESCs, we established ESC lines carrying a tetracycline (Tc)-regulatable Snail transgene (Tc-Snail), or no transgene (Tc-Empty) (Masui et al., 2005; Mishima et al., 2007).

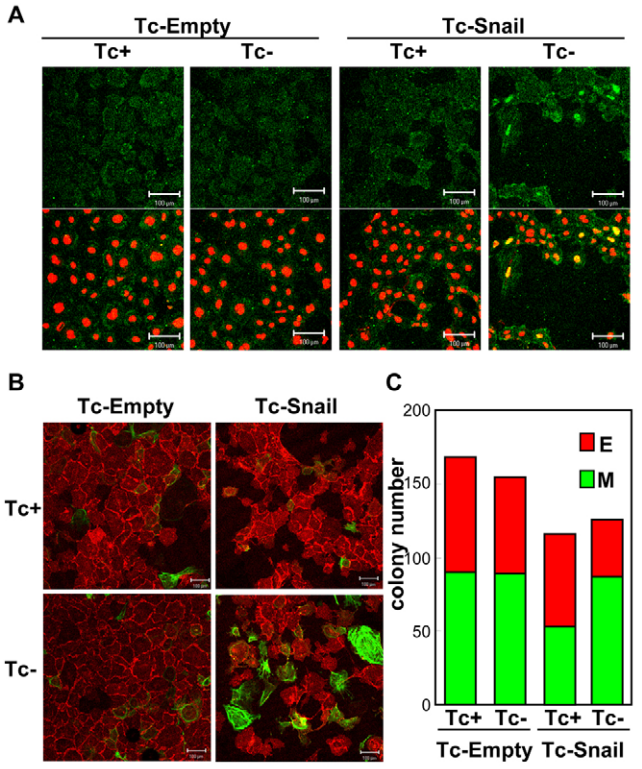

Fig. 4. Effect of tetracycline (Tc)-regulated Snail expression on MESECs. (A) MESECs were sorted from the vascular cells derived from ESCs carrying a Tc-regulated transgene encoding FLAG-epitope-tagged mouse Snail (Tc-Snail) or control transgene (Tc-Empty), and cultured in the absence (–) or presence (+) of Tc. Expression of FLAG-Snail (top and bottom rows, green) was examined, in addition to nuclear staining (bottom row, red). (B) MESECs in A were subject to immunofluorescence staining for PECAM1 (red) and SMA (green). (C) Quantitative analysis of the effects of Snail on colony formation from single MESECs, performed as described in Fig. 1F. Briefly, MESECs derived from Tc-Empty or Tc-Snail ESCs were cultured at low density with 10% FCS in the absence (–) or presence (+) of Tc for 4 days, followed by staining of colonies for PECAM1 and SMA. E, pure endothelial colony; M, mural-containing colony. Scale bars: 100 μm.

EMT has been reported to play important roles during the early stages of development, such as gastrulation. Recently, upregulation of Snail expression was detected during an early differentiation process of human ESCs, which was identified as EMT (Ullmann et al., 2007). Removal of Tc from the culture of undifferentiated Tc-Snail cells, but not that of Tc-Empty cells, induced the expression of the Snail transgene, which resulted in dramatic changes in their morphology from epithelial to mesenchymal in appearance (supplementary material Fig. S3A). We further found that E-cadherin expression was downregulated by Snail expression (supplementary material Fig. S3B). These results suggest that Snail enhances the EMT that takes place during early differentiation process of ESCs.

In order to examine the effects of Snail on vascular development, we differentiated the Tc-Empty and Tc-Snail ESCs into vascular cells in the presence of Tc, so that no transgene expression was induced until the endothelial cells were fully differentiated. MESECs were sorted using anti-CD34 antibodies, and were cultured in the presence or absence of Tc (supplementary material Fig. S1). As shown in Fig. 4A, Snail transgene expression was induced in the vascular cells derived from Tc-Snail ESCs only in the absence of Tc. The majority of control MESECs maintained their endothelial characteristics, expressing PECAM1 when Snail was not expressed (Fig. 4B). However, when Snail was expressed, many of the endothelial cells exhibited mural characteristics, including expression of SMA (Fig. 4B), as observed when MESECs were treated with TGFβ2 (Fig. 1).

To further dissect the roles of Snail in the EndMT of MESECs, we performed quantitative colony-formation assays. The frequency of colonies containing SMA^+ cells (M) was unaffected by the removal of Tc in the MESECs derived from Tc-Empty ESCs (Tc+, 53%; Tc–, 57%) (Fig. 4C). By contrast, Snail expression induced by the removal of Tc in the Tc-Snail ESC-derived MESECs significantly increased the frequency of SMA^+ colony formation (Tc+, 45%; Tc–, 69%). This suggests that Snail induces the differentiation of MESECs into SMA^+ cells.

We examined whether induction of EndMT by Snail transgene expression requires activation of TGFβ type I receptors. Addition of TβR-I inhibitor to the MESECs derived from Tc-Snail ESCs in the presence of Tc abrogated the EndMT induced by endogenous TGFβ signals (supplementary material Fig. S4A). Expression of the Snail transgene by removal of Tc induced EndMT, both in the absence and presence of TβR-I inhibitor. These results were confirmed by a quantitative colony-formation assay (supplementary material Fig. S4B) and suggest that Snail induces the EndMT of MESECs as a downstream target of TGFβ type I receptor-mediated signals.

Snail downregulates endothelial marker expression and upregulates mural marker expression

We further examined the effects of Snail on the expression of various endothelial and mural cell markers in MESECs. As shown in Fig. 5A,B, the amount of transcript for claudin 5 and for SMA was down- and upregulated, respectively, by Snail expression in the MESECs derived from Tc-Snail ESCs. Analogous changes in protein levels were confirmed by immunoblot analysis (Fig. 5E).

The expression of other mural cell markers, SM22α and calponin, was also upregulated by Snail expression in MESECs (Fig. 5C,D). These changes in expression of the various markers induced by Snail are reminiscent of those induced by TGFβ2 treatment, suggesting that Snail is involved in the TGFβ2-induced EndMT of MESECs.

Snail is required for the TGFβ2-induced EndMT of MESECs

In order to determine whether Snail is required for the TGFβ2-mediated EndMT, we used siRNA directed against *Snail* to reduce the expression of endogenous protein. Snail siRNA was transfected into MESECs, followed by stimulation of the cells with TGFβ2 (supplementary material Fig. S1). As shown in Fig. 6A, Snail siRNA successfully knocked down the expression of endogenous *Snail* mRNA while TGFβ2 still induced *Snail* expression to the endogenous level. In cells transfected with control siRNA, TGFβ2 induced the differentiation of MESECs into SMA^+ cells, whereas TGFβ2 failed to do so in the cells transfected with Snail siRNA (Fig. 6B). These results were confirmed by quantitative colony-formation assays (Fig. 6C).

Furthermore, the TGFβ2-mediated upregulation of expression of various mural cell markers (SMA, SM22α and calponin) was repressed by Snail siRNA, whereas downregulation of claudin 5 expression by TGFβ2 was partially inhibited by Snail siRNA (Fig. 7A-E). These results reveal that Snail is necessary for the EndMT induced by TGFβ2.

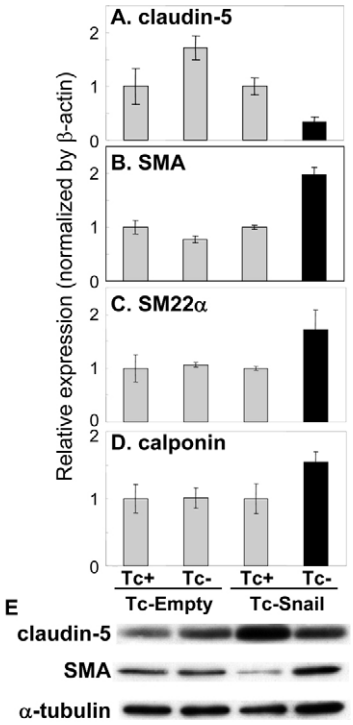

Fig. 5. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs derived from Tc-Empty or Tc-Snail ESCs cultured in the absence (–) or presence (+) of Tc were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

Smad4-independent pathways are partly involved in the TGFβ2-induced EndMT of MESECs

Upon ligand binding, TGFβ receptor complexes activate both Smad and non-Smad signaling pathways. In order to examine whether these non-Smad pathways are involved in the TGFβ-induced EndMT, we knocked down the expression of *Smad4*, the only co-Smad that is necessary for both the Smad2/3 and Smad1/5/8 pathways (Fig. 8A). In the MESECs in which *Smad4* expression was knocked down, TGFβ2 failed to induce the expression of PAI1 (SERPINE1) (Fig. 8B), a target of the Smad2/3 pathway, but partially induced Snail expression (Fig. 8C), suggesting that Snail is partially induced by Smad4-independent pathways. In accordance with the results of Snail expression, knockdown of *Smad4* expression failed to fully abrogate the TGFβ2-mediated EndMT (Fig. 8D), the suppression of claudin 5 (Fig. 8E) or the induction of SMA expression (Fig. 8F). These results suggest that TGFβ activates Smad4-dependent and -independent pathways, both of which play important roles in the induction of Snail expression that leads to EndMT.

We also examined whether the induction of EndMT by Snail transgene expression requires Smad4. The expression of the Snail transgene by removal of Tc from the culture of the MESECs derived from Tc-Snail ESCs was able to induce EndMT, both in the absence and presence of Smad4 expression (supplementary material Fig. S5A). These results were confirmed by quantitative RT-PCR (supplementary material Fig. S5B,C), and suggest that Snail induces the EndMT of MESECs as a downstream target of Smad4-mediated signals.

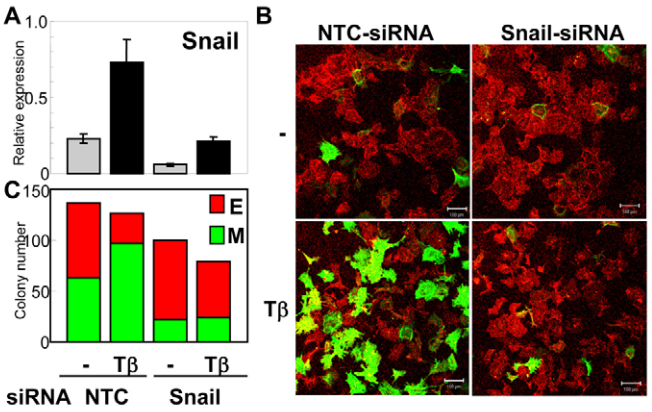

Fig. 6. Effect of *Snail* knockdown on MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Snail siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) The levels of endogenous expression of Snail in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. Black and gray bars, represent +TGFβ2 and –TGFβ2, respectively. (B) The MESECs were subjected to immunofluorescence staining for PECAM1 (red) and SMA (green). (C) Quantitative analysis of the effects of Snail on colony formation from single MESECs, performed as described in Fig. 1F. Briefly, MESECs transfected with Snail siRNA or scrambled sequence were cultured at low density with 10% FCS in the absence (–) or presence of TGFβ2 (Tβ) for 4 days, followed by staining of colonies for PECAM1 and SMA. E, pure endothelial colony; M, mural-containing colony. Scale bars: 100 μm.

Discussion

In the present study, we showed that TGFβ2 induces the differentiation of endothelial cells into mural cells, with an increase in expression of the mural markers, SMA, SM22α and calponin. Previous reports have shown that TGFβ induces various mural markers during the differentiation of neural crest stem cells into smooth muscle cells (Shah et al., 1996), and that TGFβ-induced δEF1 is involved in this process (Nishimura et al., 2006). Although TGFβ has been shown to induce the expression of Snail during EMT of kidney epithelial cells (Peinado et al., 2003), functional roles of Snail during TGFβ-induced EMT were not fully elucidated. The present findings directly show, for the first time, that Snail mediates TGFβ-induced upregulation of multiple mural markers and the downregulation of claudin 5 in endothelial cells.

We also found that loss of Smad4 expression decreases, but does not completely abolish, TGFβ-induced Snail expression and EndMT (Fig. 8). We previously showed that Snail expression is upregulated within 30 minutes of addition of TGFβ to NMuMG mammary epithelial cells, in which TGFβ induces EMT (Shirakihara et al., 2007), suggesting that Snail is a direct target of TGFβ signals. The molecular mechanisms by which Smad4-dependent and -independent signals activate the Snail promoter in endothelial cells remain to be studied in the future.

Although Snail has been shown to play important roles in EMT, the molecular mechanisms by which Snail regulates the transcription of EMT-related targets have not been elucidated. In order to examine whether Snail binds to the endogenous SMA promoters in intact chromatin, we have subjected cross-linked chromatin samples prepared from Tc-Snail ESC-derived endothelial cells to chromatin immunoprecipitation (ChIP) assays. Nishimura and colleagues previously identified a TGFβ-responsive SMA promoter region containing Smad3-binding sequences and an E-box to which

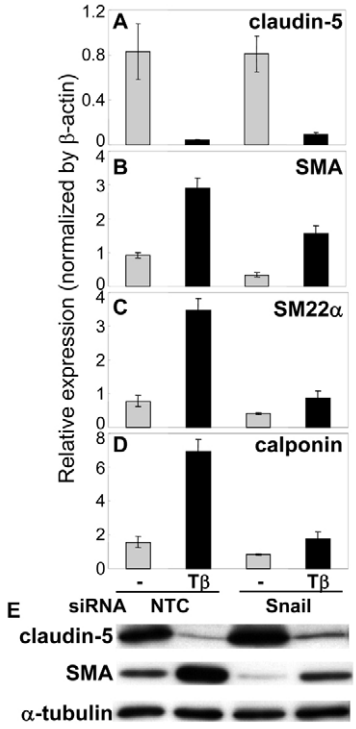

Fig. 7. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs transfected with Snail siRNA or scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ) were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

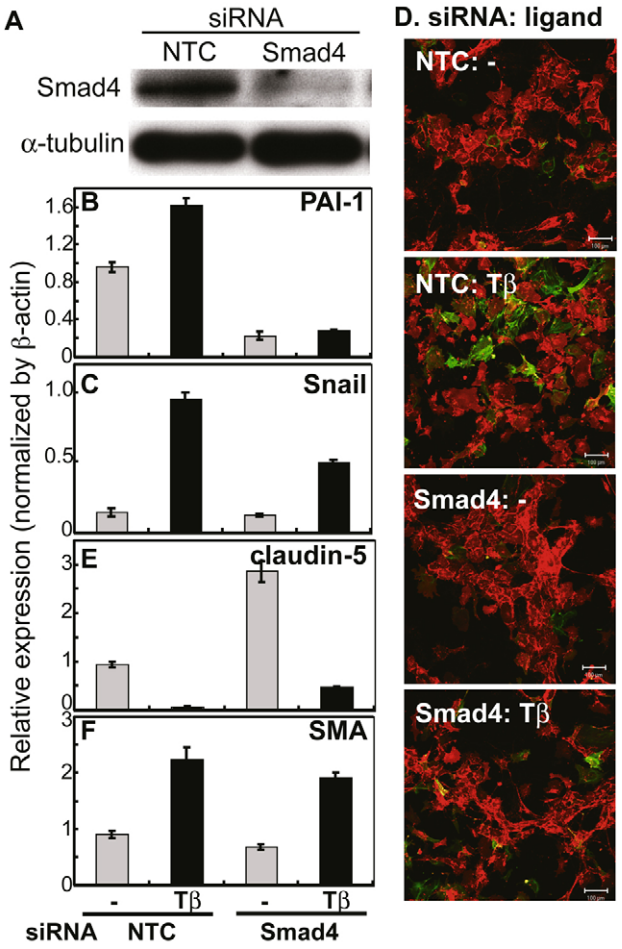

Fig. 8. Effect of *Smad4* knockdown on TGFβ-induced EndMT of MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Smad4 siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) Levels of endogenous expression of Smad4 in the MESECs were analyzed by immunoblotting. (B,C,E,F) Levels of expression of PAI1 (B), Snail (C), claudin 5 (E) and SMA (F) in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (D) The MESECs were subject to immunofluorescence staining for PECAM1 (red) and SMA (green).

Snail proteins might bind (Nishimura et al., 2006). We were also able to pull down the TGFβ-responsive element with antibodies against Smad3 in the Tc-Snail ESC-derived endothelial cells treated with TGFβ (supplementary material Fig. S6A), but not with antibodies against FLAG-Snail (supplementary material Fig. S6B). These results suggest that Snail does not bind to the TGFβ-responsive element to induce SMA expression in MESECs.

During EMT, a decrease in the expression of multiple tight-junction molecules, such as ZO1 and claudins, is accompanied by an increase in the expression of mesenchymal markers. We observed a decrease in the expression of claudin 5, an endothelium-specific tight-junction molecule, induced by TGFβ in MESECs. We previously reported that expression of claudin 5 is downregulated by TGFβ during endothelial differentiation from ESC-derived vascular progenitor cells (Watabe et al., 2003). Since claudin 1 expression is also repressed by Snail and Slug during EMT of kidney epithelial cells (Martinez-Estrada et al., 2006), downregulation of claudin family members might be a crucial event during EMT and EndMT.

During EMT and EndMT, expression of E-cadherin and VE-cadherin is, respectively, also decreased. However, VE-cadherin expression was not altered by Snail in MESECs, whereas E-cadherin expression was suppressed by Snail in undifferentiated ESCs. This might suggest that repression of VE-cadherin requires other transcription factors. We recently showed that TGFβ-induced δEF1 and SIP1, but not Snail, are involved in the downregulation of E-cadherin expression in mammary epithelial cells (Shirakihara et al., 2007). However, expression of the other EMT-related transcription factors was unaffected by TGFβ in MESECs, suggesting that other EMT-related signaling pathways are involved in the repression of VE-cadherin expression. In the embryonic heart, Notch functions to promote the TGFβ-induced EMT that results in formation of the cardiac valvular primordia (Timmerman et al., 2004). Liebner and colleagues showed that TGFβ induction of EndMT during heart cushion development is strongly inhibited in mice deficient for β-catenin, suggesting that an interaction between TGFβ and Wnt signaling pathways plays important roles in this process (Liebner et al., 2004). The roles of Notch and Wnt signals in the EndMT of MESECs remain to be elucidated in the future.

The expression of Twist, another EMT-related transcription factor, has been reported to be regulated by BMP2 (Ma et al., 2005), a member of the TGFβ family that has been implicated in cardiac cushion EndMT. In the embryos that lack BMP2 or BMP type IA receptor in AV myocardium or endocardium, respectively, cardiac

cushion formation is perturbed, with loss of expression of various transcription factors, including Twist (Ma et al., 2005). However, the present study shows that BMP4 fails to induce EndMT of MESECs. In accordance with this, Snail expression in the endocardium was unaffected by the loss of BMP2 or BMP type IA receptor (Ma et al., 2005). A recent report showed that BMP7 inhibits the TGFβ-induced EndMT of cardiac endothelial cells (Zeisberg et al., 2007a). We also found that BMP7 partially inhibits the TGFβ-mediated SMA expression in MESECs (supplementary material Fig. S7). These results suggest that certain types of BMPs play roles in the EndMT in a manner independent of TGFβ.

Recently, EndMT was implicated in two pathological situations. During cardiac fibrosis, accumulated fibroblasts cause the deposition of extracellular matrix, which can cause heart failure. Furthermore, activated fibroblasts can induce the progression of cancers. Zeisberg and colleagues reported that the TGFβ-induced EndMT plays important roles in the formation of fibroblasts from endothelial cells during cardiac fibrosis (Zeisberg et al., 2007a) and cancer progression (Zeisberg et al., 2007b). Since fibroblasts are key to both situations, EndMT is expected to be a target in the therapy of cardiac dysfunction and cancer. Therefore, the present findings might lead to a greater understanding of not only normal cardiovascular development, but also of such pathological situations, and eventually to the development of strategies to manipulate these signals for therapeutic benefit.

Materials and Methods

Cells and cell culture

The maintenance, differentiation, culture and cell sorting of mouse CCE and MGZ5TcH2 ESCs (gifts from Drs M. J. Evans and H. Niwa, respectively) were as described (Yamashita et al., 2000). Differentiated ESC-derived endothelial cells were sorted using PE-conjugated anti-CD34 antibodies (Pharmingen) and a MACS separation system (Miltenyi Biotec). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). HUVECs were obtained from Sanko Junyaku and cultured as described (Mishima et al., 2007). VEGF (R&D, 30 ng/ml), TGFβ1, 2 and 3 (R&D, 1 ng/ml), BMP4 (R&D, 50 ng/ml), BMP7 (R&D, 500 ng/ml), activin (R&D, 25 ng/ml), TβR-I inhibitor (Calbiochem LY364947, 1 μM) and tetracycline (Sigma-Aldrich, 1 μg/ml) were used.

RNA interference and oligonucleotides

siRNAs were introduced into cells as described (Shirakihara et al., 2007). The target sequences for mouse Snail and Smad4 siRNAs were 5′-UGCAGUUGAAGAUCUUCCGCGACUG-3′ and 5′-UUAAUCCUGAGAGAUCAAUUCCAGGS-3′, respectively. Control siRNAs were obtained from Ambion.

Immunohistochemistry and immunoblot analysis

Immunohistochemistry of cultured cells was performed as described (Yamashita et al., 2000) using monoclonal antibodies to PECAM1 (Mec13.3, BD Pharmingen), SMA (1A4, Sigma-Aldrich) and FLAG (M2, Sigma-Aldrich). Stained cells were photographed using a confocal microscope (LSM510 META, Carl Zeiss MicroImaging) with 10× (Plan-Neofluar 10×/0.30) objectives and LSM Image Browser. All images were taken at room temperature, and imported into Adobe Photoshop as TIFs for contrast adjustment and figure assembly. Immunoblot analyses were performed as described (Kawabata et al., 1998) using antibodies to claudin 5 (Zymed), SMA (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), Snail (Cell Signaling) and E-cadherin (BD Transduction Laboratories).

RNA isolation and RT-PCR

Total RNA was prepared using RNeasy Reagent (Qiagen) and reverse-transcribed by random priming and using a Superscript First-Strand Synthesis Kit (Invitrogen). Quantitative RT-PCR analysis was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). All expression data were normalized to those for β-actin. For primer sequences, see supplementary material Table S1.

Chromatin immunoprecipitation (ChIP) assay

Endothelial cells derived from Tc-Snail ESCs were obtained in the absence or presence of Tc, and were incubated with or without TGFβ for 3 hours. Cells were fixed by adding formaldehyde and harvested. ChIP assays were carried out as described (Nishimura et al., 2006). In order to precipitate Smad3 and FLAG-tagged Snail, anti-Smad3 antibody (Upstate Biotechnology) and anti-FLAG (M2) antibody were used. PCR of the SMA promoter around the TGFβ hypersensitivity region was performed using immunoprecipitated chromatin with primers 5′-CAGTTGTTCTGAGGGCTTAGGATGTTTATC-3′ and 5′-ACAAGGAGCAAAGACGGGCTGAAGCTGGCC-3′.

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