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RESEARCH ARTICLE

FGF2-induced Ras–MAPK signalling maintains lymphatic endothelial cell identity by upregulating endothelial-cell-specific gene expression and suppressing TGFβ signalling through Smad2

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ABSTRACT

The lymphatic endothelial cell (LEC) fate decision program during development has been described. However, the mechanism underlying the maintenance of differentiated LEC identity remains largely unknown. Here, we show that fibroblast growth factor 2 (FGF2) plays a fundamental role in maintaining a differentiated LEC trait. In addition to demonstrating the appearance of LECs expressing α-smooth muscle actin in mouse lymphedematous skin *in vivo*, we found that mouse immortalised LECs lose their characteristics and undergo endothelial-to-mesenchymal transition (EndMT) when cultured in FGF2-depleted medium. FGF2 depletion acted synergistically with transforming growth factor (TGF) β to induce EndMT. We also found that H-Ras-overexpressing LECs were resistant to EndMT. Activation of H-Ras not only upregulated FGF2-induced activation of the Erk mitogen activated protein kinases (MAPK3 and MAPK1), but also suppressed TGFβ-induced activation of Smad2 by modulating Smad2 phosphorylation by MAPKs. These results suggest that FGF2 regulates LEC-specific gene expression and suppresses TGFβ signalling in LECs through Smad2 in a Ras–MAPK-dependent manner. Taken together, our findings provide a new insight into the FGF2–Ras–MAPK-dependent mechanism that maintains and modulates the LEC trait.

KEY WORDS: Lymphatic endothelial cell, Endothelial–mesenchymal transition, FGF2, Ras, MAPK, Smad

INTRODUCTION

Lymphatic vessels play essential roles in tissue fluid homeostasis, transport of macromolecules and immune cell trafficking. Growth, maturation and regression of lymphatic vessels are associated with tissue remodelling during embryonic development, tissue repair, inflammation and tumorigenesis. In such situations, malformation or malfunction of lymphatic vessels is the primary cause of lymphedema, which leads to abnormal tissue remodelling and impaired immune responses. Therefore, it is important to understand the mechanism governing the development of adequate and functional lymphatic vessels.

Lymphatic endothelial cell (LEC) fate and subsequent lymphatic vessel morphogenesis during embryonic development are determined by genetic codes, as demonstrated by previous studies using knockout mice that lack lymphatic vessels. Briefly, a subset of blood endothelial cells (BECs) acquires LEC identity during embryonic development in a Prox1-expression-dependent manner (Wigle and Oliver, 1999), moves away from blood vessels, and then forms the lymphatic vasculature upon vascular endothelial growth factor receptor (VEGFR)-3 signalling (Zhang et al., 2010). Once the LEC fate is determined, LECs retain their cell identity. However, recent studies have suggested that LECs undergo dynamic changes from quiescent to active states and vice versa during physiological and pathological lymphangiogenesis. Gene expression levels of *Prox1* and *Vegfr3* in LECs were found to vary during inflammation and its resolution (Flister et al., 2010; Huggenberger et al., 2011; Kataru et al., 2011), suggesting that expression levels of *Prox1* and *Vegfr3* mediate appropriate lymphatic vessel formation and remodelling by changing the behaviour of LECs in response to lymphangiogenic and anti-lymphangiogenic signals.

In addition to their heterogeneity, LECs display plasticity, as evidenced by their ability to undergo differentiation into lymphatic-valve-forming cells, de-differentiation into ECs with BEC-like features, and transdifferentiation into fibroblast-like cells. A subset of LECs further differentiate into more specialised cells involved in the formation of lymphatic valve leaflets in collecting lymphatic vessels, in response to genetic and environmental cues (Bazigou et al., 2009; Kanady et al., 2011; Norrmén et al., 2009; Sabine et al., 2012). Temporally induced, conditional-knockout mice lacking Prox1 in differentiated LECs exhibit blood-filled lymphatic vessels, and siRNA-meditated knockdown of Prox1 in LECs results in BEC-like gene expression characteristics *in vitro*, suggesting that Prox1 is required for maintaining LEC identity (Johnson et al., 2008). Blood flow or shear stress might also change LECs into BEC-like cells, as suggested by incorporation of LECs into blood vessels *in vivo* and changes in LEC gene expression signature *in vitro* (Chen et al., 2012a). However, it is not known whether transdifferentiation of LECs into fibroblast-like cells (which is known as endothelial-to-mesenchymal transition or EndMT) occurs *in vivo*, although it has been suggested that metastatic Kaposi sarcoma cells in humans derive from Kaposi sarcoma herpes virus (KSHV)-infected LECs that have undergone EndMT in a Notch-signalling-dependent fashion (Cheng et al., 2011). Additionally, a previous study demonstrated that the formation of α-smooth muscle actin (αSMA)-positive lymphatic vessels is induced by attenuated regeneration of skin incisions and TGFβ stimulation, and the presence of these vessels correlated with severe lymphedema and fibrosis. The authors suggest that a subset of LECs expressing the mesenchymal and fibroblast marker αSMA

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exist in lymphedematous tail skins and contribute to pathogenesis (Clavin et al., 2008).

Collectively, these studies suggest that the maintenance and modification of LEC identity play important roles in physiological and pathological lymphatic vessel formation and function. Nevertheless, the underlying molecular mechanism of maintenance of LEC identity remains unknown. Moreover, it is still unclear whether αSMA-positive LECs exist *in vivo*. In this study, we show the existence of αSMA-expressing LECs *in vitro* and *in vivo*, and reveal an unexpected role of FGF2 in the maintenance of differentiated LEC identity. We found that FGF2 is required for the preservation of LEC-specific gene expression, and plays an inhibitory role in EndMT of LECs *in vitro*. In addition, we demonstrate that activation of Ras–MAPK signalling inhibits EndMT of LECs and compensates for the FGF2 signal. Finally, we show that Ras–MAPK signalling prevents LECs from undergoing TGFβ-induced EndMT by blocking Smad2 activation.

RESULTS

A subset of LECs expresses the fibroblast and mesenchymal cell marker αSMA during lymphatic vessel regeneration induced by experimental lymphedema in mice

A previous study demonstrated the formation of αSMA-positive lymphatic vessels in edematous tail skins of experimental lymphedema model mice, and it has been suggested that the presence of αSMA-positive LECs might be implicated in lymphatic vessel malfunction, leading to lymphedema (Clavin et al., 2008). However, macrophages have been reported to incorporate into the endothelial layer of lymphatic vessels and occasionally transdifferentiate into LEC-like cells and/or αSMA-positive fibroblast-like cells during wound healing and proliferative eye diseases (Lin et al., 2011; Maruyama et al., 2007; Maruyama et al., 2005). Therefore, it remains to be elucidated whether αSMA-positive LECs arise during lymphedematous conditions. To re-evaluate the appearance of αSMA-expressing LECs, we used genetically engineered mice harbouring the *Rosa26-mT/mG* and *Vegfr3-CreMer* knock-in alleles, in which enhanced green fluorescent protein (EGFP) expression from the mT/mG transgene on the *Rosa26* locus is switched on by tamoxifen-induced Cre recombinase activity in VEGFR-3-expressing cells (Fig. 1A). The transgene on the *Rosa26* locus is known to express EGFP stably and uniformly, so we thought it more suitable for detecting LECs and their behaviour during inflammation and tissue repair than endogenous lymphatic marker genes such as *Prox1* and *Vegfr3*, which are variably expressed in LECs during lymphatic vessel remodelling in inflammation and its resolution (Flister et al., 2010; Huggenberger et al., 2011; Kataru et al., 2011). We induced Cre-mediated recombination in adult mice by oral administration of tamoxifen, and then induced experimental lymphedema. We confirmed that the LECs in these mice were labelled with EGFP by double immunostaining with EGFP and LEC markers, namely, Lyve1 and podoplanin (Fig. 1B), and Prox1 (supplementary material Fig. S1A). It was possible that Cre-mediated recombination might induce EGFP expression in macrophages, because a subset of macrophages was found to express VEGFR-3 in pathological conditions (Schoppmann et al., 2002). However, we did not observe EGFP expression in F4/80-positive macrophages or incorporation of F4/80-positive cells into the lymphatic endothelial layer (supplementary material Fig. S1B), suggesting that the EGFP-positive cell population in lymphedematous tail skin does not include macrophage-derived cells. Under these conditions of LEC tracing, we found a subset of LECs expressing αSMA in collecting lymphatic vessels in lymphedematous tail skin (Fig. 1C). To distinguish between those LECs and smooth muscle cells that discontinuously line the collecting lymphatic vessels, we observed immunostained sections using confocal laser microscopy, and found αSMA-positive LECs, as well as peri-lymphovascular smooth muscle cells (Fig. 1D). We also found αSMA-positive LECs within the superficial, hyperplastic initial lymphatics in lymphedematous tail skin (supplementary material Fig. S1C). These results indicate that αSMA-expressing LECs emerge in lymphedematous conditions *in vivo*. Previous reports have demonstrated that αSMA-positive fibroblasts arise from BECs through EndMT and contribute to fibrosis (Zeisberg et al., 2007a; Zeisberg et al., 2007b). However, we did not observe EGFP-positive cells other than EGFP-positive LECs in lymphedematous tissue 3 weeks after experimental induction of lymphedema, suggesting that αSMA-positive LECs do not acquire fibroblast or mesenchymal cell-like features that cause fibrosis. However, these results suggest that a subset of LECs in lymphedematous skin is in the early phase of EndMT and their LEC characteristics are partially diminished. Therefore, we sought to identify similar cellular changes of LECs *in vitro*, to understand the heterogeneous LEC characteristics observed *in vivo*.

FGF2 is required for the maintenance of gene expression levels of endothelial cell markers in mLECs and inhibits EndMT of mLECs

We previously reported the use of an endothelial cell culture system from transgenic mice that express the SV40 tsA58T antigen in an endothelial cell lineage (Yamaguchi et al., 2008). tsA58T Ag-positive endothelial cells proliferate continuously and maintain endothelial cell properties at 33℃ without undergoing cell senescence. However, during our experiments designed to optimise the endothelial cell culture procedure, we found that immortalised mouse LECs (hereafter mLECs) survive and proliferate but cannot maintain their endothelial cell characteristics when cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal calf serum (FCS). In such conditions, these cells lost their endothelial cell marker expression and turned into αSMA-positive cells, suggesting that they might have undergone EndMT. However, the LEC characteristics were maintained when the cells were cultured in a commercially available medium optimised for primary ECs (an EGM2-MV Bullet Kit produced by Lonza), as described previously (Ichise et al., 2010; Ichise et al., 2012; Yamaguchi et al., 2008). These findings suggest that inhibitor(s) of EndMT might be present in the EGM2-MV complete medium (CM).

EGM2-MV CM consists of an EBM-2 basal medium (BM), FCS and defined supplements such as epidermal growth factor (EGF), FGF2, insulin-like growth factor 1 (IGF1) analogue, vascular endothelial growth factor (VEGF)-A, hydrocortisone and ascorbic acid. We hypothesised that the above growth supplements might be potential inhibitors of EndMT. To identify an EndMT-inhibiting factor present in the medium, we cultured mLECs in EGM2-MV CM, EBM2 BM or CM minus one of the supplements for 6 days, and then checked CD31 and αSMA expression by immunostaining. αSMA-positive cells and CD31-negative cells emerged among mLECs cultured in BM, whereas the cells remained positive for CD31 and negative for αSMA when cultured in CM (Fig. 2A). Also, we found that the appearance of αSMA-positive cells was induced by the depletion of FGF2, but not VEGF-A, EGF or IGF1

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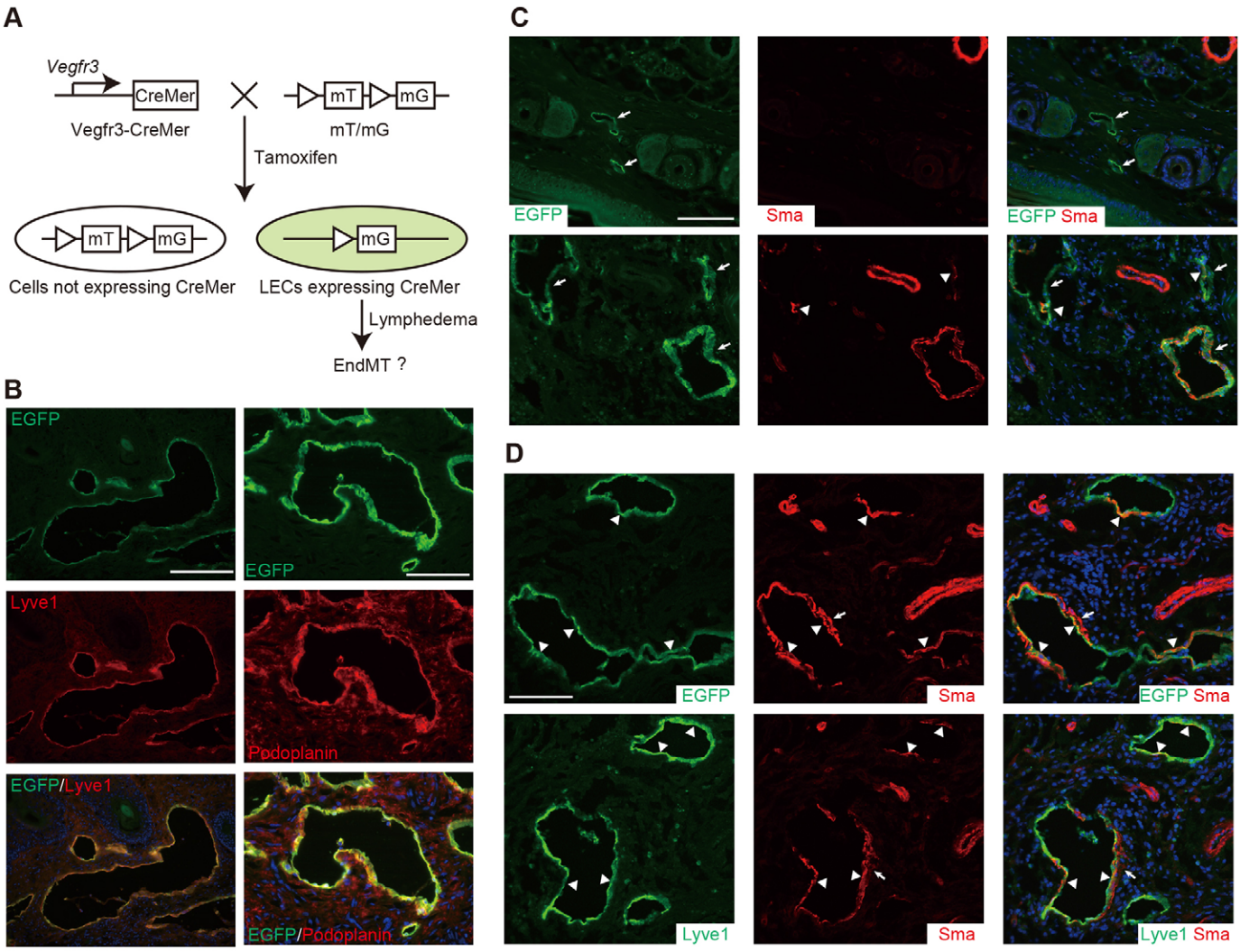

Fig. 1. αSMA-positive LECs are observed in experimental tail lymphedema *in vivo*. (A) A strategy for LEC fate tracing *in vivo*. CreMer is driven by endogenous *Vegfr3* gene, which is a lymphatic endothelial marker. Tamoxifen activates CreMer in LECs, leading to expression of membrane-bound EGFP (mG), instead of membrane-bound tdTomato (mT). We induced experimental tail lymphedema in mice expressing EGFP in LECs. (B) Transverse sections of lymphedematous tails of *mT/mG* and *Vegfr3-CreMer* knock-in mice following tamoxifen administration, immunostained for EGFP (green) and Lyve1 (red; left), or EGFP (green) and podoplanin (red; right), showing that LECs are labelled with EGFP. Representative microimages are shown. Scale bars: 200 μm (left) and 100 μm (right). (C) Transverse sections of normal and lymphedematous tails of *mT/mG* and *Vegfr3-CreMer* knock-in mice following tamoxifen administration, immunostained for EGFP (green, arrows) and αSMA (red). αSMA-positive lymphatic vessels (arrowheads) are obvious in lymphedematous tails (bottom), but not in normal tails (top). Representative microimages are shown. Scale bar: 100 μm. (D) Evaluation of αSMA-expressing LECs by confocal microscopy. Transverse sections of lymphedematous tails of *mT/mG* and *Vegfr3-CreMer* knock-in mice following tamoxifen administration, immunostained for EGFP (green) and αSMA (red, top panels) or Lyve1 (green) and αSMA (red, bottom panels). Arrows indicate αSMA-positive peri-lymphovascular smooth muscle cells and arrowheads indicate αSMA-expressing LECs. Representative microimages are shown. Scale bar: 100 μm.

analogue (Fig. 2A), and these cells showed a filamentous αSMA staining pattern (supplementary material Fig. S2). In the absence of FGF2, mRNA expression levels of lymphatic endothelial and endothelial markers (*Vegfr3* and *Lyve1* for lymphatic endothelial markers; *Vegfr2*, *VE-cad* and *Pecam1* for endothelial markers) were reduced, whereas the mesenchymal markers *Acta2*, encoding αSMA, and *Fn1* (fibronectin 1) were upregulated (Fig. 2B). The lymphangiogenic activity of FGF2 has been demonstrated *in vivo* and *in vitro* (Cao et al., 2012; Chang et al., 2004; Choi et al., 2012; Kazenwadel et al., 2012; Kubo et al., 2002; Matsuo et al., 2007; Pepper et al., 1994; Shin et al., 2006), but the role of FGF2 in EndMT of LECs has not been studied previously. Therefore, we focused on the anti-EndMT activity of FGF2 in LECs.

Having established that FGF2 is required to suppress EndMT, we next investigated whether FGF2 is sufficient for maintaining LEC characteristics. To address this, we cultured the cells in BM supplemented or not with FGF2 and examined EC marker expression. As shown in Fig. 3, FGF2 upregulated all of the EC markers examined, and suppressed αSMA and fibronectin expression (Fig. 3A,B). Compared with mLECs cultured in CM, EC marker expression was not fully recovered in mLECs cultured in FGF2-supplemented BM (supplementary material Fig. S3), implying that factors other than FGF2 might be involved in the induction of EC marker gene expression. However, the results suggest that FGF2 is the primary factor involved in maintaining endothelial cell marker expression and plays a unique role in the suppression of mesenchymal markers expression in mLECs.

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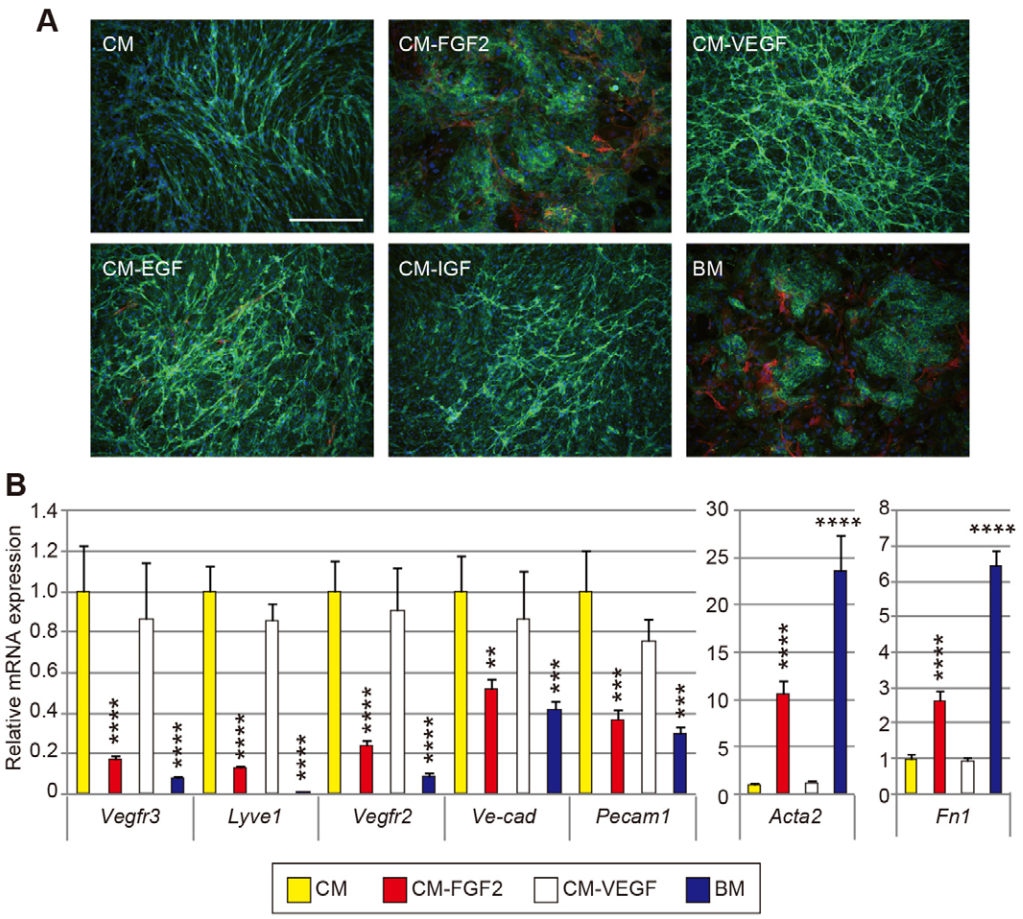

Fig. 2. FGF2 has an inhibitory role in EndMT of mLECs. (A) mLECs immunostained for Pecam1 (green) and αSMA (red). mLECs were cultured for 6 days in EGM-2MV complete medium (CM), EGM-2MV medium lacking FGF2 (CM-FGF2), VEGF (CM-VEGF), EGF (CM-EGF) or IGF1 analogue (CM-IGF), and EBM2 basal medium (BM). Representative microimages are shown. Scale bar: 400 μm. (B) Real-time RT-PCR assays for mRNA expression in mLECs cultured for 8 days in EGM-2MV CM, EGM-2MV medium lacking FGF2 (CM-FGF2) or VEGF-A (CM-VEGF), and EBM2 BM. *Vegfr3* and *Lyve1*, lymphatic endothelial markers; *Vegfr2*, *VE-cad* and *Pecam1*, endothelial markers; *Acta2* (αSMA) and *Fn1* (fibronectin), mesenchymal markers. Error bars represent s.d.; $n=3$. $^{**}P<0.01$, $^{***}P<0.005$, $^{****}P<0.001$ (versus mLECs cultured in CM).

Next, we confirmed that fibroblast growth factor receptors (FGFRs) are expressed in LECs at the mRNA level, as described previously (Cao et al., 2012; Choi et al., 2012; Kazenwadel et al., 2012; Matsuo et al., 2007; Shin et al., 2006). We checked FGFR expression by real-time RT-PCR, using mLECs cultured in EGM2, and pre-optimised TaqMan expression assays. The $\Delta\Delta Ct$ values for *Fgfr1*, *Fgfr2* and *Fgfr3* were 15.16±0.15, 21.53±0.40 and 22.34±0.16 (means ± s.d.), respectively, suggesting that the most abundantly expressed FGFR is FGFR1, followed by FGFR2 and FGFR3. We examined the effect of an FGFR kinase inhibitor,

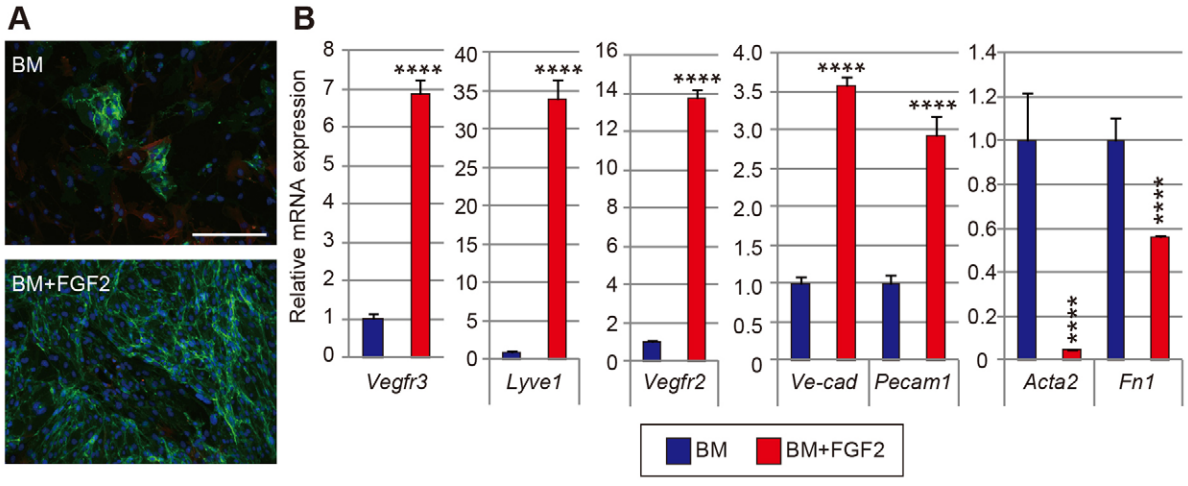

Fig. 3. FGF2 is required for inhibition of EndMT of mLECs. (A) mLECs immunostained for Pecam1 (green) and αSMA (red). mLECs were cultured for 6 days in EBM2 BM, EBM2 BM supplemented with FGF2 (BM+FGF2). Representative microimages are shown. Scale bar: 200 μm. (B) Real-time RT-PCR assays for mRNA expression in mLECs cultured for 10 days in EBM2 BM or EBM2 BM supplemented with FGF2 (BM+FGF2). *Vegfr3* and *Lyve1*, lymphatic endothelial markers; *Vegfr2*, *VE-cad* and *Pecam1*, endothelial markers; *Acta2* and *Fn1*, mesenchymal markers. Means of mRNA levels in mLECs cultured in BM were set to 1. Error bars represent s.d.; $n=3$. $^{****}P<0.001$ (versus mLECs cultured in BM).

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SU5402, which targets all FGFRs, to examine whether the kinase activities of FGFRs mediate the FGF2 signal in LECs. We found that the inhibitor led to the downregulation of endothelial marker gene expression and the upregulation of mesenchymal marker expression, not only in mLECs (supplementary material Fig. S4A), but also in human primary LECs (hereafter hLECs) (supplementary material Fig. S4B). Consistent with SU5402 treatment, knockdown of *Fgfr1* resulted in downregulation of *Vegfr3* and *Lyve1* in mLECs (supplementary material Fig. S4C). These results suggest that FGF2 directs endothelial marker gene expression and represses expression of mesenchymal marker genes through FGFR signalling in LECs.

FGF2 depletion and TGFβ1 stimulation synergistically induce EndMT of mLECs

Previous studies have demonstrated that TGFβ ligands play a pivotal role in inducing EndMT of BECs *in vivo* (Potts and Runyan, 1989) and *in vitro* (Arciniegas et al., 1992; Potts et al., 1991), implying that TGFβ is also involved in EndMT of LECs, in addition to its inhibitory roles in LEC proliferation, morphogenesis and motility, as described previously (Oka et al., 2008). To elucidate this possibility, we examined the effects of TGFβ as well as FGF2 on EndMT of mLECs. In CM supplemented with TGFβ, the appearance of αSMA-positive cells was not induced (Fig. 4A), but the gene expression of *Vegfr3*, *Lyve1* and *Vegfr2* was downregulated and *Acta2* and *Fn1* were upregulated (Fig. 4B), suggesting that TGFβ mediates EndMT processes. We further investigated whether FGF2 acts to inhibit EndMT-inducing TGFβ stimuli and found that, in FGF2-depleted CM supplemented with TGFβ, the appearance of αSMA-positive cells was induced (Fig. 4A) and the downregulation of endothelial cell marker genes and the upregulation of fibroblast and mesenchymal marker genes were significantly greater (Fig. 4B). These results indicate that FGF2 counteracts TGFβ during EndMT of LECs. To exclude the possibility that the above result was caused by proliferation of contaminating αSMA-positive cells during the 6–8 day culture experiments for EndMT, we performed an additional experiment using mLECs that were mitotically inactivated by 5000 rad X-ray irradiation. In these LECs, we found strong αSMA expression under EndMT-inducing culture conditions (Fig. 4C). These results suggest that the observed αSMA expression is due to upregulation of the *Acta2*

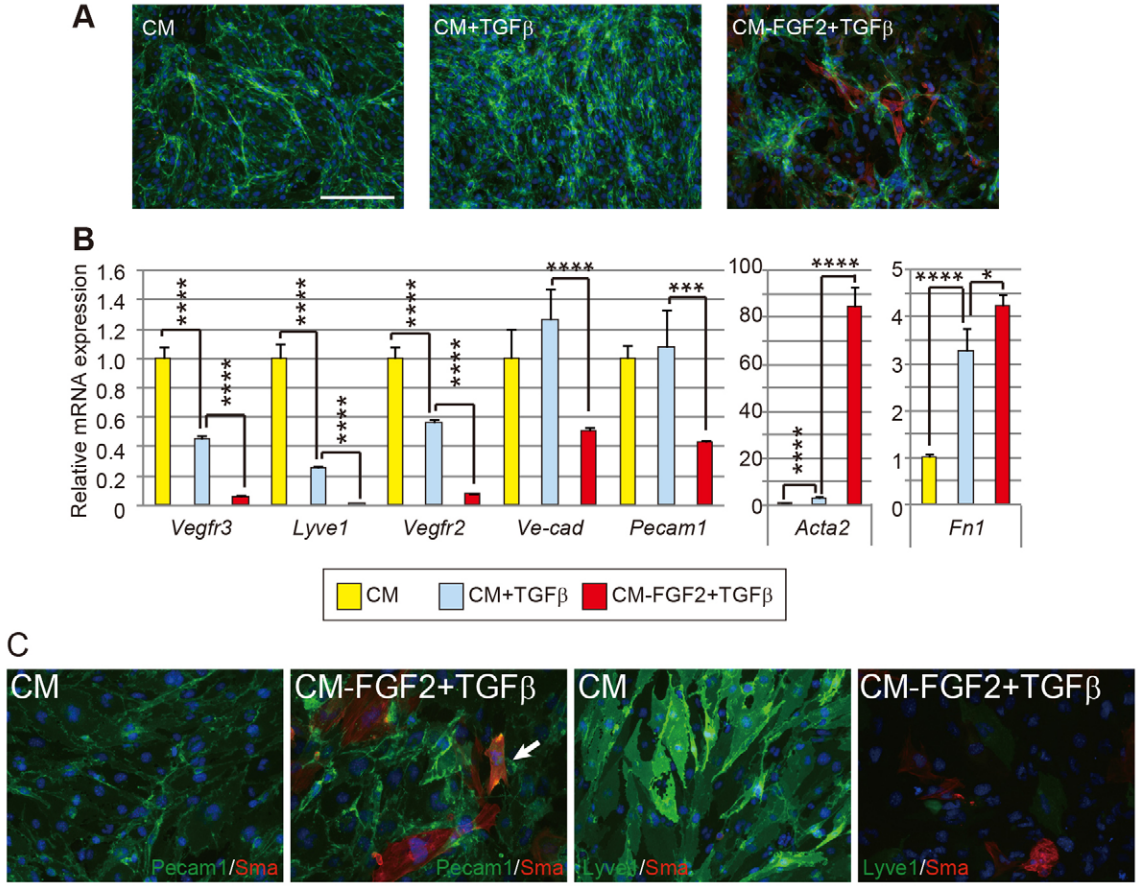

Fig. 4. FGF2 depletion and TGFβ1 stimulation synergistically induce EndMT of mLECs. (A) mLECs immunostained for Pecam1 (green) and αSMA (red). mLECs were cultured for 6 days in EGM-2MV medium (CM), EGM-2MV medium supplemented with TGFβ1 (CM+TGFβ) and FGF2-depleted EGM-2MV medium supplemented with TGFβ1 (CM-FGF2+TGFβ). Representative microimages are shown. Scale bar: 200 μm. (B) Real-time RT-PCR assays for mRNA expression in mLECs cultured for 4 days in EGM-2MV medium (CM), EGM-2MV medium supplemented with TGFβ1 (CM+TGFβ) and FGF2-depleted EGM-2MV medium supplemented with TGFβ1 (CM-FGF2+TGFβ). *Vegfr3* and *Lyve1*, lymphatic endothelial markers; *Vegfr2*, *VE-cad* and *Pecam1*, endothelial markers; *Acta2* and *Fn1*, mesenchymal markers. Error bars represent s.d.; $n=3$. *$P<0.05$, ***$P<0.005$, ****$P<0.001$. (C) Mitotically inactivated mLECs (5000 rad X-ray irradiation) immunostained for Pecam1 (green, first two panels), Lyve1 (green, second two panels) and αSMA (red). Cells were cultured for 4 days in EGM-2MV medium (CM) and FGF2-depleted EGM-2MV medium supplemented with TGFβ1 (CM-FGF2+TGFβ). Arrow indicates a Pecam1 and αSMA double-positive LEC. Representative microimages are shown.

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gene in LECs rather than due to contamination or proliferation of αSMA-expressing non-LECs.

H-Ras overexpression blocks EndMT by preventing the downregulation of endothelial markers caused by FGF2 depletion

We previously obtained and maintained H-Ras-overexpressing mLECs from transgenic mice that express wild-type H-Ras and tsA58T Ag in a subset of LECs (Ichise et al., 2010). Having established that H-Ras-overexpressing mLECs were highly resistant to EndMT during sub-culture, compared with control mLECs that do not overexpress H-Ras, we performed experiments using H-Ras-overexpressing cells to examine the role of Ras signalling in EndMT. We compared FGF2-induced MAPK phosphorylation and marker gene expression in H-Ras-overexpressing mLECs with control mLECs. Although sustained MAPK phosphorylation was significant in starved H-Ras-overexpressing mLECs, as described previously (Ichise et al., 2010), FGF2-induced MAPK phosphorylation levels were higher in H-Ras-overexpressing mLECs than in control mLECs, suggesting that overexpression of H-Ras amplifies the FGF2-driven MAPK signal (Fig. 5A). In the absence of FGF2, αSMA-expressing cells were observed within control mLECs, but not H-Ras-overexpressing mLECs, confirming our previous observation on the EndMT-resistance of those cells (Fig. 5B). Real-time RT-PCR analysis showed that mRNA levels of *Vegfr3*, *Lyve1* and *VE-cad* in H-Ras-overexpressing LECs were higher than those in control LECs when cultured in CM (Fig. 5C). Moreover, H-Ras-overexpressing LECs were resistant to EndMT induced by FGF2 depletion and TGFβ stimulation, as demonstrated by significant differences in gene expression levels in the two types of mLECs (Fig. 5C). Even when cultured in FGF2-depleted, TGFβ1-supplemented CM, endothelial marker expression in H-Ras-overexpressing mLECs remained as high as that in control mLECs cultured in CM, and

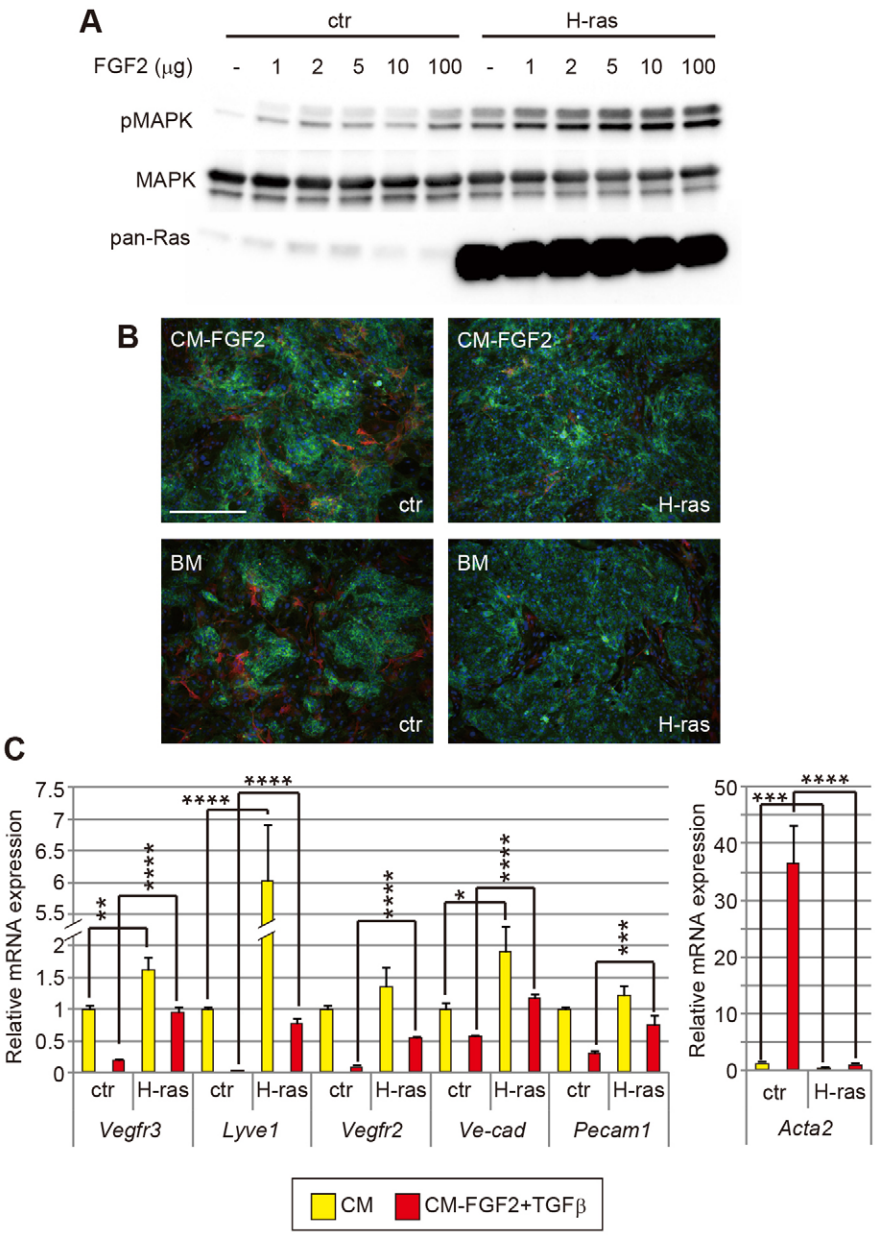

Fig. 5. H-Ras overexpression sustains FGF2-induced MAPK activation in mLECs and blocks FGF2 depletion-induced EndMT of mLECs. (A) Western blots showing MAPK phosphorylation in control and H-Ras-overexpressing mLECs stimulated by FGF2. pMAPK, phosphorylated MAPKs; MAPK, total MAPKs; pan-Ras, total Ras isoforms. (B) mLECs immunostained for Pecam1 (green) and αSMA (red). Control (ctr) and H-Ras-overexpressing (H-ras) mLECs were cultured for 6 days in EGM2-MV medium with (CM) or without FGF2 (CM-FGF). Micrographs for control mLECs in CM-FGF2 and BM (left panels) are the same as those shown in Fig. 1. Scale bar: 400 μm. (C) Real-time RT-PCR assays for mRNA expression in control and H-Ras-overexpressing mLECs cultured for 8 days in EGM-2MV (CM) or FGF2-depleted EGM-2MV supplemented with TGFβ1 (CM-FGF2+TGFβ). Error bars represent s.d.; $n=3$. *$P<0.05$, **$P<0.01$, ***$P<0.005$, ****$P<0.001$.

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αSMA expression was not induced (Fig. 5C). These results suggest that FGF2-induced, active Ras signalling counteracts the intracellular signalling that induces EndMT.

It is possible that, in Ras-overexpressing LECs, Ras-induced expression of FGF-ligand-encoding genes results in upregulated FGFR signalling in the absence of FGF2. Alternatively, Ras might downregulate TGFβ signalling in the presence of TGFβ1, by reducing the expression of TGFβ receptors on LECs. To examine these possibilities, we compared mRNA levels of *Fgf2*, *Tgfbr1* and *Tgfbr2* in control mLECs with those in H-Ras-overexpressing mLECs. We found significant, but contrasting changes in *Fgf2* and *Tgfbr1* levels that did not suggest cell-autonomous enhancement of FGFR signalling or suppression of TGFβ signalling (supplementary material Fig. S5). Although we could not exclude the possibility that one or more FGF-ligand-encoding genes other than *Fgf2* might be upregulated in H-Ras-overexpressing LECs, we postulated that Ras signalling might act downstream of the FGF2 signal and might interrupt TGFβ signalling without upregulating the expression of FGFs or downregulating the expression of TGFβRs.

Endogenous Ras proteins are involved in FGF2-induced MAPK signalling and maintenance of endothelial marker gene expression

To find out whether endogenous Ras proteins participate in cellular events involved in EndMT, we knocked down Ras expression in mLECs. Knockdown of Ras resulted in reduced phosphorylation of MAPKs in response to FGF2 (Fig. 6A) and downregulation of endothelial marker gene expression (Fig. 6B). Ras-knockdown LECs were affected so severely that they did not survive the 6 day culture period necessary for EndMT induction. Although a change in αSMA expression could not be observed, these results suggest that endogenous Ras proteins are involved in FGF-induced MAPK signalling and maintenance of endothelial marker gene expression.

H-Ras overexpression inhibits TGFβ-induced Smad2 activation

TGFβ ligands induce TGFβ type II receptor-mediated phosphorylation and activation of TGFβ type I receptors, leading to phosphorylation of receptor-regulated Smad proteins (R-Smads) at the C-termini. R-Smads that are phosphorylated at the C-terminus, together with the common mediator Smad4, act as transcription regulators of TGFβ ligand-induced responses (Goumans et al., 2002; Nakao et al., 1997).

As described above, we found no significant differences in the two types of LECs that might account for formation of FGF2−FGFR signalling circuits or downregulation of TGFβ receptor expression in H-Ras-overexpressing LECs. Therefore, we focused on the status of R-Smad activation and examined TGFβ1-induced phosphorylation of R-Smad proteins. Phosphorylation of Smad family members Smad2, Smad3 and Smad1/5 at the C-terminal region was induced in TGFβ1-stimulated, control and H-Ras-overexpressing LECs (Fig. 7A,B). However, we found that Smad2 is the only member that is differently phosphorylated between the two types of LECs examined. Smad2 was significantly less phosphorylated at the C-termini in TGFβ1-stimulated H-Ras-overexpressing LECs, whereas the linker region of Smad2 was more heavily phosphorylated at the basal level before TGFβ1 stimulation (Fig. 7A). To examine whether changes in Smad2 phosphorylation levels lead to changes in Smad2 function, we checked Smad-responsive gene expression in H-Ras-overexpressing LECs. *Snai1*, which encodes the EndMT-inducing transcription factor Snail (Kokudo et al., 2008), was upregulated in control mLECs cultured in FGF2-depleted, TGFβ1-containing EGM2-MV medium. By contrast, *Snai1* was expressed at a significantly lower level in H-Ras-overexpressing mLECs compared with control mLECs, and *Snai1* upregulation was not observed in H-Ras-overexpressing mLECs in the presence of TGFβ1 (Fig. 7C). These results suggest that the Ras signal negatively modulates Smad2-mediated gene expression by changing the phosphorylation levels of Smad2.

Ras inhibitory signal on Smad2 is mediated by Erk MAPKs

Ras and Erk MAPK-induced linker phosphorylation of Smad2/3 has been suggested to inhibit Smad2/3 function and lead to the silencing of TGFβ-driven gene expression in tumour cells

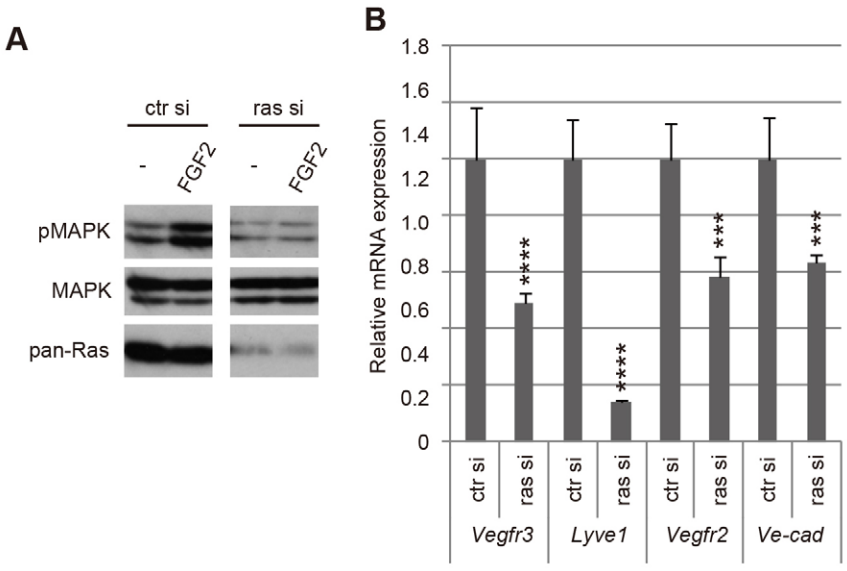

Fig. 6. Endogenous Ras proteins are involved in FGF2-induced MAPK signalling and maintenance of endothelial marker gene expression in mLECs. (A) Ras-mediated MAPK activation by FGF2 was impaired by the knockdown of Ras proteins. (B) Knockdown of Ras decreases the mRNA expression of vascular endothelial markers in mLECs in the EGM2-MV CM. Error bars represent s.d.; $n=3$. ***$P<0.005$, ****$P<0.001$ (versus mLECs transfected with control siRNA).

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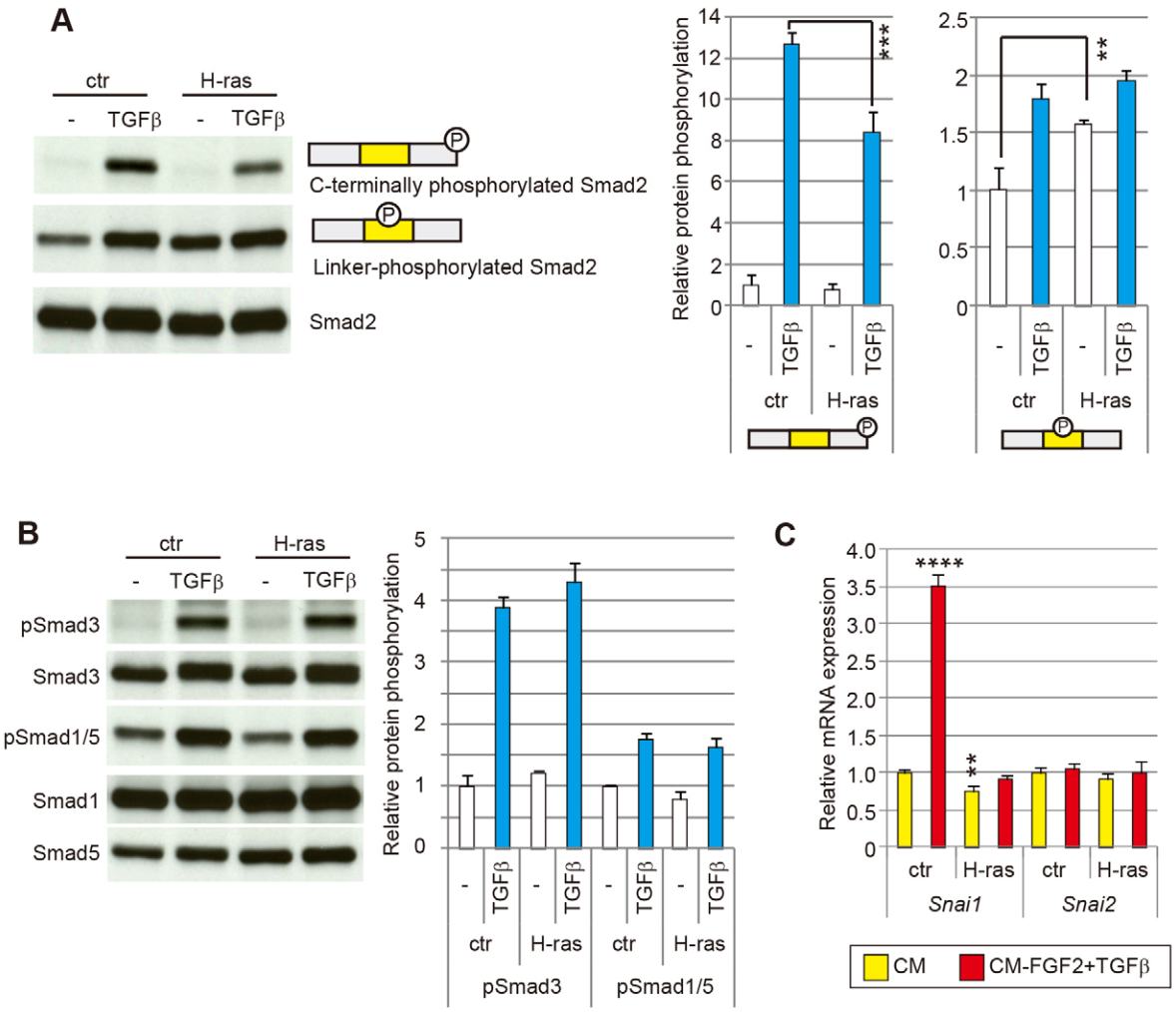

Fig. 7. H-Ras overexpression inhibits TGFβ1-induced Smad2 phosphorylation. (A) Western blots of C-terminally phosphorylated, linker phosphorylated and total Smad2 in control and H-Ras-overexpressing mLECs stimulated by TGFβ1, and quantitative analysis of western blots. Error bars s.d.; $n=3$. ***$P<0.005$ (versus TGFβ1-stimulated control mLECs), **$P<0.01$ (versus starved control mLECs). (B) Western blots of phosphorylated and total Smad1/3/5 in control and H-Ras-overexpressing mLECs stimulated with TGFβ1, and quantitative analysis of western blots. (C) Real-time RT-PCR assay for mRNA expression in control and H-Ras-overexpressing mLECs cultured in EGM2-MV CM and FGF2-depleted EGM2-MV CM supplemented with TGFβ1 (CM-FGF2+TGFβ). Error bars represent s.d.; $n=3$. **$P<0.01$, ****$P<0.001$ (versus control mLECs cultured in CM).

(Kretzschmar et al., 1999). To elucidate whether Erk MAPKs (MAPK3 and MAPK1) transduce Ras signal to Smad2 in LECs, we examined the effect of a MEK inhibitor U0126 on mLECs in the presence or absence of TGFβ1. Linker phosphorylation was reduced in U0126-treated mLECs in the absence of TGFβ1, and TGFβ1-induced C-terminal phosphorylation was higher (Fig. 8A). Consistent with the Smad2 phosphorylation, *Snai1* and *Snai2* gene expression was upregulated by MEK inhibition (Fig. 8B). We confirmed that TGFβ-driven transcription of *Snai1* was upregulated by a different MEK inhibitor, PD184352, in a dose-dependent manner (supplementary material Fig. S6). These results suggest that Erk MAPKs inhibit Smad2 transcriptional activity by phosphorylating the linker region of Smad2.

DISCUSSION

Taken together, our findings suggest that FGF2–Ras–MAPK signalling is responsible for the maintenance of LEC identity, not only by preserving EC-specific gene expression but also by suppressing Smad2 transcriptional activity by phosphorylation of the Smad2 linker region (Fig. 8C). FGF2 is a pleiotropic factor that induces proliferation, differentiation and motility of various types of cells that express multiple FGFR isoforms during embryonic development, wound healing, inflammation and tumorigenesis. FGF2 was also found as a lymphangiogenic factor *in vitro* and *in vivo* (Kubo et al., 2002; Pepper et al., 1994), but studying its roles in lymphangiogenesis is complicated by the fact that FGF2 stimulates various responses by many types of cell in and around sites of tissue remodelling. Two distinct mechanisms underlying FGF2-induced lymphangiogenesis have been suggested. One mechanism is that FGF2 directly activates FGFRs on LECs, leading to lymphangiogenic responses such as proliferation, migration and tube-like morphogenesis of LECs (Cao et al., 2012; Choi et al., 2012; Kazenwadel et al., 2012; Matsuo et al., 2007; Pepper et al., 1994; Shin et al., 2006). In addition, the use of high concentration of FGF2 in culture medium and FGF2 stimulation after starvation were associated with upregulation of pro-lymphangiogenic gene expression in LECs (Cao et al., 2012; Larrieu-Lahargue et al., 2012). A second

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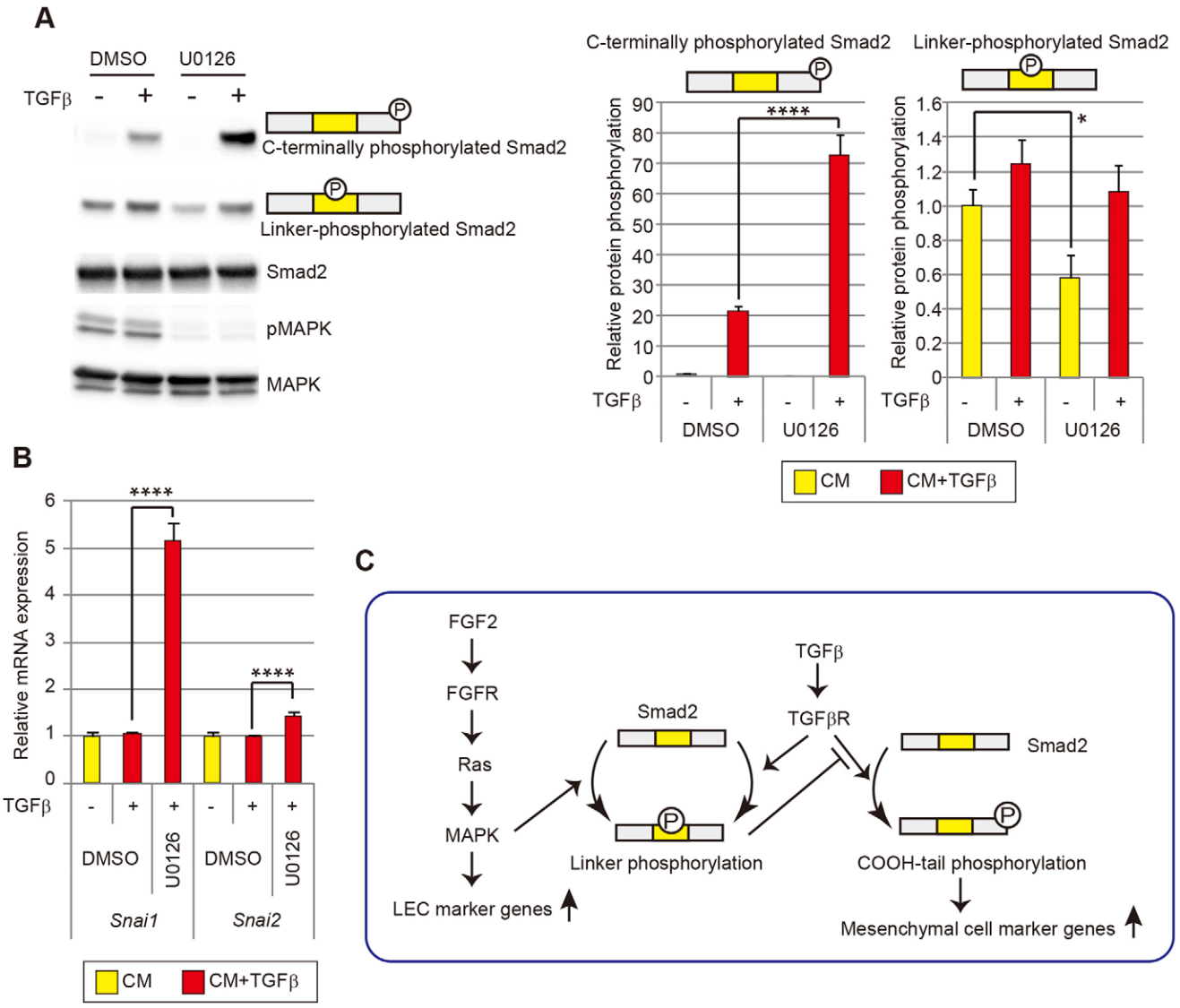

Fig. 8. MAPKs repress Smad2 activation by phosphorylating the Smad2 linker region in the absence of TGFβ1 and suppressing the TGFβ1-driven phosphorylation of Smad2 C-terminal region. (A) Western blots of Smad2 and MAPK phosphorylation in mLECs stimulated with TGFβ1 in the presence or absence of a MEK inhibitor U0126, and quantitative analysis of western blots. Error bars represent the s.d.; $n=3$. $^{*}P<0.05$, $^{****}P<0.001$. (B) Real-time RT-PCR assay for expression of *Snai1* and *Snai2* mRNA in mLECs cultured in EGM2-MV CM and TGFβ1-supplemented EGM2-MV (CM+TGFβ) in the presence or absence of U0126. Error bars represent the s.d.; $n=3$. $^{****}P<0.001$. (C) Schematic representation of the role of FGF2 in EndMT of LECs. In LECs, the FGF2–FGFR–Ras–MAPK pathway not only maintains endothelial marker gene expression, but also induces phosphorylation of the Smad2 linker, leading to suppression of TGFβ-induced Smad2 activation. FGF2 represses EndMT by maintaining endothelial marker gene expression and suppressing TGFβ–Smad2-induced activation of mesenchymal genes.

suggested mechanism is that FGF2 might induce expression and secretion of lymphangiogenic factors other than FGF2 from smooth muscle cells, BECs and perivascular cells, and then stimulate lymphangiogenesis in an indirect fashion (Bruyère et al., 2008; Chang et al., 2004; Kubo et al., 2002). In the present study, we showed that FGF2 directly stimulates LECs through FGFR signalling, which is consistent with previous findings that suggest a direct effect of FGF2 on LECs. However, FGF2-induced LEC responses were distinct from conventional FGFR-signal-induced cellular responses in proliferation, survival and motility. We found that FGF2 determines the LEC trait by maintaining LEC marker gene expression and preventing TGFβ–Smad2-dependent EndMT through Ras–MAPK signalling. These findings indicate that FGF2 indirectly changes the behaviour of LECs by regulating their characteristics. The recent findings of FGF-dependent maintenance of VEGFR2 expression in BECs (Murakami et al., 2011), and FGF2-mediated inhibition of the anti-angiogenic effect of a VEGFR kinase inhibitor (Welti et al., 2011) suggest that FGFR signals modulate VEGF–VEGFR2 signals by regulating VEGFR2 expression levels. Although we did not examine the role of VEGF-C in LECs in the presence or absence of FGFR signal, FGFR–Ras–MAPK-mediated transcriptional regulation of its receptor VEGFR-3 might change LEC responses to VEGF-C stimulation, suggesting a potential molecular mechanism of co-operative function of FGF2 and VEGF-C on LEC tips *in vivo* (Cao et al., 2012). Future *in vivo* studies are needed to determine how FGFR signalling contributes to lymphatic vessel regeneration and pathological lymphangiogenesis.

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Deissler and colleagues (Deissler et al., 2006) demonstrated that TGFβ induces αSMA expression and phenotypic alterations of immortalised bovine retinal microvascular endothelial cells (iBRECs). They also found that induction of αSMA expression was inhibited by FGF2, suggesting crosstalk between FGF2 and TGFβ signalling pathways in part during EndMT processes. However, the molecular mechanism of the crosstalk remains unknown. Our findings presented here might explain the underlying mechanism by which FGF2 counteracts the effect of TGFβ in iBRECs, and imply that EndMT of BECs might also be influenced by FGF signalling. A recent study showed that let-7 miRNA induced by FGFR signalling suppresses expression of TGFβ and TGFβR in human BECs and prevents TGFβ-dependent EndMT (Chen et al., 2012b). These findings are consistent with our study, with regard to the inhibitory role of FGF signalling in EndMT. Nevertheless, future studies are needed to address how let-7-dependent and Smad2-phosphorylation-dependent mechanisms integrate into FGF-mediated prevention of EndMT in different cell types and different cellular context.

A previous study has shown that oncogenic Ras suppresses TGFβ-induced Smad2/3 activation by MAPK-induced phosphorylation of the Smad2/3 linker region, and suggested a mechanism for the counterbalanced regulation of Smad2/3 by TGFβ and Ras signals in mitogenic cellular responses (Kretzschmar et al., 1999). However, the exact role of the counterbalanced regulation by these signals in cellular responses *in vivo* is unclear, and most studies have focused on and suggested a co-operative relationship between oncogenic Ras and epithelial-to-mesenchymal transition (EMT)-inducing TGFβ signals in tumour progression (Kretzschmar et al., 1999). Our study clearly shows the counterbalanced regulation of Smad2 by TGFβ, Ras and FGF2 signals and its importance in maintaining LEC identity. In contrast to mLECs, TGFβ-treated hLECs in the absence of FGF2 stopped proliferating and died (T.I., unpublished observation). The mLECs that we used express a weak-acting but mitogenic SV40 large T antigen that leads to continuous cell proliferation *in vitro*, suggesting that another signal that accelerates cell proliferation or cell survival is involved in the progression of EndMT of LECs. Alternatively, it is possible that environmental cues might be required for EndMT of human LECs, because KSHV-infected human LECs undergo EndMT when cultured in 3D, but not 2D, *in vitro* (Cheng et al., 2011). Future studies should address whether and how human LECs undergo EndMT. Currently, it is unclear how the MAPK-induced linker phosphorylation of Smad2 downregulates Smad2 activation, or why the activity of Smad2, but not Smad3, is suppressed by Ras–MAPK signalling in LECs. Intracellular localisation or degradation of Smad2 or its binding partner(s) might be involved in Ras–MAPK-induced Smad2 suppression in LECs. It would be interesting to examine the molecular mechanism underlying Smad2 function in LECs further.

Many studies have demonstrated that embryonic stem cells and induced pluripotent stem cells differentiate into ECs *in vitro*, and the stem-cell-derived ECs are viewed as potentially unlimited resources in regenerative medicine for the treatment of vascular diseases. However, stem-cell-derived ECs remain stable *in vitro* for only a short period of time, thus limiting the practical use of stem-cell-derived ECs in basic research, as well as in clinical research. Recently, a number of studies have demonstrated that TGFβ inhibition is highly effective in the generation and maintenance of phenotypically stable ESC-derived BECs and amniotic-cell-derived, directly reprogrammed BECs with large-scale expansion potential (Ginsberg et al., 2012; James et al., 2010). The results suggest a potential mechanism of a TGFβ-opposing signal for maintaining the cellular identity of differentiated ECs, in addition to EndMT, during developmental, physiological and pathological conditions. Although our study focused on LECs and it is unknown whether we can apply our findings to BECs at the present time, it would also be interesting to elucidate the roles of FGF2 and Ras–MAPK signals in maintaining differentiated BEC identity.

EndMT of BECs is part of the developmental process in cardiac valve formation (Markwald et al., 1977), and pathological processes implicated in fibrosis, tumorigenesis and heterotopic ossification (Medici et al., 2010; Zeisberg et al., 2007a; Zeisberg et al., 2007b). Little is known about lymphatic EndMT of LECs, except that a subset of Kaposi sarcoma cells, originated from KSHV-infected LECs, acquires invasive phenotype by EndMT in humans (Cheng et al., 2011). In our study, EGFP-labelled cell tracing did not show transdifferentiation of LECs into fibroblasts in the edematous skin 3 weeks after injury, suggesting that LECs do not undergo typical EndMT in such conditions or during such an experimental time period. However, Clavin and colleagues (Clavin et al., 2008), demonstrated that hyperplastic formation of αSMA-positive lymphatic vessels correlated positively with the severity of lymphedema, and TGFβ induced αSMA-positive lymphatic vessel formation. This finding suggests that TGFβ might induce the formation of lymphatic vessels with impaired lymphatic function, although it has also been described as an anti-lymphangiogenesis factor (Oka et al., 2008). The behaviour of αSMA-positive LECs in lymphatic vessels remains unknown, but we speculate that their altered cellular structure impairs the organised formation of lymphatic vessels with functionally specialised junctions (Baluk et al., 2007). Our findings, together with the findings of Clavin and co-workers, imply that FGF2-meditated maintenance of LEC characteristics might be important for functional lymphatic regeneration during tissue repair *in vivo*. Recent studies have reported that endothelial-cell-specific knockout mice for *Fgfr1* or *Smad2* are viable (Itoh et al., 2012; Zhao et al., 2012), suggesting that both genes, and their potential roles in lymphatic EndMT are dispensable for developmental lymphangiogenesis and lymphatic vessel function under physiological conditions. However, it would be important to examine whether these mice exhibit defects in lymphatic vessel regeneration and function during wound healing, and whether and how FGFR, Ras and TGFβ signalling contribute to lymphatic regeneration and function *in vivo*.

MATERIALS AND METHODS

Mice

C57BL/6J (CLEA Japan, Tokyo, Japan; Japan SLC, Shizuoka, Japan) and ROSA26-mT/mG knock-in mice (Muzumdar et al., 2007) (Stock No. 007676; The Jackson Laboratory, Bar Harbor, ME) were used. Generation of a new genetically engineered mouse strain is described below. All mice were housed under pathogen-free conditions. All mouse work conformed to guidelines approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Generation of *Vegfr3-CreMer* knock-in mice

We modified a targeting vector that was used previously for generating the *Vegfr3*-EGFP knock-in allele (Ichise et al., 2010). Briefly, an EGFP cDNA in the vector was replaced with a CreMer cDNA encoding a tamoxifen-inducible Cre recombinase (Zhang et al., 1996) (a gift from Dr Michael Reth, University of Freiburg, Germany). *Pac*I-digested,

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linearised vector (20 μg) was introduced into C57BL/6J×129/SvEv F1 male embryonic stem cells (ESCs). Mice were generated using two independent, correctly targeted ESC clones assessed by PCR genotyping. Prior to generation of chimeras, the neomycin-resistance gene was removed by transient expression of hFLPe in ESCs, using pxCAhFLPe (Kondo et al., 2009) (a gift from Drs Yumi Kanegae and Izumu Saito, University of Tokyo, Japan). The heterozygous mice were backcrossed at least six times to C57BL/6J mice before being used for this study.

Tamoxifen administration

Tamoxifen administration was performed according to a procedure described previously (Anastassiadis et al., 2010), with slight modifications. Briefly, tamoxifen (T5648; Sigma-Aldrich, St Louis, MO) dissolved in peanut oil (Sigma-Aldrich) containing 10% ethanol was administered to 4-week-old mice harbouring the Vegfr3-CreMer and Rosa26 mT/mG alleles once weekly for 5 weeks by oral gavage of 200 mg/kg, prior to inducing experimental lymphedema.

Mouse lymphedema model

We induced experimental lymphatic regeneration in the tails of mice, according to previously described procedures (Choi et al., 2012; Rutkowski et al., 2006). Briefly, we removed a circumferential 2-mm-wide piece of skin located ~1 cm distal of the tail base, and severed the deeper lymphatics running alongside the major blood vessels. Three weeks after skin removal, tails were surgically removed and processed for immunohistochemical analyses.

Immunohistochemistry

Mouse tails were dissected and fixed in 4% paraformaldehyde in PBS at 4℃ for 2 days. Fixed specimens were decalcified in PBS containing 0.5 M EDTA and 20% sucrose at 4℃ overnight or longer, and then processed for cryo-sectioning. 5–10 μm frozen sections were rehydrated in PBS and antigen retrieval was accomplished by incubation in PBS containing 0.1–0.25% trypsin and 0.5 mM EDTA at 37℃ or room temperature for 15 minutes. Prior to incubation with primary antibodies, all sections were incubated in PBS or methanol containing 3% H_2O_2 at room temperature for 15 minutes. A streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was also used with biotinylated antibodies. The following primary antibodies were used: rat anti-mouse Lyve1 (R&D Systems, Minneapolis, MN); rat anti-mouse CD31 (BD-Pharmingen, Franklin Lakes, NJ); rat anti-GFP (Nacalai-Tesque, Kyoto, Japan); biotinylated goat anti-mouse Lyve1 (R&D Systems); Cy3-conjugated mouse anti-human αSMA (Sigma-Aldrich) and hamster anti-mouse Podoplanin (BioLegend, San Diego, CA).

Sections were incubated with a primary antibody, followed by incubation with the Histofine reagent (Nichirei Biosciences, Tokyo, Japan). Prior to detection of the antibody complexes, sections were incubated with a biotinylated antibody for double immunostaining. Following detection of the first antibody complexes, sections were incubated with 3% H_2O_2 to quench the peroxidase activity of the Histofine reagent, and then incubated with streptavidin-HRP (NEN/Perkin-Elmer, Waltham, MA). The TSA PLUS HRP Detection System (NEN/Perkin-Elmer) was used for visualisation of immunostained sections. Fluorescence micrographs were acquired with a BioRevo BZ-9000 microscope (Keyence, Osaka, Japan) and A1 confocal laser microscope system (Nicon Instech, Tokyo, Japan). Micrographs shown in the figures are representative of two independently stained specimens from three or more mice. For immunocytochemistry, cells were fixed on ice with 4% PFA in PBS for 10 minutes, incubated in methanol at −20℃ for 20 minutes, and rehydrated in PBS. For visualisation of immunostained cells, the corresponding secondary antibodies labelled with Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes/Invitrogen) were used. Cy3-conjugated anti-αSMA was observed without using secondary antibodies. 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was used for nuclear staining.

For supplementary material Fig. S1A, frozen sections were immunostained with rabbit anti-GFP (MBL, Nagoya, Japan) and rat anti-mouse F4/80 (eBioscience, San Diego, CA), and visualised using the TSA PLUS HRP Detection System. For supplementary material Fig. S1B, 100 μm sections of fixed and decalcified tails were prepared using a vibrating microtome, and rat anti-GFP (Nacalai-Tesque) and rabbit anti-Prox1 (Acris Antibodies GmbH, Herford, Germany) were used for whole-mount immunostaining. For visualisation of immunostained cells, the corresponding fluorescently labelled secondary antibodies were used.

Cell culture

Immortalised mouse mesenteric LECs expressing the SV40 tsA58T antigen, referred to as mLECs, were described previously (Ichise et al., 2010; Yamaguchi et al., 2008). Cells were cultured in EGM-2MV medium (Lonza, Basel, Switzerland) on gelatinised culture dishes at 33℃ (a permissive temperature for the thermolabile tsA58T antigen) with 5% CO_2 in a humidified incubator. Human dermal microvascular LECs (hLECs) from pooled donors (Lonza) were cultured on gelatinised culture dishes using an EGM-2 MV bullet kit (Lonza) according to the manufacturer's protocol. The quality of the commercial human LECs was confirmed by immunocytochemistry for Prox1 (more than 99% were Prox1 positive). For Fig. 4C, mitotically inactivated mLECs were prepared by 5000 rad X-ray irradiation and used for analysis.

For experiments on the role of overexpressed H-Ras in mLECs (Fig. 5), cells were cultured in EBM-2 BM supplemented with 0.5% FCS at 33℃ for 16 hours. Prior to stimulation with growth factors, the medium was replaced with fresh EBM-2 with 0.5% FCS. Recombinant human FGF2 (Peprotech EC, London, UK) was added to the medium at final concentrations of 1–100 ng/ml. Cells were then incubated at 33℃ for 10 minutes before harvesting them for analysis. For experiments on the role of endogenous Ras in mLECs (Fig. 6), see knockdown experiments described below. For experiments on the role of overexpressed H-Ras in TGFβ signalling in mLECs (Fig. 7), cells were cultured in EBM-2 BM supplemented with 0.5% FCS at 33℃ for 16 hours. Prior to stimulation with growth factors, the medium was replaced with fresh EBM-2 with 0.5% FCS. Recombinant human TGFβ1 (Peprotech EC, London, UK) was added to the medium at final concentrations of 1 ng/ml. Cells were then incubated at 33℃ for 1 hour before harvesting them for analysis.

FGFR inhibitor SU5402 (Wako Pure Chemical Industries, Osaka, Japan), MEK inhibitor U0126 (Cell Signaling Technology, Danvers, MA) and PD184352 (Wako Pure Chemical Industries) were dissolved in DMSO and used for cell culture. DMSO was used for control experiments. For supplementary material Fig. S4, cells were cultured for 7 days (mLECs) or 8 days (hLECs) in EGM-2MV CM with DMSO or SU5402 (5 μM). For experiments on the role of Erk MAPKs in Smad2 activation, cells were cultured in the presence of 50 μM U0126 (Fig. 8) or 0.8 to 8 μM PD184352 (supplementary material Fig. S6) and 1 ng/ml TGFβ1 at 33℃ for 24 hours before harvesting them for analysis.

Quantitative real-time RT-PCR

Total RNA from LECs was obtained using TRIzol reagent (Invitrogen), and then treated with DNase I and purified. cDNA corresponding to 20 ng of total RNA was used for each reaction. Quantitative real-time RT-PCR was performed with the StepOne Real-time PCR system using a High-Capacity cDNA Reverse Transcription kit, TaqMan Gene Expression Master Mix, TaqMan Fast Advanced Master Mix, TaqMan Gene Expression Assays (*Flt4* (*Vegfr3*), Mm00433337_m1; *Lyve1*, Mm00475056_m1; *Kdr* (*Vegfr2*), Mm01222419_m1; *Cdh5* (*VEcadherin*), Mm00486938_m1; *Pecam1*, Mm01242584_m1; *Acta2*, Mm00725412_s1; *Fn1* (*Fibronectin*), Mm01256744_m1; *Snai1*, Mm00441533_g1; *Snai2*, Mm00441531_m1; *Fgfr1*, Mm00438930_m1; *Fgfr2*, Mm01269930_m1; *Fgfr3*, Mm00433294_m1; *Fgf2*, Mm00433287_m1; *Tgfbr1*, Mm00436964_m1; *Tgfbr2*, Mm00436977_m1; *FLT4*, Hs01047677_m1; *LYVE1*, Hs00272659_m1; *KDR*, Hs00911694_m1; *CDH5*, Hs00901463_m1, *PECAM1*, Hs00169777_m1; *ACTA2*, Hs00426835_g1) and TaqMan Endogenous control 18S rRNA (Applied Biosystems/Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Triplicates were run and analysed using the comparative *Ct* method, according to the manufacturer's protocol.

Western blot analysis

Cell lysates (20 μg) were resolved by SDS-PAGE, and blotted by semi-dry transfer onto a PVDF or nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Western blot analysis was performed using the following primary antibodies: mouse anti-Pan-Ras (Calbiochem/Merck, Darmstadt, Germany), rabbit anti-phosphorylated p42/44 MAPK, anti-p42/44 MAPK, anti-phosphorylated Smad2 (Ser465/467; 138D4), anti-phosphorylated Smad2 (Ser245/250/255), anti-phosphorylated Smad3 (Ser423/425; C25A9), anti-phosphorylated Smad1/5 (Ser463/465; 41D10), anti-Smad2 XP (D43B4), anti-Smad3 (C67H9), anti-Smad1 XP (D59D7) and anti-Smad5 (Cell Signaling Technology). The secondary antibodies used were anti-rabbit IgG (HRP) and anti-mouse IgG (HRP) (Cell Signaling Technology). For Figs 5, 6 and 7, signals were detected using ECL (GE Healthcare Bio-Sciences, Piscataway, NJ) and X-ray film (Fujifilm, Tokyo, Japan). For western blot analysis, three independent samples from the same experiment (triplicates) were separated on the same gel and transferred onto the same membrane. Western blot results shown in the figures are representative of triplicate experiments. For Fig. 7, western blot images were analysed using NIH ImageJ software, and quantitative analyses are represented graphically. Each western blot quantification analysis was performed by at least two independent experiments to confirm the reproducibility of the experiment. For Fig. 8, a ChemiDoc MP system (Bio-Rad Laboratories), Clarity Western ECL Substrate (Bio-Rad Laboratories) and ECL Prime (GE Healthcare Bio-Sciences) was used for chemiluminescence detection of western blots and image acquisition with data analysis and validation.

Knockdown experiments

Knockdown experiments were performed using Stealth RNAi siRNA duplexes, the corresponding Stealth RNAi Negative Control Duplexes and Lipofectamine RNAiMAX transfection reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions. *Fgfr1* Stealth Select RNAi siRNA (MSS274341) was used for *Fgfr1* knockdown. The sense strand sequences of the siRNA duplexes for *Hras*, *Nras* and *Kras* were described previously (Ichise et al., 2010). Cells were transfected with 20 nM siRNA duplexes for 24 hours and then cultured in EGM-2MV medium for 32 hours (for growth factor stimulation described below) or 48 hours (for gene expression analysis). For growth factor stimulation, cells were cultured in EBM-2 BM supplemented with 0.5% FCS at 33℃ for 16 hours. Prior to stimulation with growth factors, the medium was replaced with fresh EBM-2 with 0.5% FCS. Recombinant human FGF2 (Peprotech EC, London, UK) was added to the medium at a final concentration of 10 ng/ml. Cells were then incubated at 33℃ for 10 minutes before harvesting them for analysis. Cells cultured for 72 hours after transfection were used for analyses.

Statistics

Comparisons in this study were made using two-tailed paired Student *t*-tests with a significance level of $P \leq 0.05$.

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Author contributions

T.I. and H.I. conceived and designed the experiments, performed the experiments, analysed the data and wrote the paper. T.I., N.Y. and H.I contributed reagents, materials and analysis tools.

Competing interests

The authors declare no competing interests.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.137836/-/DC1

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