ORIGINAL ARTICLE

Smad-independent pathway involved in transforming growth factor β1-induced Nox4 expression and proliferation of endothelial cells

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Abstract NADPH oxidase-derived reactive oxygen species are important for various cellular functions, including proliferation. Endothelial cells predominantly express the Nox4 isoform of NADPH oxidase, but it is not entirely clear how it is regulated. In this study, we investigated the signalling pathways involved in transforming growth factor-β1 (TGF-β1)-induced Nox4 expression and the proliferation of human microvascular endothelial cells (HMECs). TGF-β1 stimulated Nox4 messenger RNA and protein expression in HMECs. TGF-β1-induced Nox4 also increased hydrogen peroxide production, which was inhibited by diphenyleneiodonium and EUK134. The acute treatment of HMECs with TGF-β1 enhanced the phosphorylation of Smad2 and extracellular signal-regulated kinase (ERK) 1/2, without affecting p38MAPK, Akt, or Jun N-terminal kinase 1/2 (JNK1/2) pathways. Further, inhibition of Smad2 signalling using an inhibitor of activin receptor-linked kinase 5 SB431542 reduced TGF-β1-induced Nox4 expression, while inhibition of ERK1/2 with the inhibitor of mitogen-activated protein kinase kinase 1/2 U0126 decreased both basal and TGF-β1-induced Nox4 expression. Inhibition of ERK1/2 phosphorylation with U0126 did not affect Smad2 phosphorylation. Finally, TGF-β1 enhanced endothelial cell proliferation, which was reduced by U0126 but not by SB431542. These findings suggest that the non-canonical pathway ERK1/2 regulates Nox4 expression and may be involved in TGF-β1-induced proliferation of endothelial cells, which is vital during angiogenesis and vascular development.

Keywords TGF-β1 · Nox4 · ERK1/2 · Endothelial cells · Proliferation

List of abbreviation

ALK	Activin receptor-like kinase
H₂O₂	Hydrogen peroxide
HMEC	Human dermal microvascular endothelial cells
Nox4	NADPH oxidase 4
ROS	Reactive oxygen species
TGF-β1	Transforming growth factor β1
MEK1/2	Mitogen-activated protein kinase kinase 1/2
ERK1/2	Extracellular signal-regulated kinase 1/2
JNK1/2	Jun N-terminal kinase 1/2

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Introduction

Transforming growth factor-β1 (TGF-β1) is a multifunctional growth factor that regulates many biological processes, such as embryonic development, cell proliferation, migration, extracellular matrix production and differentiation of a variety of cell types. These diverse TGF-β1 responses are regulated via activation of multiple downstream signalling pathways. The canonical pathway induced by TGF-β1 involves two

transmembrane serine/threonine kinase receptors types I and II. TGF-β1 is initiated by its binding to its type II receptor and the subsequent recruitment of the type I receptor, also known as activin receptor-like kinase 5 (ALK5). The activated ALK5 induces phosphorylation of Smad2/3, which then binds to the regulatory subunit Smad4. This complex translocates to the nucleus, where it regulates the transcription of a specific set of genes involved in cellular functions. In addition to ALK5, endothelial cells also express a type I receptor, known as ALK1, which induces phosphorylation of Smad1/5 (ten Dijke and Arthur 2007). Activation of TGF-β receptor signalling is followed by a less direct, and therefore slower, activation of other serine/threonine kinases, including the MAPKs, extracellular-signal regulated kinase 1/2 (ERK), Jun N-terminal kinase (JNK), p38 kinase, and phosphatidylinositol 3′-kinase (PI3K) Akt, (Zhang 2009; Heldin and Moustakas 2012)

TGF-β isoforms activates many regulatory pathways in cardiovascular physiology and disease. One of the major targets of TGF-β in the vasculature is endothelial cells (Goumans et al. 2009; Jakobsson and van Meeteren 2013). Disruption of TGF-β signalling in endothelial cells results in impaired vasculogenesis in the embryo (Goumans et al. 2009) and has consequences for postnatal angiogenesis (Pardali et al. 2010). In addition, TGF-β has been implicated in modulating endothelial cell proliferation, apoptosis, permeability and morphogenesis (Roberts and Sporn 1989; Myoken et al. 1990; Lee et al. 2007; Lu et al. 2009). There is evidence that TGF-β1 concentrations are elevated in the plasma of patients with risk factors for cardiovascular disease, including obesity and diabetes (Pfeiffer et al. 1996; Scaglione et al. 2003).

Interestingly, TGF-β1 has been found to stimulate the production of reactive oxygen species (ROS) in a variety of cell types, including endothelial cells, via activation of NADPH oxidases (Hu et al. 2005; Hecker et al. 2009; Carnesecchi et al. 2011; Martin-Garrido et al. 2011; Peshavariya et al. 2014), the only known enzymes whose sole function is to produce ROS. There are seven isoforms of the NADPH oxidase catalytic subunit (Nox1 to Nox5 and Duox1 and 2), among which Nox1, Nox2, Nox4, and Nox5 are expressed by human endothelial cells (Bedard and Krause 2007; Chan et al. 2009). Nox4 is unique in terms of its constitutive activity and because its major detectable product is hydrogen peroxide (H_2O_2) rather than superoxide (Takac et al. 2011). Recently, we and others have shown that TGF-β1 induces H_2O_2 formation via Nox4 in several types of vascular cells, including endothelial, vascular smooth muscle and fibroblast cells (Hecker et al. 2009; Carnesecchi et al. 2011; Martin-Garrido et al. 2011; Peshavariya et al. 2014). These studies also suggested that TGF-β-induced Nox4 expression in vascular cells occurs mainly via canonical activation of the Smad2/3-dependent pathway. However, the effect of TGF-β1-induced non-canonical pathways, such as MAPKs, and expression of Nox4 in endothelial cells remain unknown.

In the present study, we explored the regulation of Nox4 via non-canonical pathways in human microvascular endothelial cells. Previously, we and others have shown that Nox4 has an important role in serum and growth factor-induced endothelial cell proliferation and survival (Datla et al. 2007; Peshavariya et al. 2009; Craige et al. 2011; Schroder et al. 2012; Peshavariya et al. 2014). Therefore, we also investigated the effects of TGF-β1-induced non-canonical pathways on the proliferation of endothelial cells.

Materials and methods

Cell culture

The human microvascular endothelial cells (HMECs) used in this study were a kind gift from the Centre for Disease Control and Prevention, Atlanta, GA, USA. The HMECs were cultured in an EGM-MV bullet kit (hydrocortisone, gentamicin, amphotericin-B, bovine brain extract, human epidermal, human fibroblast growth factor-basic) with 15 % foetal bovine serum (FBS; Lonza, Victoria, Australia) in a 5 % CO_2 incubator at 37 °C.

Experimental setup

Unless otherwise specified, the cells were treated with TGF-β1 (10 ng/ml; Sigma-Aldrich, New South Wales, Australia) for 6 h before cell harvest. When the effects of the inhibitors were examined, the cells were pre-treated with ALK5 inhibitor SB431542 (10 μM; Sigma-Aldrich), MEK 1/2 inhibitor U0126 (10 μM, Sigma-Aldrich), superoxide/catalase mimetic EUK134 (25 μM; Cayman Chemical Company, Michigan, USA) and a flavoprotein such as NADPH oxidase inhibitor diphenyleneiodonium (DPI) (10 μM, Sigma-Aldrich) for 1 h before stimulating with TGF-β1 (10 ng/ml). In some cases, cells were treated with TGF-β1 (10 ng/ml) from 10 to 120 min.

Gene expression analysis

Endothelial cells (10^5 cells/well) were seeded in six-well plates and serum deprived overnight prior to conducting the experiments. RNA from the treated cells was extracted with the TRI reagent according to the manufacturer's instructions (Ambion, Austin, TX, USA). One hundred nanograms total RNA was reverse-transcribed to complementary DNA (cDNA) using TaqMan high-performance reverse transcription reagents (Applied Biosystems, Life Technologies, Victoria, Australia) at 25 °C for 10 min and at 37 °C for 2 h, followed by 85 °C for 5 s in a thermal cycler (BioRad-DNA Engine; Bio-Rad, New South Wales, Australia). Quantitative PCR was set up using 5 ng cDNA template from respective

samples, Taqman gene expression mastermix (Life Technologies, CA) and predesigned, gene-specific Nox4 (Hs01558199_m1) probes and primer sets. Water was used as no template control. Using StepOne Plus System (Life Technologies, CA) real-time PCR run was set up as follows: 2 min at 50 °C and then 10 min at 95 °C followed by 36 cycles of 15 s at 95 °C and 1 min at 60 °C. The cycle threshold (CT) values form all real-time PCR experiments were analysed using $^{\Delta\Delta}$CT method. The data were normalised to housekeeping gene GAPDH (human 4326317E) and expressed as fold changes over those in the control treatment group.

Western blot analysis

Cells (10^5 cells/well) were cultured in six-well plates, and protein was extracted as previously described (Peshavariya et al. 2014). Primary rabbit polyclonal anti-Nox4 (1:1000; Abcam), pSmad2 (1:1000; Calbiochem), total Smad (1:1000, Abcam), rabbit polyclonal pERK1/2 (1:1000; Cell Signalling), mouse monoclonal total ERK1/2 (1:1000; Cell Signalling), rabbit polyclonal pp38MAP (1:1000; Cell Signalling), mouse monoclonal total p38MAP (1:1000; Cell Signalling), rabbit polyclonal pJNK1/2 (1:1000; Cell Signalling), rabbit polyclonal total JNK1/2 (1:1000; Cell Signalling), rabbit polyclonal pAkt (1:1000; Cell Signalling), rabbit polyclonal total Akt (1:1000; Abcam), and mouse monoclonal β-actin (1:4000; Sigma-Aldrich) antibodies were used. Proteins were detected using an enhanced chemiluminescence detection kit (GE Healthcare, New South Wales, Australia) with horseradish peroxidase conjugated to the appropriate secondary antibodies (Bio-Rad). Gene Genius Imaging System from Syngene was used to capture the images.

Amplex red assay

Extracellular H_2O_2 levels were detected using an Amplex® Red assay kit (Molecular Probes, Life Technologies, Victoria, Australia) according to the manufacturer's instructions. Serum-deprived cells (10^4 cells/well) were seeded in a black 96-well plate and treated with and without TGF-β1 (10 ng/ml) for 6 h. Following the treatments, the cells were treated with phenol-free RPMI media containing Amplex® Red reagent (10 μM) and horseradish peroxidase (0.1 U/ml). Fluorescence was then measured with excitation and emission at 550 and 590 nm, respectively, using a Polarstar microplate reader (BMG Labtech, Germany) at 37 °C. Fluorescence values were normalised to cell numbers determined by Alamar® Blue cell viability assay according to the manufacturer's instructions (Life Technologies, Victoria, Australia).

Cell proliferation assay

Cell proliferation was measured by DNA content-based CyQUANT® NF Proliferation Assay. Serum-deprived cells (10^4 cells/well) were seeded in a 24-well plate and treated for 48 h with low serum containing (1 % FBS) EGM-MV media (hydrocortisone, bovine brain extract, human epidermal, human fibroblast growth factor basic) and TGF-β1 (10 ng/ml). Following the incubation, a CyQUANT® NF Proliferation Assay solution (250 μl) was added to each well and incubated for 1 h at 37 °C/5 % CO_2. Fluorescence was measured with excitation and emission wavelengths of 480 and 520 nm, respectively, using the Polarstar microplate reader, at 37 °C.

Results

### TGF-β1-induced Nox4 expression and activity in endothelial cells

We previously showed that TGF-β1-stimulated Nox4 expression in endothelial cells derived from different vascular beds (Peshavariya et al. 2014) and confirmed here that TGF-β1 increased the expression of Nox4 messenger RNA (mRNA) (Fig. 1a) and protein (Fig. 1b). Importantly, TGF-β1 also stimulated H_2O_2 production after Nox4 induction, and this was blocked by DPI (1 μM) and the superoxide/catalase mimetic EUK 134 (10 μM).

TGF-β1-induced canonical and non-canonical pathways in endothelial cells

One of the earliest events of TGF-β1 signalling is to activate canonical and non-canonical pathways. TGF-β1 induced the canonical pathway and enhanced the phosphorylation of Smad2 within 10 min of treatment, effects that were sustained up to 60 min without affecting total Smad2 levels (Fig. 2a). Similarly, TGF-β1 activated the non-canonical pathway and increased the phosphorylation of ERK1/2 within only 10 min of treatment, and these returned to basal levels within 30 min (Fig. 2b). However, TGF-β1 did not change phosphorylation of p38 MAPK, Akt, or JNK1/2. These findings suggest that TGF-β1 activates both Smad2 and ERK1/2 pathways in endothelial cells and both may be involved in the regulation of Nox4 expression.

ERK1/2 pathway regulates Nox4 expression in endothelial cells

In accordance with our previous study, inhibition of TGF-β1-activated canonical pathways using the ALK5 inhibitor reduced Smad2 phosphorylation and Nox4 expression in
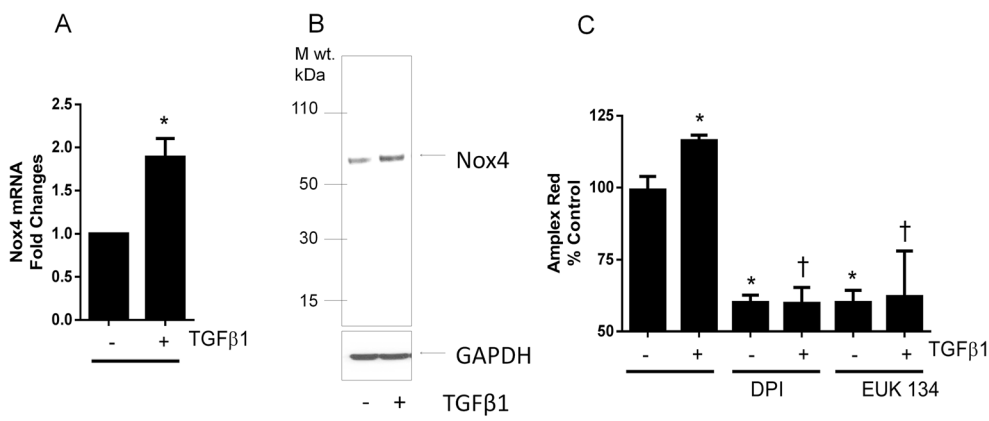
Fig. 1 TGFβ1 increases Nox4 mRNA and protein levels and H₂O₂ generation in HMECs. **a** TGFβ1 (10 ng/ml) induced Nox4 mRNA levels at 6 h in HMECs. **b** TGF-β1 (10 ng/ml) enhanced Nox4 protein levels compared to the control in HMECs, as shown in a representative Western blot. **c** Treatment of HMECs with TGFβ1 (10 ng/ml) induced H₂O₂ formation, which was blocked by the flavin inhibitor diphenyleneiodonium (DPI; 1 μM) and the superoxide /catalase mimetic EUK 34 (10 μM) at 6 h. All data are mean±SEM from three to five experiments. *$P<0.05$ from control (*Ctrl*) and *dagger* from TGFβ1

HMECs (Figure S1) (Peshavariya et al. 2014). Given that TGF-β1 is involved in the early activation of ERK1/2, we decided to test the effect of MEK1/2 inhibitor U0126 on Smad2 phosphorylation and Nox4 expression. U0126 reduced TGF-β1-induced phosphorylation of ERK1/2 without affecting the phosphorylation of Smad2 (Fig. 3a). Next, we examined the effect of U0126 on Nox4 expression and found that ERK1/2 inhibition decreased both the basal and TGF-β1-induced expression of Nox4 mRNA (Fig. 3b) and protein (Fig. 3c). Interestingly, the TGF-β1-induced Nox4 response was substantially lower but still present after the inhibition of

ERK1/2 (Fig. 3b, c). Thus, ERK1/2 activity is required for basal Nox4 expression in endothelial cells.
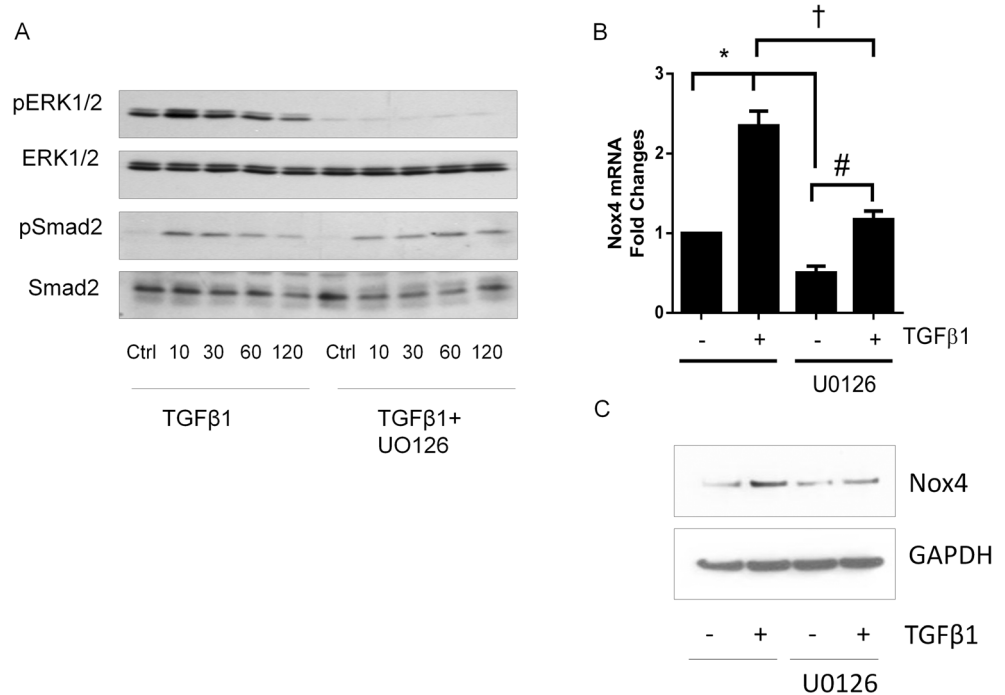
TGF-β1-induced ERK1/2 regulates proliferation of endothelial cells

Finally, we examined the effects of ALK5 inhibitor SB431542 and MEK1/2 inhibitor U0126 on TGF-β1-induced endothelial cell proliferation. The inhibition of canonical Smad2 signalling pathway using SB431542 did not inhibit either basal or TGF-β1-induced proliferation of HMECs (Fig. 4a). In

Fig. 2 Effect of TGFβ1 on cell signalling pathway in HMECs. Effects of TGFβ1 (10 ng/ml) on phosphorylation of Smad2 (**a**), ERK1/2 (**b**), P38 MAPK (**c**), Akt (**d**) and JNK1/2 (**e**). Quantitative densitometry data expressed in arbitrary unit (*AU*; measured by ImageJ software) are indicated by the *bar graphs* below. The combined density of ERK1 and ERK2 bands was taken for quantitation. Similarly, in case of JNK1 and JNK2, the combined density of top and bottom bands, respectively, was taken for quantitation. All data are mean± SEM. *$P<0.05$ from control (*Ctrl*)
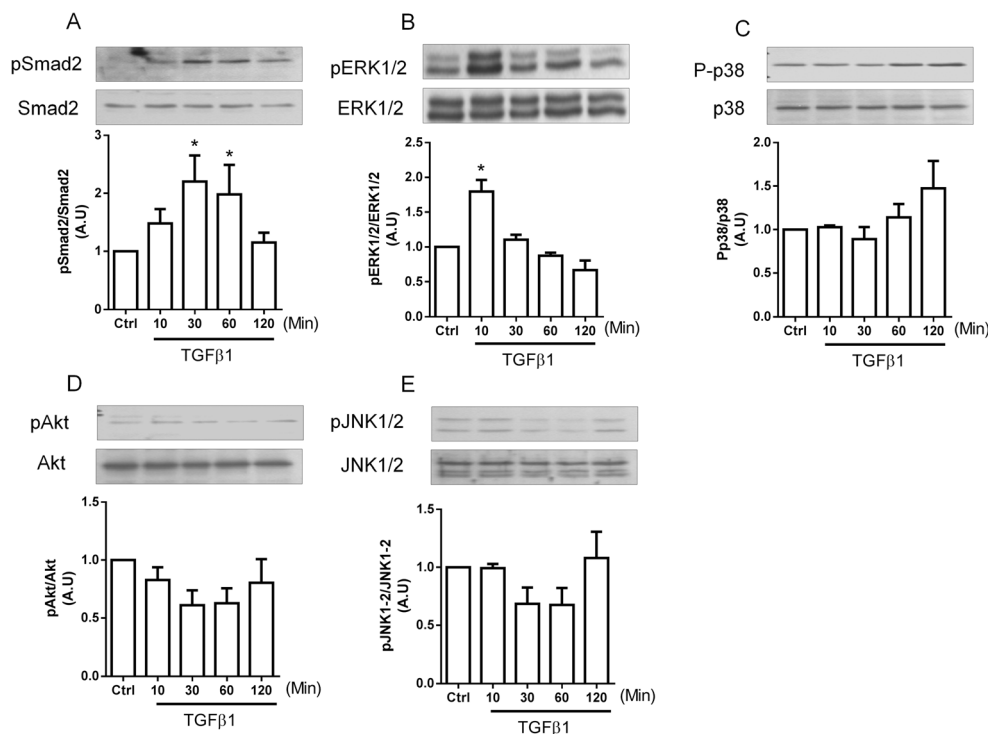
Fig. 3 TGFβ1stimulated ERK1/2 activation, which is required for Nox4 expression. **a** MEK1/2 inhibitor U0126 blocked phosphorylation of ERK1/2 without affecting Smad phosphorylation. Inhibition of ERK1/2 reduced both basal and TGFβ1 (10 ng/ml) induced Nox4 mRNA (**b**) and Nox4 Protein (**c**) expression in HMECs. All data are mean±SEM from three to five experiments, *$P<0.05$ from control (*Ctrl*) and *dagger* from TGFβ1 whereas *number sign* from U0126

contrast, U0126 decreased the TGF-β1-induced proliferation of HMECs (Fig. 4b). These findings suggest that TGF-β1-induced early activation of ERK1/2 signalling is required for endothelial cell proliferation.

Discussion

TGF-β1-induced signalling pathways are important for the regulation of several genes involved in endothelial cell functions, such as proliferation, wound healing and angiogenesis. In the present study, we showed that TGF-β1-induced

canonical Smad2 and non-canonical ERK1/2 pathways are both involved in regulation of Nox4 expression in microvascular endothelial cells. Activation of the TGF-β1-mediated non-canonical ERK1/2 pathway is crucial for endothelial cell proliferation.

It is well known that TGF-β1 mediates the activation of canonical and non-canonical pathways in a variety of cells, including endothelial cells (Vinals and Pouyssegur 2001; Lee and Blobe 2007; Zhang 2009; Joko et al. 2013). Interestingly, we observed in our study that the TGF-β1-activated phosphorylation of Smad2 occurred 10 min post-treatment and remain active for 1 h, whereas the phosphorylation of ERK1/2 reached its peak within 10 min and returned to basal
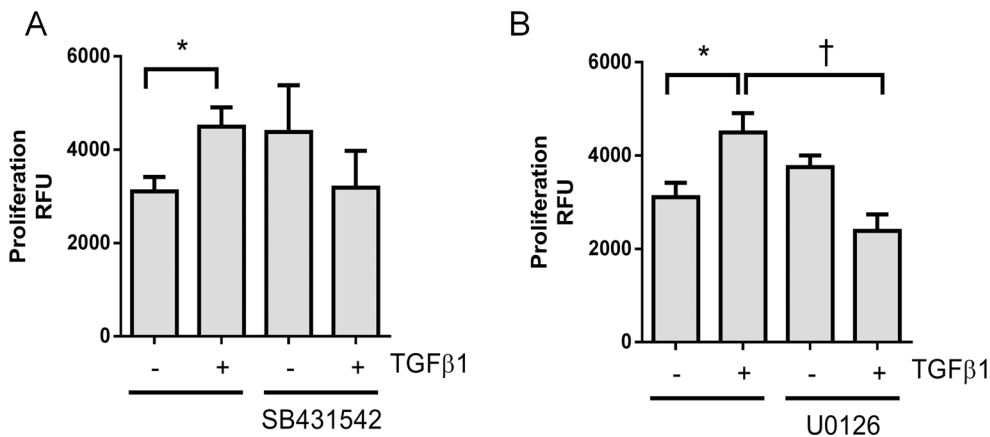
Fig. 4 ERK1/2 activation required for TGFβ1-stimulated HMEC proliferation TGFβ1 (10 ng/ml) increased the cell proliferation of HMECs measured by the cellular DNA content-based CyQUANT® NF proliferation method. **a** AKL5 inhibitor SB431542 (10 μM) does not inhibit TGFβ1-induced cell proliferation, whereas **b** inhibition of

ERK1/2 activation by UO126 (10 μM) suppresses TGFβ1-induced cell proliferation. Fluorescent value is expressed as RFU, and data are expressed as mean±SEM from four to eight experiments. *$P<0.05$ from control (*Ctrl*)

level by 30 min. In contrast, TGF-β1 did not activate the phosphorylation of p38 MAP, JNK1/2 or Akt up to 120 min. Early activation of the ERK1/2 pathway might prolong activation of Smad via phosphorylation at the linker site of Smad and enhance its stability (Kamato et al. 2013). However, we did not observe any destabilisation of pSmad2 in the presence of MEK1/2 inhibitor U0126 following TGF-β1 stimulation compared to untreated cells (Fig. 3a). In addition, it has been shown that ALK5 inhibitor SB431542 selectively inhibits phosphorylation of Smad2/3 without interfering in MAP kinase signalling pathways, such as ERK1/2 (Laping et al. 2002). These findings suggest that TGF-β1-mediated Smad2 and ERK1/2 signalling pathways are regulated independently in endothelial cells and that early activation of ERK1/2 does not interfere with Smad2 activity.

We and others have demonstrated that inhibition of the Smad2/3 signalling pathway using ALK5 inhibitor SB431542 decreased TGF-β1-induced Nox4 expression (Hecker et al. 2009; Peshavariya et al. 2014) but not expression at rest. Surprisingly, inhibition of MEK1/2 by U0126 decreased the basal expression of Nox4 and TGF-β1 had less of an effect on HMECs than untreated cells. It has also been demonstrated that TGF-β1 regulates basic fibroblast growth factor (bFGF) expression via ERK1/2-mediated induction of AP-1 binding to bFGF promoter in human lung fibroblasts (Finlay et al. 2000). A recent study also indicated that the human NOX4 promoter has close AP-1 to Smad binding sites and that deletions of either of these sites result in attenuation of TGF-β1-induced NOX4 promoter activity, suggesting that AP-1/Smad binding sites are required for Nox4 expression (Bai et al. 2014). It is plausible that AP-1 and Smad binding sites synergistically regulate NOX4 promoter activity in the presence of TGF-β1 and that inhibiting ERK1/2 may reduce AP-1 binding activity and basal and TGF-β1-induced Nox4 expression in endothelial cells. Our results are consistent with a previous report that indicated that oxidised low-density lipoprotein (OxLDL) required activation of the ERK1/2 pathway for Nox4 expression in human monocyte-derived macrophage and that inhibition of the ERK1/2 pathway reduced OxLDL-induced Nox4 expression in these cells (Lee et al. 2010).

TGF-β1 is a well-known inducer of Nox4 expression and TGF-β1-induced Nox4 has roles in a number of pathophysiological processes such as angiogenesis (Peshavariya et al. 2014), inflammation (Lee et al. 2010), cancer progression, (Sampson et al. 2011; Boudreau et al. 2012) and fibrosis (Hecker et al. 2009; Carnesecchi et al. 2011). These studies highlight that TGF-β1 induces Nox4 expression via Smad2/3-dependent canonical pathways, but the effects on cellular functions are highly cell context dependent. For instance TGF-β1-induced Smad2/3 and Nox4 are involved in the proliferation of lung fibroblast (Hecker et al. 2009) and pulmonary vascular smooth muscle (Sturrock et al. 2006) and endothelial cells (Peshavariya et al. 2014). On the other hand,

TGF-β1-induced endothelial cell proliferation is poorly understood. Several factors might influence the effect of TGF-β1-induced endothelial cell proliferation, such as culture conditions, expression level of endothelial-specific TGF-β type III receptor endoglin, origin of the endothelial cells, and activation of Smad-independent MAPK pathways. While in our study it appears that no involvement of the canonical Smad2 pathway using ALK5 inhibitor SB431542 on endothelial cell proliferation, MEK1/2 inhibitor U0126 abolished the TGF-β1-induced proliferation of HMECs. Consistent with this observation, Vinals et al. showed that in microvascular endothelial cells, TGF-β1 induced the autocrine secretion of TGF-α, a pro-survival and pro-angiogenic factor, via Smad-independent MAPK and PI3K pathways (Vinals and Pouyssegur 2001). It should be noted that ERK1/2 has a well-established role in cell proliferation and survival (Chambard et al. 2007; Mebratu and Tesfaigzi 2009). In addition, recent reports have suggested that induction of Nox4-derived H_2O_2 formation activates several pro-survival signalling activities, such as induction of HO-1(Schroder et al. 2012), VEGF (Zhang et al. 2010) and eNOS (Craige et al. 2011; Peshavariya et al. 2014) expression, and it regulates endothelial cell survival and angiogenesis. Indeed, we previously showed that TGF-β1 required Nox4 for in vitro and in vivo angiogenesis (Peshavariya et al. 2014). We have also illustrated the pro-apoptotic effect of TGF-β1-induced Nox4 in Human umbilical vein endothelial cells (HUVECs) and how it induced phenotypic changes to arterial endothelial cells, which are the main cells involved in angiogenesis (Yan et al. 2014). It has become evident that early activation of the pro-apoptotic mediators, such as caspase-3, caspase-8 and cytochrome C, are required for angiogenesis and inhibition of caspases reduces angiogenesis both in vitro and in vivo (Segura et al. 2002). Thus, it is likely that the Smad-independent ERK1/2 pathway regulates Nox4 expression and may be involved in the TGF-β1-induced proliferation of endothelial cells, which might have a significant impact on angiogenesis and vascular development. Understanding these novel TGF-β1-mediated canonical and non-canonical mechanisms is critical in the regulation of Nox4-depedent redox signalling and may have broader implications in endothelial function and the pathophysiology of cardiovascular diseases.

Conflicts of interest The authors declare no conflict of interest.

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