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Highlights

Endothelial selective deletion of Smad4 impairs angiogenesis in beige fat induction

Inhibition of angiogenesis due to Smad4 deletion attenuates beige fat induction

Free fatty acid activates Smad4 and enhances angiogenesis

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Smad4-mediated angiogenesis facilitates the beiging of white adipose tissue in mice

Chenguang Wang,1,7 Yalan Wu,1,7 Yangxian Li,1 Yang Zhang,2 Yee Lok Fung,1 Ka Kui Tong,1 Chi Wai Lau,1 Li Xiang,1 Kin Ming Kwan,3 Li-Ru You,4 Yu Huang,1,5,6,* and Xiao Yu Tian1,6,8,*

SUMMARY

Beige adipocytes are thermogenic with high expression of uncoupling protein 1 in the white adipose tissue (WAT), accompanied by angiogenesis. Previous studies showed that Smad4 is important for angiogenesis. Here we studied whether endothelial Smad4-mediated angiogenesis is involved in WAT beiging. Inducible knockout of endothelial cell (EC) selective Smad4 (Smad4iEC-KO) was achieved by using the Smad4iFlox/Flox and Tie2CreERT2 mice. Beige fat induction achieved by cold or adrenergic agonist, and angiogenesis were attenuated in WAT of Smad4iEC-KO mice, with the less proliferation of ECs and adipogenic precursors. RNA sequencing of human ECs showed that Smad4 is involved in angiogenesis-related pathways. Knockdown of Smad4 attenuated the upregulation of VEGFA, PDGFA, and angiogenesis in vitro. Treatment of human ECs with palmitic acid-induced Smad1/5 phosphorylation and the upregulation of core endothelial genes. Our study shows that endothelial Smad4 is involved in WAT beiging through angiogenesis and the expansion of adipose precursors into beige adipocytes.

INTRODUCTION

Obesity is a global health issue, which is a risk factor for diabetes, and cardiovascular diseases.1 There is a need to look for effective new therapies which help to burn fat, increase energy expenditure, and reduce adiposity. The white adipose tissue (WAT) not only stores energy in the form of lipids, but also responds to various environmental and endogenous signals, and subsequently modulates its metabolic activity. In response to cold temperature, hunger, or sympathetic stimulation, WAT releases fatty acids which can be oxidized to produce ATP and generate heat.1 As a result, WAT undergoes this process of beiging or browning characterized by multiple small lipid droplets and more mitochondrial content in the beige adipocytes, which is similar to brown adipose tissue (BAT). During this process, beige fat becomes metabolically active to generate heat through mostly uncoupling protein 1 (UCP1)-dependent mechanism,3–5 as well as UCP1-independent thermogenesis.6–8 This beiging response is important for thermoregulation against cold exposure but also serves as a potential therapeutic target for weight loss by increasing energy expenditure. Many adipocyte-derived intrinsic factors such as TGFβ family,9 as well as extrinsic factors such as immune cells and cytokines,10 have been identified to be involved in the beiging of WAT.

WAT contains different non-adipocyte cell types including endothelial cell (EC), pericyte, peripheral nerve, fibroblast, resident immune cell, and adipogenic precursor (AP). The highly vascularized WAT produces and releases vascular function-modulating growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and many others.11,12 Among them, VEGFA plays an essential role in mediating angiogenesis in WAT through its receptors VEGFR2 (KDR) to stimulate EC proliferation and migration.13 Previous studies showed that during WAT beiging, VEGFA-mediated angiogenesis helps remodel vasculature and facilitates nutrient exchange, while the inhibition of VEGFR2 signaling or VEGFA-mediated angiogenesis abolished WAT beiging in mice.14 Other angiogenic factors, such as angiopoietin-2 also have a similar effect on WAT beiging.15 In addition, deletion of transcription factor that are essential for endothelial cell function, such as Foxo1, also interferes with the VEGF signaling on metabolic homeostasis in adipose tissues.16 On the contrary, the overexpression or treatment with these angiogenic factors attenuates high fat diet-induced expansion of adipose tissue and subsequently improves insulin sensitivity by enhancing angiogenesis in mice.14,15 Apart from these factors, little is known about the regulation of angiogenesis during the induction of beige adipocytes.
TGFβ-Smad signaling is involved in developmental processes, particularly in the stabilization of vessel development. Members of the TGFβ superfamily, bone morphogenic proteins (BMPs), such as BMP4 and BMP7 are important for adipogenesis, while overexpression of BMP4 induces WAT beiging and stimulates angiogenesis. Both TGFβ and BMP use Smad2/3 and Smad1/5/8 signaling, respectively, but converge on Smad4, which then translocates to the nucleus to induce gene expression. Both global and endothelium-selective knockout of Smad4 are embryonically lethal due to developmental defects, particularly the abnormality of cardiovascular development. Mutation of Smad4 in human and mouse shows arteriovenous malformations in several vascular beds. These evidence support the important role of Smad4 in angiogenesis and vasculogenesis. However, whether Smad4 is equally important in angiogenesis within WAT and is therefore involved in beiging of WAT is still unknown.

RESULTS
Selective deletion of endothelial Smad4 attenuated beiging of white adipose tissue in mice
To examine the role of endothelium-specific Smad4, Tie2-Cre-mediated deletion of Smad4 allele in endothelial cells (ECs) in vivo was performed. Inducible CreERT2 was used because Smad4 plays a crucial role in Tie2-expressing endothelium during development. Successful knockout of Smad4 gene in ECs was induced by tamoxifen in the Smad4EC-KO compared to Smad4EC-WT mice. The expression of Smad4 was validated in several organs. In both aorta and lung tissues, diminished SMAD4 expression was observed in parallel with PECAM1 (CD31, as EC marker) in the Smad4EC-KO (Figure S1A (panel A and C)), compared to Smad4EC-WT mice (Figure S1A (panel B and D)). The CD31-positive endothelial cells collected from the lung of Smad4EC-KO also showed low expression of SMAD4 protein (Figure S1B). SMAD4 protein expression was also validated by immunofluorescence staining to co-localize with EC marker VE-cadherin in subcutaneous WAT (iSWAT) in the Smad4EC-WT and Smad4EC-KO mice (Figure S1C).

To reveal the role of endothelial Smad4 in the induction of beige fat, two models to induce beiging were used. Cold exposure at 6 °C for 4 days was used in mice. In the preliminary experiments, we used 4 °C cold exposure for 2 days. However, the survival of Smad4EC-KO mice was <50% (data not shown, about 4/9 died within 2 days), while all Smad4EC-WT mice survived. Due to animal ethics, 6 °C was used for cold exposure instead. Injection of CL316,243 (the β3-adrenergic receptor agonist) at 1 mg/kg/day for 10 days was used to induce beige fat as an alternative model. We observed a robust induction of UCP1 protein and mRNA in both models (Figures 1A–1C), which was attenuated significantly in sWAT from Smad4EC-KO mice (Figures 1A–1C, S2A, and S2B). In addition, another beige fat marker Tbx1 was also reduced in Smad4EC-KO mice (Figures 1D and 1E), although Tmem26 did not show a significant difference (Figures S2C and S2D). Histological analysis of sWAT showed typical beige fat morphology in both models of Smad4EC-WT mice with increased cytosolic content in smaller adipocytes (Figure 1F). Such change was attenuated in sWAT from Smad4EC-KO mice. Importantly, UCP1 immunohistochemistry showed less UCP1 expression in larger adipocytes in sWAT from Smad4EC-KO mice both after cold exposure and CL316,243 treatment (Figure 1G). In addition, no significant change was detected regarding UCP1 expression in either brown adipose tissue (BAT) or epididymal WAT (eWAT) from these Smad4EC-KO mice (Figures S2E–S2H).

Selective deletion of endothelial Smad4 inhibited angiogenesis during beiging
Smad4 is a transcription factor participating in EC function, especially vascular development and angiogenesis. We, therefore, wondered whether the impaired induction of beige fat is related to angiogenesis. First, we verified whether angiogenic genes increased during the process of beiging in wildtype C57BL/6 mice (Figures S3A and S3B). A time-dependent upregulation of genes responsible for angiogenesis, including Vegfa, the pro-angiogenic growth factor; its receptor and a marker of neovessel formation Vegfr2 in both cold exposed- and CL316243-treated mice (Figures S3C–S3F). No significant change at the mRNA level was detected for Pdgfa, which is another growth factor produced by ECs to promote both angiogenesis and AP proliferation (Figures S3G and S3H). Similarly, a moderate upregulation of VEGFR2 protein was detected accompanied by UCP1 upregulation in sWAT of mice after cold exposure, while little change was found with PDGFA level (Figures S3I and S3J).

Both cold- and CL316,243-induced upregulation of EC markers related to angiogenesis, including Vegfa (Figures 2A and 2B), Vegfr2 (Figures 2C and 2D), and Pdgfa (Figures 2E and 2F), were all attenuated in sWAT from Smad4EC-KO mice. We then performed whole-mount tissue staining for better observation of micro-vasculature in sWAT. In both models, the expression of CD31 as an EC marker and VEGFR2 as a marker for angiogenesis showed robust induction with higher densities of capillary structures
surrounding smaller adipocytes, which was also attenuated in Smad4<sup>iEC-KO</sup> mice (Figures 2 Ga n d2 H ). We s t -ern blotting also showed a similar reduced VEGFR2 expression in both models (Figure S4 A). To confirm the increase of capillary density and the change in adipocyte morphology, we also used BODIPY which stains lipid droplets, and CD31 to stain ECs together. The co-stain of CD31 with BODIPY showed increased capillary density with smaller lipid droplets in the sWAT from both the cold exposed and CL316,243 treated Smad4<sup>iEC-WT</sup> mice, which were attenuated in Smad4<sup>iEC-KO</sup> mice (Figures S4 B and S4C). Meanwhile, the morphology of arterioles indicated by α-SMA, was similar among all groups (Figures S4 D and S4E), suggesting that the remodeling of vasculature in sWAT in response to cold or beta3-adrenergic stimulation was mostly focused on capillary density. Meanwhile, the overall size and weight of sWAT, as well as

Figure 1. Endothelial selective deletion of Smad4 attenuated beiging of sWAT

Both Smad4<sup>iEC-WT</sup> (black) and Smad4<sup>iEC-KO</sup> (red) mice were either used for cold exposure at 6 °C vs 30 °C for 4 days or treated with CL316,243 at 1 mg/kg/day vs vehicle intraperitoneally for 10 days.

(A) Western blotting of UCP1 expression in sWAT. UCP1: 33 kDa; β-tubulin: 65 kDa. Note that additional samples were presented in Figures S2 A and S2B.

(B–E) Quantification of qRT-PCR showing Ucp1 and Tbx1 mRNA expressions in the sWAT. Data are mean ± SEM. *p < 0.05; **p < 0.01 between two groups.

(F) H&E staining of sWAT.

(G) Immunohistochemistry of UCP1 in sWAT. Scale bar = 50 μm for F-G. Images are representative of 4-5 mice from each group.
body weight, after beige fat induction, were similar between the two genotypes (Figures S5A–S5F), indicating that the overall reduction of lipid content in the beige fat was unaffected by the deletion of endothelial Smad4.

To provide a better quantification of EC and angiogenesis in the beige fat, we then profiled the stromal vascular fraction from sWAT using flow cytometric analysis. ECs were gated as viable CD45<sup>-</sup>/CD31<sup>+</sup>/CD144<sup>+</sup> cells (Figure S5G). Increased EC number in the sWAT from both models to induce beige fat in the Smad4<sup>iEC-WT</sup> mice, was attenuated in Smad4<sup>iEC-KO</sup> mice (Figures 3A and 3B). The increased EC number is probably due to increased proliferation as indicated by Ki67 staining, which showed that the increase of Ki67 signal was attenuated in ECs from sWAT of Smad4<sup>iEC-KO</sup> mice (Figures 3C and 3D, representative flow plots in Figure 3I). BrdU incorporation assay also showed a similar change as Ki67 in CL316,243-treated mice (Figure S5H).

Figure 2. Endothelial selective deletion of Smad4 inhibited angiogenesis induced during beiging
(A–F) Quantification of qRT-PCR showing mRNA expressions of genes related to angiogenesis in the sWAT of Smad4<sup>EC-WT</sup> (black) and Smad4<sup>EC-KO</sup> (red) mice. Data are mean ± SEM. *p < 0.05; **p < 0.01 between two groups indicated by the line.
(G and H) Whole mount immunofluorescence of VEGFR2 (green) co-stained with CD31 (red), merged with nucleus (blue) of sWAT for CL316,243 vs vehicle-treated mice (G), and mice housed at 6°C vs 30°C (H). Images were representative from 4 to 5 mice from each group. Scale bar = 100 μm.
In addition to angiogenesis, ECs also produce secretory factors to regulate other cell functions in the adipose tissue. PDGFA is one of the growth factors produced by ECs, which is regulated by the BMP-Smad signaling as showed in our previous study. PDGFA is the ligand for PDGFRα, which is highly expressed in

Figure 3. Endothelial selective deletion of Smad4 decreased the cell number and proliferation of ECs and APs in the beige sWAT from the Smad4EC-WT (black) and Smad4EC-KO (red) mice measured by flow cytometry.

(A and B) total EC cell count per fat in sWAT.
(C and D) % of Ki67-positive ECs out of all the ECs.
(E and F) total AP cell count per fat in sWAT.
(G and H) % of Ki67-positive ECs out of all the APs. Data are mean ± SEM. *p < 0.05 between two groups.
(I) representative flow plots of Ki67⁺ proportion in ECs of both models.
(J) representative flow plots of Ki67⁺ proportion in APs of both models.
Figure 4. Smad4-mediated angiogenesis enhanced by fatty acid in endothelial cells

(A) Fold change of genes related to angiogenesis in SMAD4-shRNA vs scramble-treated HUVECs, measured by RNA-seq. n=3 experiments.

(B–F) Quantification of qRT-PCR showing mRNA expressions in Scramble (black) and SMAD4-shRNA (red) treated HUVECs. PA: palmitic acid 0.2 mmol/L. Data are mean ± SEM. *p < 0.05; **p < 0.01 between two groups.
APs responding to beige adipocyte induction. We, therefore, asked whether Smad4 signaling-induced PDGFA expression also contributed to AP proliferation and commitment to beige adipocytes in the process of beige fat induction. By profiling the AP population in sWAT, which was gated as viable CD45-CD31-CD144-Sca1+PDGFRα+ cells (Figure S5G), we found that the deletion of endothelial Smad4 might have a trend to attenuate the increase of AP numbers (Figures 3E and 3F), and also possibly the proliferation of APs indicated by Ki67 (Figures 3G and 3H, representative flow plots in Figure 3J), in both models to induce beige sWAT. In addition, the sWAT from Smad4ECKO mice also had less proliferation of APs suggested by BrdU signal after CL316,243 treatment (Figure S5I).

Endothelial Smad4 facilitated angiogenesis in response to fatty acid and protein kinase C activation

Based on the in vivo evidence and previous publications showing that angiogenesis is important for beige fat induction, we first examined whether Smad4 is critical for angiogenesis. Knockdown of Smad4 was achieved in HUVECs by lentiviral SMAD4 shRNA. After transfection, cells were subjected to RNA sequencing, which showed that several key pathways related to angiogenesis were suppressed in SMAD4 knockdown ECs, including angiogenic growth factors and receptors related to VEGF signaling; ligands and receptors in the BMP/Notch signaling; and angiopoietin signaling (Figure 4A). The reduced expression of genes by Smad4 knockdown was further confirmed by qRT-PCR which showed that VEGFA and PDGFA were downregulated in SMAD4 knockdown HUVECs (Figures 4B–4F), whereas the overexpression of SMAD4 upregulated these genes (Figure S6A). The effect on genes that are related to angiogenesis is also consistent with the results from the tube formation assay showing that tube formation was inhibited in HUVECs with SMAD4 knockdown, which was improved with the addition of VEGF (Figure 4G). These results indicate the importance of Smad4 in angiogenesis.

Next, we attempted to dissect the signaling pathway leading to the activation of Smad4 and the subsequent downstream expression of angiogenic factors. BMP4 is one of the signals to activate Smad signaling, which is also involved in the beiging of WAT. We first detected the expression of BMP4 in the sWAT from both cold-exposed and CL316,243-treated mice, which did not show significant upregulation at the protein level, whereas Smad1/5 phosphorylation gradually increased with time in both models of beige fat induction (Figure S3I and S3J). We also measured other BMPs involved in adipogenesis at the mRNA level, including two other more abundant Bmp2 and Bmp7. Both genes were similar although Bmp7 showed a slightly increasing trend which was insignificant (Figures S3K and S3L). We, therefore, looked into the possibility of secretory factors which might stimulate Smad signaling, while also being produced during beige fat induction. During beiging, fatty acids were released and metabolized for heat generation or in response to sympathetic stimulation. We first profiled the circulating level of several fatty acid species, including both saturated and unsaturated fatty acids, which showed that both cold exposure and CL315,243 treatment resulted in an obvious reduction of free fatty acids, indicating increased consumption. Meanwhile, the levels were comparable between the two genotypes (Figures S7A–S7J).

We then asked whether one of the most abundant fatty acids, palmitic acid (PA) which is released into the bloodstream during beiging, could enhance angiogenesis. We first found that the expressions of angiogenic growth factors were upregulated by PA treatment (Figures 4B–4E). Tube formation was also enhanced by PA in control HUVECs (Figure 4G). Importantly, SMAD4 knockdown attenuated PA-induced upregulation of angiogenic genes (Figures 4B–4E), as well as tube formation (Figure 4F). Several core EC genes were also upregulated by PA at the protein level, including VEGFR2, CD144, and CD31 (Figure 4H). Likewise, unsaturated fatty acids, oleic acid, and linoleic acid (OA + LA) were able to upregulate core EC genes, such as VEGFR2 (Figures S6B–S6D), suggesting that fatty acids were able to increase angiogenesis.

To further dissect the signaling pathways leading to PA-induced Smad activation, we first showed that PA was able to activate Smad1/5 as showed by the increased phosphorylation of Smad1/5 transiently after the
addition of PA (Figure 4I). Meanwhile, the protein kinase C (PKC) signaling, which is known to be activated by fatty acids, also increased, especially the PKCα/β isoform (Figure 4J). We next examined whether PKC is upstream of Smad activation in response to PA. Western blotting showed that the co-treatment with a PKC inhibitor GF109203X attenuated Smad1/5 phosphorylation induced by PA (Figure 4K). GF109203X was also able to inhibit the phosphorylation of both PKCα/β and PKCδ induced by PA (Figure S6E). In addition, both GF109203X and another inhibitor Go9386 were able to attenuate PA-induced upregulation of CD144 and CD31 (Figure 4H). These results suggest that fatty acid released upon lipolysis activates PKC (most likely PKCα/β), which then enhanced Smad1/5 phosphorylation and Smad4 signaling to facilitate angiogenesis during the beiging of WAT.

DISCUSSION
In this study, we show that the selective deletion of Smad4 in endothelium in adult mice attenuates the induction of beige adipose tissue due to impaired angiogenesis, suggesting that endothelial Smad4 activation, and its downstream expression of angiogenic factors, are critical for the beiging of WAT. Using both cold exposure and treatment with β3-adrenergic agonist models in mice, we showed that the deletion of endothelial Smad4 diminished UCP1 upregulation and angiogenesis in mouse sWAT. We also showed that free fatty acid most likely from lipolysis of WAT is able to activate Smad1/5 and Smad4 signaling in ECs, suggesting the crosstalk between adipocytes and ECs.

The role of Smad4 and its upstream trigger TGFβ signaling was first studied in tumor angiogenesis. However, an opposite effect of Smad4 was also reported in some cancer cell models to suppress angiogenesis and cancer metastasis probably due to a complicated function of TGFβ and its effectors at different stages and different types of cancers. More recently, Smad4, was identified as one of the endothelial genes linked to arteriovenous malformation, which is the pathology underlying hereditary hemorrhagic telangiectasia, a clinical condition associated with endothelial misalignment and defective migration.

Knockout of Smad4 in endothelium induces arteriovenous shunt. Conversely, increased Smad4 expression is likely to be associated with pathological angiogenesis demonstrated by the neovascularization in oxygen-induced retinopathy. The role of ligands upstream of the Smad signaling, including mainly the TGFβ and BMP family, have been well studied in vascular development. TGFβ induces the expression of angiogenic genes such as VEGF and VEGFR1 through Smad2/3 and Smad4 in various cell types. A recent study showed that the deletion of ALK1, the receptor for BMP family ligand, induces arteriovenous malformation, through VEGFR2. The deletion of ALK1 ligands such as BMP10, results in vascular malformation and early death of zebrafish embryos. Other BMPs, such as BMP2 and BMP4, are known to regulate VEGF expression in ECs. Overall, the Smad signaling plays an important role in both developmental and pathological angiogenesis.

Angiogenesis in the adult adipose tissue is not well understood like in the other organs. Studies have demonstrated that the vascular niche is a source of progenitor cells that can be committed to preadipocytes, therefore, facilitating adipogenesis. Angiogenesis is essential for both the expansion of adipose tissue and the browning/beiging process under different conditions. However, in addition to VEGFA, the transcriptional regulation of angiogenesis during beiging is not well understood. Here we showed that, as a core factor controlling vascular development and pathological neovascularization, Smad4 is also important for angiogenesis of the WAT during beiging, suggesting the physiological importance of Smad4 especially in maintaining vascular homeostasis in response to the metabolic challenge at the adult stage.

In the present study, we found that the proliferation of AP, which is the source of preadipocytes is also attenuated by endothelial selective Smad4 knockout. In our previous study, we found that BMP4 and Smad control the expression of PDGFA produced by ECs. It is likely that PDGFA acts as a paracrine factor to stimulate the proliferation and commitment of APs toward beige adipocytes. However, we were not able to tell whether Smad4 is responsible for beige adipocytes originating from vascular cells or APs.

Based on the results showing an important role of endothelial Smad4 at the beiging of WAT, we also wondered how Smad4 was activated in the ECs. We found that Smad1/5 phosphorylation increased during sWAT beiging under cold exposure. However, we did not observe an obvious increase of ligands such BMPs. We then explored whether other factors released by adipocytes could be involved in activating Smad. After cold exposure and CL316,243 treatment, the plasma level of free fatty acids decreased due...
to its oxidation to generate heat. Fatty acids, liberating from lipoprotein-associated triglycerides stored in the lipid droplets from adipocytes, are released from WAT and transported through the endothelium. During this process, ECs are exposed to abundant amounts of fatty acids. We, therefore, explored whether fatty acids could activate Smad signaling. We found that palmitic acid (PA) can increase Smad1/5 phosphorylation in HUVECs. Meanwhile, PA also increases the phosphorylation of PKCa/β, which could be a direct or indirect effect of PA through ceramide. Interestingly, in our hands, pharmacological inhibition of PKC attenuated PA-induced upregulation of EC genes as well as the phosphorylation of Smad1/5 in HUVECs, suggesting that PKC might be induced by PA and is upstream of Smad activation, leading to the induction of angiogenic genes. However, the interaction between PKC and Smad has not been well characterized in EC, but mostly in other cell types, involving a different PKC subtype. In the present study, PKCa/β, but not PKCd is more likely to be induced by PA. In addition, we also tested other free fatty acid species including OA+LA, which are unsaturated fatty acids also detected in the plasma. OA+LA also upregulated endothelial genes, which were not suppressed by PKC inhibitors (Figure S6B). However, OA and LA also increase Smad1/5 phosphorylation (Figures S6C and S6D), suggesting that there might be other mechanisms involved in Smad activation. Further dissection of the mechanism, especially on the regulation of Smad by PKC, can be studied in future studies.

**Limitations of the study**

In addition to what was discussed above, there are also several limitations of the present study. Based on the available transgenic mouse strains we used, we were not able to distinguish between different EC subtypes, and the involvement of other Tie2-expressing cells such as macrophages, which are known to play an important role in metabolic homeostasis of adipose tissue. Although we analyzed the expression of some BMPs which showed no difference, it is possible that there may be other BMPs and TGFβ ligands involved or interaction of its receptors such as ALKs with other membrane or matrix proteins. Regarding the effect of fatty acid, we only measured the circulating fatty acid. However, it is uncertain whether the concentration is relevant to the ECs within sWAT under both cold and adrenergic agonist treatment conditions. The effect of Smad4-mediated angiogenesis in high-fat diet-induced obesity in mice could also be tested in the future. We will explore all these possibilities in future studies.

**STAR METHODS**

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Supplemental information can be found online at [https://doi.org/10.1016/j.isci.2023.106272](https://doi.org/10.1016/j.isci.2023.106272).

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REFERENCES


### KEY RESOURCES TABLE

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(Continued on next page)
Continued

**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
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Anti-PKCα antibody | Cell Signaling Technology | Catalog # 2056, RRID:AB_2284227
Anti-phospho-PKCα/βII antibody | Cell Signaling Technology | Catalog # 9375, RRID:AB_2284224
Anti-PKCδ antibody | Cell Signaling Technology | Catalog # 9616, RRID:AB_10949973
Anti-phospho-PKCδ antibody | Cell Signaling Technology | Catalog # 9374, RRID:AB_2168837
Anti-Smad1 antibody | Cell Signaling Technology | Catalog # 6944, RRID:AB_10858882
Anti-phospho-Smad1 antibody | Cell Signaling Technology | Catalog # 5753, RRID:AB_10706171
Anti-Smad1/5 antibody | Cell Signaling Technology | Catalog # 12534, RRID:AB_2797946
Anti-beta Actin antibody | Abcam | Catalog # ab8227, RRID:AB_2305186

**Chemicals, peptides, and recombinant proteins**

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<td>CL316,243</td>
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<td>collagenase I</td>
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<td>BrdU</td>
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<td>Go6983</td>
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<td>GF109203X</td>
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**Critical commercial assays**

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<td>LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation</td>
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<td>Smad 1/5/9 Antibody Sampler Kit</td>
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**Experimental models: Cell lines**

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<tr>
<th>Name</th>
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<td>Human umbilical vein endothelial cells</td>
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<td>HEK293FT cells</td>
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**Experimental models: Organisms/strains**

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<td>Smad4flx/flx</td>
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<tr>
<td>Tie2CreERT2tg/+ mice</td>
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**Oligonucleotides**

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<td>Human SMAD4 mRNA</td>
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Additional Primer sequences are provided in Table S1

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by lead contact, Xiao Yu Tian (xytian@cuhk.edu.hk).

**Materials availability**
No new materials were generated in this study.

**Data and code availability**
All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animal models**
All animal experimentations are in compliance with the approval from the Department of Health of Hong Kong Government, and the University Animal Experimentation Ethics Committee of Chinese University of Hong Kong. All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. All these mice were on a C57BL/6 background. Adult male mice (8–10 weeks old) were housed in a controlled environment (12-h light-dark cycle, 23 °C ± 1 °C) with ad libitum access to water and standard chow diet (LabDiet 5053). Inducible endothelium-selective deletion of Smad4 was achieved by a transgene Tie2-CreERT2-mediated deletion of the Smad4 allele, with the approach of crossbreeding the Tie2CreERT2tg/+ mice with the Smad4flox/flox (or Smad4f/f) mice. Genotyping of the Smad4f/f mice and Tie2CreERT2tg/+ mice was performed using a standard PCR to amplify genomic DNA extracted from mouse tail (genotyping primers in Table S1).46

The Smad4f/f;Tie2CreERT2tg/+ mice (or Smad4EC-KO) was used as the endothelium-selective Smad4 knockout; while the Smad4f/f mice (or Smad4EC-WT) were used as genetic controls. Smad4f/f mice and Tie2CreERT2tg/+ mice were generated as described previously.46 Intraperitoneal injection of tamoxifen (1mg/mouse, dissolved in sunflower oil, Sigma-Aldrich T5648) on five consecutive days was performed on 8-week-old mice for inducing the Cre activity of the mice. For cold exposure, 10-week-old male mice were housed at 6 °C (cold exposure) in a temperature-controlled chamber for 4 days, while the control mice were maintained at 30 °C (thermonuclear) in another chamber. For β3-adrenergic receptor agonist treatment, CL316,243 (1 mg/kg/day dissolved in saline, from Tocris Bioscience) was injected intraperitoneally for 10 days, while saline was used as the vehicle control.
METHOD DETAILS

Histological analysis
Inguinal adipose depot, which was used as the subcutaneous WAT (sWAT), was dissected immediately after the mice were euthanized by CO₂, and fixed in formalin for preparation of paraffin section at 5-μm thickness. Sections were dewaxed and rehydrated following standard procedures. Some sections were used for H&E staining. Some sections were treated with 10 mmol/L citric acid solution and boiled at 95 °C for 20 min for antigen retrieval, followed by incubation with 3% H₂O₂ to quench endogenous peroxidase for 15 min. Sections were then washed, blocked with normal goat serum (Abcam), and stained with anti-UCP1 antibody (Abcam, 1:200) for 2 h at room temperature, followed by incubation with goat anti-rabbit biotin-conjugated antibody, then with HRP. Sections were then stained with DAP (Dako) to develop positive signal, washed and counterstained with hematoxylin, and blued by Scott's tap water. Images were taken using upright Olympus IX83 microscope.

Whole mount immunofluorescence
Fresh sWAT was fixed overnight and cut with a scalpel into smaller slices. Following a protocol adopted from a previous report, the sWAT slices were incubated with 20 μg/mL proteinase K in 10 mmol/L Tris-HCl buffer for 5 min at room temperature, followed by methanol for 30 min, blocked with serum and stained with antibodies including: anti-CD31 (Abcam), anti-VEGFR2 (R&D system), anti-αSMA (Abcam), all at 1:200 dilution. After overnight incubation, donkey anti-rabbit AlexaFluor 546 and anti-goat AlexaFluor 488 secondary antibodies (ThermoFisher) were used. For visualising adipocytes, some slides were then incubated with BODIDY (1:200 from ThermoFisher) for 30 min. Images were taken by Olympus FV1200 confocal microscope.

Flow cytometric analysis
After euthanasia, sWAT was cut into smaller pieces and digested with enzymes cocktail containing collagenase I (450 U/mL), hyaluronidase (60 U/ml), DNAse (60 U/ml), F12 medium at 37 °C for 15 mins with shaking (200 rpm), then minced by gentleMACS™ dissociator, and incubated for 10 min at 37 °C with gentle shaking as previously described. The digest was ceased by adding an equal volume of FACS buffer (2 mM EDTA and 5% FBS in PBS), filtered through a 40 μm strainer, pelleted and resuspended to obtain the single-cell suspension of the stromal vascular fraction. The cells were then incubated with Live/Dead Aqua (Thermo, 1:1000), blocked by anti-CD16/32 antibody (1:500) for 30 min, followed by staining with different fluorochrome-conjugated antibodies including: FITC/BV605 anti-CD31 (BD), BV605 anti-CD45, PE-Cy7 anti-CD140a (PDGFRα), PerCP-Cy5.5 anti-Sca1, PE anti-VEGFR2 from BioLegend; APC anti-Ki67 from eBioScience. For analysis of Ki67 expression, after surface staining, the cells were treated with BD Cytofix/Cytoperm solution, followed by BD Perm/Wash buffer, and stained with anti-Ki67. Analysis was performed on BD LSR Fortessa or FACS Fusion (BD Biosciences) and data were analyzed using FlowJo (Tree Star, Inc.).

BrdU staining analyzed by flow cytometry
APC anti-BrdU was from eBioScience. BrdU (0.1 mg/g body weight, Sigma) was injected intraperitoneally 4 h before the mice were euthanized. For analysis of BrdU incorporation, after surface staining, the cells were treated with BD Cytofix/Cytoperm solution, followed by BD Perm/Wash buffer, and stained with anti-BrdU. Analysis was performed on BD LSR Fortessa or FACS Fusion (BD Biosciences) and data were analyzed using FlowJo (Tree Star, Inc.).

Cell culture and treatments
Human umbilical vein endothelial cells (HUVECs, from Lonza) were cultured in EGM2 (Lonza). Cell were used at the passage between 7-9. Palmitic acid (PA) (0.2 mmol/L) was prepared as previously described was used to treated HUVECs for 30 mins (in Figures 4H and 4K) or indicated time (as illustrated in Figures 4I and 4J). Linoleic Acid-Oleic Acid-Albumin (LA+OA) (Sigma) was added at indicated time within 48 h with concentration of 260 μmol/L (in Figure S6C) or for 12 h at indicated concentrations (as illustrated in Figure S6D). Equal volume/concentration of BSA was used as control. Go6983 (1 μmol/L) and GF109203X (5 μmol/L) were from Tocris. GF109203X (5 μmol/L) was added at 30 mins before PA or LA+OA treatment. For tube formation, HUVECs were plated on solidified growth factor reduced Matrigel (Corning) in EGM2 at 5 × 10⁴/well in 48-well plate for 12 h. Images were taken with upright phase contrast microscope (IX83, Olympus). For knockdown of SMAD4 in HUVECs, lentiviral PLKO.1 vector (GeneChem, Shanghai) carrying 4 shRNA sequences targeting human SMAD4 mRNA was co-transfected with psPAX2 and pMD2.G plasmid.
into HEK293FT cells. 48 h post-transfection, culture medium was collected for purification of recombinant lentivirus released from HEK293FT cells. Concentrated lentivirus was added to HUVECs at passage 4.

**Western blotting**

Proteins were prepared by homogenizing sWAT or cells in ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing PhosSTOP™ and cComplete™ (Roche, Switzerland) using TissueLyser LT (Qiagen, German). The protein sample were separated by SDS-PAGE, transferred onto PVDF membranes (Bio-Rad, USA), and then probed with primary antibodies followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Denmark). The protein bands were visualized with chemiluminescence substrates (Thermo, USA), captured by either by X-ray films or ChemiDoc™ MP imaging system (Bio-Rad, USA) and quantified by using the NIH ImageJ software. GAPDH or β-tubulin were used as the internal control. Primary antibodies include: anti-UCP1 (1:1000, Abcam, ab10983), anti-BMP4 (1:1000, Abcam, ab39973), anti-PDGFA (1:500, SantaCruz, sc-128), anti-VEGFR2 (1:500, R&D, AF644); anti-C1D144 (1:1000, VE-cadherin, R&D, MAB9381), anti-CD31 (Abcam, ab28364), GAPDH (1:5000, Thermofisher MA5-15738), β-tubulin (Thermofisher, MA5-16308), anti-PKCα (#2056), anti-phospho-PKCα/βII (#9375), anti-PKCδ (#9374), anti-Smad1 (#6944), phospho-Smad1 (#5753), Smad1/5 (12534), phospho-Smad1/5 (12656), (all 8 antibodies at 1:1000, from Cell Signaling Technology). Uncut blots and quantification are presented in Data S1.

**RNA extraction and real-time quantitative PCR**

Total RNA was extracted from tissues and cells with RNAiso Plus (Takara). High capacity cDNA reverse transcription kit (Thermo) was used for cDNA synthesis. The TB Green Premix Ex Taq II (Takara, Japan) was used to accurate detection the expression of target genes in intercalator-based real-time PCR. The real-time PCR was performed on Viia™ 7 System. Expression levels of all target genes were normalized with the β-actin gene for mouse tissue, and GAPDH for human cells respectively. Primers are listed in Table S1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All analyses were performed with GraphPad Prism 7 (GraphPad, San Diego, CA). Data were presented as means ± SEM of n separate experiments. Statistical significance was determined by one-way ANOVA and post-hoc Tukey test between two groups. Significance was indicated as *p < 0.05, **p < 0.01.