• Basic Research •

Role of *Smad4* from ocular surface ectoderm in retinal vasculature development

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Received: 2019-10-13 Accepted: 2019-12-09

Abstract
● **AIM:** To investigate how signals from lens regulate retinal vascular development and neovascularization.
● **METHODS:** Le-Cre transgenic mouse line was employed to inactivate *Smad4* in the surface ectoderm selectively. Standard histological and whole-mount retina staining were employed to reveal morphological changes of retinal vasculature in *Smad4* defective eye. cDNA microarray and subsequent analyses were conducted to investigate the molecular mechanism underlying the vascular phenotype. Quantitative polymerase chain reaction (qPCR) was carried out to verify the microarrays results.
● **RESULTS:** We found that inactivation of *Smad4* specifically on surface ectoderm leads to a variety of retinal vasculature anomalies. Microarray analyses and qPCR revealed that Sema3c, Sema3e, Nrp1, Tie1, Sox7, Sox17, and Sox18 are significantly affected in the knockout retinas at different developmental stages, suggesting that ocular surface ectoderm-derived Smad4 can signal to the retina and regulates various angiogenic signaling in the retina.
● **CONCLUSION:** Our data suggest that the cross-talk between ocular surface ectoderm and retina is important for retinal vasculature development, and *Smad4* regulates various signaling associated with sprouting angiogenesis, vascular remodeling and maturation in the retina of mice.
● **KEYWORDS:** *Smad4*; retinal vasculature; surface ectoderm

DOI:10.18240/ijo.2020.02.05

Citation: Li J, Zhang JS, Zhao JY, Han GG. Role of *Smad4* from ocular surface ectoderm in retinal vasculature development. *Int J Ophthalmol* 2020;13(2):231-238

INTRODUCTION
Retinal vascular abnormalities can cause severe vision impairment or even blindness. Many inherited and acquired retinal vascular abnormalities, such as Norrie disease (ND), familial exudative vitreoretinopathy, retinopathy of prematurity and diabetic retinopathy (DR), are the leading causes of blindness in the world[1-2]. The underlying cellular and molecular mechanism of pathogenesis of retinal vascular diseases remains unclear. The essential pathogenesis of these diseases include aberrant vascular development as well as pathological neovascularization, which subsequently leading to retinal detachment and visual impairment. Although current treatment strategies, such as retinal laser photocoagulation as well as intraocular injection of anti-vascular endothelial growth factor (VEGF) therapies, can relieve retinal neovascularization[3-4]. These treatments also have deleterious side effects, and many patients can not improve eyesight after treatment[5]. Therefore, researches on mechanisms of retinal angiogenesis may pave the way to more effective treatment strategies for retinal vascular diseases.

Numerous signaling pathways, including axon guidance pathway, transforming growth factor-β (TGF-β) signaling pathway, Wnt signaling pathway, Notch signaling pathway, and VEGF signaling pathway[6-12], are required for retinal vasculature development. Furthermore, signals from lens are essential for development of the eye especially retina[13-18]. However, how signals from lens regulate these signaling pathways in retinal vasculature development remains unclear. *Smad4* is a key intracellular effector of TGF-β superfamily. Loss of *Smad4* specifically on endothelial cells results in formation of retinal arteriovenous malformations, increased blood vessel diameters and reduced vascular outgrowth[19-20]. Our previous research has shown that inactivation of *Smad4* on surface ectoderm leads to microphthalmia, congenital cataracts, retinal folds and retinal detachment in mice[21]. As retinal

vascular abnormality is one of the most common etiology of retinal folds and retinal detachment. Therefore, we considered whether *Smad4* from surface ectoderm is required for retinal vascular development. Here we fully characterized the retinal vascular pathologies in mice when *Smad4* is knocked out only in the ocular surface ectoderm. Mechanistically, *Smad4* in the ocular surface ectoderm may regulates various angiogenic signaling in the retina, especially signaling of axon guidance pathway and Wnt pathways.

MATERIALS AND METHODS

Ethical Approval All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Le-Cre transgenic mice[22] were crossed with *Smad4*$^{flox/flox}$ mice[23] were to get Le-Cre; *Smad4*$^{flox/flox}$ (*Smad4*-cKO). The transgenic mice were kind gifts of Dr. Yi-Hsin Liu (University of Southern California, Los Angeles, USA). Generation of *Smad4*-cKO mice have been described previously[21].

Hematoxylin and Eosin Staining and Immunostaining Eyeballs were fixed with Davidson's fixative, then underwent dehydration and embedded in paraffin. Paraffin sections (4 μm) were processed for hematoxylin and eosin staining and immunohistochemical staining as previously described[21]. Primary antibody used was goat anti-CD31 antibody (sc-1506, Santa Cruz) and secondary antibody was donkey alexa 488-conjugated anti-goat IgG (A-32814, Life Technologies).

Whole-mount Retina Staining Eyeballs were fixed in 4% paraformaldehyde for 10min. Retinas were isolated by dissection, and fixed in 4% paraformaldehyde for 30min. After washed by PBS, the retinas were incubated in rabbit anti-collagen IV antibody (ab19808, abcam) at 4℃ overnight. Then retinas were incubated in the donkey alexa 555-conjugated anti-rabbit IgG (A-31572, Life Technologies) or FITC conjugated isolectin B4 (L 2895, Sigma-Aldrich, St. Louis, MO, USA) for 1h. TUNEL assay was performed by using Fluorescein *in situ* Cell Death Detection Kit (TMR red, Roche, Basel, Switzerland) .

Retinal vessel growth was evaluated by measuring the ratio of vascularized area to the whole retina. The capillary density, vascular branch points, vascular diameter, vessel ghosts and apoptosis cells were measured in a 100×100 μm^2 area using Image J software. Six to eight areas were analysed per retina, obtained from at least four independent animals of different litters.

Microarrays and Quantitative Polymerase Chain Reaction of Retinal Tissue Retinal RNA specimens of control and *Smad4*-cKO mice of P0 were used for microarrays, which was performed by China National Engineering Center for Biochip at Shanghai. Pathway analysis and gene ontology analysis were conducted subsequently. Quantitative polymerase chain reaction (qPCR) was performed by using SYBR Premix Ex TaqTM II (Takara, Dalian, China). The sequences of real-time qPCR primers are listed in Table 1.

Statistical Analyses Statistical evaluations between control and *Smad4*-cKO samples were performed using the unpaired Student's *t*-test (two-tailed).

RESULTS

Conditional Inactivation of *Smad4* on Surface Ectoderm Affects Retinal Sprouting Angiogenesis Le-Cre transgenic mouse line and *Smad4*$^{flox/flox}$ mouse line was used to inactivate *Smad4* in the surface ectoderm selectively. The efficiency of *Smad4* knockout was confirmed in our previous studies[21]. We also found that *Smad4*-cKO mice developed microphthalmia, congenital cataracts, retinal detachment and retinal folds in our previous studies. As retinal detachment and retinal folds were observed in most retinas of adult cKO mice. And retinal vascular abnormality is one of the most common etiology of secondary retinal folds and retinal detachment. Hence, we further explored the retinal angiogenesis in the cKO mice.

After birth, retinal vessels grow radially from the optic disk and reach peripheral retina in mice by P10. Postnatal *Smad4*-cKO mice displayed significantly accelerated vascular sprouting compared with the wide type (WT) mice (Figure 1A-1F and 1K). At P8, the superficial retinal vasculature extends to inner and outer plexiform layers to form the two deeper capillary networks in mice. The vascular migration into the inner retina was not affected in the *Smad4*-cKO mice at P9 (Figure 1G-1J).

Conditional Inactivation of *Smad4* on Surface Ectoderm Results in Defective Retinal Vascular Remodeling and Maturation At P18, the morphology of vascular plexus in the superficial, intermediate and deep layers of retinal vasculature can clearly be distinguished. Retinal flat-mounts showed modestly less vascular coverage in the superficial, intermediate and deep layers of retinal vasculature, and slightly fewer branching points in the superficial vascular plexus in the *Smad4*-cKO mice compared with the controls at P18 (Figure 2A, 2C and 2D). The number of arteries and veins were decreased in *Smad4*-cKO than in WT mice retinas at P18, and was still apparent in adult retinas (Figure 3A, 3E and 3I). The widths of retinal arterioles and venules were increased in the *Smad4*-cKO mice than that of control, but the ratio of arteriole/venule width showed no difference at P18 (Figure 2B, 2E and 2F).

We subsequently demonstrated a loss of endothelial cells by co-staining these cells (Isolectin IB4) with the basement membrane (collagen IV). In the *Smad4* defective mice, abundant vessel ghosts were observed at P18, and there was significantly more increase in the number of vessel ghosts compared to the controls at 1mo (Figure 3C, 3G and 3J).

Table 1 Primary sequences used for real-time PCR

Genes	Sense (5'- 3')	Antisense (5'- 3')
Dll4	GAAAGGCTCTGGAGCAAGCA	ACGTGAGACATTACCTGCGG
Notch1	CTTGCCAGGTTTTGCTGGAC	CTTTGCCGTTGACAGGGTTG
VEGFA	GCAGCGACAAGGCAGACTAT	AAGAGCCCAGAAGTTGGACG
Sema3a	GCAGTATAATGAGCATGGACTGT	CACCCCAGAAAAGACAGGCA
Sema3c	GAAGGCCTCTGATAGTCCGC	GGGAAACCCCCTCATTGGAG
Sema3d	GTTGCTAGCAGGAAGGGTGA	GCTCCAGTTCCACACACGTA
Sema3e	TGTGCGTGAATGACATGGGA	GATGGCGTCATCGGGGTAAT
Plxnd1	CGAGAGCAAGCGCAACATAC	ACCAACCACAGTAGGCATCG
Nrp1	TGGCACAGGTGATGACTTCC	CATGAGAGCCGGACATGTGA
Bmp9	ATTACGGCTCCAGCTCATAGTG	CCCTTGGCCTACTCACCAAT
Bmp10	TGACCCTTTGCTGGTTGTGT	TTCATACCCAGGAGGAGCGA
Tgfb1	CACTCCCGTGGCTTCTAGTG	GCGGGTGACCTCTTTAGCAT
Tgfbr2	GATGCATCCATCCACCTAAGC	AGAAGCGGCATCTTCCAGAG
Norrin	ATTGTGGTTTGGGTGCCTGA	CATCCATGGACTGGGTGTCC
Fzd4	GACGTCTGATATCCCGCACA	TCCTCCTCGTCCCCGAAC
Lrp5	CTGCATAGCATTGAACGGGC	GGTCCAGCGTGTAGTGTGAA
Tspan12	GAATCTCTCTGGGGTGTCACT	AGCAATCATGACGGGGTGAA
Sox17	AGTTTTCCCCAAGGCTAGCTTCC	CGTCTTTGGCCCACACCATA
Ang1	TGGCTTGGATGTGCAACCTT	TTTGCAGAGCGTTGGTGTTG
Ang2	AAGGAAGCCCTTATGGACGA	CCAGCCATTCTCACAGCCAA
Tie1	CCATCCTGGCTGCCCTTTTA	GGGGGCGTATTCGATAGCAA
Tie2	AGCTGTAGAGCAAGTCACTCTC	ATTCGTTGGAGAACTTGGCAC
Foxo1	CGGAAAATCACCCCGGAGAA	TACACCAGGGAATGCACGTC
Sox7	CTTGGAATCCCAGGCACCAT	ATACACGTGTCCAAGGGCAG
Sox18	CCCGTTTCCCAATCCTCTGT	TAGCATCAGACAGCGCAGAA

PCR: Polymerase chain reaction.

These results indicated precocious vessel regression in *Smad4*-cKO retinas. We subsequently examined vascular endothelial cells death using TUNEL assay. The apoptosis number of vascular endothelial cells was significantly higher in *Smad4*-cKO retinas than in control retinas at P18 (Figure 3D, 3H and 3K), which caused instability of formed vessels. Furthermore, aberrant growth of neovascular tufts and retinal vascular leakage were observed in *Smad4*-cKO mice at 1mo (Figure 3B and 3F).

Taken together, loss of *Smad4* on surface ectoderm resulted in a variety of retinal vasculature anomalies such as accelerated vascular sprouting, arteriovenous malformations, less vascular coverage, increased vessel ghosts and apoptosis of vascular endothelial cells. Regression of the developing retinal vasculature and microvascular damage may subsequently lead to the appearance of retinal detachment and retinal folds.

Microarray Analysis of *Smad4*-cKO and Control Retina To understand the molecular mechanism of the defective retinal angiogenesis in *Smad4*-cKO mice, we performed cDNA microarray analyses with retinal RNA specimens of control and *Smad4*-cKO mice of P0. As expected, *Smad4* expression showed no difference between control and *Smad4*-cKO retina (Figure 4A). Microarray analyses showed 614 genes were upregulated and 1284 genes were downregulated in the cKO retinas compared to the WT controls using a cutoff of 3-fold. Gene expression change lower than three fold was considered as no evident difference.

Pathway Analysis of the Differentially Expressed Genes Pathway analysis was performed by using the SBS Analysis System (http://sas.ebioservice.com/). Several signaling pathways were significantly regulated by *Smad4*, among which five pathways associated with retinal vasculature development were: axon guidance pathway, TGF-β signaling pathway, Wnt signaling pathway, Notch signaling pathway and VEGF signaling pathway (Figure 5A). In axon guidance pathway, class-3 semaphorins, Nrp1 and ephrin families implicate in the regulation of retinal vasculature development. The microarray results showed Sema3c, Sema3e and Nrp1 expression were significantly affected in *Smad4*-cKO retina, while Eph receptors and their ligands expression showed no evident difference (Figure 5B). Among genes of Wnt signaling pathway, Sox17 expression was significantly down-regulated in *Smad4*-cKO retina, whilst Norrin, Fzd4, Lrp5 and Tspan12, which were reported to play a vital role in

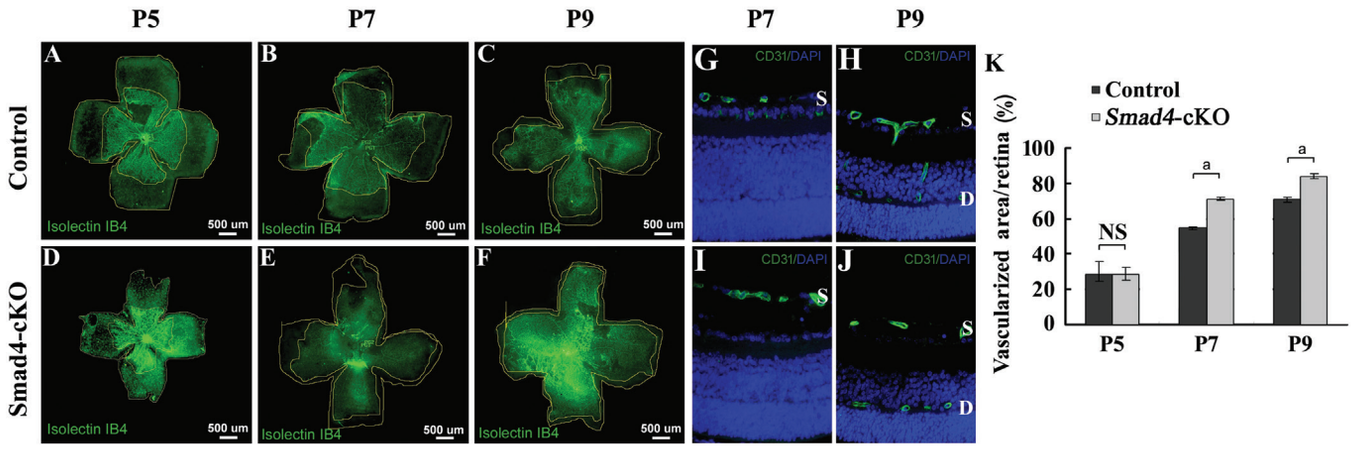

Figure 1 Conditional inactivation of *Smad4* on surface ectoderm affects retinal sprouting angiogenesis A-F: Photographs of retinal flat-mounts showed the radially extension of vessel sprouts from the optic disk of cKO mice and control mice of the indicated stages. Vascular endothelial cells were stained with isolectin IB4 (green). Outer yellow lines indicated retina edge. Inner yellow lines indicated the vascularized area edge. G-J: Photographs of histological sections showed retinal vasculature of superficial and deep capillary networks of cKO mice and control mice of the indicated stages. Vascular endothelial cells were stained with CD31 (green). S: Superficial vasculature; D: Deep vasculature. K: Quantification of the vascularized retinal area normalized to the whole retinal area during extension of vascular sprouts. n=4 per group; $^{a}P<0.05$.

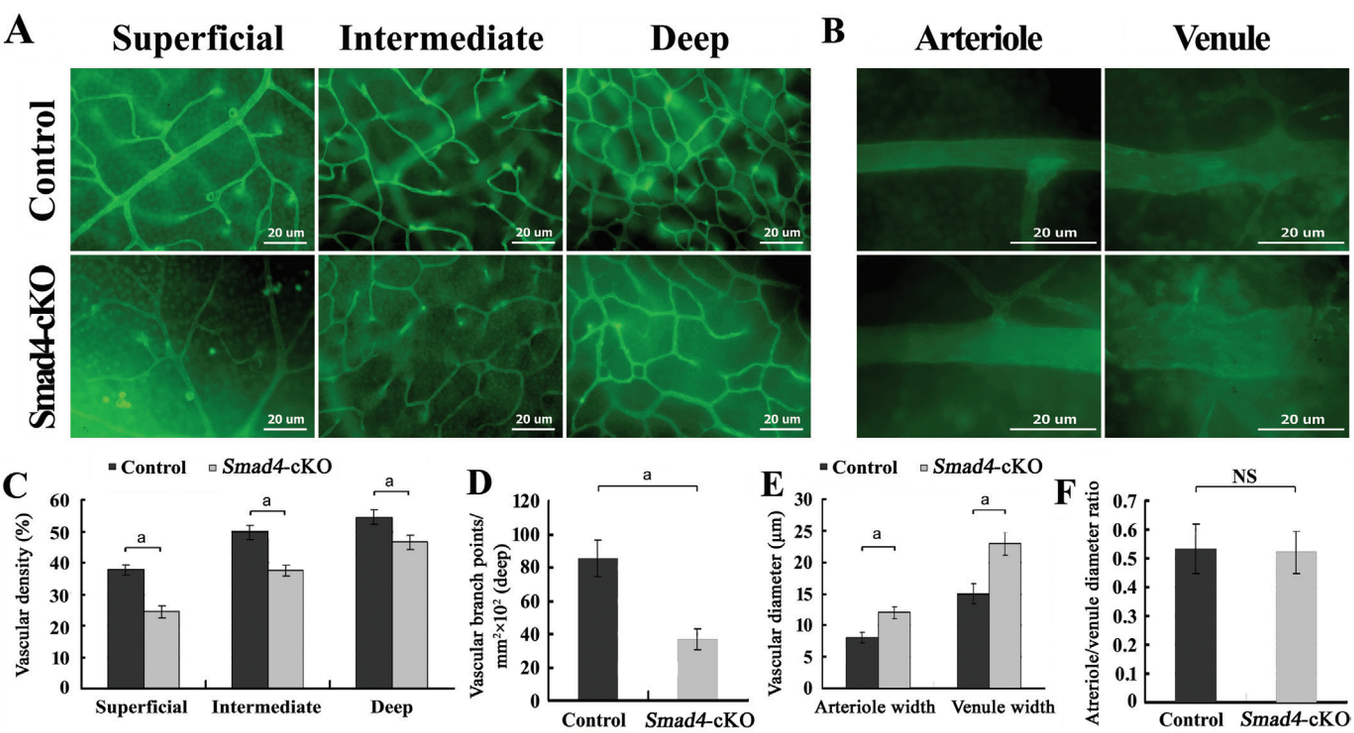

Figure 2 Conditional inactivation of *Smad4* on surface ectoderm results in arteriovenous malformations and less vascular coverage in retina A: Representative images of selected areas of the superficial, intermediate and deep retinal vascular plexus at P18. Vascular endothelial cells were stained with isolectin IB4 (green). B: Representative images of retinal arterioles and venules of cKO mice and control mice at P18. Vascular endothelial cells were stained with isolectin IB4 (green). C: Quantification of the vascular density of the superficial, intermediate and deep retinal vascular plexus per field in the selected areas of cKO mice and control mice at P18. D: Quantification of branching points per field in the selected areas of cKO mice and control mice at P18. E: Quantification of the vascular diameter of retinal arterioles and venules of cKO mice and control mice at P18. F: Quantification of the retinal arterioles/venules diameter ratio of cKO mice and control mice at P18 (6-8 sections per retina, 4 retinas from different animals. $^{a}P<0.05$. P: Postnatal).

angiogenesis, showed no obvious expression changes between control and *Smad4*-cKO retina (Figure 5C). The expression of genes of TGF-β signaling pathway, Notch signaling pathway and VEGF signaling pathway, which were associated with

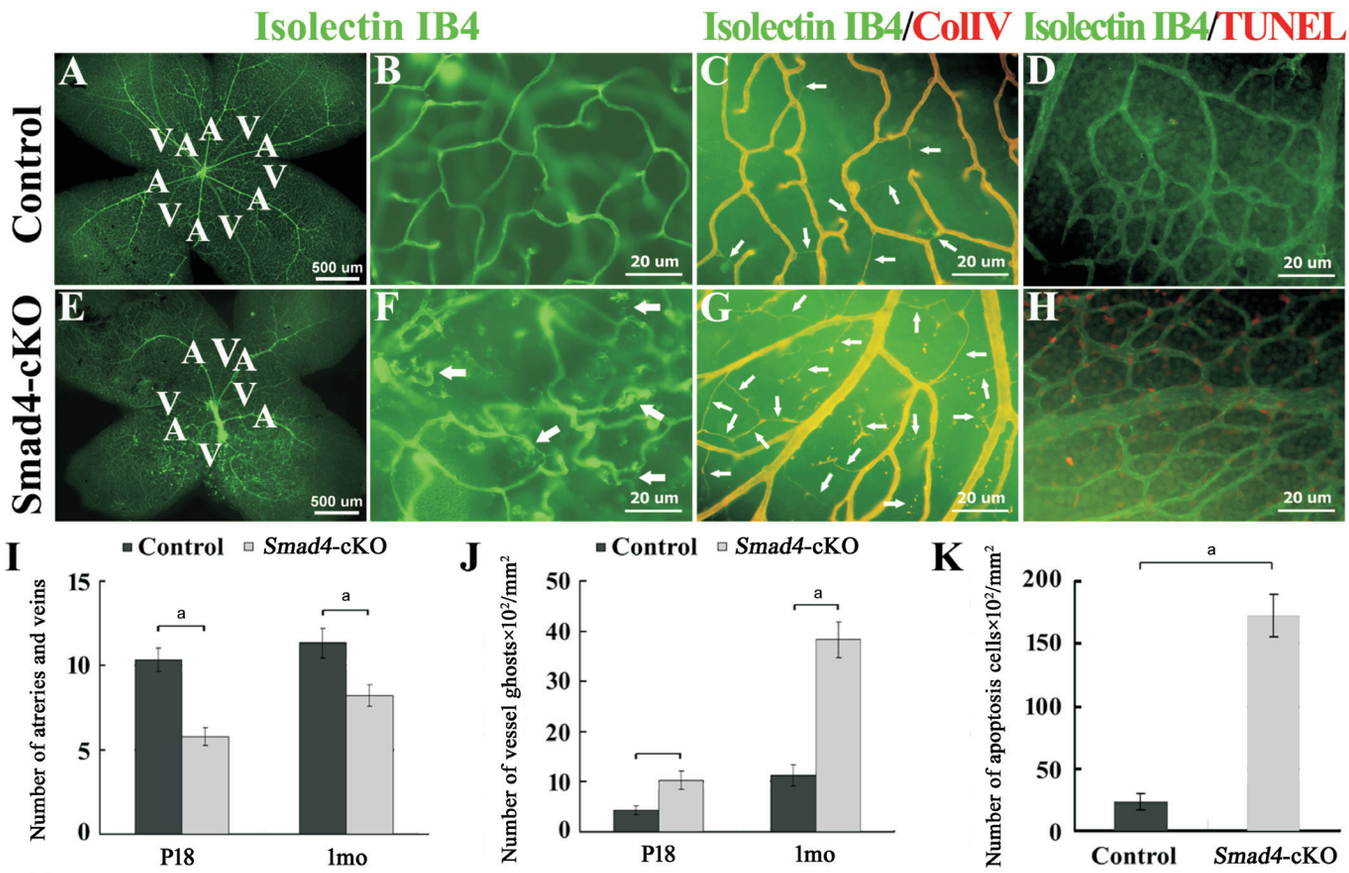

Figure 3 Conditional inactivation of *Smad4* on surface ectoderm results in regression of the developing retinal vasculature and microvascular damage A, E: Representative images of central portion of retinal flat mounts of cKO mice and control mice at P18. Vascular endothelial cells were stained with isolectin IB4 (green). A: Artery; V: Vein. B, F: Representative images of selected areas of cKO mice and control mice at 1mo. Vascular endothelial cells were stained with isolectin IB4 (green). Aberrant growth of neovascular tufts and retinal vascular leakage (arrows) were observed in Smad4-cKO mice. C, G: Representative images of selected areas of double immunostaining on retinal flat-mounts using collagen IV and isolectin IB4 showed pericyte ghost (arrows) of cKO mice and control mice at P18. D, H: Representative images of selected areas of TUNEL assay on retinal flat-mounts in Smad4-cKO mice and control mice at P18. I: The number of retinal veins and arteries of cKO mice and control mice at P18 and 1mo. J: Quantification of vessel ghosts per field in the selected areas of cKO mice and control mice at P18 and 1mo. K: Quantification of apoptosis endothelial cells per field in the selected areas of cKO mice and control mice at P18 (6-8 sections per retina, 4 retinas from different animals. $^aP<0.05$. P: Postnatal).

retinal vasculature development, were not markedly affected in *Smad4*-cKO retina (Figure 4A-4C). Taken together, gene expression change in the axon guidance pathway and Wnt pathways in the *Smad4*-cKO retinas may underlie the phenotype in *Smad4*-cKO retinas.

Gene Expression Changes of Signals Related to Sprouting Angiogenesis in *Smad4*-cKO Retina Gene ontology analysis revealed that the differentially expressed genes associated with sprouting angiogenesis were Dll4, Notch1, VEGFA, Sema3a, Sema3c, Sema3d, Sema3e, Plxnd1, Nrp1, Bmp9, Bmp10, Tgfb1, Tgfbr2, Norrin, Fzd4, Lrp5, Tspan12 and Sox17. The expression of the regulated genes was confirmed by qPCR with mRNA isolated from control and *Smad4*-cKO retina of P0 (Figures 4D, 5D). Sema3c, Sema3e and Sox17 were down-regulated, whilst Nrp1 were up-regulated in the cKO retina (Figure 5B-5D). The expression of Dll4, Notch1, VEGFA, Sema3a, Sema3d, Plxnd1, Bmp9, Bmp10, Tgfb1, Tgfbr2, Norrin, Fzd4, Lrp5 and Tspan12 showed no difference between control and *Smad4*-cKO retina (Figure 4A-4D). These gene expression changes in *Smad4*-cKO retina were associated with accelerated vascular sprouting, revealing a role of *Smad4* from surface ectoderm in retinal angiogenesis by regulating the expression of Sema3c, Sema3e, Nrp1 and Sox17.

Gene Expression Changes of Signals Related to Vascular Remodeling and Maturation in *Smad4*-cKO Retina We also investigated the expression changes of the genes related to vascular remodeling and maturation in control and *Smad4*-cKO retina. The expression of Ang1, Ang2, Tie1, Tie2, Foxo1, Dll4, Notch1, Sox7, Sox17, and Sox18 of P18 was detected using qPCR. Tie1, Sox7, Sox17, and Sox18 were down-

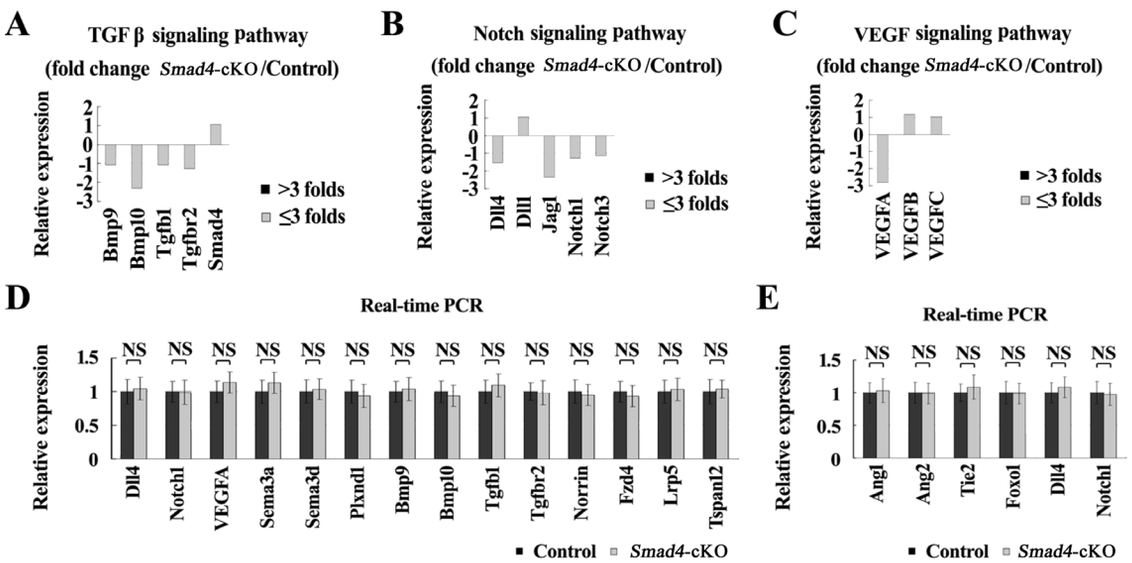

Figure 4 Microarray analysis and real-time qPCR showed the differentially expressed genes in control and *Smad4*-cKO retina A-C: Charts showed the expression changes of genes related to retinal vasculature development in TGF-β signaling pathway, Notch signaling pathway and VEGF signaling pathway detected by microarray within retina, respectively. Gene expression change lower than three fold was considered as no evident difference. D: Real-time qPCR was performed to detect the expression of genes associated with sprouting angiogenesis within retina at P0. E: Real-time qPCR was performed to detect the expression of genes associated with vascular remodeling and maturation within retina at P18 (*n*=4. P: Postnatal.)

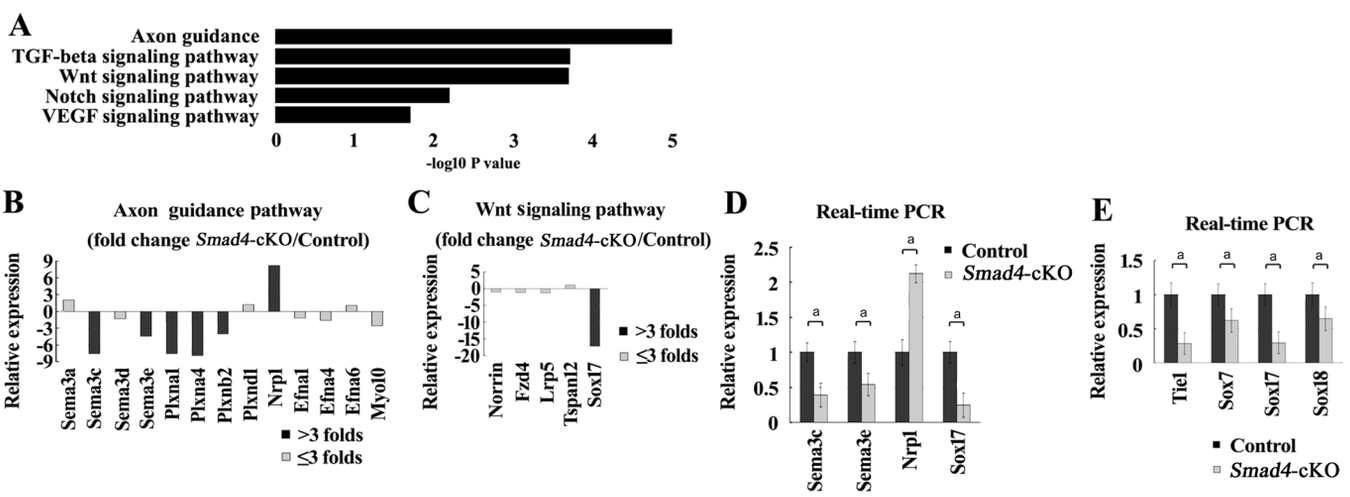

Figure 5 Microarray analysis and real-time qPCR showed the differentially expressed genes in control and *Smad4*-cKO retina A: Five signaling pathways associated with retinal vasculature development were affected by *Smad4* defined by the SBS Analysis System (http://sas.ebioservice.com/). B, C: Charts showed the expression changes of genes related to retinal vasculature development in axon guidance pathway and Wnt signaling pathway detected by microarray within retina, respectively. Gene expression change lower than three fold was considered as no evident difference. D: Real-time qPCR was performed to detect the expression of Sema3c, Sema3e, Nrp1 and Sox17 within retina at P0. The expression of Sema3c, Sema3e and Sox17 was down-regulated in *Smad4*-cKO retina. The expression of Nrp1 was up-regulated in *Smad4*-cKO retina. E: Real-time qPCR was performed to detect the expression of Tie1, Sox7, Sox17, and Sox18 within retina at P18. The expression of Tie1, Sox7, Sox17, and Sox18 was down-regulated in *Smad4*-cKO retina (*n*=4; $^{a}P<0.05$. P: Postnatal).

regulated in the cKO retina, whilst the expression of Ang1, Ang2, Tie2, Foxo1, Dll4 and Notch1 showed no difference between control and *Smad4*-cKO retina (Figures 4E, 5E). The results were associated with retinal vasculature phenotypes such as arteriovenous malformations, less vascular coverage, increased vessel ghosts and apoptosis of vascular endothelial cells, suggesting the change of Tie1, Sox7, Sox17, and Sox18 may underlie the vascular phenotype in *Smad4*-cKO retinas.

DISCUSSION

Interaction between neuroepithelium and ectoderm plays an essential role in vertebrate eye development[13-18]. It is well established that signals from lens are essential for

multiple development stages in eye tissues, such as optic vesicle invagination, expansion of the developing eye, neural retina identity, retinal vascular development and retinal maintenance[13-18,21]. How lens cross-talks with retina in the retinal vasculature development remains unclear. Our results show loss of *Smad4* on surface ectoderm resulted in a variety of retinal vasculature anomalies such as accelerated vascular sprouting, arteriovenous malformations, less vascular coverage, increased vessel ghosts and apoptosis of vascular endothelial cells. These data suggest that the cross-talk between ocular surface ectoderm and retina is important for retinal vasculature development, and *Smad4* regulates various signalings associated with sprouting angiogenesis, vascular remodeling and maturation in the retina.

Numerous signalings have been known to play essential roles in normal retinal vasculature development. Among them including signalings of axon guidance pathway, Wnt signalings, TGF-β signalings, Notch signalings, and VEGF signalings[6-12]. In our results, the expression of Sema3c, Sema3e, Nrp1 and Sox17 were significantly affected in *Smad4*-cKO retina, suggesting the change of axon guidance pathway and Wnt pathways may underlie the vascular phenotype in *Smad4*-cKO retinas.

We performed detailed analysis of changes of stimulatory and inhibitory signals for sprouting angiogenesis in retinas of *Smad4*-cKO mice. Previous studies have shown that pro-angiogenic molecules, including VEGFA[24], Nrp1[25], Tgfb1, Tgfbr2[11,26], Norrin, Fzd4, Lrp5 and Tspan12[27-28], normally supports the tip state during retinal vasculature development, whist anti-angiogenic molecules, including Sema3a[11,29], Sema3c[11], Sema3d[11], Sema3e, Plxnd1[11,30], Dll4/Notch1 signalling[31], Bmp9, Bmp10[32] and Sox17[33], suppress the tip state, leading to decreased branching in the primary plexus of the retinal vasculature. In our results, Sema3c, Sema3e and Sox17 were down-regulated, whilst Nrp1 were up-regulated in the *Smad4*-cKO retina. The expression of Dll4, Notch1, VEGFA, Sema3a, Sema3d, Plxnd1, Bmp9, Bmp10, Tgfb1, Tgfbr2, Norrin, Fzd4, Lrp5 and Tspan12 showed no difference between control and cKO retina. Consequently, the accelerated retinal vascular sprouting could be caused by inhibition of Sema3c, Sema3e and Sox17, and activation of Nrp1 in *Smad4*-cKO mice after birth.

We also observed changes in the expression of genes related to vascular remodeling and maturation in the retina of control and *Smad4*-cKO mice. At P18, Tie1, Sox7, Sox17, and Sox18 were down-regulated in the cKO retina, whilst the expression of Ang1, Ang2, Tie2, Foxo1, Dll4 and Notch1 showed no difference between control and *Smad4*-cKO retina. From previous research, SoxF family members Sox7, Sox17, and Sox18 have been implicated in regulating vascular growth, differentiation, remodeling, and maintenance in a redundant fashion[11,34]. Tie1 has been shown to be important in vascular remodeling and maintenance, and loss of Tie1 increases vessel regression and endothelial apoptosis in mice[35]. In our results, abundant vessel ghosts were observed at P18, and there was significantly more increase in the number of vessel ghosts at 1mo in the *Smad4* defective mice compared to the controls. The apoptosis number of vascular endothelial cells was significantly higher in *Smad4*-cKO retinas than in control retinas at P18. Moreover, the number of arteries and veins were decreased in *Smad4*-cKO than in WT mice retinas at P18, and was still apparent in adult retinas. These are consistent with the changes of Tie1, Sox7, Sox17, and Sox18 in the cKO retina at P18. Less vascular coverage and fewer branching points in the *Smad4*-cKO mice compared with the controls at P18, probably caused by the excessive endothelial apoptosis and increases vessel regression. Retinal hypoxia and ischemia leads to aberrant growth of neovascular tufts and retinal vascular leakage, and finally resulted in retinal detachment and visual impairment in *Smad4*-cKO mice.

In conclusion, our present work provides evidence of pathological retinal vasculature change in mice when *Smad4* is inactivated only in the ocular surface ectoderm, and proposes that the pathological change of retina vasculature is caused by signaling molecules released from lens that interrupt the expression of a series of angiogenic molecules in retina. Our studies address the importance of the cross-talk between ocular surface ectoderm and retina in retinal vascular development, reminding us to concern with the prevention of phacogenic retinal vascular disease. Future research on modulation of *Smad4* expression may provide an effective mechanism for regulation of ocular angiogenesis.

ACKNOWLEDGEMENTS

Foundation: Supported by Tianjin Natural Science Funds (No.18JCQNJC10600).

Conflicts of Interest: Li J, None; **Zhang JS,** None; **Zhao JY,** None; **Han GG,** None.

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