Thromboxane Governs the Differentiation of Adipose-Derived Stromal Cells Toward Endothelial Cells In Vitro and In Vivo

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Rationale: Autologous adipose-derived stromal cells (ASCs) offer great promise as angiogenic cell therapy for ischemic diseases. Because of their limited self-renewal capacity and pluripotentiality, the therapeutic efficacy of ASCs is still relatively low. Thromboxane has been shown to play an important role in the maintenance of vascular homeostasis. However, little is known about the effects of thromboxane on ASC-mediated angiogenesis.

Objective: To explore the role of the thromboxane-prostanoid receptor (TP) in mediating the angiogenic capacity of ASCs in vivo.

Methods and Results: ASCs were prepared from mouse epididymal fat pads and induced to differentiate into endothelial cells (ECs) by vascular endothelial growth factor. Cyclooxygenase-2 expression, thromboxane production, and TP expression were upregulated in ASCs on vascular endothelial growth factor treatment. Genetic deletion or pharmacological inhibition of TP in mouse or human ASCs accelerated EC differentiation and increased tube formation in vitro, enhanced angiogenesis in vivo Matrigel plugs and ischemic mouse hindlimbs. TP deficiency resulted in a significant cellular accumulation of β-catenin by suppression of calpain-mediated degradation in ASCs. Knockdown of β-catenin completely abrogated the enhanced EC differentiation of TP-deficient ASCs, whereas inhibition of calpain reversed the suppressed angiogenic capacity of TP re-expressed ASCs. Moreover, TP was coupled with Gαq to induce calpain-mediated suppression of β-catenin signaling through calcium influx in ASCs.

Conclusion: Thromboxane restrained EC differentiation of ASCs through TP-mediated repression of the calpain-dependent β-catenin signaling pathway. These results indicate that TP inhibition could be a promising strategy for therapy utilizing ASCs in the treatment of ischemic diseases. (Circ Res. 2016;118:1194-1207. DOI: 10.1161/CIRCRESAHA.115.307853.)

Key Words: adipose-derived stromal cell ■ angiogenesis ■ calcium ■ calpain ■ thromboxane receptor

Angiogenesis, the formation of new blood vessels, is a central process involved in diverse physiological and pathological conditions, including organ development, wound healing, ischemia, inflammatory diseases, and even cancer. Insufficient angiogenesis underlies conditions such as ischemic heart disease and peripheral vascular disease, where inadequate growth of blood vessels leads to cumulative tissue damage. Therapeutic angiogenesis, including transplantation of stem cells, which is a strategy for treating ischemic tissue by stimulating the generation of collateral vessels, has emerged as a promising therapy for the treatment of various conditions. Mesenchymal stem cells have the potential to differentiate into multiple lineages of tissues, such as vasculature, skeletal muscle, cartilage, and fat, under lineage-specific culture conditions. Mesenchymal stem cells have been isolated from many tissue sources, including the bone marrow, skin, umbilical cord, adipose tissue, and heart. Owing to the ease...
and reproducibility of the isolation procedure, adipose tissue has become an attractive reservoir of autologous cells for regenerative medicine.\(^4\) Adipose tissue is rich in pluripotent adipose-derived stromal cells (ASCs). In recent years, intensive efforts have been made to use ASCs for the improvement of revascularization and tissue repair in ischemic tissues.\(^7,8\)

The administration of ASCs can favorably promote neovascularization in animal models of ischemic limbs\(^9,10\) and can effectively improve left ventricular function in animal models of acute and chronic myocardial infarction.\(^11,12\) The proangiogenic benefits of ASC transplantation may be ascribed to their differentiation capability toward different mature lineages within ischemic tissues, especially CD31\(^+\) endothelial cells (ECs)\(^13,14,15\) and paracrine secretion of angiogenic growth factors produced by ASCs, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1.\(^14,15\) VEGF-A was an essential factor for endothelial differentiation from ASCs,\(^16\) probably through acting on kinase insert domain receptor,\(^17,18\) However, compared with induced pluripotent stem cells and embryonic stem cells, the self-renewal capacity and multipotency of ASCs are still low, which limits their applications in angiogenic therapy. In addition, the key transcription factors, molecular events, and their relevant signaling pathways that determine ASCs in vivo toward specific lineages or fates remain to be explored.

Thromboxane, a bioactive metabolite of arachidonic acid produced through the cyclooxygenase pathway, exerts diverse physiological/pathophysiological functions through the thromboxane receptor (TP) in autocrine or paracrine manner, such as platelet aggregation and vascular smooth muscle contraction.\(^19\) Low-dose aspirin is widely used to prevent stroke and heart attack by blocking COX-1–derived thromboxane in platelets.\(^20\) The TP receptor exists in 2 alternatively spliced isoforms (TP\(\alpha\) and TP\(\beta\)) in humans,\(^21\) but only 1 form is present in rodents, homologous to the human TP\(\beta\) isoform.\(^22\) It is broadly distributed throughout the body, with higher expression observed in the immune organs.\(^23\) Thromboxane/TP has been implicated in allergic inflammation,\(^24\) blood pressure regulation,\(^25\) vascular remodeling,\(^26\) and atherosclerosis.\(^27\) TP receptor antagonists display multiple beneficial effects in cardiovascular diseases.\(^28,29\) Moreover, thromboxane mimetics antagonized fibroblast growth factor-2 (FGF-2)– and VEGF-induced EC migration in vitro and angiogenesis in vivo.\(^30,31\) However, the role of thromboxane/TP in the differentiation of ASCs toward ECs and their potential application in angiogenic therapy remains unknown.

In this study, we observed that the COX-2/thromboxane/TP axis was elevated during EC differentiation of ASCs induced by VEGF. Disruption of TP receptors augmented directional EC differentiation of ASCs and improved ASC-mediated neovascularization in Matrigel plugs and ischemic mouse hindlimbs. Moreover, we demonstrated that TP deletion suppressed calpain-mediated \(\beta\)-catenin degradation in ASCs by coupling G\(\text{tq}\) and subsequently increased angiogenic capacity of ASCs by activation of Wnt signaling. In addition, we observed that pharmacological inhibition of TP enhanced angiogenic potential of human ASCs. Our findings suggested that TP could be a potential target for ASC-based therapy for ischemic diseases.

**Methods**

The detailed Methods are available in the Online Data Supplement.

**Results**

**COX-2/Thromboxane/TP Axis Is Upregulated in ASCs On VEGF Stimulation**

Mouse ASCs were prepared from epididymal fat pads and expanded in culture. At the second passage, ASCs expressed the stroma-associated markers CD29 (85.5±1.1%), CD90 (73.0±0.8%), and Sca-1 (26.7±0.8%), but few EC markers such as CD31 and CD34 (Online Table I), indicating that ASCs were a population of mesenchymal stem cells and not from hematopoietic origin. To explore whether prostanooids are involved in the regulation of VEGF-induced EC differentiation of ASCs, we first examined the expression of all prostanooid receptors in ASCs by real-time-polymerase chain reaction. All of the prostanooid receptors were expressed at varying levels in ASCs, with the exception of the \(\nu\)-prostanoid receptor (Figure 1A). In contrast to the constitutive expression of COX-1, COX-2 was minimally expressed in ASCs but was dramatically induced by VEGF after 7 days (Figure 1B). We profiled the production of prostanooids during VEGF stimulation, both thromboxane (Figure 1C) and PGI\(_2\) (prostaglandin I\(_2\)) were significantly elevated (Online Figure IA). In addition, thromboxane synthase (TxAS; Figure 1D) and TP expression were also upregulated in ASCs after VEGF stimulation (Figure 1E).

The induction of COX-2, TxAS, and TP expression as well as thromboxane production of ASCs peaked at about day 7 in response to VEGF stimulation, and intracellular calcium levels in ASCs peaked at day 1 (Online Figure IIA–IID). Therefore, these results indicated that the COX-2/thromboxane/TP axis was upregulated during EC differentiation of ASCs.

**TP Deficiency Enhances VEGF-Induced EC Differentiation of ASCs In Vitro and In Vivo**

To further explore the potential role of thromboxane/TP in ASC differentiation toward ECs, ASCs were prepared from both TP-knockout (KO) and wild-type (WT) control mice. The number of CD34+/CD31\(^+\) cells in TP-KO ASCs was higher than that of WT ASCs (TP-KO, 283±19 \(\mu\)m\(^2\)/field versus WT, 126±7 \(\mu\)m\(^2\)/field; \(P<0.05\); Online Figure IIIA and IIIB) under basal conditions, and this difference became more prominent in the presence of VEGF (TP-KO, 1037±90 \(\mu\)m\(^2\)/field
versus WT, 431±42 μm²/field; P<0.05; Online Figure IIIA and IIIB). Accordingly, the mRNA levels of the EC markers Tie2, VE-cadherin, NOS3, CXCR4, and VEGFR2 were also significantly upregulated in TP-KO ASCs (Online Figure IIIC). Strikingly, CD31 expression in TP-KO ASCs increased 10-fold in the presence of VEGF compared with that in WT ASCs (Online Figure IIIC). Consistent with CD31 expression, the population of CD31⁺ cells was significantly higher in VEGF-treated TP-KO ASCs (14 days of culture) than that in WT ASCs (22.1±0.7% versus 12.8±1.2%, respectively; P<0.05; Figure 1F and 1G). In addition, we also found markedly elevated transcription of proangiogenic factors, such as VEGF-A and basic fibroblast growth factor (bFGF), in TP-KO ASCs compared with WT ASCs (Figure 1H).

Tube formation assays were then performed to test the angiogenic capacity of TP-KO ASCs. After 14 days of VEGF stimulation, TP-KO ASCs exhibited more intensive tube-like structures compared with WT ASCs (Figure 1I and 1J). Notably, tube-like structures could be observed in the normal cultures of TP-KO ASCs (Figure 1I). To confirm whether TP
deficiency enhanced the potency of ASC differentiation toward ECs in vivo, Matrigel plug assays were conducted using donor ASCs derived from enhanced green fluorescent protein (EGFP) transgenic mice. As shown in Figure 1K, plugs with TP-KO ASCs exhibited more extensive dark-red colored new blood vessel formation than plugs with WT ASCs, and immunofluorescence staining revealed more profound CD31+ vascular architecture in Matrigel plugs with TP-KO ASCs (Figure 1L and 1M).

To exclude any interference of mature ECs pre-existing in ASCs, we prepared ASCs lacking CD31 expression (CD31−ASCs) by using CD31 negative immunomagnetic selection. Interestingly, no significant differences in expression of cell surface markers were detected after 2 passages in culture between the original preparation and CD31−ASCs (Online Table II), suggesting a few CD31+ ASCs (≈2%) observed in culture may be because of spontaneous differentiation.2,3,33 CD31−ASCs at second passage displayed comparable capacity for EC differentiation with nonselected ASCs in response to VEGF stimulation (Online Figure IV). Again, TP deficiency significantly enhanced differentiation of CD31−ASCs toward ECs in vitro (Online Figure IVA–IVD), expression of angiogenic factors (Online Figure IVE) and tube-like structures in response to VEGF treatment (Online Figure IVF and IVG), and increased in vivo angiogenesis in Matrigel plugs (Online Figure IVH–IVJ).

In addition, a cotton thread was implanted into the epididymal fat pad to induce granulation tissue formation with angiogenesis in both TP-KO and WT mice (Online Figure VA). Histological analysis of the granulation tissues revealed the presence of a fibroblastic layer around the implanted thread, which was thicker in TP-KO mice than in WT mice (Online Figure VB). Consistently, more CD31+ vessel-like structures were observed in the fibroblastic layer of TP-KO mice than that of WT mice (Online Figure VC and VD). Taken together, these data indicated that TP deficiency may promote the EC differentiation of ASCs in vivo.

TP Deficiency Enhances the Angiogenic Efficacy of ASC Therapy in a Mouse Model of Hindlimb Ischemia

To determine whether TP deletion increased the therapeutic efficacy of ASCs in treating ischemic diseases, we implanted ASCs directly into ischemic mouse hindlimbs. To trace donor cell fates, primary ASCs were isolated from red fluorescent protein (RFP) transgenic mice. Laser Doppler perfusion imaging analysis revealed that blood reperfusion in ischemic limbs was significantly better in the TP-KO ASCs transplantation group than in the WT ASCs group at both 7 and 14 days post therapy (Laser Doppler perfusion imaging ratio at day 7, 0.54±0.03 versus 0.42±0.02; P<0.05; Laser Doppler perfusion imaging ratio at day 14, 0.74±0.02 versus 0.59±0.03; P<0.01; Figure 2A and 2B). CD31 staining was used to assess capillary density in the gastrocnemius muscle at 14 days after ASCs delivery. As shown in Figure 2C and 2D, transplantation of TP-KO ASCs significantly enhanced overall capillary formation compared with transplantation of WT ASCs. Moreover, the proliferation index, as measured by PCNA+ cells, was significantly higher in the TP-KO group (Figure 2E). Importantly, RFP+/PCNA+/CD31+ cells, indicating the transplanted ASC-derived ECs with proliferative activity, were more frequently observed in the TP-KO ASCs group than in the control group (2.21±0.12 versus 0.62±0.01×10^3 µm^2/field, respectively; P<0.01; Figure 2F).

Interestingly, RFP+/PCNA+/CD31+ cells, indicating recipient proliferative ECs, were almost tripled in the TP-KO ASCs transplantation group when compared with the WT group (Figure 2G). These results were consistent with the increased paracrine effects of TP-KO ASCs observed in vitro (Figure 1H). In addition, no significant differences of inflammatory cell infiltration (data not shown), SMA+ cells (Online Figure VIA and VIB) and NG2+ mural cells (Online Figure VIC and VID) were observed in ischemic limbs between WT and TP-KO ASCs treatment. A small proportion of mural cell differentiation from ASCs was detected (NG2+RFP+/CD31+ cells; Online Figure VIE), despite TP deficiency–increased CD31 differentiation of ASCs (Online Figure VIF).

Intracellular β-Catenin Protein Is Upregulated in TP-KO ASCs Treated With VEGF

Because Wnt and Notch signaling pathways are critical for self-renewal and differentiation of mammary stem cells,34,35 we next examined alterations of Wnt and Notch signaling in ASCs. Among all the Notch proteins and their ligands, we found that the expression of Notch1,Dll4, and Jagged2 were significantly increased in ASCs on VEGF stimulation (Online Figure VIIA). Some Notch target genes, including NRP1, Hes1, Hey1, and Hey2, were also upregulated during EC differentiation (Online Figure VIIB). However, no significant differences were found between TP-KO and WT ASCs. Notably, much higher expression of cytoplasmic β-catenin protein, the key mediator of Wnt signaling, was detected in TP-KO ASCs than in WT ASCs after VEGF stimulation (Figure 3A and 3B). Accordingly, the Wnt target gene c-myc (Figure 3C) and endothelial marker CD31 (Figure 3D) were also markedly upregulated in VEGF-treated TP-KO ASCs. Moreover, the mRNA levels of Wnt target genes, such as c-myc, cyclin D1, and fra-1, were significantly upregulated in TP-KO ASCs in the absence or presence of VEGF (Figure 3E). In addition, re-expression/overexpression of TP in TP-KO ASCs (Figure 3F) suppressed the expression of cytoplasmic β-catenin and its target genes (Figure 3G–3I). To investigate whether suppressing Wnt signaling could block angiogenic potential in TP-KO ASCs, β-catenin expression was silenced by small interfering RNA (si-β-cat), as also evidenced by marked downregulation of its target genes, including c-myc (Figure 3K). Notably, β-catenin knockdown completely abolished the differences in endothelial markers (VE-cadherin and CD31; Figure 3K) and proangiogenic factors (VEGF-A and bFGF; Figure 3L and 3M) on VEGF-induced differentiation between WT and TP-KO ASCs.

Knockdown of β-Catenin Attenuates the Enhanced Angiogenic Capacity of TP-KO ASCs In Vitro and In Vivo

Next, we examined the influence of β-catenin knockdown on the angiogenic capacity of TP-KO ASCs. As shown in
Figure 4A and 4B, the enhanced tube-like structure formation of TP-deficient ASCs was inhibited by transfection of si-β-catenin both in the presence or in the absence of VEGF. Similarly, pretreatment with si-β-catenin significantly suppressed the increased blood perfusion in Matrigel plugs formed by TP-KO ASCs at 14 days after implantation (Figure 4C). Immunofluorescence staining confirmed that the profound CD31+/EGFP+ vascular networks in the Matrigel plugs were dramatically attenuated in the TP-deficient ASCs group by knockdown of β-catenin (Figure 4D and 4E). We also delivered si-β-catenin-pretreated ASCs into the muscles of ischemic mice hindlimbs and showed that the enhanced blood flow recovery in TP-KO ASCs at both 7 and 14 days after therapy was suppressed by si-β-catenin transfection (Figure 5A and 5B). Likewise, the enhanced CD31 (Figure 5C and 5D) and PCNA stainings (Figure 5E) in the TP-KO ASCs transplantation group were markedly suppressed by si-β-catenin treatment. Importantly, the augmentation of RFP+/PCNA+/CD31+ cells, representing ECs derived from transplanted ASCs, was blocked in the si-β-catenin-pretreated TP-KO ASCs transplantation group (Figure 5F), and staining of RFP-/PCNA+/CD31+ cells, indicating the paracrine effects of transplanted ASCs, was also decreased after silencing of β-catenin (Figure 5G). Thus, these results suggested that the enhanced angiogenic capacity of TP-KO ASCs was dependent on β-catenin signaling.

Reduced Calpain Activity Leads to Cellular Accumulation of β-Catenin in TP-KO ASCs

In the canonical Wnt pathway, glycogen synthase kinase 3β (GSK-3β) phosphorylates β-catenin, promoting its degradation by the proteasome. The activity of GSK-3β can be suppressed by phosphorylation at serine 9. Interestingly, in our study, inhibitory phosphorylation of GSK-3β was not altered between WT and TP-KO ASCs (Online Figure VIIIA). These results suggested that the accumulation of β-catenin in TP-KO ASCs was independent of the canonical Wnt signaling pathway. Moreover, we failed to detect any changes in the transcription of β-catenin mRNA in TP-KO ASCs (Online Figure VIIIB).

Accumulating evidence has shown that calpain is involved in intracellular β-catenin degradation by direct cleavage. Using specific antibodies against the C-terminal region of β-catenin, we demonstrated that cytoplasmic β-catenin was recognized as several smaller fragments (Figure 6A), suggesting that the N terminus of β-catenin had been cleaved. In TP-KO ASCs, the cleaved β-catenin portion was markedly decreased, whereas full-length β-catenin was...
relevant increase when compared with that in WT ASCs (Figure 6A). Moreover, re-expression of TP in TP-KO ASCs restored β-catenin cleavage and accordingly reduced total full-length β-catenin protein (Figure 6B). In addition, dramatically low calpain activity was observed in TP-KO ASCs compared with WT ASCs. After stimulation with U46619 (a TP-specific agonist), calpain activity was significantly elevated in WT ASCs but not in TP-deficient ASCs (Figure 6C).

In contrast, re-expression of TP allowed for the recovery of basal and U46619-induced calpain activity in TP-KO ASCs (Figure 6D), suggesting that calpain activity could be modulated by thromboxane/TP signaling.

To test whether calpain mediated the degradation of cytoplasmic β-catenin and angiogenesis in ASCs, we treated ASCs re-expressing TP with the calpain inhibitor calpeptin (CPT). We found that CPT dramatically restored the levels of full-length β-catenin in ASCs re-expressing TP by suppressing calpain-mediated cleavage of β-catenin (Figure 6E). Accordingly, cyclin D1 and CD31 expressions in ASCs re-expressing TP were significantly induced by CPT in a concentration-dependent manner (Figure 6E). Again, the suppression of EC differentiation (GFP+/CD31+ cells) of ASCs by TP overexpression was markedly restored by CPT pretreatment in a concentration-dependent manner, as evidenced by immunofluorescence staining (Online Figure IXA and IXB) and flow cytometry (Online Figure IXC and IXD). Consistent with the results in vitro, in vivo Matrigel plug angiogenesis assays demonstrated that TP re-expression markedly attenuated the formation of vascular networks in ASCs; this effect could be rescued by CPT (Figure 6F–6H). Moreover, CPT significantly restored the decreased expression of VEGF-A and bFGF in ASCs re-expressing TP (Figure 6I and 6J). These data indicate that reduced calpain-mediated degradation of β-catenin may contribute to the increased angiogenic capacity of TP-KO ASCs.
Stimulation of Gq receptors activates phospholipase C; thus, Activity in ASCs (Figure 6K and 6L), indicating that m-calpain was elevated during VEGF-induced EC differentiation in ASCs. We also examined the effects of TP inhibition on the angiogenic capacity of primary human ASCs. Treatment with the TP-specific inhibitor SQ29548 significantly increased directional differentiation of human ASCs toward ECs (CD31+ population; Figure 8A and 8B) and enhanced the formation of vessel-like network structures in culture in the presence of VEGF (Figure 8C and 8D). In addition, upregulated expression of VEGF-A and bFGF was also observed in SQ29548-treated human ASCs (Figure 8E and 8F). In the implanted Matrigel plugs, SQ29548 administration also promoted more blood perfusion (Figure 8G) and more CD31+ vascular structure formation in both the presence and the absence of VEGF (Figure 8H and 8I). Interestingly, recipient-derived capillary structures (mouse CD31+) that infiltrated into the Matrigel plugs were also augmented after SQ29548 treatment (Figure 8H and 8I), indicating that SQ29548 enhanced the paracrine effects of human ASCs.

Discussion

Thromboxane and its receptor (TP) play important roles in cardiovascular homeostasis, affecting platelet activation and vascular tone. In this study, we demonstrated that COX-2 expression, thromboxane production, and TP expression were elevated during VEGF-induced EC differentiation in ASCs. Disruption of this pathway by genetic deletion or pharmacological inhibition of TP increased the angiogenic capacity of...
ASCs in vitro and in vivo. Furthermore, we found that thromboxane/TP suppressed ASC differentiation toward ECs by modulating the calpain-dependent degradation of β-catenin. These findings indicate that inhibition of thromboxane/TP signaling may increase the therapeutic efficacy of ASCs for the treatment of ischemic diseases.

Adult stem cells, residing in most mammalian tissues and organs, including ASCs in fat tissues, play important roles in the maintenance of tissue homeostasis, such as repairing dead or damaged cells. The surrounding microenvironment (or the niche)- supporting cells, extracellular matrices, and autocrine and paracrine hormonal signals, can modulate the self-renewal and differentiation of stem cells. G-protein–coupled receptor activation plays important roles in the regulation of biological properties of stem cells. G-protein–coupled receptor expression levels were markedly altered during somatic reprogramming to induced pluripotent stem cells and self-renewal of induced pluripotent stem cells, and distinct G-protein–coupled receptor genes are specifically expressed at each differentiation stage. We found that the expression of COX-2, TxAS, and thromboxane production, as well as TP receptor was upregulated in ASCs differentiated into ECs. Moreover, inhibition or genetic disruption of the thromboxane/TP axis enhanced the angiogenic potential of ASCs, whereas forced ectopic expression of TP suppressed VEGF-induced EC differentiation of ASCs. These observations indicate that thromboxane/TP signaling likely acted as an endogenous inhibitor of ASC differentiation toward ECs under (patho)physiological conditions. However, others reported that TP blockers suppressed migration and angiogenesis of human umbilical vein ECs and human dermal microvascular ECs, and activation of TP induced the differentiation of human ASCs to smooth muscle–like cells. The discrepancies are probably ascribed to different cells and different approaches used, and perhaps different downstream signaling is mediated by TP receptors.

Angiogenic therapy with ASCs is believed to be effective through both self-differentiation of ASCs into ECs and secretion of angiogenic factors, such as VEGF. We observed that the transplanted ASCs were incorporated in the vessels (RFP+/PCNA+/CD31+) in ischemic hindlimbs of mice, implying that some ASCs can differentiate into ECs in vivo. Moreover, the transplanted ASCs may also differentiate into vascular smooth muscle cells and even pericyte-like cells in vivo to stabilize the neovascularature and exert proangiogenic paracrine effects as well. Indeed, both TP-KO ASC (RFP+/PCNA+/CD31+) and recipient-cell-derived (RFP+/PCNA+/CD31+) neovascularization were more than doubled than that in hindlimbs transplanted with WT ASCs, indicating enhanced...
differentiation toward ECs and paracrine activity in TP-KO ASCs. These results were consistent with the enhanced vascular structure formation in vivo matrigel plug assays, accelerated differentiation of ECs in response to VEGF, and significant elevation of VEGF-A and bFGF expression in TP-KO ASCs in vitro. Thus, thromboxane/TP signaling may suppress the angiogenic potential of ASCs by inhibiting both EC differentiation and paracrine signaling.

Both Wnt/β-catenin and Notch signaling are involved in the regulation of maintenance, self-renewal, and directional differentiation of adult tissue-derived stem cells.34,35 Dual activation of Wnt/β-catenin and Notch signaling, together with VEGF, is necessary for the determination of EC fate from vascular progenitors.57 Indeed, both Notch signaling and β-catenin target genes were upregulated during VEGF-induced ASC differentiation into ECs. However, in VEGF-treated TP-KO ASCs, we observed markedly enhanced expression of β-catenin and its target genes. In contrast, TP overexpression suppressed β-catenin activity in ASCs. Knockdown of β-catenin by siRNAs completely abrogated the augmented in vitro EC differentiation potential and in vivo neovascularization capacity of TP-KO ASCs, suggesting that hyperactivity of Wnt/β-catenin signaling induced by TP deficiency enhanced the angiogenic efficacy of ASCs. Consistent with these observations, the
pharmacological activation of β-catenin signaling has been shown to increase endothelial differentiation from embryonic stem cells, whereas conditional inactivation of β-catenin in ECs causes a defective vascular pattern and increases vascular fragility during embryogenesis. Both VEGF-A and bFGF can stimulate β-catenin signaling in ECs. Interestingly, β-catenin also promotes the expression of VEGF-A and VEGF-C by increasing their promoter activities, indicating that VEGF-A may be a β-catenin target gene. Thus, along with accumulation of cytosolic β-catenin, increased expression of VEGF-A was detected in TP-KO ASCs, which, in turn, may contribute to the paracrine effects on neovascularization in TP-KO ASC–transplanted ischemic hindlimbs. Moreover, other angiogenic regulators, such as bFGF and endothelin-1, have previously been reported as β-catenin target genes. In agreement with our observations, CD133+ progenitor cells mediate paracrine stimulation of angiogenesis and would be healing through activation of Wnt signaling.

In the canonical Wnt signaling pathway, Wnt and GSK3β act upstream of the β-catenin-T-cell factor–dependent gene regulation machinery. GSK3β mediates the phosphorylation of β-catenin and subsequently leads to the degradation of β-catenin. However, no differences in GSK3β activity, phosphorylation of β-catenin, and β-catenin mRNA expression were observed between WT and TP-KO ASCs, indicating that β-catenin accumulation in TP-KO ASCs was not because of changes in gene transcription or GSK3β-mediated degradation. Calpain, a member of the family of intracellular Ca2+-dependent cysteine proteases, has been reported to mediate β-catenin degradation. Using a C-terminal–specific antibody, we detected a substantial reduction in cleaved fragments of β-catenin in TP-KO ASCs. Accordingly, calpain
activity was also suppressed in TP-KO ASCs, and re-expression of TP in TP-KO ASCs restored calpain activity and increased β-catenin fragmentation. Inhibition of calpain protease in ASCs re-expressing TP suppressed β-catenin degradation and increased β-catenin/T-cell factor transcriptional activity and angiogenic potential in ASCs, indicating that the reduction in calpain-mediated β-catenin degradation contributed to the elevated endothelial differentiation of TP-KO ASCs. Moreover, calpain is involved in the suppression of Wnt/β-catenin signaling, which is in agreement with our observations and calpain suppressed the differentiation of stem cells toward neurons and skeletal muscle cells. However, calpain-mediated N-terminal truncation of β-catenin may enhance T-cell factor–dependent transcription by increasing accumulation of active 75-kDa β-catenin protein in some tissues or cancers. The different roles of calpain in different tissues could be because of multiple cleavage sites in β-catenin or different molecular complexes formed by β-catenin in different tissues. In ASCs, we observed the accumulation of smaller degradation fragments of β-catenin. The detailed molecular basis of these differences requires further investigations. In addition, m-calpain, but not μ-calpain, seems to mediate β-catenin degradation in ASCs, which is analogous to previous observations in neurovascular ECs. There are many different calpain targets in different cells, such as β-catenin in colon cancer cells, CD31 in platelets, and caspase 12 in cardiomyocytes and neurons. We found that the activation of TP receptors promotes calpain cleavage of β-catenin in ASCs on VEGF treatment, without overt influence of CD31 and caspase 12. Thus, calpain-mediated substrate degradation is cell type specific and context dependent.

In summary, the deletion or inhibition of TP increased EC differentiation of ASCs via the suppression of calpain-mediated β-catenin degradation in mice and humans. The potential applications of TP pathway inhibition for enhancing therapeutic efficacy of ASCs in human ischemic diseases warrant further investigations.

Figure 8. Thromboxane-prostanoid receptor (TP) inhibition enhances endothelial cell (EC) differentiation in human adipose-derived stromal cells (ASCs). A, Representative flow charts for CD31+ subsets in SQ29548-treated human ASCs with or without vascular endothelial growth factor (VEGF) stimulation. B, Quantification of CD31+ cells. *P<0.05 vs vehicle, #P<0.05 (Mann–Whitney U test); n=6. Experiments were repeated 3 times. C, Tube formation in SQ29548-treated human ASCs with or without VEGF stimulation. D, Quantification of tube-like structures. Data represent mean±SEM. *P<0.05 vs vehicle (Mann–Whitney U test); n=6. Experiments were repeated twice. E and F, Relative mRNA expression levels of VEGF-A and basic fibroblast growth factor (bFGF) in human ASCs with or without SQ29548 treatment during EC induction. *P<0.05 vs vehicle (unpaired 2-tailed t test); n=9–10. G, In vivo Matrigel plug angiogenesis assays in SQ29548-treated human ASCs. H, Representative images of vascular network formation in SQ29548-treated human ASCs in the Matrigel plug 2 wk after implantation. Scale bar, 50 μm. I, Quantification of human (h) and mouse (m) CD31+ cells. Data represent mean±SEM. *P<0.05 vs vehicle (unpaired 2-tailed t test); n=6.
Acknowledgments

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Therapeutic angiogenesis, including autologous transplantation of mesenchymal stromal cells, has emerged as a promising strategy for the treatment of ischemic diseases.
- Adipose tissue is rich in pluripotent adipose-derived stromal cells (ASCs).
- Thromboxane exerts multiple physiological functions by acting on its receptor (thromboxane-prostanoid [TP]), low-dose aspirin is widely used to prevent cardiovascular events by blocking platelet-derived thromboxane.

**What New Information Does This Article Contribute?**

- Thromboxane/TP axis is upregulated during endothelial cell differentiation of ASCs.
- Genetic deficiency or pharmacological inhibition of TP receptor increases vascular endothelial growth factor–induced endothelial cell differentiation of ASCs in vitro and in vivo.

- TP deficiency enhances the angiogenic efficacy of ASC therapy in mice through suppression of calpain-dependent degradation of β-catenin.

Autologous ASCs offer great promise as angiogenic cell therapy for ischemic diseases. However, the self-renewal capacity and multipotency of ASCs are still relatively low, which limits their applications in angiogenic therapy. In this study, we show that cyclooxygenase-2/thromboxane/TP axis is elevated during endothelial cell differentiation of ASCs induced by vascular endothelial growth factor. Disruption of this pathway by genetic deletion or pharmacological inhibition of TP receptor increases the angiogenic capacity of both mouse and human ASCs in vitro and in vivo. TP deletion suppresses calpain-mediated β-catenin degradation in ASCs to facilitate endothelial cell differentiation. Thus, our observations indicate that inhibition of thromboxane/TP signaling may increase the therapeutic efficacy of autologous ASCs for the treatment of ischemic diseases.
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Supplemental Materials

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Materials and Methods

Animals

All mice were maintained on a C57BL/6 genetic background. Wild-type (WT) littermates were generated from thromboxane-prostanoid receptor (TP) heterozygous matings as experimental controls. dsRed and enhanced green fluorescent protein (EGFP) transgenic mice were crossed with TP-knockout (KO) mice to generate dsRed or EGFP/TP-KO mice, respectively. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

Reagents

Calpeptin, U46619, arachidonic acid and SQ29548 were obtained from Cayman Chemical Company (Cayman Chemical, Ann Arbor, MI, USA). Mouse and human vascular endothelial growth factor (VEGF)165 and basic fibroblast growth factor (bFGF) were purchased from Peprotech (Peprotech Inc., Rocky Hill, NJ, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA).

Isolation and culture of adipose-derived stem cells (ASCs)

Human ASCs (second passage) were purchased from Cyagen Biosciences (cat. no. HUXMD-01001) and cultured in OriCell human ASC growth medium (cat. no. HUXMD-90011). Mouse ASCs were isolated from epididymal fat pads as previously described1. Briefly, adipose tissues were cut into small pieces, which were then digested with 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 60 min. The resultant cell suspension was filtered through a 100-µm mesh (BD Biosciences) to remove tissue debris. The cell suspension was then diluted with phosphate-buffered saline (PBS) and centrifuged to remove collagenase. The cell pellet (also called the stromal vascular fraction [SVF]) was resuspended in 160 mM NH4Cl and incubated at room temperature for 10 min to lyse red blood cells. SVF were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C in an atmosphere with 5% CO2. Medium is changed the day after seeding and then every second day. All mouse ASCs used for subsequent experiments were prepared from the second passage.

For endothelial cell differentiation experiments, 200 μL Matrigel was coated on each well in a 12-well plate and incubated for 1 h at 37°C to allow the gel to solidify. Next, 1 × 10^5 ASCs were seeded in base culture medium or medium with 50 ng/mL VEGF and 2 ng/mL bFGF.

Isolation of CD31− ASCs by negative selection

Negative immunomagnetic selection of CD31− ASCs was achieved by “anti-CD31 magnetic particles” in combination with the related cell separation system (EasySep™) from StemCell Technologies following manufacturer's instructions.

Flow cytometric analysis

Mouse ASCs were analyzed using a BD flow cytometer (FACScan, BD Biosciences). In brief, the
cells were harvested and incubated for 30 min on ice with 1% bovine serum albumin (BSA) in PBS containing primary antibodies directed against the following cell surface markers: CD29, CD90, Sca-1, CD34, and CD31 (eBioscience, San Diego, CA, USA). The cells were then washed twice before analysis. FCS files were exported and analyzed using FlowJo 8.3.3 software (Tree Star Inc., Ashland, OR, USA).

**Immunofluorescence staining**

For immunofluorescence staining, the frozen sections from tissues or glass coverslips with cells were fixed in cold acetone and washed with PBS. After treatment with PBS containing 0.25% Triton X-100 for 10 min for permeabilization, the samples were incubated with 3% BSA/PBS for 30 min to block nonspecific binding of the antibodies. Subsequently, the slides were incubated with primary antibodies specific to mouse CD34 (diluted 1:50; eBioscience), CD31 (diluted 1:50; eBioscience), CD31 (diluted 1:500; BD Biosciences), CD31 (diluted 1:500; Abcam, Cambridge, MA, USA), GFP (diluted 1:1000; Abcam, Cambridge, MA, USA), RFP (diluted 1:1000; Abcam), α-SMA (diluted 1:1000; Abcam), NG2 (diluted 1:200; R&D Systems, Minneapolis, MN, USA) and PCNA (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA) and human CD31 (diluted 1:200; R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Slides were then washed with PBS three times and incubated with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 633 (Invitrogen) for 1 h at room temperature. ProLong Gold antifade reagent with DAPI (Invitrogen) was applied to mount and counterstain the slides. All of the fluorescence images were captured and analyzed under a laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). ASC-induced angiogenesis was quantified as previously described, and the staining area was measured using Image-Pro Plus software 6.0 (Media Cybernetics, Rockville, MD, USA). In the hindlimb ischemia model, at least 10 random images were taken in the central regions of gastrocnemius muscle sections under a fluorescence microscope, and at least three different sections were obtained from each animal. In the in vivo Matrigel plug model, at least three different sections were cut from each plug, and five random images were taken in the central region of each section. For cells growing on the glass slide, at least five random images were taken in the central region of each slide.

**PG extraction and analysis**

Cultured ASCs were incubated with arachidonic acid (30 μmol/L), and culture supernatants were then collected and centrifuged for 15 min at 12,000 × g at 4°C. PGs (6-keto-PGF₁α and TxB₂) were extracted as described previously and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses.

**Tube formation assays**

For tube formation assays, 500 μL Matrigel was coated on each well of a 12-well plate and incubated for 1 h at 37°C to allow the gel to solidify. Next, 2 × 10⁵ ASCs were seeded in base culture medium. After incubation for 12 h, the cells were visualized using a light microscope (IX51; Olympus, Center Valley, PA, USA). Each well was photographed (3 random images). Numbers of meshes were quantitatively analyzed using ‘HUVEC angiogenesis analyzer’ plugin for Image J software (NIH, USA).
**In vivo Matrigel plug angiogenesis assay**
The Matrigel implantation assay was performed as previously described. In brief, 100 μL of Matrigel supplemented with mouse ASCs (0.5 × 10^6) was injected subcutaneously into the flank region of C57BL/6 mice. Human ASCs (1 × 10^6) were mixed with 500 ng/mL VEGF (Peprotech) and 2 μM SQ29548 (Cayman Chemical) in a total volume of 100 μL and then injected subcutaneously into the flank region of nude mice. The same volume of Matrigel alone was used as a negative control. After 2 weeks, Matrigel implants were isolated from the surrounding tissues, photographed, and subjected to histological analyses. To get better images of vasculature structures, a Z-stack of optical sections, 100 μm of total thickness, was captured from the Matrigel plugs using confocal microscopy (50 z-sections collected at 2-μm intervals). Final images were created by overlaying 50 z-sections.

**Cell delivery in a hindlimb ischemia model**
To assess the survival and localization, and therapeutic potential of ASCs, we used a murine model of hindlimb ischemia, as described in previous reports. Briefly, unilateral hindlimb ischemia in the left leg was introduced in male mice by ligation at two points within the proximal and distal left femoral artery. The arterial segment between the ligatures was cut off. The mice were randomized into several groups, each receiving 10^6 ASCs by gastrocnemius intramuscular injection 1 day after surgery.

**Laser Doppler blood perfusion (LDBP)**
Blood flow to the ischemic or normal (nonischemic) hindlimb was assessed using a laser Doppler perfusion imager (LDI; Moor Instruments Ltd., UK) as described previously. Each animal was maintained at a core temperature of 37°C, and hindlimb blood flow was measured on days 7 and 14. Laser Doppler perfusion imaging (LDPI) was performed for the ischemic (left leg) and nonischemic (sham surgery, right leg) hindlimbs as described in the manufacturer’s protocol.

**Cotton thread-induced formation of granulation tissue**
Cotton thread-induced formation of granulation tissue was stimulated as previous described. Cotton threads (Araiwa Co., Sendai, Japan) were washed overnight with ethyl acetate and dried at room temperature before being cut to a length of 1 cm (3 mg weight) and sterilized by dry heat at 180°C for 2 h. The cotton threads were implanted into the epididymal fat pad of anesthetized mice using a 13-gauge implant needle (Natume, Tokyo, Japan). After 2 weeks, the mice were anesthetized and humanely euthanized. The epididymal fat pads were then dissected together with the cotton threads and photographed. After dissection, part of the epididymal fat pad from each mouse was fixed, dehydrated, and embedded in paraffin for histological analysis; the remaining fat pads were frozen for sectioning and other experiments.

**Adenovirus generation and transduction**
Adenovirus was produced using the pAd-Track-CMV-GFP vector, containing cDNA encoding full-length mouse TP, and generated in the 293A viral packaging cell line. After several rounds of propagation, recombinant adenovirus was purified by ultracentrifugation in a cesium chloride gradient. The transduction efficiency was estimated by determining the fluorescence of GFP.

**RNA extraction and real-time quantitative polymerase chain reaction (PCR)**
Total RNA from ASCs was extracted using TRIzol reagent (Invitrogen) according to the
manufacturer’s instructions. Briefly, total RNA was reverse-transcribed to cDNA using a Reverse Transcription Reagent kit (Takara, Dalian, China) according to the manufacturer’s protocol. Real-time PCR was performed using SYBR Green mix (Takara). Target gene expression was normalized to the level of actin mRNA. The PCR protocol was as follows: 5 min at 95°C for one cycle, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. A dissociation curve was obtained for each PCR product. The primer sequences are summarized in Supplementary Table III.

RNA interference
ASCs were transfected using RNAiFect Transfection Reagent (Qiagen, Crawley, UK) according to the manufacturer’s instructions with either specific sequences for β-catenin (100 nM) or a scrambled siRNA (Genepharma, Shanghai, PR China). The mouse siRNAs were designed to target 21-nucleotide sequences (Supplementary Table IV). Control siRNAs included scrambled derivatives of the β-catenin siRNA sequences, an unrelated siRNA labeled by FAM, and a positive control GAPDH siRNA. One microgram of siRNA and RNAiFect Transfection Reagent were mixed and incubated for 10–15 min at room temperature to allow formation of transfection complexes before delivery to the cells. Knockdown efficiency was assessed by Western blot analysis 48 h later.

Calpain activity assay
Calpain activity was examined using a kit according to the manufacturer’s instructions (Calbiochem, San Diego, CA, USA). ASCs were treated with U46619 (1 µM) 1 min before the activity test. Fluorescence was recorded using a fluorescence plate reader at an excitation wavelength of ~360–380 nm and an emission wavelength of ~440–460 nm. Relative fluorescence units were calculated.

Confocal calcium imaging
Ca²⁺ transients were recorded in ASCs using a laser scanning confocal microscope (Carl Zeiss, Inc., Germany)⁸.⁹. Briefly, cells were loaded with 6 µg/mL Fluo-3 or Rhod 4 (Dojindo Laboratories, Kumamoto, Japan) in Hank’s Balanced Salt Solution [HBSS, Invitrogen, Carlsbad, CA, USA. Composition: 1.3 mM calcium chloride, 0.5 mM magnesium chloride, 0.4 mM magnesium sulfate, 5.3 mM potassium chloride, 0.4 mM potassium phosphate monobasic (KH₂PO₄), 4.2 mM sodium bicarbonate (NaHCO₃), 137.9 mM sodium chloride, 0.3 mM sodium phosphate dibasic (Na₂HPO₄), 5.6 mM D-glucose (dextrose), pH, 7.4] for 30 min at 37°C. Cells were incubated in HBSS after washing during calcium measurement. Fluo-3 was excited at 488 nm and Rhod 4 at 594 nm. Images were acquired in the line-scan (X-T) mode with 512 pixels per line at a rate of 5 ms per scan. Two-dimensional images were obtained with the confocal microscope operating in the frame-scan mode (X-Y, 512 × 512 pixels). SR Ca²⁺ load was evaluated by rapid application of 1 µM U46619 (final concentration) with a multichannel rapid application system.

Co-immunoprecipitation
ASCs were transduced with adenovirus harboring empty vector or TP cDNA. TP receptors were immunoprecipitated by incubating 1 mg of whole cell lysates in immunoprecipitation buffer with 5 µL HA-tag antibody or normal IgG (Cell Signaling Technology) control at 4°C for 3 h, followed by incubation with protein A/G agarose (Invitrogen) at 4°C overnight with gentle agitation. After extensive washing, the immune complexes were recovered by boiling in loading buffer and were
subjected to Western blot analysis with HA-tagged antibodies or anti-G_q/11 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Western blot analysis**
Protein from ASCs was extracted in lysis buffer containing protease inhibitors. Membrane proteins were extracted using a Plasma Membrane Protein Extraction Kit (BioVision, Mountain View, CA, USA). The cytosolic fractions were collected just after first centrifugation at 10,000 \( \times g \) for 30 min. Total cellular protein was determined by the BCA method using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal quantities of proteins were denatured and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels, transferred to nitrocellulose membranes, incubated with 5% skim milk for 1–1.5 h, and then incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted as follows: anti- mouse actin (1:2000; Sigma-Aldrich), anti-c-myc (1:1000; geneTex), anti-cyclin D1 (1:1000; Abcam), anti- CD31 (1:500; Abgent), anti-HA-tag, anti-m-calpain, anti-µ-calpain, anti-VE-cadherin, anti-phospho-GSK3β, anti-total-GSK3β, anti-phospho-β-catenin\(^{33}\) (1:1000; Cell Signaling Technology), anti-C-terminal β-catenin (1:1000; BD Biosciences), anti-N-terminal β-catenin (1:1000; Abcam), and anti-β-catenin (1:1000; Millipore). The membranes were then conjugated with a horseradish peroxidase (HRP)-labeled secondary antibody in blocking buffer for 2 h at room temperature. Blots were developed using enhanced chemiluminescence reagents (Pierce), followed by densitometric quantification using ImageJ.

**Statistical analysis**
Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed with SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) using either Student’s \( t \) test, Mann-Whitney \( U \) test or one-way analysis of variance followed by Bonferroni post-hoc test where appropriate. \( P \) values of less than 0.05 were considered statistically significant.

**References**
CD, Yu Y. Cyclooxygenase-2-derived prostaglandin e(2) promotes injury-induced vascular neointimal hyperplasia through the e-prostanoid 3 receptor. Circ Res. 2013;113:104-114


Supplementary Figure legends

Supplementary Figure Ⅰ Production of prostanoids in ASCs after VEGF stimulation. (A) PGI₂(6-keto-PGF₁α). (B) PGE₂. (C) PGD₂. (D) PGF₂α. *P < 0.05 vs. Vehicle (unpaired two-tailed t-test), n = 4-6.

Supplementary Figure Ⅱ TP signaling axis is desensitized in ASCs during VEGF induction. (A) Western blot analysis of COX-2, TxAS and TP in ASCs in response to VEGF treatment for 14 days. (B) Quantitative analysis of COX-2, TxAS and TP protein levels. Data represent mean±s.e.m.*P < 0.05 vs. day 0 (Mann-Whitney U test), n = 3. (C) TxB₂ production in cultured ASCs with VEGF treatment. Data represent mean±s.e.m. *P < 0.05 vs. day 0 (Mann-Whitney U test), n = 3. (D) Kinetics of changes in intracellular calcium concentration in ASCs measured with Fluo-3 during VEGF induction. *P < 0.05 vs. day 0 (unpaired two-tailed t-test), n = 8-10.

Supplementary Figure Ⅲ TP deficiency enhances VEGF-induced EC differentiation of ASCs in vitro. (A) Representative images of immunostaining of CD34 (green), CD31 (red), and nuclei (DAPI, blue) in ASCs in the absence or presence of VEGF. (B) Quantification of CD34⁺/CD31⁺ cells. Data represent mean±s.e.m. *P < 0.05 vs. WT; #P < 0.05 (Mann-Whitney U test); n = 6. The experiments were repeated one time. (C) Relative mRNA levels of endothelial markers of ASCs in the absence or presence of VEGF. *P < 0.05 vs. WT (Mann-Whitney U test); n = 4-6.

Supplementary Figure Ⅳ TP deficiency enhances the ability of CD31⁻ ASCs to differentiate into endothelial cells. (A) Representative flow charts for CD31⁺, Tie2⁺, CD144⁺ and CD34⁺ subsets in ASCs in the absence or presence of VEGF. (B) Quantification of CD31⁺, Tie2⁺, CD144⁺ and CD34⁺ cells. Data represent mean±s.e.m. *P < 0.05 vs. WT, #P < 0.05 (Mann-Whitney U test), n = 6-8. (C) Relative mRNA levels of endothelial markers of ASCs in the absence or presence of VEGF. Data represent mean±s.e.m.*P < 0.05 vs. WT (Mann-Whitney U test), n = 5-6. (D) Western blot analysis of CD31 in ASCs in response to VEGF treatment. (E) mRNA expression levels of pro-angiogenic growth factors in WT and TP-KO ASCs in the absence or presence of VEGF. Data represent mean±s.e.m.*P < 0.05 vs. WT (Mann-Whitney U test), n = 4-6. (F) Tube formation of WT and TP-KO ASCs in the absence or presence of VEGF. (G) Quantification of tube-like structures. Data represent mean±s.e.m. *P < 0.05 vs. WT, #P < 0.05 (Mann-Whitney U test), n = 8-10. (H) In vivo Matrigel plug angiogenesis assay in WT and TP-KO ASCs. (I) Representative images of vascular network formation in WT and TP-KO ASCs in the Matrigel plug 2 weeks after implantation. Scale bar, 50 µm. (J) Quantification of EGFP⁺/CD31⁺ blood vessels. EGFP⁺, cells-derived from EGFP transgenic mice. *P < 0.05 vs. WT (unpaired two-tailed t-test), n = 6. Data represent mean±s.e.m.

Supplementary Figure Ⅴ Cotton thread-induced formation of granulation tissue and angiogenesis in TP-KO and WT mice. (A) Representative cotton thread-implanted epididymal fat pads from TP-KO and WT mice. Green arrowheads, outgrowth of new blood vessels. (B) Histological analysis of cotton thread-induced granulation tissues. T indicates cotton thread. (C) Immunofluorescence staining of CD31 (red) and nuclei (DAPI, blue) in the fibroblast layer. Scale bar, 50 µm. (D) Quantitative analysis of total capillary density (CD31⁺) in the fibroblast layer. Data represent mean±s.e.m. *P < 0.05 vs. WT (unpaired two-tailed t-test), n = 6.
Supplementary Figure VI Formation of arterioles in a mouse model of hindlimb ischemia after WT and TP KO ASC treatment (A) Immunofluorescence staining of α-SMA and CD31 in RFP+ ASC-treated ischemic hindlimbs. RFP+, cells from RFP transgenic mice; yellow arrow, RFP+/CD31+ cells; scale bar, 50 μm. (B) Quantitative analysis of total α-SMA+ cells in the ischemic limbs. (C) Immunofluorescence staining of NG2 and CD31 in RFP+ ASC-treated ischemic hindlimbs. RFP+, cells from RFP transgenic mice; yellow arrow, NG2-/RFP+/CD31+ cells; white arrow, NG2+/RFP+/CD31- cells; scale bar, 50 μm. (D) Quantitative analysis of total NG2+ cells in the ischemic limbs. (E) Quantitative analysis of total NG2+/RFP+/CD31- cells in the ischemic limbs. (F) Quantitative analysis of total NG2-/RFP+/CD31+ cells in the ischemic limbs. *P < 0.05 vs. WT (unpaired two-tailed t-test). n = 6.

Supplementary Figure VII Effects of TP deficiency on Notch signaling in ASCs. (A) Relative mRNA levels of Notch receptors and ligands in WT and TP-KO ASCs in response to VEGF treatment. Data represent mean±s.e.m. #P < 0.05 vs. Vehicle (Mann-Whitney U test); n = 4-6. (B) Relative mRNA levels of Notch target genes in WT and TP-KO ASCs in response to VEGF treatment. Data represent mean±s.e.m. #P < 0.05 vs. Vehicle (Mann-Whitney U test); n = 4-6.

Supplementary Figure VIII TP deficiency promotes β-catenin deficiency independent of GSK3β activity. (A) Western blot analysis of phospho (p)-GSK3βS9, total (t)-GSK3β, p-β-cateninS33, and total (t)-β-catenin in WT and TP KO ASCs in response to VEGF treatment. (B) Relative mRNA expression levels of β-catenin in WT and TP-KO ASCs in response to VEGF. Data represent mean±s.e.m. P > 0.05 vs. WT (Mann-Whitney U test); n = 6.

Supplementary Figure IX Effects of calpain treatment on EC differentiation of TP-KO ASCs transduced by TP-expressing adenovirus. (A) Immunofluorescence staining of CD31 in calpain-treated ASCs re-expressing TP. CPT, calpeptin. Scale bar, 50 μm. (B) Quantification of GFP+/CD31+ cells. Data represent mean±s.e.m. *P < 0.05 vs. GFP vectors, #P < 0.05, &P < 0.05 as indicated (One way ANOVA, followed by Bonferroni post-hoc test); n = 6. (C) Representative flow charts for GFP+/CD31+ subsets in calpain-treated ASCs re-expressing TP. (D) Quantification of GFP+/CD31+ cells. Data represent mean±s.e.m. *P < 0.05 vs. GFP vectors, #P < 0.05, &P < 0.05 as indicated (One way ANOVA, followed by Bonferroni post-hoc test); n = 6.

Supplementary Figure X Expression of calpain isoforms and endogenous inhibitors of calpain in ASCs. (A) Relative mRNA levels of calpain isoforms in ASCs. Data represent mean±s.e.m. n = 6. (B) Western blot analysis of endogenous inhibitors of calpain in TP KO and WT ASCs.
Supplementary Figure III

A

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B

CD34* CD31* (×10^2 μm²/field)

WT   TP KO
Vehicle VEGF

C

Relative mRNA level

Tie2  CD31  VE-cad  NOS3  CXCR4  VEGFR2

Vehicle VEGF
Supplementary Figure V

A

B

C

D

WT  TP KO

WT  TP KO

WT  TP KO

WT  TP KO

CD31

DAPI

Merged

CD31+ (×10^3 μm²/field)

0  5  10  15  20  25

*
Supplementary Figure X

A

Relative mRNA level

μ-calpain
m-calpain
Calpain 3
Calpain 5
Calpain 6
Calpain 7
Calpain 8
Calpain 9
Calpain 10

B

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Supplementary Table I  Characterization of original prepared ASCs.

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* P < 0.05 vs. WT, #P < 0.05 vs. vehicle (unpaired two-tailed t-test), n = 6-8.

Supplementary Table II  Characterization of original prepared ASCs and CD31− ASCs.

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<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Tie2</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
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<tr>
<td>CD144</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.2</td>
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## Supplementary Table III  Primers for real-time PCR analysis

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<th>Gene</th>
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<td>GGTCGCCGTAGTGGGCAACTTCTTGA</td>
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<td>VEGF-A</td>
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<td>VE-Cadherin</td>
<td>CAATGACAAACTTCCCGTCTGT</td>
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<td>NOS3</td>
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<td>Notch2</td>
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<td>m-Calpain</td>
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<td>Calpain 8</td>
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<td>GCTCATGCGTGATTTCTCCA</td>
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<td>Calpain 9</td>
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<td>Calpain 10</td>
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## Supplementary Table IV  siRNA sequences

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