TEAD1 (TEA Domain Transcription Factor 1) Promotes Smooth Muscle Cell Proliferation Through Upregulating SLC1A5 (Solute Carrier Family 1 Member 5)-Mediated Glutamine Uptake

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Rationale: TEAD (TEA domain transcription factor) 1—a major effector of the Hippo signaling pathway—acts as an oncoprotein in a variety of tumors. However, the function of TEAD1 in vascular smooth muscle cells (VSMCs) remains unclear.

Objective: To assess the role of TEAD1 in vascular injury–induced smooth muscle proliferation and delineate the mechanisms underlying its action.

Methods and Results: We found that TEAD1 expression is enhanced in mouse femoral artery after wire injury and correlates with the activation of mTORC1 (mechanistic target of rapamycin complex 1) signaling in vivo. Using an inducible smooth muscle–specific Tead1 KO (knockout) mouse model, we found that specific deletion of Tead1 in adult VSMCs is sufficient to attenuate arterial injury–induced neointima formation due to inhibition of mTORC1 activation and VSMC proliferation. Furthermore, we found that TEAD1 plays a unique role in VSMCs, where it not only downregulates VSMC differentiation markers but also activates mTORC1 signaling, leading to enhanced VSMC proliferation. Using whole-transcriptome sequencing analysis, we identified Slc1a5 (solute carrier family 1 member 5)—a key glutamine transporter—as a novel TEAD1 target gene. SLC1A5 overexpression mimicked TEAD1 in promoting mTORC1 activation and VSMC proliferation. Moreover, depletion of SLC1A5 by silencing RNA or blocking SLC1A5-mediated glutamine uptake attenuated TEAD1-dependent mTORC1 activation and VSMC proliferation.

Conclusions: Our study unravels a novel mechanism by which TEAD1 promotes VSMC proliferation via transcriptional induction of SLC1A5, thereby activating mTORC1 signaling and promoting neointima formation. (Circ Res. 2019;124:1309-1322. DOI: 10.1161/CIRCRESAHA.118.314187.)

Key Words: animals • downregulation • glutamine • mice • myocytes, smooth muscle • neoplasms • transcription factors

Vascular smooth muscle cells (VSMCs) are the major cell type in blood vessels. Unlike skeletal or cardiac myocytes that are terminally differentiated, VSMCs retain a remarkable plasticity. In response to environmental cues such as vascular injury, VSMCs may modulate from a quiescent status to a hyperproliferative phenotype that contributes to vascular remodeling and neointima formation.1 Abnormal proliferation of VSMCs is key to a number of occlusive vascular diseases in humans, such as atherosclerosis, intimal hyperplasia associated with restenosis, and vein graft stenosis.1 Although substantial progress has been made with unraveling the origin of intimal VSMCs in arterial injury and atherosclerosis,2–4 the mechanisms involved in triggering VSMC proliferation are far from being completely understood.

As major downstream nuclear effectors of Hippo signaling, TEAD (TEA domain transcription factor) proteins are a family of 4 transcription factors (TEAD1–4) that bind to a...
What Is Known?

- Vascular smooth muscle (VSMC) proliferation is key to a number of occlusive vascular diseases that block the blood flow in arteries.
- TEAD (TEA domain transcription factor) 1 is a transcription factor that has been shown to promote cancer cell proliferation.
- mTORC1 (mechanistic target of rapamycin complex 1) is a master cell growth regulator, and its inhibitors (eg, rapamycin) are commonly used in the clinical setting to limit VSMC overgrowth-induced blood vessel blockage.
- SLC1A5 (solute carrier family 1 member 5)—a high-affinity L-glutamine transporter that is highly expressed in several cancer types—promotes the uptake of L-glutamine, leading to the downstream effects of activated mTORC1 signaling and subsequent cell growth are mediated through the phosphorylation of p70S6K (p70 ribosomal protein S6 kinase-1), S6 (ribosomal protein S6), and 4EBP1 (eukaryotic initiation factor-4E–binding protein-1), ultimately leading to enhanced cell growth and proliferation by promoting protein synthesis and triggering proteasome-mediated degradation of the key cell cycle inhibitor p27kip1. Furthermore, expression of TEAD proteins is upregulated in many cancer types and correlates with poor survival in patients with cancer. In addition to their recognized roles in cancer, TEADs have been shown to regulate the expression of multiple smooth muscle (SM)–specific and cardiac muscle–specific genes during cardiovascular development. In SM, while TEADs are required for the initial transcriptional activation of key SM differentiation genes such as Myocardin (Myocardin) and Acta2 (SMA [SM α-actin]) during embryonic development, we previously demonstrated that in adult SM, TEAD1 represses SM-specific gene expression by disrupting the interaction of myocardin with the key transcription factor SRF (serum response factor). However, whether TEAD1 regulates VSMC proliferation and neointima formation in vivo remains unknown.

mTORC1 (mechanistic target of rapamycin complex 1) is a master growth regulator that can be activated by various signals, such as growth factors and amino acids. The downstream effects of activated mTORC1 signaling and subsequent cell growth are mediated through the phosphorylation of p70S6K (p70 ribosomal protein S6 kinase-1), S6 (ribosomal protein S6), and 4EBP1 (eukaryotic initiation factor-4E–binding protein-1), ultimately leading to enhanced cell growth and proliferation by promoting protein synthesis and triggering proteasome-mediated degradation of the key cell cycle inhibitor p27kip1. The key role of mTORC1 in regulating VSMC proliferation and neointima formation is supported by the fact that mTORC1 inhibitors (eg, rapamycin) are commonly used in the clinical setting to limit intimal hyperplasia associated with restenosis after angioplasty. However, how mTORC1 is activated in VSMCs after vascular injury is largely unknown. Recent studies have demonstrated that SLC1A5 (solute carrier family 1 member 5)—a high-affinity L-glutamine transporter that is highly expressed in several cancer types—promotes the uptake of L-glutamine, leading to the growth of cancer cells. 

What New Information Does This Article Contribute?

- TEAD1 deficiency in VSMCs inhibits vascular injury–induced arterial occlusion due to impaired VSMC proliferation.
- TEAD1 is upregulated after arterial injury and transcriptionally induces SLC1A5 expression, leading to mTORC1 signaling activation, thereby promoting VSMC proliferation and arterial occlusion.

In many occlusive vascular diseases, VSMCs undergo phenotypic switching to a hyperproliferative state. Identification of the mechanisms involved in VSMC proliferation will lead us toward better understanding the pathology of these diseases and ultimately designing new therapeutic agents for their treatment and prevention. Our study uncovers a previously unrecognized role of the transcription factor TEAD1 in VSMC proliferation. Our data demonstrate that vascular injury induces TEAD1 expression. The injury-induced TEAD1 expression, in turn, transcriptionally promotes the key glutamine transporter SLC1A5 expression, leading to enhanced glutamine uptake and activation of mTORC1 signaling, which results in enhanced VSMC proliferation that underlies the robust neointima formation in response to vascular injury. Our novel findings suggest that TEAD1-SLC1A5-glutamine uptake is a promising therapeutic axis to ameliorate occlusive vascular diseases in humans.

Nonstandard Abbreviations and Acronyms

- 4EBP1: eukaryotic initiation factor-4E–binding protein-1
- EC: endothelial cell
- EdU: 5-ethynyl-2′-deoxyuridine
- F: flox
- GPNA: γ-L-glutamyl-p-nitroanilide
- HCASMC: human coronary artery smooth muscle cell
- HET: heterozygous
- Hic-5: hydrogen peroxide–inducible clone 5
- iKO: inducible knockout
- K0: knockout
- LFA: left femoral artery
- MCAT: muscle CAT element
- MHC: myosin heavy chain
- MLCK: myosin light-chain kinase
- mTORC: mechanistic target of rapamycin complex
- p70S6K: p70 ribosomal protein S6 kinase
- PCNA: proliferating cell nuclear antigen
- pHi: phospho-histone 3
- pS6: phospho-ribosomal protein S6
- S6: ribosomal protein S6
- SLC1A5: solute carrier family 1 member 5
- SM: smooth muscle
- SMA: smooth muscle α-actin
- SRF: serum response factor
- TEAD: TEA domain transcription factor
- VSMC: vascular smooth muscle cell
- WT: wild type
- YAP: yes-associated protein

Consensus DNA sequence 5′-CATTCC-3′, named the muscle-specific cytidine-adenosine-thymidine (MCAT) element. Through interaction with a variety of cofactors, such as YAP (yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif), TEADs bind to MCAT-containing genes that regulate cell growth, thereby leading to oncogenic transformation. Furthermore, the expression of TEAD proteins is upregulated in many cancer types and correlates with poor survival in patients with cancer. In addition to their recognized roles in cancer, TEADs have been shown to regulate the expression of multiple smooth muscle (SM)–specific and cardiac muscle–specific genes during cardiovascular development. In SM, while TEADs are required for the initial transcriptional activation of key SM differentiation genes such as Myocardin (Myocardin) and Acta2 (SMA [SM α-actin]) during embryonic development, we previously demonstrated that in adult SM, TEAD1 represses SM-specific gene expression by disrupting the interaction of myocardin with the key transcription factor SRF (serum response factor). However, whether TEAD1 regulates VSMC proliferation and neointima formation in vivo remains unknown. mTORC1 (mechanistic target of rapamycin complex 1) is a master growth regulator that can be activated by various signals, such as growth factors and amino acids. The downstream effects of activated mTORC1 signaling and subsequent cell growth are mediated through the phosphorylation of p70S6K (p70 ribosomal protein S6 kinase-1), S6 (ribosomal protein S6), and 4EBP1 (eukaryotic initiation factor-4E–binding protein-1), ultimately leading to enhanced cell growth and proliferation by promoting protein synthesis and triggering proteasome-mediated degradation of the key cell cycle inhibitor p27kip1. The key role of mTORC1 in regulating VSMC proliferation and neointima formation is supported by the fact that mTORC1 inhibitors (eg, rapamycin) are commonly used in the clinical setting to limit intimal hyperplasia associated with restenosis after angioplasty. However, how mTORC1 is activated in VSMCs after vascular injury is largely unknown. Recent studies have demonstrated that SLC1A5 (solute carrier family 1 member 5)—a high-affinity L-glutamine transporter that is highly expressed in several cancer types—promotes the uptake of L-glutamine, leading to the
activation of mTORC1 signaling and thus promotes cancer cell proliferation. However, to date, the role of SLC1A5 in VSMC proliferation and the upstream regulatory mechanism of SLC1A5 expression in VSMCs have not been examined.

In this study, we aimed to identify the role and the downstream actions of upregulated expression of TEAD1 in VSMC proliferation and neointima formation. We found that TEAD1 expression is enhanced in mouse femoral artery after wire injury and correlates with the activation of mTORC1 signaling in vivo. Because Tead1 global KO (knockout) mice are embryonic lethal, we recently generated Tead1 F (flox) mice to circumvent this embryonic lethality and to allow us to study TEAD1 function specifically in postnatal VSMCs. Using an inducible SM-specific Tead1 KO mouse model, we found that specific deletion of Tead1 in adult VSMCs is sufficient to attenuate arterial injury–induced neointima formation by inhibiting mTORC1 activation and attenuating VSMC proliferation. Using whole-transcriptome sequencing analysis, we identified Slc1a5 as a novel TEAD1 transcriptional target that mediates, at least in part, TEAD1-induced mTORC1 signaling activation and VSMC proliferation. Our study suggests that TEAD1-SLC1A5-glutamine uptake is a promising therapeutic axis to ameliorate occlusive vascular disease.

Methods
The authors declare that all supporting data are available within the article and its Online Data Supplement. The RNA-sequencing (seq) data generated in this study have been deposited in the Sequences Read Archive at the NCBI (National Center for Biotechnology Information) under accession number PRJNA492803.

Tead1 global heterozygote mice were generated as we recently reported. SM-specific Tead1 iKO (inducible KO) mice were generated by crossing female Tead1 F mice with male mice expressing tamoxifen-inducible Cre driven by the SM-specific gene Myh11 (SM myosin heavy chain [SM MHC]). Myh11-CreER(T2) Only male mice were used in this study because SM MHC-CreER2 transgene is only localized in the Y chromosome. At 10 weeks of age, male mice (Myh11-CreER(T2)/Tead1(+/−)) were randomly assigned to 2 groups. These 2 groups of mice were then intraperitoneally injected with vehicle sunflower oil (served as control) or tamoxifen (1 mg per mouse, iKO), respectively, once a day for 10 days with 2 days’ break after the first 5 injections. Two weeks after the last injection, wire injury was performed in the left femoral artery (LFA) as described previously. The right femoral artery in the same mouse served as sham control. All mice used in this study were maintained on a C57BL/6 background. The use of experimental mouse for arterial injury procedures and BSL-2 (biosafety level 2) viral work was approved by the Institutional Animal Care and Use Committee and Biosafety Committees at Augusta University. A detailed, expanded Methods section is included in the Online Data Supplement.

Results
TEAD1 Expression Is Induced After Arterial Injury and Positively Correlates With mTORC1 Activation in VSMCs In Vivo
Before investigating TEAD1 function in the vasculature in vivo, we examined the relative mRNA abundance of all 4 TEAD family genes in cultured vascular cells, including VSMCs, endothelial cells (ECs), and macrophages. Quantitative polymerase chain reaction data revealed that in comparison to other TEAD genes, TEAD1 is the most abundant member of the TEAD family in human coronary artery SM cells (HCASMCs; Online Figure IA through IC). Furthermore, Western blotting data revealed that TEAD1 protein is readily detectable in VSMCs, across all the examined species (Online Figure ID). Next, we examined the expression of TEAD1 after arterial injury, where VSMCs actively proliferate in vivo, thereby contributing to neointima formation. We found that, compared with control right femoral arteries, the expression levels of TEAD1 and YAP—a key TEAD transcriptional cofactor—are enhanced in the LFA s after wire injury, a mouse arterial injury model that mimics postangioplasty restenosis in humans (Figure 1A and 1B). Furthermore, TEAD1 was highly expressed in both the neointimal area and the expanding adventitial area after arterial injury (Figure 1C and 1D). Notably, the enhanced expression of TEAD1 not only correlated with upregulation of the proliferative markers pH3 (phospho-histone H3) and PCNA (proliferating cell nuclear antigen) and downregulation of contractile SM markers (SM MHC [myosin heavy chain], MLCK [myosin light-chain kinase], calponin, and SM22α [smooth muscle protein 22-alpha]; Figure 1A and 1B) but also correlated with enhanced phosphorylation of S6 and 4EBP1 (functional readouts of activated mTORC1) in VSMCs (Figure 1A, 1B, 1E, and 1F). These data demonstrate that TEAD1 induction, in response to vascular injury, correlates with SM proliferation and mTORC1 activation in vivo.

SM-Specific Deletion of Tead1 Attenuates Neointima Formation and Decreases VSMC Proliferation In Vivo
To determine the functional role of TEAD1 in vivo, we first performed wire injury in LFA in Tead1 global HET (heterozygous) mice because Tead1 global KO mouse is embryonic lethal. Characterization of the Tead1 HET mice demonstrated that TEAD1 protein expression in HET mice is reduced to ~50% in both aortic and heart tissues compared with WT (wild type) control mice. This decreased TEAD1 expression did not affect SM or cardiac contractile protein expression or gross morphology of intact right femoral artery (Online Figure IIA through IID). However, compared with littermate WT control mice, Tead1 HET mice exhibited markedly reduced intimal area and intima-to-media ratio in injured LFA by ~58% and ~61%, respectively, without affecting the relative medial area in LFA at day 14 post-wire injury (Online Figure IIE through IIIH). These results demonstrate that heterozygous deletion of Tead1 attenuates neointima formation after arterial injury.

To study TEAD1 function specifically in postnatal VSMCs in vivo, we generated SM-specific Tead1 iKO mice. Compared with control mice, iKO mice have markedly reduced TEAD1 mRNA and protein expression specifically in the medial VSMCs (Online Figure IIIA through IIIF). Similar to the global Tead1 HET mice, deletion of Tead1 in adult VSMCs did not alter SM contractile gene expression, overall vessel morphology, or VSMC numbers (Online Figure IIIIC, IIIE, and IIIF), indicating that TEAD1 is not required for the maintenance of quiescent VSMCs in adult mice. To test whether SM-specific deletion of Tead1 attenuates neointima formation, we performed LFA wire injury in iKO mice versus control mice. We found that SM-specific Tead1 KO markedly reduced the intimal area and the intima-to-media ratio in injured LFA by ~70% and ~72%, respectively, without affecting the relative
medial area at day 14 post-injury (Figure 2A through 2D). Furthermore, SM-specific Tead1 iKO significantly decreased the total number of EdU (5-ethynyl-2′-deoxyuridine)- and Ki67-positive VSMCs in the neointimal area (Figure 2E through 2H). These data suggest that SM-specific deletion of Tead1 attenuates neointima formation because of impaired VSMC proliferation.

SM-Specific Deletion of Tead1 Inhibits mTORC1 Activation After Vascular Injury

Data described above demonstrated that TEAD1 expression correlates with enhanced mTORC1 signaling in VSMCs after arterial injury (Figure 1). We next tested the effects of SM-specific Tead1 iKO on vascular injury-induced mTORC1 activation. Western blot and immunofluorescence analysis demonstrated that compared with control mice, SM-specific Tead1 iKO significantly attenuated the induction of pS6 (phospho-S6; mTORC1 activation marker) and the proliferative marker pH3 in injured LFA (Figure 3A through 3C). However, SM-specific Tead1 iKO did not rescue the injury-induced downregulation of multiple contractile SM markers in LFA (Figure 3A and 3B; Online Figure IV). Together, these results suggest that specific deletion of Tead1 in VSMCs inhibits vascular injury–induced neointima formation by attenuating mTORC1 activation and VSMC proliferation.

Glutamine Transporter Slc1a5 Is a Direct Target Gene of TEAD1

To gain further insight on the mechanism underlying TEAD1-mediated mTORC1 activation and VSMC proliferation, we performed whole-transcriptome analysis by RNA-seq of intact aortic tissues isolated from SM-Tead1 iKO mice versus control mice. Data from the RNA-seq analysis demonstrated that SM-specific Tead1 iKO significantly affected the expression of genes involved in cell proliferation, differentiation,
Figure 2. Smooth muscle (SM)–specific deletion of Tead (TEA domain transcription factor) 1 attenuates neointima formation by inhibiting vascular SM cell proliferation. A, Control or SM-specific Tead1 iKO (inducible knockout) mice were subjected to left femoral artery (LFA) wire injury. At 14 d post-injury, LFAs were harvested, and neointimal area was analyzed by hematoxylin and eosin (HE) or Elastic Van Gieson (EVG) staining. n=9 per group. (Continued)
and migration (Figure 4B). Further analysis of the downregulated mRNAs revealed that SM-specific Tead1 iKO inhibited the expression of Slc1a5—a major glutamine transporter that was previously linked to mTORC1 activation in nonvascular cells (Figure 4C; Online Table I). Western blotting confirmed that SM-specific deletion of TEAD1 downregulated SLC1A5 expression at the protein level in intact aortic tissues (Figure 4D). Consistently, siRNA-mediated knockdown of endogenous TEAD1, but not other TEAD family genes, in HCASMCs specifically downregulated SLC1A5 mRNA expression levels by ≈50%, without affecting the expression of SLC7A5 and SLC38A1 (Figure 4E; Online Figure V). The TEAD1-dependent expression of SLC1A5 was confirmed at the protein level in HCASMCs (Figure 4F). Furthermore, we found that Slc1a5 expression was upregulated at the mRNA level in injured LFA 7 days post-injury, which was accompanied with upregulation of the proliferative marker PcnA and downregulation of SM contractile markers Acta2 (SMA) and Cnn1 (calponin; Figure 4G). Next, we attempted to test whether SLC1A5 is upregulated at the protein level in injured vessels. Because of the limited amount of protein that can be extracted from the relatively small-sized mouse femoral artery
Figure 4. The glutamine transporter SLC1A5 (solute carrier family 1 member 5) is a direct target gene of TEAD (TEA domain transcription factor) 1. A. Gene ontology analysis was performed to show the biological functions related with the differentially expressed genes following ablation of Tead1 in adult vascular SM cells in mice. B. Heat map to show the expression of Tead1 and SLC (solute carrier family) family genes in control or SM-specific Tead1 iKO mouse aortic tissues. C. Embryonic morphogenesis, Circadian rhythm regulation of cell proliferation, Regulation of cell differentiation, Muscle contraction, Muscle cell differentiation, Regulated cell migration. D. Aortic tissue lysates harvested from control or SM-specific Tead1 iKO mouse aortic tissues. E. Human coronary artery SM cells (HCASMCs) were transfected with scrambled control siRNA (siControl) or TEAD1 siRNA (siTEAD1) for 48 h. Total RNA was isolated for qRT-PCR (quantitative real-time polymerase chain reaction) analysis to determine mRNA expression as indicated. Two primer sets (PS) were used to assess SLC1A5 expression as shown. F. Similar to E, except that HCASMCs were harvested for Western blot analysis. G. Control right femoral arteries (RFAs) or wire-injured left femoral arteries (LFAs) from adult male C57BL/6 mice at 7 days post-injury were harvested, and total RNA was extracted for qRT-PCR analysis to determine mRNA expression using the gene-specific primers as indicated. The mRNA expression in control RFA was set to 1 (red dashed line). H. HCASMCs were transduced with GFP (green fluorescent protein) or TEAD1 adenovirus, and total RNA was isolated for qRT-PCR analysis to determine the relative mRNA expression. I. Similar to H, except that HCASMCs were harvested for Western blot analysis. J. Schematic diagram to show generation of WT (wild type) or muscle CAT element (MCAT) mutation human SLC1A5 promoter–driven luciferase reporters. K. TEAD1 adenovirus was transduced into HCASMCs, and then chromatin was harvested for immunoprecipitation with TEAD1 antibody or IgG control. The precipitated DNA was amplified by real-time polymerase chain reaction using SLC1A5 promoter-specific primers that span the MCAT region depicted in J or primers localized in SLC1A5 exon 8 region. L. The luciferase reporter constructs harboring WT SLC1A5 promoter or mutation of predicted TEAD1-binding site in SLC1A5 promoter were cotransfected with TEAD1 expression plasmid into 10T1/2 cells. Fourty-eight hours post-transfection, cells were harvested for dual luciferase assays. Reporter activity was normalized to a renilla luciferase internal control and expressed relative to the transfection with control empty plasmid (set to 1). FPKM indicates fragments per kilobase of transcript per million mapped reads; L, long exposure; and S, short exposure. *P<0.05.
or lack of sensitivity of the SLC1A5 antibody for small amount of protein, we switched to utilize a rat carotid artery balloon injury model that is similar to mouse wire injury in resembling human postangioplasty restenosis,30 while providing a larger amount of protein. Using the protein lysate harvested from the balloon-injured rat carotid artery 7 days post-injury or uninjured contralateral controls, data from Western blotting assays demonstrated that TEAD1 was upregulated in injured arteries and correlated with induction of SLC1A5, pS6, PCNA, and pH3, with accompanying downregulation of contractile SM markers (Hic-5 [hydrogen peroxide-inducible clone 5], calponin, and SM22α; Online Figure VIA and VIB).

Because arterial injury can induce both TEAD1 and SLC1A5 expression (Figures 1A and 4G; Online Figure VI), we next tested whether overexpression of TEAD1 in VSMCs can promote SLC1A5 expression. Indeed, adenovirus-mediated overexpression of TEAD1 in HCASMCs specifically upregulated SLC1A5 mRNA (Figure 4H) and protein (Figure 4I) expression, respectively. We next sought to delineate the mechanism by which TEAD1 regulates SLC1A5 gene expression in VSMCs. Bioinformatic analysis identified an evolutionarily conserved TEAD-binding element (MCAT) in the SLC1A5 gene promoter region (Figure 4J). Chromatin immunoprecipitation assays demonstrated an ≈2.4-fold enrichment of TEAD1 in the promoter region of SLC1A5 harboring the MCAT element but not in the SLC1A5 exon 8 region (Figure 4K). To test whether TEAD1 can directly activate SLC1A5 gene promoter, we generated luciferase reporters containing SLC1A5 promoter region harboring the WT or mutated MCAT site (Figure 4J). Data from luciferase assays demonstrated that TEAD1 significantly enhanced SLC1A5 promoter activity by ≈2.4-fold. Mutation of the TEAD-binding MCAT site not only inhibited the basal activity of SLC1A5 gene reporter but also abolished TEAD1-dependent SLC1A5 promoter activation (Figure 4L). Together, these results demonstrate that SLC1A5 is a direct transcriptional target of TEAD1 and suggest that SLC1A5 may mediate the effects of TEAD1 on mTORC1 activation and VSMC proliferation.

TEAD1 Is Sufficient and Required to Promote VSMC Proliferation, Induce SLC1A5, and Activate mTORC1 Signaling

Data described above demonstrated that wire injury–induced and balloon injury–induced TEAD1 expression positively correlates with mTORC1 activation (Figure 1; Online Figure VI) and that SM-specific Tead1 iKO attenuates mTORC1 activation and neointimal cell proliferation in vivo (Figure 2). Therefore, we next sought to test the direct effects of TEAD1 on VSMC proliferation and mTORC1 activation in HCASMCs in vitro. Data from WST-1 (water soluble tetrazolium-1) colorimetric proliferation assay, EdU incorporation assay, and cell number counting revealed that overexpression of TEAD1 in HCASMCs is sufficient to promote VSMC proliferation (Figure 5A through 5D). Furthermore, TEAD1 overexpression specifically induced SLC1A5 expression and enhanced mTORC1 signaling as indicated by the hyperphosphorylation of mTORC1 substrates S6 and 4EBP1 (Figure 5E). Furthermore, overexpression of TEAD1 in HCASMCs neither affected the basal nor the activated Akt (protein kinase B) or ERK (extracellular signal-regulated kinase) signaling induced by acute treatment (30 minutes) with the potent mitogenic growth factor PDGF (platelet-derived growth factor)-BB,31 suggesting a selective function of TEAD1 on mTORC1 signaling activation (Figure 5F). Western blot data from our initial time course experiments revealed that PDGF-BB promoted a maximal expression of the proliferative marker pH3 by 2-day treatment, whereas it downregulated multiple SM contractile proteins after 5-day treatment (Online Figure VII). Therefore, we chose 2-day treatment of PDGF-BB to examine the effects of overexpression of TEAD1 on PDGF-BB–induced VSMC proliferation. We found that overexpression of TEAD1 had a slightly additive effect on the induced expression of the proliferative marker pH3 and downregulated expression of the cell cycle inhibitor p27 in the presence of PDGF-BB for 2 days (Figure 5F). Furthermore, to confirm the reproducibility of our findings above, the functional effects of overexpression of TEAD1 on SLC1A5 expression and mTORC1 activation were validated in an independent isolate of HCASMCs derived from a different human donor (Online Figure VIII). Together, these data demonstrate that TEAD1 is sufficient to promote VSMC proliferation, induce SLC1A5 expression, and activate mTORC1 signaling independent of PDGF-BB (Figure 5A through 5F). Consistent with the data from gain-of-function assays, knocking down endogenous TEAD1 in HCASMCs using specific siRNA against TEAD1 resulted in inhibition of VSMC proliferation as indicated by WST-1 proliferation assay and cell count analysis (Figure 5G and 5H). Furthermore, silencing TEAD1 led to downregulation of SLC1A5 protein expression, inhibition of 4EBP1 phosphorylation, and downregulation of the proliferative marker pH3. Meanwhile, silencing TEAD1 promoted the expression of the cell cycle inhibitor p27 and expression of the VSMC differentiation markers, Hic-5, SMA, and calponin (Figure 5I). Taken together, our TEAD1 gain-of-function and loss-of-function assays in HCASMCs demonstrate that TEAD1 is sufficient and required to induce SLC1A5 expression, activate mTORC1 signaling, and promote a differentiated-to-proliferative phenotypic switching of VSMCs in vitro.

SLC1A5 Mimics TEAD1 in Activating mTORC1 Signaling and Promoting VSMC Proliferation

Our data described above demonstrated that TEAD1 directly targets SLC1A5, activates mTORC1 signaling, and promotes VSMC proliferation (Figures 4 and 5). We next sought to determine whether SLC1A5 can mimic the function of TEAD1 in promoting mTORC1 activation and VSMC proliferation. Similar to overexpression of TEAD1, data from proliferation assays revealed that overexpression of SLC1A5 in HCASMCs promotes VSMC proliferation (Figure 6A through 6D). Furthermore, adenovirus-mediated overexpression of SLC1A5 was sufficient to enhance mTORC1 activity as indicated by enhanced phosphorylation of p70S6K and S6, without affecting the phosphorylation of Akt or ERK (Figure 6E). SLC1A5 overexpression also enhanced the expression of the proliferative marker pH3, while having no effect on the expression of VSMC differentiation markers or TEAD1 expression (Figure 6E). Conversely, depletion of endogenous
Figure 5. TEAD (TEA domain transcription factor) 1 is required and sufficient to promote vascular smooth muscle cell proliferation, upregulate SLC1A5 (solute carrier family 1 member 5), and activate mTORC1 (mechanistic target of rapamycin complex 1) signaling in vitro. A. Human coronary artery smooth muscle cells (HCASMCs) were transduced with GFP (green fluorescent protein) or TEAD1 adenovirus for WST-1 (water soluble tetrazolium-1) proliferation assay at day 5 post-transduction. B. HCASMCs were transduced with GFP or TEAD1 adenovirus for 48 h and then incubated with EdU (5-ethynyl-2′-deoxyuridine) overnight. Immunofluorescence staining was performed to visualize EdU incorporation (red). Cell nuclei were costained with DAPI (4′,6-diamidino-2-phenylindole; blue). C, The percentage of EdU+ cells vs total number of cells is plotted. D, After transduction with GFP control or TEAD1 adenovirus, HCASMCs were plated at equal number (day 0) and harvested for cell counting analysis at day 3 or day 5 post-transduction. n=3 per group. E, HCASMCs were transduced with GFP or TEAD1 adenovirus in the absence or presence of PDGF (platelet-derived growth factor)-BB (Continued)
SLC1A5 by siRNA resulted in inhibition of VSMC proliferation (Figure 6F and 6G), inhibition of 4EBP1 phosphorylation, and reduced the expression of the proliferative marker pH3 (Figure 6H). Together, these SLC1A5 gain-of-function and loss-of-function studies demonstrate that SLC1A5 mimics the effects of TEAD1 in promoting mTORC1 activation and VSMC proliferation.

**Discussion**

Our study uncovers a previously unrecognized role of TEAD1 in VSMC proliferation. Our data demonstrate that vascular injury induces TEAD1 expression. The injury-induced TEAD1 expression, in turn, transcriptionally promotes SLC1A5 expression, leading to enhanced glutamine uptake and activation of mTORC1 signaling, which results in enhanced VSMC proliferation that underlies the robust neointima formation in response to vascular injury (Figure 7G). Previous studies demonstrated that TEAD proteins are upregulated in many cancer types, which correlates with poor clinical outcome in patients with cancer. Consistent with these observations in cancer tissues, this study demonstrates that TEAD1 expression is induced in the neointimal area in response to arterial injury (Figure 1; Online Figure VI), suggesting that TEAD1 induction positively correlates with enhanced cell proliferation across different cell types. It will be intriguing to delineate the underlying mechanism of TEAD1 induction after arterial injury. It is likely that YAP acts as an upstream transactivator for TEAD1 expression since YAP expression was induced after arterial injury. Further investigation is needed to test this novel hypothesis. Mechanistically, multiple targets have been identified that mediate the growth promoting effects of TEADs/YAP in cancer cells or in VSMCs, including CTGF (connective tissue growth factor), cyr61 (cysteine-rich angiogenic inducer 61), and c-myc. In contrast to these earlier studies, data from RNA-sequencing analysis revealed that none of the genes listed above were altered in the SM-specific TEAD1 KO aortic tissues (Online Table I), indicating that the underlying mechanism of TEAD1-mediated cell growth is cell context dependent.

To our knowledge, to date, there are no reports showing a direct association between TEAD1, SLC1A5 expression/mutation, or glutamine uptake with human vascular diseases. However, TEADs have been implicated to play roles in regulating cell cycle suppressor genes, CDKN2A (p16 and p14) and CDKN2B (p15), which were identified as human coronary artery disease risk alleles in 9p21.3. Furthermore, as revealed by ChIP-seq (chromatin immunoprecipitation sequencing) with the coronary artery disease–associated transcription factor TCF21 (transcription factor 21) in HACMSCs, TEADs co-occupy with TCF21 on many gene loci related to other independent coronary artery disease loci. Although our work provides a direct evidence that TEAD1 promotes VSMC proliferation, further investigations are needed to establish the causative role of TEAD1 in human vascular diseases.

A recent lineage-tracing study revealed that vascular injury–induced VSMC proliferation contributes far more than VSMC migration to the neointimal VSMC accumulation in vascular diseases. Our study demonstrates a critical role of TEAD1 in promoting VSMC proliferation and neointima formation. However, it appears that TEAD1 may also play a role in VSMC migration as demonstrated by the gene ontology analysis from our RNA-seq data (Figure 4B). Future work is needed to identify the potential target genes of TEAD1 and their roles in migration in VSMCs.
Several studies have shown that amino acid transporters are critical for cell proliferation, but the mechanisms regulating the expression of these transporters are poorly understood. A recent study demonstrated that silencing YAP—a major transcriptional cofactor of TEADs—reduces the expression of SLC1A5 in cancer cells. However, the direct role of TEAD proteins in mediating YAP effects on SLC1A5 has not been explored. In this study, we demonstrate, for the first time, that TEAD1 directly binds and activates the SLC1A5 promoter in VSMCs, increasing SLC1A5 expression, thereby leading to the activation of mTORC1 signaling and enhanced VSMC proliferation (Figures 4 and 5). In addition to SLC1A5, Park et al recently demonstrated that YAP regulates the expression of 2 other amino acid transporters, namely SLC38A1 and SLC7A5, to activate mTORC1 signaling. Similarly, Hansen et al demonstrated that YAP induces the promoter activity and the expression of the high-affinity leucine transporter SLC7A5 to enhance mTORC1 signaling. However, in this study, we found that TEAD1 did not regulate the expression of SLC38A1 or SLC7A5 in HCASMCs (Figures 4E, 4H, and 5E). Together, these studies suggest that TEAD/YAP proteins may regulate different amino acid transporters in a cell context–dependent manner. Notably, while overexpression of TEAD1 per se is sufficient to activate SLC1A5/mTORC1 signaling pathway (Figure 5E; Online Figure VIII), TEAD1 modestly induces SLC1A5 mRNA expression (Figure 4H). We speculate there are 2 possibilities for this modest induction: (1) the endogenous TEAD1 and SLC1A5 are expressed at high levels in cultured HCASMCs to maintain the proliferative phenotype. Therefore, adenosine-mediated overexpression of TEAD1 is unable to further dramatically potentiate its target SLC1A5 expression; or (2) it is likely that TEAD1 requires other cofactors such as YAP to cooperatively drive SLC1A5 expression in VSMCs. We previously reported that similar to TEAD1, YAP can promote VSMC proliferation in vitro and in vivo. Consistently, we found that YAP expression is also upregulated after wire injury (Figure 1A and 1B). These data suggest that TEAD1–dependent maximal activation of SLC1A5 likely requires binding to YAP or other unidentified cofactors. Future studies are required to test this possibility.

Previous studies have demonstrated a key role of SLC1A5 in enhancing mTORC1 activity in nonvascular cells that is mediated via enhanced glutamine uptake. Our current study extends these findings and reveals a previously unrecognized role of SLC1A5 and glutamine uptake in VSMC proliferation in vitro. Furthermore, our data suggest that SLC1A5 may mediate, at least in part, the effects of TEAD1 on mTORC1 activation and VSMC proliferation but does not mediate the effects of TEAD1 on VSMC dedifferentiation (Figure 6E). Future studies are required to test the specific role of SLC1A5 in vascular injury–induced neointima formation in vivo.

Although the role of TEADs in regulating VSMC proliferation seems clear, the role of TEADs in regulating VSMC differentiation is far more complex. During embryonic development, TEADs are required for the initial transcriptional activation of key SM differentiation genes, such as myocardin and Acta2, but are not required for the promoter activity of Acta2 in adult differentiated SM cells in vivo. Conversely,
in adult SM, we previously demonstrated that TEAD1 promotes dedifferentiation of rat aortic VSMCs by disrupting myocardin/SRF interactions,\(^12\) suggesting that TEADs may enhance or downregulate SM differentiation genes in a temporal manner. In this study, we demonstrate that TEAD1 promotes dedifferentiation in HCASMCs in vitro (Figure 5F and Figure 7).

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Figure 7. SLC1A5 (solute carrier family 1 member 5) and SLC1A5-mediated glutamine uptake are required for TEAD (TEA domain transcription factor) 1-dependent mTORC1 (mechanistic target of rapamycin complex 1) activation and vascular smooth muscle cell (VSMC) proliferation. A. Human coronary artery smooth muscle cells (HCASMCs) were transduced with GFP (green fluorescent protein) or TEAD1 adenovirus in the absence or presence of GPNA (\(\gamma\)-L-glutamyl-p-nitroanilide; 3 mmol/L)—a blocker for SLC1A5 transporter-mediated glutamine uptake, for 72 h and then cells were harvested for Western blot analysis or (B) WST-1 (water soluble tetrazolium-1) proliferation assay. *\(P<0.05\), vs GFP transduction without GPNA treatment; #\(P<0.05\), vs TEAD1 transduction without GPNA treatment. C, HCASMCs were transfected with scrambled control siRNA (siControl) or SLC1A5 siRNA (siSLC1A5) for 2 d and then transduced with control GFP or TEAD1 adenovirus for an additional 2 d, then VSMCs were harvested for Western blot. D, HCASMCs were transduced with siControl or siSLC1A5 for 2 d and then transduced with control GFP or TEAD1 adenovirus for an additional 3 d for WST-1 proliferation assay or (E) cell count analysis. *\(P<0.05\), vs GFP transduction and siControl transfection; #\(P<0.05\), vs TEAD1 transduction and siControl transfection. F, HCASMCs were transduced with GFP or TEAD1 adenovirus in the absence or presence of rapamycin (50 nM)—an inhibitor for mTORC1—for 72 h and then cells were harvested for Western blot analysis. G, Schematic diagram depicting the key findings of this study. We found that vascular injury induces TEAD1 expression, which in turn transcriptionally activates the glutamine transporter SLC1A5 expression in VSMCs. The overexpressed SLC1A5 enhances glutamine uptake, leading to mTORC1 activation, VSMC proliferation, and neointima formation in response to vascular injury. Blocking SLC1A5-mediated glutamine uptake by GPNA or inhibiting mTORC1 activation by rapamycin abolishes TEAD1-induced VSMC proliferation. L indicates long exposure; and S, short exposure.
51). On the contrary, we found that specific deletion of Tead1 in VSMCs of adult mice does not significantly affect the expression of SM differentiation markers in intact aortic tissues (Online Figure IIC) or rescue their downregulated expression after arterial injury (Figure 3A and 3B). These discrepancies between our in vitro and in vivo observations may be attributed to a difference in the differentiation and proliferation states of VSMCs in culture versus intact vessels. In addition, after arterial injury, many factors other than TEAD1, such as PDGF-BB, are induced/release that likely contribute to de-differentiation of VSMCs.51,52

Previous studies have demonstrated that rapamycin-eluting stents inhibit mTORC1 activation in both ECs and VSMCs, leading to disruption of re-endothelialization of injured vessels, and thus, are accompanied with an increased risk of instant thrombosis.53,54 Accordingly, in future studies, it will be important to determine whether TEAD1 has differential effects on VSMCs versus ECs in terms of mTORC1 signaling and proliferation. Because TEAD1 mRNA and protein levels are more abundant in human VSMCs than in human ECs (Online Figure I), the axis of TEAD1-SLC1A5-glutamine uptake could be a more favorable therapeutic target for treatment of restenosis without affecting recovery of ECs. Furthermore, the critical role of glutamine in cancer cell growth and homeostasis suggests potential for developing new therapies that target glutamine metabolism.55 Our study suggests that antagonizing cellular glutamine uptake, which could potentially abrogate glutamine metabolism, may represent a novel efficacious approach for treating VSMC-driven vascular diseases.

In summary, our study unravels a novel mechanism of TEAD1-mediated VSMC proliferation and neointima formation via activation of the SLC1A5-glutamine uptake axis. Our study further suggests that targeting this axis is a promising therapeutic strategy to ameliorate vascular occlusive disease.

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Disclosures

None.

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