The Induction of Yes-Associated Protein Expression After Arterial Injury Is Crucial for Smooth Muscle Phenotypic Modulation and Neointima Formation

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Objective—Abnormal proliferation and migration of vascular smooth muscle cells (SMCs) are the key events in the progression of neointima formation in response to vascular injury. The goal of this study is to investigate the functional role of a potent oncogene yes-associated protein (YAP) in SM phenotypic modulation in vitro and in vivo.

Methods and Results—In vitro cell culture and in vivo in both mouse and rat arterial injury models YAP expression is significantly induced and correlated with the vascular SMC synthetic phenotype. Overexpression of YAP promotes SMC migration and proliferation while attenuating SM contractile gene expression. Conversely, knocking down endogenous YAP in SMCs upregulates SM gene expression but attenuates SMC proliferation and migration. Consistent with this, knocking down YAP expression in a rat carotid balloon injury model and genetic deletion of YAP, specifically, in vascular SMCs in mouse after carotid artery ligation injury attenuates injury-induced SM phenotypic switch and neointima formation.

Conclusion—YAP plays a novel integrative role in SM phenotypic modulation by inhibiting SM-specific gene expression while promoting SM proliferation and migration in vitro and in vivo. Blocking the induction of YAP would be a potential therapeutic approach for ameliorating vascular occlusive diseases. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: yes-associated protein ■ smooth muscle ■ phenotypic modulation ■ neointima formation
phenotype (synthetic phenotype) that contributes to vascular remodeling and lesion formation. Although tremendous progress has been made in defining specific transcriptional mechanisms involved in regulating SMC contractile gene expression, the mechanisms that coordinate SMC contractile and synthetic phenotype switching are poorly explored.

Several studies suggest that cancer and SM-related diseases such as neointimal formation during atherosclerosis or after arterial injury share several common fundamental biological mechanisms. For example, like tumor cells, after vascular injury SMCs are highly proliferative and migratory with loss of expression of differentiation markers. Abnormal proliferation and migration of SMCs are the key events in atherosclerosis, neointimal hyperplasia, and in response to vascular injury. The SMC proliferation and migration are critical for early plaque development and, therefore, plaques can be considered as benign SMC tumors of the arterial wall. Given that YAP has been demonstrated to be a potent oncoprotein to promote cell proliferation and migration, we hypothesize that YAP plays an essential role in SM phenotypic switching.

In this report, we demonstrate that expression of YAP is dramatically induced during SM phenotypic modulation to the synthetic state in vitro and in vivo. We show that YAP plays a critical role in coordinating the phenotypic modulation of SMCs, by inhibiting expression of SM differentiation genes and by promoting proliferative and migratory functions. Depletion of YAP expression after vascular injury alleviates the injury-induced SM–specific gene downregulation and attenuates neointima formation in vivo. This study suggests that components of the Hippo–YAP pathway may be appropriate therapeutic targets for ameliorating SM-related vascular diseases.

Materials and Methods

Rat aortic SMCs were prepared as previously reported. Mouse carotid artery ligation and rat carotid artery balloon injury were performed as described in our previous reports. A detailed Materials and Methods section is provided in the online-only Data Supplement.

Results

YAP Expression Is Induced During SM Phenotypic Modulation

To explore the potential function of YAP in SMCs, first we examined the expression of YAP during SM phenotypic modulation. During phenotypic switch of vascular SMCs induced by cell culture, YAP expression was dramatically induced in parallel with the expression of the synthetic SMC marker nonmuscle myosin heavy chain IIb, whereas expression of contractile SMC markers, including SM22-α, SM α-actin, and Hic-5, was significantly reduced (Figure 1A in the online-only Data Supplement). The phosphorylated YAP (p-YAP, S127) signal was elevated as well, suggesting the YAP upstream kinase pathway was activated after vascular SMC cultured in growth medium. Furthermore, immunostaining experiments revealed that YAP primarily localized in the nuclei of cultured vascular SMCs, although consistent with the observed high levels of p-YAP by Western blot, YAP signal was also seen in cytoplasm (Figure 1B in the online-only Data Supplement). We next sought to examine YAP expression during SM phenotypic modulation in vivo. Consistent with the in vitro data, we found that YAP was significantly elevated in a rat carotid artery balloon injury model that resembles angioplasty in humans, both 3 and 14 days after injury (Figure 1A, 1B, and 1D). YAP expression coincides with increased expression of proliferative SM marker, proliferating cell nuclear Ag (Figure 1B), and downregulation of SM contractile genes (Figure 1A and 1B). Similarly, elevated YAP expression was observed 3, 7, and 21 days after mouse carotid ligation injury that is characterized by phenotypic modulation of medial SMCs, as indicated by the data from quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, and immunohistological staining assays (Figure IIA–IIC in the online-only Data Supplement). In contrast, genes for SM contractile phenotype, including SM α-actin, Hic-5, and SM22α, were significantly reduced (Figure 1B in the online-only Data Supplement). In agreement with a previous study showing that arterial injury can induce and activate Mst1, an upstream Hippo kinase of YAP, we found balloon injury increases p-YAP level (Figure 1B). However, total YAP expression increased significantly more than the p-YAP signal as the percentage of YAP phosphorylated after injury actually decreased by 50% (Figure 1C). Consistent with this finding, immunohistochemical staining demonstrated that YAP is highly expressed in the synthetic neointima SMCs where it is localized in the nuclei of ≈50% of the SMCs (Figure 2D and 2E). Furthermore, the expression YAP was significantly increased in the nuclear fractions from balloon-injured carotid arteries (Figure III in the online-only Data Supplement). These data suggested that the excessive induction of YAP can override the phosphorylation-induced repression of YAP mediated by upstream Hippo pathway kinases, suggesting a feedback regulatory loop. Taken together, these in vitro and in vivo data demonstrate that YAP expression and nuclear localization is positively correlated with the synthetic SM phenotype.

YAP Represses Smooth Muscle–Specific Gene Expression

As YAP expression is significantly induced during SM phenotypic modulation in vitro and in vivo, we then sought to investigate the function of YAP in regulating expression of SM-specific genes by gain- or loss-of-function assays. Retrovirus encoding wild-type (WT) YAP, constitutively active YAP 5SA, or empty control vector were transduced into rat aortic SMCs. We first performed phalloidin staining to examine the effects of overexpression of YAP and 5SA on SMC cytoskeleton organization. Data from this experiment demonstrated that overexpression WT YAP and YAP 5SA displayed dramatic cell morphological alteration. Moreover, cells expressing YAP 5SA lost cell–cell contact inhibition and grow on top of each other (Figure IV in the online-only Data Supplement). To further characterize YAP functions in SMCs, protein or RNA from rat aortic SMCs that were transduced by WT YAP, YAP 5SA, or empty control vector were harvested and SM gene expression quantitated by Western blotting and quantitative real-time polymerase chain reaction. All SM-specific genes examined were significantly downregulated 50% to 90% by overexpression of WT YAP or the 5SA YAP mutant at both the protein and mRNA level.
The YAP 5SA mutant, which is resistant to inhibition induced by the Hippo pathway, was more potent in abrogating SM-specific gene expression compared with WT YAP (Figure 2A and 2B). Moreover, data from reporter assays revealed that WT YAP or YAP 5SA significantly inhibit SM-specific Hic-5 and SM myosin heavy chain promoter activity (Figure 2C), suggesting the inhibitory effect on SM-specific gene expression by WT YAP or 5SA mutant YAP is at the transcriptional level. Consistently, depletion of YAP using short interfering RNA significantly increased expression of SM markers in rat aortic SMCs (Figure 2D and 2E). Finally, overexpression YAP or YAP 5SA strongly induced the expression of cyclin D1, a cell cycle-regulating gene that was previously shown to promote cell proliferation19 (Figure 2A), whereas knockdown YAP significantly downregulated cyclin D1 expression at both protein and mRNA levels in cultured vascular SMCs (Figure 2D and 2E). Silencing YAP has no effects on the expression of serum response factor (SRF), a key regulator for SM differentiation.20 Together, these data demonstrate that YAP is a potent repressor for SM-specific gene expression, whereas YAP is a strong inducer for cell proliferation gene expression.

YAP Promotes SMC Proliferation

Proliferation of vascular SMCs plays a key role in the development of neointima thickening. Because YAP is induced in cell culture and after arterial injury where modulated SMCs are known to be proliferative and migratory, we directly tested whether YAP is able to affect SMC proliferation by overexpressing YAP or 5SA mutant in rat aortic SMCs (Figure 3A and 3B) and silencing endogenous YAP with short interfering RNA (Figure 3C and 3D). Data from these experiments revealed that the rates of SMC proliferation were stimulated by YAP overexpression while inhibited by silencing endogenous YAP expression, supporting a positive role for the YAP in regulating vascular SMC proliferation.

YAP Promotes SMC Migration

In response, arterial injury SMC migration from medial layer is another key event to build neointima. Using a scratch wound
healing assay, we found that overexpression of YAP in rat aortic SMCs significantly promotes wound closure that is known to be dependent primarily on cell migration over this time period (Figure 4A and 4B). Boyden chamber assays further confirmed that YAP can promote SMC migration, whereas 5SA mutant has a stronger effect (Figure 4C). In contrast, silencing endogenous YAP impaired wound closure (Figure 4D), suggesting that YAP plays an important role in SMC migration.

**Knockdown or Knockout of YAP In Vivo Prevents Arterial Injury–Induced SM Phenotypic Modulation and Attenuates Neointima Formation**

We next sought to determine the role of YAP in SM phenotypic modulation and neointima hyperplasia in vivo by transduction with lentivirus encoding short hairpin RNA against YAP (shYAP) to attenuate YAP gene upregulation after rat carotid artery balloon injury. We first confirmed that shYAP lentivirus efficiently knocked down expression of YAP after rat carotid artery balloon injury (Figure VA in the online-only Data Supplement) and that the lentivirus efficiently transduced into the injured vascular wall after arterial injury (Figure VB in the online-only Data Supplement). Short hairpin luciferase lentivirus–transduced animals served as controls. We chose D14 postinjury to assess the effects of silencing YAP on SM gene expression and neointima formation, as previous study has shown that the highest SMC fraction within neointima was seen at this time point. Data from Western blotting and immunostaining revealed that in vivo knockdown of YAP after...
rat carotid balloon injury significantly rescued injury-induced downregulation of SM-specific genes (Figure 5A and 5B), including the most restricted SM marker, SM myosin heavy chain (Figure VI in the online-only Data Supplement). Treatment with shYAP significantly decreased the neointima/media ratio and attenuated neointima area as compared with control shLuciferase virus–transduced carotid arteries (Figure 5C–5E). Moreover, knockdown of YAP significantly decreased neointima SMC proliferation by 50% as indicated by Ki67 staining (Figure VII in the online-only Data Supplement). Taken together, these data demonstrated that blocking YAP induction after rat carotid artery balloon injury can reduce the arterial injury–induced decrease in expression of SM-specific genes and attenuate neointima formation by inhibiting SMC proliferation. To further determine the specific contribution of SMC-derived YAP on neointima formation, a carotid artery ligation injury was performed on SMC-specific YAP heterozygote knockout mice. For these experiments, we could not use homozygous SMC-specific YAP knockout mice as they die perinatally (data not shown). First, we demonstrated that YAP expression in SMC-specific YAP heterozygotes (SM22α-Cre<sup>−/+</sup>/YAP<sup>fl/fl</sup>, YAP SM Het) was reduced in SMCs by 50%, with no change of expression in other non-SM tissues, such as brain (Figure VIII in the online-only Data Supplement). Twenty-one days after ligation injury there was an attenuated reduction of SM-specific gene expression and decreased SMC proliferation in YAP heterozygous mice (Figure 6A). This resulted in a 40% to 50% decrease in neointima formation in the SM-specific heterozygote YAP mice (Figure 6B–6D). Taken together, these data demonstrated that inactivation of YAP within SMCs is sufficient to upregulate SM-specific gene expression and attenuate neointima formation after arterial ligation injury.

**Discussion**

The Hippo signaling pathway is evolutionarily conserved from *Drosophila* to mammals and plays a critical role in controlling...
organ size and tumorigenesis by regulating cell proliferation. In this report, we demonstrated that YAP plays a novel role in vascular SMCs. We found that overexpression of YAP promotes SMC migration and proliferation while attenuating SM contractile gene expression (Figures 2–4). Conversely, knocking down endogenous YAP in SMCs upregulates SM-specific gene expression while attenuating SMC proliferation and migration (Figures 2–4) in vitro. During the preparation of the manuscript, we noticed that a recent study showed similar results demonstrating that the YAP is induced upon stimuli of SM phenotypic modulation and knockdown YAP can increase SM-specific gene expression in vitro. However, this study was performed in cell culture settings, whereas YAP function in vascular SMCs in vivo is completely unknown. In the current report, we provide the first evidence demonstrating that knocking down YAP expression in vivo in rats and mice is sufficient to prevent vascular injury–induced decreases in SM-specific genes and attenuates neointima formation (Figures 5 and 6).

YAP SSA, a mutant that is insensitive to Hippo inhibition, more dramatically abrogated SM gene expression in vitro and had a stronger ability to promote SMC proliferation and migration (Figures 2–4). Furthermore, arterial injury and cell culture activates the endogenous Hippo kinase cascade to inactivate a portion of YAP by retaining it in the cytoplasm (Figure I in the online-only Data Supplement and Figure 1). This activated Hippo signaling can thus be viewed as a natural protective response to the detrimental stimuli to prevent excessive SMC proliferation and lesion formation. Although, both cell culture and arterial injury stimulate activation of YAP upstream kinases to phosphorylate and inactivate YAP, the excessive induction of total YAP expression overwhelms this regulatory system resulting in unphosphorylated YAP translocating into the SMC nuclei. This nuclear YAP promotes SM migration and proliferation while inhibiting SM differentiation by attenuating SM-specific gene expression. In support of the notion that the Hippo pathway plays a critical role in the vasculature after injury, a previous study showed that arterial injury induces and activates Mst1 kinase and infection with adenovirus-encoding Mst1 in balloon-injured rat carotid artery suppressed neointimal formation. Additionally, cardiac-specific deletion of Mst kinase–associated activator salvador, or deletion of Hippo kinases Mst1/2 and Lats1/2 in mouse, led to activated YAP, resulting in large hearts with elevated cardiomyocyte proliferation. Although the endogenous ligands stimulating this pathway are poorly characterized in mammals, one recognized stimulus is cell contact, a stimulus that is known to activate Hippo kinases but negatively affect SMC proliferation and promote differentiation. Taken together, these studies suggested that Hippo signaling is crucial for regulating YAP function in cardiomyocytes and vascular SMCs. Although our data showed that YAP phosphorylation is elevated during SM phenotypic modulation, it is unknown...
which Hippo signaling components are involved in this process. Future studies are needed to explore the precise mechanisms by which the Hippo pathway is activated in response to arterial injury and screen the stimuli that are involved in YAP induction during SM phenotypic modulation.

It is interesting to speculate on how YAP may promote SM cell proliferation while inhibiting differentiation. YAP lacks a DNA-binding domain but functions as a potent transcriptional cofactor through interaction with TEADs or PPxy (PY)-motif containing transcription factors via its N-terminal TEAD-binding domain or through a C terminus WW domain, respectively. Both TEAD-binding domain and WW domains have been shown to be required for YAP-mediated oncogenic activity. For instance, TEAD-binding element within cyclin D1 gene promoter is crucial for YAP-mediated malignant mesothelioma cell proliferation. Consistent with this, we found cyclin D1 is significantly induced by overexpression YAP whereas knockdown of YAP decreases cyclin D1 expression, suggesting the positive effects of YAP on SMC proliferation are at least partially through induction of cell cycle–regulating genes. Previous studies have also shown that TEAD-binding elements within the SM α-actin promoter, that bind TEAD proteins, are required for activity of the promoter in embryonic SMCs but not in adult SMCs. TEAD1 has also been shown to directly interact with SRF, a transcription factor required for expression of most SM-specific genes. SRF also plays an important role in regulating SMC migration and proliferation by interaction with a variety of cofactors. The Hippo pathway may, thus, regulate expression of SM-specific genes and affect SM proliferation and migration through modulating the activity of SRF. Further studies are needed to investigate this possibility. Furthermore, YAP also physically and functionally interacts with PY motif–containing transcription factors, such as SMAD7, p73, Runx1, and Runx2, through its WW domain. Interestingly, we also identified a conserved PY motif in the myocardin family proteins (data not shown), a group of factors that are potent activators of SM-specific genes through interaction with SRF. Consistent with this, YAP can interact with myocardin and disrupt myocardin binding to SRF, thereby downregulating SM-specific gene expression. Together these findings suggest that YAP may regulate the phenotype of vascular SMCs by altering the activity of the SRF axis. Additionally, it has been shown that transforming growth factor-β and Notch signaling are involved in the SM differentiation further investigation is needed to test the possible cross talk between Hippo–YAP pathway with transforming growth factor-β and Notch signaling. Elucidating the mechanisms controlling SM phenotypic modulation is critical for understanding the pathogenesis and
progression of many vascular diseases and ultimately identifying new therapeutic targets to treat these diseases. A number of serious clinical conditions, including atherosclerosis, intimal hyperplasia associated with restenosis, and vein graft stenosis, are largely dependent upon SMC phenotype modulation contributing to progression of intimal lesions, resulting in occlusions of vessels. These phenotypically switched SMCs in the neointima, in turn, exacerbate lesion development by increasing production of growth factors and extracellular matrix. In this report, we demonstrate that preventing YAP induction after arterial injury can significantly attenuate neointima formation in vivo at least in part through inhibiting SMC proliferation and migration (Figures 5 and 6). As neointimal formation after arterial injury includes multiple cellular processes, such as SMC death, matrix production, and endothelial regeneration, it will be intriguing to investigate whether YAP can affect these aspects. In summary, this exciting study not only provides completely novel insights into the mechanisms controlling SMC phenotypic modulation but also identifies YAP as a potential therapeutic target for ameliorating vascular diseases.

Acknowledgments

We thank Drs Paul Herring and Harold Singer for a critical reading of the manuscript. We also thank Dr Duojia Pan for sharing YAP flox mice. We are thankful to Christina Rotondi at the AMC Histology Core for her excellent technical support with immunohistochemistry staining.

Sources of Funding

The project described was supported by a grant (1R01HL109605-01A1) from the National Heart, Lung, and Blood Institute, National Institutes of Health to J. Zhou and a Postdoctoral Fellowship from American Heart Association to X. Wang.

Disclosures

The content is solely the responsibility of the authors and does not necessarily represent the official views the National Institutes of Health.

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Arterioscler Thromb Vasc Biol. published online August 23, 2012; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Methods

**Preparation of rat aortic SMCs.** Rat arterial tissues were harvested and cultured rat aortic SMCs were prepared as described in our previous reports. Briefly, male Sprague-Dawley rats (200-250 grams; Taconic Farms, Germantown, NY) were euthanized by CO\(_2\) and the aorta was dissected to remove adhering periadventitial tissue and the endothelium denuded with a catheter. The aorta was then digested with a Blend enzyme III solution (Roche, 0.5U/ml) for 10 min at 37°C followed by dissection to remove the adventitial layer, then the remaining medial layer was minced into small pieces for a second digestion with Blend enzyme III for 2 hours at 37°C. Following removal of digestion solution and resuspending in 10% FBS DMEM medium, cells were gently liberated with a pipette and transferred into culture dishes.

**Immunocytochemistry.** Rat aortic SMCs (passage 3) were grown on coverslips overnight and then fixed, permeabilized, and incubated with monoclonal anti-SM α-actin antibody (1:500, Sigma, clone 1A4) and rabbit polyclonal anti-YAP antibody (1:50, Santa Cruz, H125), followed by incubation with Alexa Fluor 594-conjugated anti-mouse and Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibodies (1:250) (Molecular Probes). Cells were then immersed in ProLong Gold mounting medium with DAPI (Invitrogen) to visualize nuclei. FITC-conjugated phalloidin was utilized to stain F-actin filaments in YAP over-expressing cells essentially following manufacture’s protocol (Sigma).

**Luciferase reporter assays.** Transient transfection and reporter assays were carried out with
Fugene6 transfection reagent (Roche) as previously described\textsuperscript{3,4}. The level of promoter activity was evaluated by measurement of firefly luciferase activity relative to the internal control TK-renilla luciferase activity using the Dual Luciferase Assay System as described by the manufacturer (Promega). A minimum of six independent transfections was performed and all assays were replicated at least twice. Results are reported as the mean ± SEM.

**Retroviral construction and cell infection.** Expression plasmids encoding YAP and YAP 5SA were kindly provided by Dr. Kun-liang Guan, UCSD\textsuperscript{5} and subcloned into pBabe retroviral vector with a Flag tag. The recombinant viral DNA was transfected into Amp HEK293 cells for viral packaging and the retroviral particles were harvested for infection into cultured rat aortic SMCs as described previously\textsuperscript{1,6,7}. Empty retrovirus served as negative control.

**Delivery of silence RNA (siRNA) duplex into SMCs.** Rat SMCs were transfected with siRNA duplexes using the Neon Transfection system (Invitrogen). Briefly, cultured rat aortic SMCs were detached from dish by trypsin digestion. Following a wash in PBS, cells were then suspended in R buffer and mixed with siRNA duplexes (100 nM). Finally, electroporation was performed with the parameters of 1700V, 1 pulse of 20 milli-second width. After electroporation, the cells then were plated in 10% FBS antibiotic-free medium for 48 hours. Scrambled control siRNA and siRNA targeting YAP were designed and purchased from Ambion. The sequence of the siRNA targeting YAP is: 5'- AGAGATACTTCTTTAATCA -3'.

**Quantitative real time RT-PCR (qRT-PCR) analysis.** Total RNA from tissue or SMCs was isolated with TRIzol reagent and qRT-PCR was performed with respective gene-specific primers as we previously reported\textsuperscript{1,2,6,7}. All samples were amplified in duplicate and every experiment was repeated independently 2 times. Relative gene expression was converted using the $2^{\Delta\Delta ct}$
method against the internal control acidic ribosomal phosphoprotein P0 (RPLP0) house-keeping gene.

**Protein extraction and Western blotting.** Mouse or rat carotid arteries were harvested by cutting open longitudinally and removing off the adventitia as described in our recent report². Tissues were cut into small pieces and ground with a glass homogenizer in RIPA buffer (Fisher) with 1% proteinase inhibitor cocktail (Pierce) and 1% PMSF. After sonication and centrifugation of the cell lysate, proteins were quantified by BCA assay and then loaded in a 6-9% SDS-PAGE gel at 5-20 μg per lane. Protein was extracted from cultured rat aortic SMCs as previously described¹. Cytoplasmic and nuclear protein were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) from 3 combined uninjured control or balloon inflation injured rat carotid arteries. Antibodies used in this study were: YAP (Santa Cruz, H125, 1:1000), p-YAP (Cell signaling, 1:1000), MLCK (Sigma, clone K36, 1:5000), SMα-actin (Sigma, AC-74, 1:2000), cyclin D1 (Cell signaling, #2926, 1:1000), calponin (Sigma, 1:5000; Santa Cruz, N-15R, 1:1000), GAPDH (Santa Cruz, 1:2000), Hic-5 (BD, 1:5000), lamin A/C (Cell Signaling, 1:2000), NMHCII-B (Covance, 1:2000), PCNA (Santa Cruz, 1:500), SMα-actin (Sigma, 1:10000), SM22α³ (1:5000), SRF (Santa Cruz, G-20, 1:3000), vinculin (Sigma, 1:5000). Images were taken by ImageQuan LAS4000 Imaging Station (GE) and band densities were quantified using the ImageQuant TL software (GE).

**SMC proliferation assay.** Proliferation of rat aortic SMCs was measured by cell proliferation WST-1 kit (Roche) in 96-well format. Following silencing or over-expression of YAP, rat aortic SMCs were maintained in 0.2% FBS medium for additional 48 hours to allow growth arrest. Cells were then treated with DMEM medium, 50 ng/ml PDGF-BB or 10% FBS as indicated in
figures. After 24 hours the rates of proliferation were determined with incubation of 10 μl/well WST-1 for 4 hours, and then measured the absorbance at 440 nm with a plate reader. For SMC counting, YAP over-expressing or knock-down cells were seeded in 60mm dish at equal density and then cell numbers were manually counted for 3 constitutive days by a hemocytometer.

**SMC migration assay.** For the wound healing assay, rat aortic SMCs transduced with retrovirus encoding YAP or control vector were seeded in 6-well plates at confluent density (1X10⁶ cell/well). Cell migration was assayed 8 or 12-hour after P10 tip scratching and the relative closure distance were measured. Five fields were measured by taking images under a microscope. Boyden chamber assays were carried out as previously described¹. Briefly, rat aortic SMCs were grown in medium containing 10% FBS for 48 hours post infection with YAP, 5SA or control retrovirus. Subsequently, the infected cells were trypsinized and seeded into Boyden chambers (PET track-etched, 8-μm pores, 24-well format; Becton Dickinson) in serum-free DMEM medium. Chambers were then immersed in 10% FBS medium for 5 hours. The top side of the membranes was swabbed to remove cells, and then cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde, stained with DAPI to visualize nuclei, and counted under fluorescence microscopy. Five identically located fields per membrane were averaged for quantification of migrated cell numbers. The cell numbers on each field were counted by Image J software (NIH).

**Mouse carotid artery ligation model.** Mouse carotid artery ligation was performed as previously described²,⁶,⁸. Briefly, to examine YAP expression following mouse carotid artery ligation injury, C57BL/6 male mice (Taconic Farms, 12-14 weeks old) were anesthetized by intraperitoneal injection of a mixed solution of xylazine (5mg/kg body weight) and ketamine (80mg/kg body weight). The left common carotid artery was dissected and completely ligated
just proximal to the carotid bifurcation. The right carotid artery served as an uninjured contralateral control. The left and right carotid arteries were harvested at the time point as indicated in the figures either for qRT-PCR, Western blot or histological analysis. To determine the effects of SMC-specific inactivation of YAP on neointima formation following carotid artery ligation injury, we first crossed female homozygous floxed allele YAP mice \(^9\) (YAP\(^{ff}\), generously provided by Dr. Duojia Pan, Johns Hopkins University School of Medicine) with male SM22\(\alpha\)-Cre transgenic mice \(^{10}\) (purchased from Jackson Laboratory) to create a mouse line SM22\(\alpha\)-Cre\(^{+/0}\)/YAP\(^{flWT}\). Then male SM22\(\alpha\)-Cre\(^{+/0}\)/YAP\(^{flWT}\) mice were crossed with female YAP\(^{ff}\) mice to obtain SM22\(\alpha\)-Cre\(^{+/0}\)/YAP\(^{flWT}\) experimental mice (YAP SM Het) and SM22\(\alpha\)-Cre\(^{0/0}\)/YAP\(^{flWT}\) littermate (flox control) control mice. Three-month old gender-matched YAP smooth muscle-specific heterozygotes and their littermate control mice were then used to perform carotid artery ligation injury. Post injury 21 days, carotid artery tissues were harvested for Western blot or embedded transversely in paraffin for histological analysis as described below. Cross-sections of carotid arteries (7\(\mu\)m thickness) were prepared from proximal to ligature to the aortic arch. Morphometric analysis was performed using 6 sections from each artery and these sections were located at around 250\(\mu\)m proximal to ligature. At least 3 mice per genotype were analyzed for each assay.

**shRNA lentiviral construct and infection in rat carotid balloon angioplasty.** shRNA lentivirus targeting rat YAP have been designed and generated in pSIH1-H1-copGFP-Puro lentiviral backbone by System Biosciences. The sequence of shRNA targeting rat YAP 3'-UTR is: 5'-GCTGCCACCAAGTT-3'. shRNA lentivirus against luciferase was served as a negative control \(^8\). As this vector contains an independent CMV promoter-driven GFP expression cassette the viral transduction efficiency can be conveniently monitored by examining GFP expression. Rat balloon angioplasty was carried out as previously described \(^8,^{11}\). Briefly, male
Sprague-Dawley rats (350 g; Taconic Farms, Germantown, NY) were anesthetized with xylazine 4.6 mg/kg and ketamine 70 mg/kg via intraperitoneal injection. Following a midline cervical incision and muscular tissues separation, the left common carotid artery was exposed and blunt dissection was performed alongside the artery by dull forceps to expose the carotid artery bifurcation into the internal/external branches. Blood flow cessation was achieved by arterial clamps and a small arteriotomy was made in the external carotid artery. A 2F Fogarty balloon embolectomy (Edwards) was inserted through the small cut and passed into the common carotid artery. After balloon inflation at 1.6-2.0 atm of pressure, the catheter was partially withdrawn and reinserted 3 times. Concentrated lentiviral solutions encoding shYAP or shLuciferase (100µl) were infused into the injured segment of the common carotid artery and incubated for 30 minutes. After the viral treatment, residual viral solutions in lumen were aspirated to avoid viral solutions into the systemic circulation. A permanent ligation was then placed in the external carotid artery, and blood flow in the common carotid artery and its internal branch was restored by releasing arterial clamps. The right intact carotid artery served as a contralateral control. The use of experimental animals (mouse and rat) for arterial injury procedures, including BSL-2 viral work has been approved by the IACUC and Biosafety committees at Albany Medical College.

Sections, Hematoxylin/Eosin (HE) staining, immunofluorescence (IF) and immunohistochemistry (IHC). Rats or mice were euthanized by asphyxiation with CO₂ and fragments of carotid arteries were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin for all histological assays as described in our previous report. Sections were cut at 7-um thickness, deparaffinized and antigen retrieval was done by using microwave to heat at 98°C for 2 minutes in citric acid buffer (10mM, pH6.0). After goat serum (10%, invitrogen) blocking for 30 minutes, sections were then incubated with anti YAP (Santa Cruz,
1:30), anti SM α-actin (Sigma, mouse, 1:600), anti GFP (Abcam, rabbit, 1:200), anti SM MHC (Biomedical Technologies Inc, rabbit 1:30), anti Ki-67 (Thermo Scientific, rabbit, 1:30) antibodies.

For IHC, sections were stained using the Vectastain Elite ABC Kit (Vector Laboratories) as described in our recent report. For IF, the sections were stained with secondary antibody (488nm anti-rabbit secondary antibody or 647nm anti-mouse secondary antibody, 1:250 dilution, Invitrogen) diluted in blocking buffer for 1 hour at room temperature. Sections were immersed with mounting medium (ProLong Gold anti-fade reagent with DAPI, Invitrogen) to visualize nuclei. Sections stained with IF were imaged using confocal microscopy (LS510 meta, Zeiss) at 63x magnification and Imaris software (Bitplane, CT) was utilized for YAP/DAPI co-localization analysis in Z-stack scanning photos. HE staining was performed following standard protocol. Phase contrast images were acquired using an Olympus BX51 inverted microscope. The areas of intima and media were measured by ImageJ software (NIH).

**Statistical analysis.** Data are expressed as means ± SE, and statistical analysis using One-way ANOVA was done with Prism software (Graphpad). Differences with p values < 0.05 were considered significant.
References


Supplemental Figure Legends

Supplemental Figure I. YAP expression is elevated during smooth muscle phenotypic modulation. A. Western blots of rat aortic tissue or 2nd passage enzymatically dispersed aortic SMC lysates. Cell culture dramatically induces YAP expression while down-regulates smooth muscle-specific gene expression. B. Rat aortic SMCs were plated on cover slips and stained with anti-YAP (green) and anti-SM α-actin (red) antibodies to detect YAP subcellular localization in cultured vascular SMCs. DAPI staining was utilized to visualize nuclei (blue). Scale bar, 50μm.

Supplemental Figure II. YAP expression is induced in mouse carotid artery ligation injury. A. qRT-PCR was performed to assess YAP mRNA expression in the carotid arteries post ligation injury day 3 and day 10 in 12 week-old C57BL/6 male mice. The YAP expression in contralateral artery tissues was set to 1 (red line). N=3. B. Western blotting of YAP or smooth muscle-specific gene expression in contralateral control or injured carotid artery that post-operation 3 and 7 days of carotid artery ligation injury. A representative blot is shown from 4 independent experiments. C. 21 days after mouse carotid artery ligation serial sections from injured and contralateral control artery vessels were stained for YAP or SM α-actin (brown). Sections treated with secondary antibody alone served as negative control. All sections were counterstained by hematoxylin to visualize nuclei (blue). An arrow points to internal elastic lamina. NI: neointima, M: media, AD: adventitia layer. Scale bar, 100μm.

Supplemental Figure III. Expression of YAP is elevated in vascular SMC nuclei after injury. Cytoplasmic and nuclear protein were harvested by using NE-PER Nuclear and
Cytoplasmic Extraction Kit (Thermo Scientific) from uninjured control or balloon inflation injured rat carotid arteries (post-operation 14 days). Equal amount of nuclear and cytoplasmic fractions were immunoblotted with anti-YAP, anti-GAPDH and MLCK (cytoplasmic markers), or anti-lamin A/C (nuclear marker) antibodies, as indicated. C.E.: cytosolic extract; N.E.: nuclear extract.

Supplemental Figure IV. SMCs over-expressing of YAP and YAP 5SA mutant display a dramatic cell morphological change. Retrovirus encoding WT YAP, constitutively active YAP 5SA mutant or empty control vector were transduced into rat aortic SMCs. Phalloidin staining (green) was performed to stain F-actin filament to examine the effects of over-expression of YAP and 5SA on SMC cytoskeleton organization. Scale bar: 50μm.

Supplemental Figure V. Validation of YAP shRNA lentivirus knock-down efficacy in vitro and infection efficiency in vivo. A. Rat aortic SMCs that infected shRNA lentivirus against YAP (shYAP) or control luciferase (shLuc) were harvested for Western blotting. Numbers below each lane indicate relative expression YAP normalized to GAPDH. As this shRNA vector co-expresses GFP the viral transduction efficiency in vivo can be monitored by detecting GFP signal. B. IF was performed using anti-GFP antibody (green) in the rat carotid arteries that balloon injured (14 days post injury) with local delivery of either shLuci or shYAP virus and their corresponding contralateral vessels. Sections that incubated with 2nd antibody were served as a negative control. An arrow points to internal elastic lamina. NI: neointima, M: media. Scale bar: 20μm.

Supplemental Figure VI. Silencing YAP in vivo attenuates vascular injury-induced down regulation of SM MHC expression. IF was performed using anti-SM MHC antibody (red) in the rat carotid arteries that balloon injured (14 days post injury) with infection of shLuci or shYAP
virus and their corresponding contralateral vessels. Sections that incubated with 2nd antibody were served as a negative control. An arrow points to internal elastic lamina. NI: neointima, M: media. Scale bar: 20μm.

**Supplemental Figure VII. Silencing YAP in vivo attenuates vascular injury-induced SMC proliferation.** A. Ki67 staining (green) was performed in the rat carotid arteries that balloon injured (14 days post injury) with infusion of shLuci or shYAP virus and their corresponding contralateral vessels. Sections that incubated with 2nd antibody were served as a negative control. Arrow-heads point to the representative Ki67 positive cells in the injured vessels. An arrow points to internal elastic lamina. NI: neointima, M: media. Scale bar: 20μm. B. Quantification of the percentage of Ki67 positive cells in the shLuci or shYAP virus infected vessels as shown in panel “A”. N=4. *P < 0.05.

**Supplemental Figure VIII. YAP expression is specifically reduced in the aorta of smooth muscle specific YAP-deficient heterozygous mice.** Aorta and brain were harvested either from flox control (SM22α-Cre0/0/YAPf/WT) or from YAP smooth muscle-specific heterozygous (SM22α-Cre+/0/YAPf/WT, YAP SM Het) mice to assess YAP expression by Western blot. Numbers below each lane indicate relative expression YAP normalized to GAPDH.
Supplemental Figure I

A

B

YAP

SM α-actin

DAPI

merge
Supplemental Figure III
Supplemental Figure IV
Supplemental Figure VI

![Supplemental Figure VI](image_url)
Supplemental Figure VIII

![Image of a gel blot with bands for YAP and GAPDH in aorta and brain samples labeled as flop control, Yap-Sm. Het, and YAP SM. Het]