Yap1 regulates vascular smooth muscle cell phenotypic switch by interaction with myocardin

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Running title: Yap1 modulates VSMC phenotypic switch

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**Background:** Hippo-Yap signaling pathway is one of the critical pathways regulating cell proliferation, differentiation and apoptosis.

**Results:** Knockdown of endogenous Yap1 impairs VSMC proliferation and enhances VSMC contractile phenotype by promoting the association of the myocardin/ SRF/CArG complex.

**Conclusion:** Yap1 signaling pathway is a central regulator of phenotypic switch of VSMCs

**Significance:** Phenotypic switch of VSMCs and vessel injury response can be controlled by modulation of Hippo-Yap signaling.

**SUMMARY**

The Hippo-Yap (Yes-associated protein, Yap) signaling pathway has emerged as one of the critical pathways regulating cell proliferation, differentiation and apoptosis in response to environmental and developmental cues. However, Yap1 roles in vascular smooth muscle cell (VSMC) biology have not been investigated. VSMC undergo phenotypic switch, a process characterized by decreased gene expression of VSMC contractile markers and increased proliferation, migration, and matrix synthesis. The goals of the present studies were to investigate the relationship between Yap1 and VSMC phenotypic switch and determine the molecular mechanisms by which Yap1 affects this essential process in VSMC biology. Results demonstrated that the expression of Yap1 was rapidly up-regulated by stimulation with PDGF-BB (a known inducer of phenotypic switch in VSMCs) and in the injured vessel wall. Knockdown of Yap1 impaired VSMC proliferation in vitro and enhanced the expression of VSMC contractile genes as well by increasing serum response factor (SRF) binding to CArG containing regions of VSMC specific contractile genes within intact chromatin. Conversely, the interaction between
serum response factor and its co-activator myocardin was reduced by overexpression of Yap1 in a dose-dependent manner. Taken together, these results indicate that down-regulation of Yap1 promotes VSMC contractile phenotype by both up-regulating myocardin expression and promoting the association of the serum response factor/myocardin complex with VSMC contractile gene promoters, and suggest that Yap1 signaling pathway is a central regulator of phenotypic switch of VSMCs.

Vascular smooth muscle cells (VSMCs) within the vessels retain remarkable plasticity and are characterized in part by their ability to modulate their phenotypes in response to the environmental stimuli through a process characterized by decreased gene expression of VSMC contractile markers and increased proliferation, migration, and matrix synthesis(1). As a result, VSMCs proliferate and synthesize collagens and matrix metalloproteinases(1,2). Phenotypic switch, thus, is one of the major cellular events underlying many VSMC-related pathological conditions such as atherosclerosis, post-angioplasty restenosis, hypertension and tumor angiogenesis(1). Unraveling the mechanisms involved in VSMC phenotypic switch is an important step towards better understanding the pathology of these diseases and ultimately designing therapeutic agents for their treatment and prevention.

The switch between the contractile and synthetic VSMC phenotypes is tightly controlled through a synergistic and coordinated molecular regulatory network (3-8). Within these molecules, serum response factor (SRF), a ubiquitously expressed transcription factor that executes multiple functions through binding to evolutionarily conserved cis-elements –the CArG box (CC(A/T)6GG)-, plays a central role(9,10). Nearly all of the VSMC-specific contractile protein genes and many other genes involved in migration, proliferation, and extracellular matrix production during the process of VSMC phenotypic switch contain CArG boxes within their promoters(1,9,10). The communication between SRF and CArG boxes in response to environmental changes is mostly controlled by the interaction of SRF with additional transcription factors, co-activators, including myocardin(11), myocardin-related transcription factors(12), PRX1(13), and GATA factors(14,15), and co-repressors, including ELK-1(6,16), KLF4(7), YY1(17), Foxo factors(18), amongst others. The balance between those positive and negative SRF cofactors instructs the dynamic VSMC gene expression in response to environmental cues (1,19). Among these regulatory components, myocardin is perhaps the most potent transcriptional co-activator of SRF identified in nature so far and strongly transactivates VSMC-specific gene expression by physically interacting with SRF(11,20).

In the past decade, the Hippo-Yap signaling pathway has emerged as one of the critical conserved pathways that regulate cell proliferation and apoptosis in response to environmental and developmental signals (21-23). This signaling pathway was discovered in Drosophila(22) with many genes involved in the Hippo pathway
evolutionarily conserved in *Drosophila* and vertebrates. The activation of macrophage stimulating 1/2 (Mst1/2) kinases by certain environmental cues phosphorylate and subsequently activate large tumor suppressor, homolog 1/2 (Lats1/2) kinase, which in turn directly phosphorylates Yap1. Phosphorylated Yap1 is retained in the cytoplasm and, as a result, Yap1 function is inhibited (24,25). Analysis of Hippo-Yap signaling in mammalian organisms has revealed its role in the regulation of maintenance of stem cell pluripotency, tumorigenesis and organ size (21,22,25-31).

However, the function of the Hippo-Yap pathway in VSMC biology remains largely unexplored. In particular, the involvement of Yap1 in VSMC phenotypic switch and the molecular mechanisms underlying such potential link are unknown. Here, we uncover for the first time a role for Yap1 in VSMC phenotypic switch. We found up-regulation of Yap1 expression during VSMC phenotypic switch induced by PDGF-BB *in vitro* and during balloon injury-induced vessel lesion formation *in vivo*. Conversely, the knockdown of Yap1 expression not only led to impaired proliferation of VSMCs but also enhanced expression of SMC contractile-specific markers. Furthermore, Yap1 affects VSMC phenotypic switch *via* interference with myocardin/SRF/CArG box tertiary complex formation. This study provides new insights into the mechanisms controlling phenotypic switch of VSMC and identify a new potential therapeutic target for ameliorating VSMC-related diseases and a potential tool for accelerating viable approaches to vascular engineering.

**EXPERIMENTAL PROCEDURES**

*Rat Aortic SMC (RASMC) Culture*-The culture of RASMCs was performed following a previous report (32). Briefly, primary RASMCs were cultured in DMEM/F12 supplemented with 10% (vol/vol) FBS in a 5% CO₂ humidified atmosphere at 37°C.

*Infection with shRNA lentivirus*-Knockdown of Yap1 was achieved by infection with a lentivirus expressing the scrambled control or Yap1 shRNA (generous gift from Dr. Kunliang Guan, University of California, San Diego)(21,25,33). Cultured passage 3 RASMCs were infected with these viruses and selected with 5 μg/mL puromycin in culture medium for one week before using them in the experimental procedures. For simplicity, the resulting cells will be referred to as “scrambled” VSMCs and “shYap1” VSMCs throughout the text.

*RNA extraction and quantitative Real-Time Polymerase Chain Reaction*-Total RNA from cultured cells was extracted by using the RNaseasy mini kit (Qiagen) and cDNA was synthesized with the Superscript III first-strand synthesis system (Invitrogen). Quantitative RT-PCR amplification using custom primers (listed in Supplemental Table 1) was conducted on a Bio-Rad MyIQ detection system (Bio-Rad), as in our previous reports(34,35). 18S RNA served as an internal standard.

*VSMC proliferation assays*-Cell proliferation was determined by growth curves of VSMCs derived...
from cell counting as described before (36). Passage-matched scrambled and shYap1 VSMCs were seeded at approximately $6 \times 10^5$ cells/ml in growth medium. Cell numbers were determined for the indicated periods with the hemocytometer measurement method in a blinded unbiased fashion. Each count was an average of three repeats, whereas each data point was the average of four experiments.

**Western blot analysis and co-immunoprecipitation**—Western blot analysis was carried out as we described before (35,37). Whole cell lysate samples were prepared using the mammalian protein extraction reagent M-Per (Promega) supplemented with a protease inhibitor cocktail (Roche). Antibodies against GAPDH (Santa Cruz Biotech, 1:1000), β-tubulin (Santa Cruz Biotech, 1:500), SM α-actin (SMA, Millipore, 1:5000), SM-myosin heavy chain (SMMHC, BTI, 1:2000), SM22α (Abcam, 1:2000), p27 (Santa Cruz Biotech, 1:1000), Cyclin D1 (CCND1, Santa Cruz Biotech, 1:1000) , cyclin-dependent kinase 4 (CDK4, santa Cruz Biotech, 1:1000), proliferating cell nuclear antigen (PCNA, Santa Cruz Biotech, 1:1000) and Yap1 (Santa Cruz Biotech, 1:1000) were used to examine individual protein expression. For co-immunoprecipitation (co-IP) assay, pcDNA4-Yap1-Myc (Addgene), pCGN-SRF (Addgene) were cotransfected into Hek293 cells with either pcDNA3.1 vehicle or pcDNA3.1Flag-myocardin (generous gift from Dr. Li Li, Wayne State University) and cells were harvested 48h post-transfection. The co-IP assay was performed as described in a previous report(38). Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.8, 137 mM NaCl, 1 mM EDTA, 0.1% Triton-X-100 with a protease inhibitor mixture (Roche Applied Science), the supernatants were collected after centrifugation and pre-cleared with protein G agarose for 1 hr at 4°C, and then incubated with antibodies as indicated overnight at 4°C. Normal IgG was used for a negative control. Immunoreactivity and band density were visualized by the Odyssey system (LI-COR Biosciences, Lincoln, NE), according to the manufacturer’s instructions.

**Luciferase activity assay**—HEK293 cells seeded in 96-well plates for 24 hrs were transiently transfected with pGL3-SMMHC-luciferase reporter (generous gift from Dr. GK Owens, University of Virginia), TK Renilla luciferase control reporter vector and pcDNA4-Yap1 plasmids in the presence of pcDNA3.1 vector to keep DNA constant or pcDNA3.1Flag-myocardin using lipofectamine 2000 (Invitrogen). Forty eight hours post-transfection, cells were lysed and luciferase activity was detected with the Dual-luciferase Reporter Assay System (Promega) in a Wallac 1420 workstation. The activity of TK Renilla served as an internal standard.

**Chromatin Immunoprecipitation (ChIP) assay**—ChIP assay was performed as we described previously (34). Briefly, RASMCs carrying either lentiviral scrambled or shYap1 were cultured to 70-80% confluence. Cellular proteins were cross-linked and ChIP assay was performed by using EZ-ChIP assay kit (Millipore). Purified chromatin was immunoprecipitated using anti-SRF antibody (Santa Cruz Biotech). Eluted DNA fragments were
purified to serve as templates for the PCR amplification. Rabbit IgG control (Santa Cruz Biotech) serves as negative control to the SRF antibody. The primer sets used to amplify the area containing SRF binding sites on the CrAG boxes of promoters of SMC-specific markers were described before(8) and are included in supplemental table 1. Data represents the relative enrichment of IP DNA samples as compared with input DNA.

**Rat Carotid Artery Injury Model** - The rat carotid artery (CA) balloon injury model was performed in male Sprague-Dawley rats (230 to 300g) following a previous report (32). The arteries were harvested at day 14 after operation and grinded for protein analysis (32).

**Statistical analysis** - All Data, expressed as the mean ± SD, was analyzed for statistical significance by student’s T test with values of p<0.05 considered to be significant. All experiments were independently repeated at least three times.

**RESULTS**

**Yap1 expression correlates with VSMC synthetic phenotype** - Previous publications established that PDGF-BB is a potent mediator of the VSMC phenotype switch from a contractile to a synthetic state by repressing VSMC marker gene expression as well as promoting VSMC proliferation(1,4). Our data uncovered that the level of Yap1 mRNA in RASMCs treated with 10ng/ml PDGF-BB was significantly elevated, 2 to 4-fold, compared to the vehicle-treated control (Figure 1A). Remarkably, Yap1 expression correlated with down-regulation in either the mRNA or protein levels of VSMC specific genes associated with a contractile phenotype (including myocardin, SMA, SMMHC and SM22α) and enhanced pro-proliferation gene expression (e.g. cyclin D1 and cyclin D2) (Figure 1A-1B), which is consistent with the effects of PDGF-BB on VSMC phenotypic switch and proliferation(1,4). In accordance with the *in vitro* results, Yap1 expression is up-regulated in the well-established balloon-injury induced vessel lesion formation for 14 days, when phenotypic switch is extensive *in vivo*(39). This correlates with decreased expression of VSMC contractile apparatus SMA and SM22α and increased proliferation marker PCNA (Fig 1C). These data suggest that up-regulation of the Hippo-Yap signaling pathway components is positively correlated with the synthetic VSMC phenotype both *in vitro* and *in vivo*.

**Yap1 promotes SMC proliferation** - Yap1 is known to enhance cell proliferation in other systems (26,30,31,40-43). We next tested whether knocking-down of Yap1 is able to impair VSMC proliferation. Lentiviruses containing two different target shYap1 sets were tested for their ability to knockdown endogenous Yap1 and all subsequent experiments were performed with set 2 showing dramatic down-regulation of Yap1 protein (supplemental S1A, set 2, and S1B). Knockdown of Yap1 in RASMCs reduced cell proliferation compared to scrambled controls as shown in Figure 2A. Furthermore, consistent with impaired RASMC proliferation, protein levels of the proliferative marker cyclin D1 decreased while p27 protein, an inhibitor of cyclin-dependent
kinases, was up-regulated in shYap1 RASMCs (Figure 2B). Similarly, cell proliferation-associated cyclin D1 mRNA was also down-regulated in these conditions (Supplemental figure S1B). The proliferation response to PDGF-BB is impaired by Yap1 knockdown, as evidenced by failure in up-regulation of the proliferation marker CDK4 (supplemental figure S1B). Thus, these data indicates that proliferation of RASMCs is Yap1-dependent and without the impairment of CDK4.

**Yap1 suppresses the expression of VSMC-specific contractile protein genes** -To investigate the effect of loss-function of Yap1 on VSMC contractile-specific gene expression, RASMCs were infected with lentiviral shYap1 or scrambled control as above, and total protein or mRNAs were harvested to measure SMC gene expression by Western blot or qRT-PCR (Supplemental figure S1B and Figure 3). Data from these experiments demonstrated that all examined VSMC-specific genes associated with a contractile phenotype (including myocardin, SMA, SM22α and SMMHC) were significantly up-regulated by the knockdown of Yap1 at both protein and mRNA levels (Supplemental figure S1B and Figure 3A-3B). Interestingly, the mRNA expression level of SRF in RASMCs was not affected by knockdown of Yap, indicating SRF mRNA expression is Yap1-independent. In addition, the mRNA expression of smoothelin B, which is one of the CArG-independent SMC contractile markers (44), was not significantly changed by knock-down of Yap1. Moreover, over-expression of Yap1 in HEK 293 cells attenuated the myocardin-driven activation of an SMMHC-promoter luciferase reporter (Figure 3C). Together, these data demonstrate that endogenous Yap1 is a repressor of CArG-dependent VSMC contractile-specific genes probably by antagonizing myocardin mediated activation.

**Yap1 modulates the SMC phenotype through interaction with the myocardin/SRF complex** -To assess the possible mechanism underlying this effect, a series of co-IP assays were performed. Results showed that Yap1 specifically interacts with myocardin in HEK 293 cells transiently transfected with plasmids expressing Flag-myocardin and Yap1 (Figure 4A). Thus, IP of myocardin with anti-Flag pulls down Yap1 as evidenced by western blot with anti-Myc. In addition, over-expression of Yap1 attenuated the interaction between myocardin and SRF in co-IP experiments in a dose-dependent manner in HEK 293 cells (Figure 4B) as evidenced by reduced SRF in the complexes pulled down with anti-Flag.

In order to address the functional relevance of these interactions we conducted ChIP assays on the SMMHC and SMA promoters. Significantly, results of quantitative ChIP assays showed that Yap1 knockdown in RASMCs cultured in regular conditions was associated with marked enhancement in SRF binding to CArG-containing regions of the promoter of SMA and SMMHC within intact chromatin (Figure 5). Importantly, these effects were selective in that SRF binding to the c-fos CArG was unchanged by Yap1 knockdown, which is consistent with previous studies showing that SRF binding to the c-fos
The promoter is independent of myocardin or phenotypic switch (8,45,46) (Figure 5). Taken together, these results provide compelling evidence that Yap1 may interact with both myocardin to mediate the down-regulation of contractile-specific genes in VSMC and promote proliferation.

In summary, these studies support a functional positive role of an inducible Hippo-Yap pathway in coordinating phenotypic switch changes associated with a VSMC proliferative phenotype in response to vascular injury through direct interaction with myocardin and negative regulation of its transcriptional activity on promoters of genes specific to the contractile phenotype of VMSC (Figure 6).

DISCUSSION

Previous studies demonstrated that Yap1 upstream regulators Mst1/2 and Lats1/2 act as tumor suppressors by restricting cell proliferation and promoting cell apoptosis (47). Recent studies reported that cardiac-specific knockdown of Yap1 causes hyperplastic hearts as a result of elevated cardiomyocyte proliferation and with the ensuing impaired cardiac function (31,43). In addition, Yap1-null mice die at E8.5 with defects in yolk sac vasculogenesis, suggesting Yap1 may play distinct roles during vasculogenesis (48). Yet, the specific involvement of Yap1 in vascular biology and disease remains unexplored.

In the present study, the first report examining the function of Yap1 on VSMC phenotypic switch, we show that the expression of Yap1 could be enhanced by PDGF-BB, a well-accepted driver of this phenomenon in vitro. More importantly, the in vivo expression of Yap1 is up-regulated in the injured vessel walls induced by balloon angioplasty (Figure 1), which indicates a potential active involvement of Yap1 on VSMC phenotypic switch in vivo. The knockdown of endogenous Yap1 in VSMCs results in up-regulation of contractile-specific VSMC gene expression but attenuates VSMC proliferation in vitro (Figure 2-3, Supplemental Figure S1), indicating that endogenous Yap1 is required for both proliferation and the concomitant repression of the contractile phenotype. Furthermore, the effect of Yap1 on VSMC phenotypic switch involves interference with myocardin/SRF/CArG ternary complex, one of the essential transcriptional regulatory effectors in this VSMC response (1). These results clearly suggest that Yap1 acts as an effector for PDGF-BB-induced proliferation and repression of VSMC contractile-specific genes thus operating as a central molecular switch for the phenotypic modulation of VSMC and, therefore, suggest that Yap1 is causally involved in SMC phenotypic switch associated with vascular injury and other vascular pathologies.

Our observations on Yap1 roles are consistent with a recent publication suggesting a relevant role for the Hippo-Yap signaling pathway in VSMC (49), in that over-expression of Mst1, a negative regulator of Yap1 function, enhanced VSMC apoptosis in vitro and in vivo and suppressed neointimal formation in arteries in a balloon-injury model. Although these results are of extensive interest, the precise mechanism for
Hippo-Yap signaling in vascular remodeling remained poorly understood. With vascular remodeling being highly regulated by mitogenic factors and involving phenotypic switch, further studies, as these presented here, are necessary to uncover the mechanisms underlying the effects of Hippo-Yap signaling in mitogen-treated VSMCs.

Intriguingly, knockdown of endogenous Yap1 in VSMCs selectively enhanced expression of an entire subset of VSMC-specific genes associated with the contractile phenotype, as shown in Figure 3. It is well established that virtually almost all VSMC-selective contractile marker genes characterized to date are CArG/SRF-dependent (1,2). As such, it is likely that results of the present studies are relevant to mechanisms that induce the coordinated down-regulation of multiple VSMC contractile marker genes in response to PDGF-BB treatment and the ensuing proliferation, thus defining Yap1 as a potential molecular switch controlling SMC phenotypic modulation.

Yap1 has been identified as a Ty1 enhancer activity (TEA) domain family member (TEAD)-interacting protein (50). Moreover, the interaction of Yap1 and TEAD proteins is essential for Yap1 to modulate cell activities in other cell types (33,51,52). Previous studies have shown the presence of functional muscle-specific cytidine-adenosine-thymidine sequence (MCAT) elements within the SMA promoter that bind TEAD proteins and repress the activity of the promoter during VSMC differentiation throughout embryonic development. However, this machinery is not required for SMA promoter activity in adult mature VSMCs (53,54), suggesting that the effects we observe in markers like SMA in the context of VSMCs may not be mediated by Yap1 interaction with TEAD proteins.

It has been shown that SRF plays a central function in the expression of many different VSMC specific genes(9). SRF is a multifunctional protein which not only binds a highly conserved CArG box that can be found in most SMC-specific contractile genes, but also provides a docking surface for interaction with a wide variety of accessory cofactors(9). Of the SRF-associated proteins, myocardin is the most potent natural activator for CArG box-containing VSMC–specific contractile genes and is a strong inhibitor for VSMC migration and proliferation (11,55-57). Interestingly, our data here strongly indicates that Yap1 binds myocardin in a complex, while the binding of SRF to myocardin is decreased by Yap1 over-expression in a dose-dependent manner (Figure 4). Although the nature of these complexes, the dynamics of the interaction and the domains in Yap1 responsible for this effects remain to be explored, these data in conjunction with our ChIP results (Figure 5) supports that, in adult SMC, Yap1 suppresses expression of contractile phenotype genes via inhibition of the recruitment of the essential SRF/myocardin/CArG ternary complex to the promoter of the co-regulated genes. This is likely facilitated through Yap1 decreasing expression of myocardin itself (Figure 3), as previous studies have shown that myocardin is necessary and sufficient for contractile-specific VSMC gene expression(58,59). As shown in
Figure 2, our data also suggests Yap1 is necessary for VSMC proliferation. Myocardin is a strong inhibitor of VSMC migration and proliferation(11,55-57) when VSMC cells are in the contractile phenotype. Although the detailed molecular mechanism for Yap1 effects on VSMC proliferation is still unclear and deserves in-depth investigation (Figure 6), the interaction between myocardin and Yap1 is likely to be a primary and essential component within this network to regulate VSMC proliferation by facilitating the phenotypic switch from contractile to synthetic phenotype by interfering with the myocardin-dependent activation of contractile specific genes.

Prior reports in cardiomyocytes identified a TEAD binding element in the myocardin promoter and TEAD2 could significantly activate a myocardin promoter reporter gene (60). It is possible that knockdown of Yap1 attenuates the formation of Yap1/TEAD complexes and subsequently enhance TEAD function resulting up-regulation of myocardin expression thereby potentiating SMC gene expression in VSMCs. This would require that, unlike what has been reported for similar elements in the SMA promoter, the TEAD element in the myocardin promoter be functional in adult VSMC, a possibility that remains to be investigated. It is also possible that these two mechanisms may co-exist and act coordinately to account for the up-regulation of VSMC contractile-specific gene expression resulting from the down-regulation of Yap1. The present studies support a mechanism whereby down-regulation of Yap1 selectively increases SMC contractile gene expression by allowing increased expression of the SRF co-activator myocardin (Figure 3) and simultaneously releasing the direct inhibition on myocardin/SRF/CArG ternary complex with the subsequent activation of contractile-specific gene expression mediated by this complex (Figure 4-5). Therefore, the results presented here provide evidence for an emerging model revealing a molecular mechanism whereby the up-regulation of Yap1 in the presence of PDGF-BB selectively inhibits VSMC contractile-specific genes via both inhibition of myocardin expression and concurrent direct inhibition of myocardin-mediated activation of CArG-dependent transcription by selectively attenuating SRF binding to CArG box regions of these genes within the intact chromatin (Figure 6). Additionally, endogenous Yap1 is clearly required for optimal proliferation of VSMC (Figure 2A and B), an effect previously described for other cell types(26,30,31,40-43), which, in the context of the new data presented here places Yap1 at the critical cross-regulatory junction linking proliferation and synthetic phenotypic switch(1).

This phenotypic switch of VSMC from a quiescent, contractile phenotype to a proliferative state may contribute to the repair of vascular injury. Conversely, in the normal vessels, quiescent, contractile phenotypes are maintained through repression of Yap1 which, in turn results in up-regulation of myocardin expression and synergistic direct potentiation of SRF binding to CArG box of the co-regulated contractile targets. Here, we have shown the up-regulation of Yap1 in the balloon-injury induced vessel lesion (Figure 1C). However, there is currently no direct
evidence that Yap1 plays this role in vivo because, as mentioned above, Yap1-null mice die at E8.5 with, most interestingly, defects in yolk sac vasculogenesis(48). Additional studies involving VSMC-selective conditional knockout of this gene are needed to directly test whether Yap1 contributes to VSMC phenotypic switch in vivo during vascular injury and atherosclerosis pathogenesis, as well as to further dissect the mechanisms by which Yap1 suppresses myocardin expression and selectively controls SRF binding to CArG elements within the intact chromatin.

There is extensive evidence indicating that VSMC phenotypic switch by mitogens in response to vascular injury occurs through the interaction of a transcriptional network, which contributes to overall phenotypic switching, including stimulation of VSMC growth(61-63). Among others, KLF4 (7) and ELK1 (6,16) have been reported to mediate PDGF-BB effects on phenotypic switch. The present studies are the first to provide direct evidence identifying Yap1 as a novel effector of PDGF-BB-induced repression of VSMC differentiation marker genes. Yet, it is still possible that PDGF-BB may also induce suppression of VSMC contractile genes through Yap1-independent mechanisms or that Hippo-Yap signaling may cross-talk with other PDGF-BB dependent pathways. For instance, Hippo-Yap signaling may cooperate synergistically with the inhibitory effect of KLF4 and ELK1 on the binding of myocardin to modulate VSMC phenotypic switch in response to environmental stimulation.

The ability to manipulate the phenotypic switch of VSMC in vitro and in vivo will prove essential as a therapeutic intervention to prevent proliferative vascular disorders as well as the complications of atherosclerosis and development of aneurysms(1,2). This study provides new insights into the mechanisms controlling phenotypic modulation of VSMC and defines Yap1 as a central molecular switch controlling this process, thus identifying a new potential therapeutic target for ameliorating VSMC-related diseases and a potential tool for accelerating viable approaches to stem cell differentiation and vascular engineering.

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FOOTNOTES

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3the abbreviations used are: Yap, Yes-associated protein; VSMC, vascular smooth muscle cells; PDGF, platelet derived growth factor; SRF, serum response factor; SMMHC, SM-myosin heavy chain; SMA, SM α-actin; CCND1, Cyclin D1; CCND2, cyclin D2; TEAD, TEA domain family member; ChIP, Chromatin Immunoprecipitation

FIGURES AND LEGENDS

FIGURE 1. Yap1 expression is increased during VSMC phenotypic switch induced by PDGF-BB and in vessel injury. A. RASMCs grown to 60-70% confluence underwent mitogenic quiescence by serum starvation (DMEM+0.5% FBS) for 48 hrs and were subsequently stimulated with 10ng/ml PDGF-BB for the indicated periods. Time-point expression of Yap1 and markers of VSMC proliferation and contractile phenotype were analyzed by qRT-PCR. 18S RNA served as internal control. CCND1, cyclin D1; CCND2, cyclin D2; SMA, SM α-actin; MyoCD, myocardin; SMMHC, smooth muscle myosin heavy chain. N=3. Data is expressed as mean ± SD. * p<0.05. B. Representative Western blots analysis of total protein lysates of cultured RASMCs. RASMCs were treated with 10ng/ml PDGF-BB for 24 hrs after serum starvation for 48hrs and harvested for Western blot. C. Representative Western blot analysis of total protein lysates of carotid arteries. Rat carotid arteries subjected to balloon injury and sham surgery controls were harvested 14 days after surgery and grinded for Western blot analysis as indicated in the Figure. N=3.

FIGURE 2. Knockdown of Yap1 attenuates VSMC proliferation. A. RASMCs infected with lentiviruses encoding Yap1 shRNA (shYap1) or control scrambled shRNA were seeded in 6-well plates and cell proliferation was measured by counting cell numbers at each time point as described in the text.
N=4. Data is expressed as mean ± SD. *p<0.05. **B.** Representative Western blot analysis of total protein lysates from RASMCs infected with lentiviruses encoding Yap1 shRNA (shYap1) or control scrambled shRNA actively growing in 10% FBS-containing growth medium using the indicated antibodies. GAPDH served as internal control.

**FIGURE 3. Knockdown of Yap1 enhances contractile phenotype specific gene expression in VSMCs.** A-B. RASMCs infected with shYap1 were cultured in 10% FBS-containing DMEM and harvested for qRT PCR (A) or Western blotting (B) to detect gene expression as indicated. Cells infected with lentiviral scrambled vector served as a control. C. A luciferase reporter for the SMMHC gene promoter was co-transfected with myocardin (MyoCD) and/or Yap1 expression plasmids in HEK293 cells and the luciferase activity was measured 48hrs after transfection as described in the methods. TK Renilla served as internal control. The basal activity of pcDNA3.1 empty vector on SMMHC promoter activity was normalized to 1. Data is expressed as mean ± SD. N=3. *p<0.05.

**FIGURE 4. Yap1 binds myocardin.** A. HEK 293 cells were transfected with pcDNA-myocardin-Flag and/or pcDNA4-Yap1-Myc expression plasmids. The specificity of binding between Yap1 and myocardin was confirmed by precipitation with Flag antibody and further immunoblot with anti-Myc antibody. B. HEK 293 cells were transfected with pcDNA-myocardin-Flag and SRF while pcDNA4-Yap1-Myc expression plasmid was introduced with increased dosage. Total cell lysates were precipitated with Flag antibody and further immunoblot with anti-SRF antibodies, Flag and Yap1 as indicated. IP: immunoprecipitation. IB: immunoblotting.

**FIGURE 5. Yap1 attenuates the SRF-dependent transcriptional activation of CArG box containing contractile VSMC specific genes.** ChIP assay were performed on crossed linked chromatin from RASMCs with Yap1 knockdown (shYap1) immunoprecipitated with anti-SRF antibody. The precipitated DNA was amplified by real-time PCR with VSMC gene-specific primers spanning the CArG region. Data represent the relative enrichment of IP DNA samples as compared to input DNA and is expressed as mean ± SD. N=3. *p<0.05.

**FIGURE 6.** Working hypothesis whereby Yap1 regulate VSMC phenotypic switch via myocardin. Upon stimuli driving VSMC phenotypic switch from contractile to synthetic, like PDGF-BB, Yap1 interferes with myocardin activity resulting in reduced-expression of VSMC contractile genes and, through a yet undefined mechanism, increased proliferation markers in the VSMCs.
Xie, et al. Fig. 1
Xie, et al. Fig. 1
2A

Scrambled

- shYap1

Cell Number (X10^5)

D0 D2 D3

2B

Scrambled shYap1

Yap1

CCND1

p27

GAPDH

Xie, et al. Fig. 2
Xie, et al. Fig. 3
Xie, et al. Fig. 3
4A

Yap1-Myc  -  +  +  
MyoCD-Flag  +  -  +

IP: Flag  
IB: Myc

Input

Myc  Flag

4B

SRF  +  +  +  
MyoCD-Flag  +  +  +  
Yap1-Myc

IP: Flag  
IB: SRF

Input

Flag  SRF  Yap1

Xie, et al. Fig. 4
Relative SRF binding

Xie, et al. Fig. 5
Xie, et al. Fig. 6

PDGF-BB → Yap1

MyoCD

Yap1

SRF

MyoCD

SRF

CArG

Contractile gene

SM α-actin, SMMHC, SM22α, etc..

Proliferation gene

Cyclin D1, Cyclin D2, etc..

Contractile Phenotype → Synthetic Phenotype

SM α-actin, SMMHC, SM22α, etc..
Yap1 regulates vascular smooth muscle cell phenotypic switch by interaction with myocardin
Changqing Xie, Yanhong Guo, Tianqing Zhu, Jifeng Zhang, Peter X. Ma and Y. Eugene Chen

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