Adiponectin Induces Vascular Smooth Muscle Cell Differentiation via Repression of Mammalian Target of Rapamycin Complex 1 and FoxO4

Min Ding, Yi Xie, Robert J. Wagner, Yu Jin, Ana Catarina Carrao, Lucinda S. Liu, Anthony K. Guzman, Richard J. Powell, John Hwa, Eva M. Rzucidlo, Kathleen A. Martin

Objective—The adipocyte-secreted hormone adiponectin exerts important cardioprotective and antidiabetic effects. Little is known about its effect on vascular smooth muscle cells (VSMC), key cells in restenosis, hypertension, and atherosclerosis.

Methods and Results—Using human coronary artery VSMC, we found that recombinant adiponectin in the high-molecular-weight or trimeric forms but not the globular form induced VSMC differentiation through a mechanism similar to the classic feedback signaling used by rapamycin, a drug known to effectively inhibit restenosis on drug-eluting stents. Using a combination of pharmacological agents, small interfering RNA, and overexpression approaches, we demonstrated that adiponectin activated 5′-AMP-activated protein kinase α2 isoform, leading to inhibition of mammalian target of rapamycin complex 1 and S6K1. This in turn stabilized insulin receptor substrate-1, driving Akt2-mediated inhibition of FoxO4 and subsequent contractile protein induction. Although adiponectin and rapamycin have similarly beneficial effects on VSMC phenotype in both cell and organ culture, a direct comparison of the effects of rapamycin versus adiponectin on endothelial cells revealed distinct differences: rapamycin inhibited Akt phosphorylation, whereas adiponectin maintained it. Importantly, Akt activity preserves endothelial function.

Conclusion—Adiponectin promotes VSMC differentiation and preserves endothelial cell Akt signaling, suggesting that targeting the adiponectin pathway may have advantages over rapamycin in developing new drug-eluting stent therapeutics. (Arterioscler Thromb Vasc Biol. 2011;31:1403-1410.)

Key Words: adiponectin ■ differentiation ■ mTOR ■ rapamycin ■ vascular smooth muscle

Restenosis is a frequent complication of vascular interventions, including bypass grafts, angioplasty, and stenting. Endothelial injury and vascular smooth muscle cell (VSMC) phenotypic modulation contribute to this intimal hyperplastic response.1 Rapamycin-eluting stents dramatically reduce the incidence of coronary restenosis but have recently been associated with late-stent thrombosis, a potentially fatal complication.2,3 Although the underlying mechanisms are still emerging, rapamycin inhibition of reendothelialization may contribute to late-stent thrombosis.4

Recent studies have revealed that low adiponectin levels can predict late in-stent restenosis5 and increase cardiovascular disease risk.6 Adiponectin is a 30-kDa hormone produced by white adipose tissue in inverse proportion to fat mass.7 Downstream signaling through its known receptors, AdipoR1, AdipoR2,8 and T-cadherin,9 remains poorly understood. Adiponectin mediates multiple cardioprotective effects, as adiponectin knockout mice exhibit increased neointimal formation and thrombus formation postinjury,10,11 higher blood pressure,12 and impaired recovery from hindlimb ischemia.13 They also develop increased myocardial infarct size,14 increased cardiac hypertrophy with pressure overload,15 and exacerbated left ventricular dilation and dysfunction following myocardial infarction.16 Adiponectin also exerts antiinflammatory and antidiabetic effects.17 Although several studies have documented beneficial adiponectin signaling in endothelial cells (EC),13,18–20 few have assessed the effects of the hormone on VSMC.

VSMC in mature vessels retain remarkable phenotypic plasticity. VSMC dedifferentiation contributes to intimal hyperplasia, atherosclerosis, and hypertension.21 Normal mature VSMC are differentiated, quiescent, and contractile, whereas injured VSMC exhibit a proliferative, dedifferentiated, synthetic phenotype. Differentiation markers include the contractile proteins smooth muscle myosin heavy chain (SM-MHC), SM-α-actin, calponin, and h-caldesmon. Differentiated VSMC lose these markers and upregulate extracellular matrix synthesis.21

We previously showed that rapamycin promotes VSMC differentiation via inhibition of mammalian target of rapamycin...
complex 1 (mTORC1) and its effector S6 kinase-1 (S6K1).22,23 Herein, we tested the hypothesis that adiponectin promotes VSMC differentiation through AMPK-mediated inhibition of mTORC1. As late stent thrombosis may be associated with mTORC1 inhibition,2,3 we also conduct a direct comparison of the effects of adiponectin versus rapamycin in human EC.

Materials and Methods

All cell culture experiments use human coronary artery smooth muscle cells purchased from Cascade Biologics (Portland, OR). Transfections and Western blot analysis were performed using standard methods as previously published.22–24 Detailed Supplemental Materials and Methods are available online at http://atvb.ahajournals.org.

Results

Adiponectin Induces VSMC Differentiation

To determine the effects of recombinant human adiponectin on VSMC phenotypic modulation, we used a human coronary artery VSMC culture model. These cells display a synthetic, proliferative phenotype similar to VSMC in intimal hyperplastic lesions. We have previously reported that rapamycin or prostacyclin analogs can induce VSMC differentiation in this system.22–24 Adiponectin exists in multiple oligomeric forms, including trimeric, hexameric, high-molecular-weight (HMW), and truncated globular forms in vivo.25 We examined the effects of different oligomeric preparations on VSMC differentiation. Treatment with a preparation of full-length adiponectin enriched in HMW oligomers (12 to 18mer, ~360 to 540 kDa) or with trimeric adiponectin (~90 kDa) induced expression of contractile protein markers of VSMC differentiation, including SM-MHC (SM2 isoform), h-caldesmon, calponin, and SM-α-actin (Figure 1A). Trimeric adiponectin was slightly less potent than the HMW preparation. Conversely, the truncated globular form did not efficiently induce contractile protein expression (Figure 1A).

AMPK Is Necessary for Adiponectin-Induced Differentiation

AMPK has been implicated as a key effector of adiponectin signaling in multiple other cell types.18,25,26 To determine
whether adiponectin induces VSMC differentiation via AMPK, we pretreated VSMC with compound C, a specific inhibitor of AMPK, before adiponectin treatment. Compound C inhibited adiponectin-induced AMPK and acetyl-CoA carboxylase phosphorylation, as well as contractile protein expression (Figure 2A and Supplemental Figure II). We confirmed this result using small interfering RNA (siRNA) to knock down the AMPK catalytic subunit isoforms \( \alpha_1 \) or \( \alpha_2 \). Notably, only AMPK\( \alpha_2 \) appears to be required for adiponectin-induced differentiation (Figure 2B). An increase in contractile proteins was noted on AMPK\( \alpha_1 \) knockdown, suggesting that it may potentially inhibit contractile protein expression. However, treating VSMC with 5-aminimidazole-4-carboxamide-1-b-riboside, a pharmacological activator of AMPK (which is not known to exert an AMPK isoform-specific effect), inhibited mTORC1, activated Akt and induced contractile protein expression in a time-dependent manner (Figure 2C). These data indicate that AMPK\( \alpha_2 \) activation is necessary for adiponectin-induced VSMC differentiation and that AMPK activation is sufficient to induce differentiation.

### Adiponectin Induces Differentiation via mTORC1/S6K1 Inhibition

AMPK regulates cellular energy homeostasis by repressing energy-consuming processes, including protein synthesis, while simultaneously enhancing energy-producing processes.\(^{27}\) Through phosphorylation of the tuberous sclerosis complex 2 (TSC2) protein, AMPK inhibits mTORC1 activity and protein synthesis in skeletal muscle.\(^{27}\) Because we previously reported that mTORC1 inhibition with rapamycin induces VSMC differentiation,\(^{22,23}\) we next determined whether mTORC1 pathway inhibition is required for adiponectin-induced differentiation. We infected VSMC with an adenovirus encoding green fluorescent protein alone (control) or green fluorescent protein and a hemagglutinin (HA)-tagged rapamycin-resistant constitutively active S6K1 mutant (S6K1-ED3E). This mutant is sufficient to block rapamycin-induced VSMC differentiation, IRS-1 stabilization, and Akt activation.\(^{23}\) This mutant S6K1 also inhibited adiponectin-induced VSMC differentiation (Figure 3), suggesting that adiponectin inhibition of S6K1 is required for this response. Replicate experiments using S6K1 plasmid verified these findings (data not shown).

### Akt-2 Inhibition of FoxO4 Is Required for Adiponectin-Induced Differentiation

Increasing evidence reveals that Akt1 and Akt2 differentially regulate cell migration and metabolism.\(^{29}\) We reported that rapamycin specifically activates Akt2 and that Akt2, but not Akt1, is required for rapamycin-induced VSMC differentiation.\(^{23}\) Similarly, siRNA knockdown of Akt2, but not Akt1, inhibited adiponectin-induced contractile protein expression (Figure 4). Interestingly, adiponectin increased Akt phosphorylation (Ser473) whether Akt1 or Akt2 was knocked down, suggesting that, unlike rapamycin, adiponectin may activate both Akt isoforms (Figure 4). In other experiments, we were able to detect a modest but selective activation of Akt2 using an isoform-specific immunoprecipitation approach (Supplemental Figure IIIA). We previously found that only Akt2 activity is sufficient to induce contractile protein expression.\(^{23}\) Adiponectin may activate both Akt1 and Akt2,
but it appears that the actions of Akt2 predominate given the net adiponectin effect on differentiation. Our siRNA data suggest that Akt1 may inhibit contractile protein expression, as Akt1 knockdown increases baseline MHC expression (Figure 4). We report that overexpression of Akt1 does not inhibit basal levels of contractile proteins but does inhibit their induction by adiponectin or rapamycin (Supplemental Figure IIIB). Finally, we note a compensatory increase in Akt2 expression and phosphorylated Akt2 when Akt1 is knocked down, suggesting another potential mechanism for opposing actions of these Akt isoforms (see Figures 4 and 5B and Supplemental Figure IIIC).

Figure 3. Adiponectin induces VSMC differentiation via S6K1 inhibition. VSMC were infected with control green fluorescent protein (Con (GFP)) or rapamycin-resistant mutant S6K1 adenovirus overnight and then treated with vehicle or 5 μg/mL adiponectin for 24 hours before Western analysis. Bar graphs were prepared from 3 separate experiments as described in Figure 2A. Probability values (Newman-Keuls multiple comparison post hoc tests) are indicated above the bars. Adpn indicates adiponectin; HA, hemagglutinin.

Figure 4. Adiponectin induces differentiation via Akt2. VSMC were transfected with small interfering (si) control, siAkt-1, or siAkt-2 RNAs for 24 hours and then treated with vehicle or 5 μg/mL adiponectin for 24 hours before Western analysis. A representative experiment is shown. Bar graphs were prepared from 3 separate experiments as described in Figure 2A. Adpn indicates adiponectin.

N=3, *p<0.05, **p<0.001

Figure 4A. SM2-MHC

Figure 4B. SM-α-actin

Figure 4C. P-Akt (S473) Light

Figure 4D. P-Akt (S473) Dark

Figure 4E. Akt-1

Figure 4F. Akt-2

Figure 4G. GAPDH

Figure 4H. Adiponectin 24h

Figure 4I. Con (GFP) HA-S6K1 ED3E

Figure 4J. siCon siAkt1 siAkt2

Figure 4K. h-Caldesmon

Figure 4L. Calponin

Figure 4M. HA (S6K1 ED3E)

Figure 4N. β-tubulin

Figure 4O. Adiponectin 5 μg/ml 24h

Ironically, FoxO proteins are known substrates of Akt. FoxO4, in particular, has been shown to associate with and inhibit the activity of myocardin, a critical transcriptional coactivator in VSMC differentiation.29,30 Akt phosphorylation of FoxO4 inactivates this transcription factor by promoting its nuclear exclusion.31 We report that FoxO4 prevents adiponectin-induced VSMC differentiation (Figure 5C).
Adiponectin and Rapamycin Similarly Promote VSMC Differentiation, but Have Distinct Effects on EC

As both adiponectin and rapamycin promote VSMC differentiation via mTORC1 inhibition, we conducted experiments to compare their effects on VSMC and EC in parallel. Adiponectin and rapamycin induced SM2-MHC expression to a similar extent and with similar (although nonidentical) kinetics in VSMC (Supplemental Figure VA). Notably, both adiponectin and rapamycin also prevented culture-induced contractile protein downregulation in intact vessel segments in an organ culture model (Figure 6A). We found that rapamycin also induced FoxO4 phosphorylation and translocation in a time- and dose-dependent manner (Supplemental Figure VB and VC).

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Importantly, the effects of adiponectin and rapamycin differed when directly compared in human EC. Rapamycin significantly inhibited Akt phosphorylation and completely inhibited mTORC1 in human umbilical artery or vein EC. Notably, adiponectin did not inhibit Akt in human umbilical artery EC and slightly activated Akt in human umbilical vein EC. In both EC types, adiponectin only modestly inhibited mTORC1 (Figure 6B). Because Akt/mTOR signaling is cytotoxic and promotes EC survival, the divergent effects of rapamycin and adiponectin in EC suggest that the adiponectin pathway may be a preferable route to promote VSMC differentiation while preventing EC injury.

Discussion

We report the novel findings that (1) HMW or trimeric adiponectin induces VSMC differentiation via mTORC1 inhibition, (2) adiponectin transduces this signal via AMPKα2 and Akt2 suppression of FoxO4, (3) although adiponectin and rapamycin similarly promote VSMC differentiation, only adiponectin promotes protective signaling in EC. Our study suggests that maintenance of VSMC phenotype may be an additional cardioprotective effect of endogenous adiponectin. In uncovering these roles of adiponectin, we have identified signaling mechanisms that underlie its actions.

Adiponectin Induces VSMC Differentiation via AMPK/mTORC1

We report that adiponectin promotes differentiation in human VSMC via AMPK-mediated mTORC1 inhibition (see model in Supplemental Figure VI). AMPK has emerged as a critical mediator of adiponectin’s beneficial effects on metabolism and insulin sensitivity in other tissues and on the stimulation of angiogenesis. We found that AMPK activation with 5-aminoimidazole-4-carboxamide-1-b-riboside was sufficient to inhibit mTORC1 and induce VSMC differentiation. Notably, we believe we are the first to specifically implicate the AMPKα2 isofrom as a key adiponectin effector. In contrast to other studies of adiponectin signaling in VSMC, ours is the first using multiple methods to manipulate endogenous levels of AMPK, as opposed to adenoviral overexpression. Our siRNA data interestingly suggest that AMPKα1 may oppose contractile protein expression, as contractile proteins basally increase after AMPKα1 knockdown. AMPKα2 may be the predominant effector of adiponectin-induced VSMC differentiation and may therefore be a potential therapeutic target for future stent drug development.

Of the 2 mTOR cellular complexes, only mTORC1 is inhibited by AMPK. mTORC1 and its substrate S6K1 participate in a well-documented feedback loop in which growth factor and nutrient signaling limits the activity of the phosphatidylinositol 3-kinase/Akt pathway: S6K1 phosphorylates IRS-1 on serine residues that promote its ubiquitination and degradation. This pathway modulates cell growth and insulin sensitivity, and its dysregulation is linked to cancer, diabetes, and obesity. We now report that adiponectin-induced and rapamycin-induced VSMC differentiation similarly require S6K1 inhibition. Interestingly, adiponectin inhibits mTORC1 to a lesser degree than rapamycin in both VSMC and EC, perhaps because adiponectin inhibits mTORC1 indirectly via AMPK: AMPK phosphorylates TSC2, which, in turn, inhibits Rheb, a requisite mTORC1 activator. In contrast, rapamycin/FKBP12 directly binds and inhibits mTORC1. Our work suggests that adiponectin may provide an endogenous hormonal signal that limits mTORC1 activity in helping to maintain a healthy VSMC phenotype.

We find that adiponectin, like rapamycin, requires Akt2 activation to promote VSMC differentiation. We now report that FoxO4 is an Akt2 substrate and a critical effecter of adiponectin in VSMC, as FoxO4 overexpression inhibits adiponectin-induced differentiation. As FoxO4 is known to interact with and inhibit the activity of myocardin and promote intimal hyperplasia, this provides a likely mechanism for transcriptional regulation of contractile protein expression. Unlike rapamycin, our data suggest that adiponectin may also activate Akt1, but Akt1 does not appear to be required for regulation of FoxO4 or differentiation in this model. Our data suggest that Akt1 opposes contractile protein expression, and we will pursue the mechanism in future studies. It will be interesting to determine whether adiponectin might inhibit Akt3, as this isoform has been suggested to promote VSMC proliferation. We also note potential feedback regulation between Akt isoforms, as Akt1 knockdown increases both total and phospho-Akt2. However, as this Akt2 upregulation did not significantly increase FoxO4 phosphorylation, we hypothesize that there may be Akt2 substrates in addition to FoxO4 that also contribute to contractile protein regulation.

Although adiponectin was previously shown to inhibit VSMC proliferation and migration by directly binding platelet-derived growth factor-BB and inhibiting growth factor-stimulated ERK signaling, our observations in low-serum conditions support a role for intracellular signaling mechanisms in adiponectin-induced differentiation. We found that adiponectin also efficiently induces VSMC differentiation in 10% serum conditions in cell (data not shown) and organ culture. Adiponectin is also known to inhibit VSMC and EC apoptosis via AMPK. In vivo, it is likely that adiponectin exerts pleiotropic effects on VSMC, as well as EC, that contribute to its antirestenotic effects. Depending on the success of rapamycin in combating coronary artery restenosis, recent concerns have been raised regarding sudden death from late stent thrombosis, which is not yet clearly understood, but it is likely mediated by incomplete reendothelialization and diabetes confers a greater risk. Rapamycin and other stent drugs have been shown to inhibit reendothelialization, which may contribute to this pathogenesis. A fundamental understanding of rapamycin’s actions on both VSMC and EC will be required to address this problem. An ideal stent agent would inhibit VSMC phenotypic modulation while promoting reendothelialization. We report that the natural hormone adiponectin may fulfill this dual role. Our data also demonstrate a careful direct comparison that rapamycin inhibits Akt/mTORC1 in human EC, whereas adiponectin preserves Akt/mTORC1 activity. Adiponectin-induced Akt signaling
has been shown to be proangiogenic in EC.18 Akt promotes endothelial nitric oxide synthase phosphorylation and activity, which is essential for EC migration, and has antiapoptotic effects in EC.13,18 Adiponectin additionally benefits endothelial function by promoting EC migration,18 inhibiting EC apoptosis19 and inflammatory activation,45 inducing endothelial nitric oxide production20 and decreasing reactive oxygen species production,42 suggesting a global EC protective function. Moreover, adiponectin inhibits thrombus formation,11 whereas rapamycin promotes platelet aggregation.43 We found that in contrast to rapamycin, adiponectin only modestly inhibited mTORC1 activity in EC. Notably, mTORC1 inhibition is antiangiogenic and inhibits EC migration.44 The ability of adiponectin to mimic the beneficial effects of rapamycin in VSMC while sparing cytoprotective Akt/mTORC1 signaling in EC suggests that the adiponectin pathway may be a desirable alternative for future drug-eluting stent therapeutic development. As a whole, the adiponectin pathway presents exceptional potential for future antistenotic therapy through its unique combination of anti-inflammatory effects, local antidiabetic properties, and overall promotion of arterial health.

The reasons for the opposing effects of rapamycin on Akt in VSMC versus EC are not yet known, but indirect inhibition of mTORC2 suggests a potential mechanism (see Supplemental Figure VIB). When the mTOR protein resides in mTOR complex 1 with raptor and other accessory proteins, this complex is directly sensitive to rapamycin and regulates processes including protein synthesis. A more recently identified complex, mTORC2, containing mTOR, rictor, and other proteins, is required for the phosphorylation of Akt at Ser473, as well as of other kinases, including serum/glucocorticoid regulated kinase and protein kinase Cα.36 The kinase activity of mTORC2 is itself insensitive to rapamycin, and mTORC2 assembly can be inhibited because of sequestration of newly synthesized mTOR protein in mTORC1/rapamycin-inhibited complexes. This “chronic” rapamycin inhibition of mTORC2 shows dramatic cell type specificity and has been observed in EC.45 Given the long half-life of rapamycin, this effect can be substantial in some cell types and could potentially underlie rapamycin inhibition of Akt in EC. Adiponectin does not inhibit mTORC1 activity to the complete extent that rapamycin does in VSMC or EC. Furthermore, rapamycin inhibits mTORC1 by directly binding the complex, whereas adiponectin inhibition mediated by AMPK occurs through a distinct, TSC-mediated mechanism. It is therefore likely that adiponectin inhibition of mTORC1 does not result in inhibition of mTORC2. Furthermore, we propose that adiponectin may act through as yet undefined signals to activate Akt in EC. Finally, although it is active in VSMC, the insulin-like growth factor 1/insulin-dependent feedback activation of Akt by rapamycin does not occur in all cell types, perhaps because of cell type-specific patterns of phosphorylation and expression of IRS family proteins.

Remaining Challenges
The physical properties of adiponectin, including its short half-life, rapid turnover, and multiple oligomeric forms,25 limit its current utility as a drug-eluting stent agent. Understanding the adiponectin-activated pathways in human cells that drive stent pathology is critical for identification of novel targets for development of improved therapeutics. Our data provide important signaling insights, as well as identifying the HMW and trimeric forms but not the globular form of adiponectin as potent inducers of VSMC differentiation. This is in contrast to skeletal muscle, where globular adiponectin enhances insulin sensitivity.26 Interestingly, Kobayashi et al noted that only the HMW form activates AMPK and inhibits apoptosis in EC and that it is primarily this HMW form that increased on weight loss in obese patients.19 Whether there are specific receptors and functions for each oligomeric form of adiponectin remains a subject of intense investigation. A small molecule adiponectin receptor agonist may prove to be an ideal agent for drug-eluting stents.

In summary, we report that adiponectin, through AMPK inhibition of mTORC1 and feedback activation of Akt2 and FoxO4 inhibition, promotes VSMC differentiation, in a manner similar to the stent drug rapamycin. In contrast to rapamycin, adiponectin preserves EC Akt/mTORC1 activity. This work suggests that adiponectin pathway-based therapeutics may have the potential to avoid prothrombotic adverse effects associated with rapamycin while maintaining antistenotic efficacy.

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Disclosures
None.

References


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Supplement Material

Materials and Methods

Cell culture

Human coronary artery VSMC (Cascade Biologics, Portland, OR) (passages 3-6) were cultured in M199 media as described\(^1\). HUVEC and HUAEC (Lonza Walkersville Inc, MD, up to passage 8) were cultured on fibronectin in M199 media with 20% FBS, 50 μg/ml endothelial cell growth supplement (ECGS), 100 μg/ml porcine heparin (Sigma, St. Louis, MO) and penicillin-streptomycin. Cells were cultured in media with 2.5% (VSMC) or 0.5% FBS (HUVEC or HUAEC) for 16-24 hours prior to drug treatment. Untreated VSMC proliferate and do not spontaneously differentiate in media containing 2.5% FBS\(^1\). Vehicle was added for the maximum duration of the experimental treatment if not specified. Unless specified, human full-length recombinant adiponectin (HMW enriched) produced in HEK293 cells (RD172023100, BioVendor, Candler, NC) was utilized. Trimeric (RD172091100) and globular (RD172112100) recombinant adiponectin preparations were used where indicated. Rapamycin was purchased from LC Laboratories (Woburn, MA), AICAR from Toronto Research Chemicals Inc (Ontario, Canada) and Compound C from Calbiochem (San Diego, CA).

Cell lysis and western blotting

Cells were lysed as previously described\(^1\). Equal amounts of protein per lane were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and immunoblotted using primary and secondary antibodies as described\(^2\), and primary antibodies against phospho-Thr 172 AMPK, AMPK-α (pan), AMPK-α1, AMPK-α2, phospho-ACC (Ser 79), total ACC, phospho-Ser 193 FoxO4 (Cell Signaling, Boston, MA) and total FoxO4 (Abcam, Cambridge, MA). Blots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL) and quantitated using
Contractile proteins were normalized to GAPDH or β-tubulin. Phosphorylated proteins were normalized to corresponding total protein.

**Transient transfection of siRNA and plasmid**

Transient transfection of small interfering RNA (siRNA) in VSMC was performed via Nucleofector (Lonza Walkersville Inc, MD) as described\(^2\). For gene knockdown, 1 to 1.5 million cells were transfected with 2.5 to 3 μg siRNA and cultured in 2.5% FBS for 48 hours. Cells were treated with vehicle or adiponectin and harvested for Western blotting analysis as above. Akt1 (SMARTpool), Akt2 (4 different siGENOME duplexes) and nonsilencing siRNA (si\(\text{CONTROL}\)) were purchased from Dharmacon (Lafayette, CO), and AMPK\(\alpha1\), AMPK\(\alpha2\) and a matching nonsilencing control siRNA were purchased from Qiagen (Valencia, CA).

Transfection of HA-S6K1 or HA-Akt1 plasmids were as published\(^2\)-\(^3\). Wild-type Flag-tagged FoxO4 plasmid was purchased from Addgene (Cambridge, MA). For gene overexpression, 1 million human VSMC were transfected with 10ug FoxO4 plasmid via Nucleofector and cultured in 10% FBS for 24 hours. VSMC were then cultured in 2.5% FBS and subjected to vehicle or adiponectin treatment for another 24 hours before harvesting for Western analysis. Transfection efficiencies routinely range from 40-70% based on a GFP control vector.

**Adenoviral expression of rapamycin-resistant mutant S6K1**

Infection of human VSMC with adenovirus encoding an HA-tagged, rapamycin-resistant, constitutively active mutant of S6K1 (ED3E) and green fluorescent protein (GFP), or with a control adenovirus (GFP), was performed as described\(^3\). Cells were infected with virus overnight and then washed, and media was changed to M199 with 2.5%FBS prior to adiponectin treatment.
Organ culture model
Pig femoral arteries were harvested and cut into 2 mm pieces (day 1), and maintained in M199 with 10% FBS. Drug or adiponectin was added on day 3. Media and rapamycin were refreshed every other day while fresh adiponectin was added daily. Tissue was homogenized on day 7 using our cell lysis buffer\textsuperscript{1} for western blotting analysis.

Immunofluorescence
Human VSMC were cultured on glass coverslips and subjected to adiponectin or rapamycin treatment for the indicated time points, then washed with PBS and fixed in 1:1 methanol/acetone, blocked with 3% BSA in TBS-T, and incubated with anti-phospho-Ser 193 FoxO4 primary antibody in TBS-T with 1% BSA at 4ºC overnight. Cells were then incubated with AlexaFluor568-conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR) in TBS-T with 1% BSA for one hour, and DAPI staining was performed before mounting slides. Slides were analyzed using spinning disk confocal microscopy (Perkin-Elmer, Waltham, MA).

Statistical analysis
Densitometry results from triplicates or more were analyzed by GraphPad Prism software, and presented as the mean± standard error of the mean (SEM) unless otherwise specified. Significance of differences among groups was tested using one-way ANOVA with post-hoc Newman-Keuls test for multiple comparisons or paired t-test where indicated. A p value less than 0.05 was considered significant.
Supplemental Figure Legends

Supplemental figure I: Adiponectin induces VSMC differentiation marker expression and modulates the mTORC1 signaling pathway. A, VSMC were treated with HMW-enriched adiponectin (Adpn) at the indicated concentrations for 24h and subjected to western blotting analysis. B, Bar graphs represent densitometric quantification (mean fold induction plus standard error mean) of at least 3 separate replicates of Fig. 1B (main text). The number of replicates is indicated at the base of each bar. p-values (Newman-Keuls multiple comparison post hoc tests) are indicated above the bars: *p<0.05; **p<0.01, ***p<0.001 vs. vehicle.

Supplemental figure II: Adiponectin-induced VSMC differentiation requires AMPK. VSMC were pretreated with compound C (20 μM) for 30 min, followed by treatment with 5 μg/ml adiponectin for 24h and western blotting with the indicated antibodies. Blots shown are from a replicate experiment of Fig. 2A.

Supplemental figure III: A, hCASMC were starved in 2.5% FBS for 24 hrs, followed by treatment with vehicle or 5 μg/ml adiponectin for 8 hrs. The cells were then harvested and subjected to immunoprecipitation with specific Akt1 or Akt2 isoform antibodies. Each Akt isoform bound on the sepharose beads was eluted in loading buffer and analyzed by western using pan p- Akt (S473) antibody. Bar graphs represent densitometric quantification (mean fold induction plus standard deviation) of 2 separate experiments. B, VSMC were transfected with either pcDNA vector control or pcDNA-wt Akt1 plasmids and incubated for 48 hrs. Cells were then harvested and subject to western analysis with primary antibodies to contractile proteins and GAPDH. Bar graphs represent densitometric quantification (mean fold induction plus standard error of the mean) of SM-MHC or h-caldesmon normalized to GAPDH from 3 separate experiments. C, VSMC were transfected, treated, and analyzed as in Fig. 4. Bar graphs represent densitometric quantification (mean fold induction plus standard error mean) of 9 and 5 separate replicates of Fig. 4, respectively. *p<0.05 and ***p<0.001 vs. siControl.
**Supplemental figure IV:** Adiponectin induces FoxO4 phosphorylation and nuclear exclusion. VSMC were treated with 5 μg/ml adiponectin for the indicated time points, and subjected to immunohistochemistry staining using anti-phospho-Ser 193 FoxO4 primary and AlexaFluor568-conjugated secondary antibodies. Nuclei are stained with DAPI. Photographs from spinning disk confocal immunofluorescence microscopy are shown.

**Supplemental figure V:** Adiponectin and rapamycin have similar effects on VSMC phenotype. A, VSMC treated with 20nM rapamycin or 5 μg/ml adiponectin for the indicated time points were harvested and immunoblotted as in Fig. 1. B, VSMC were treated with 20nM rapamycin for the indicated time points, or with different concentrations of rapamycin for 24h prior to western blotting analysis as above. C, VSMC were treated with 50 nM rapamycin for the indicated time points and subjected to immunohistochemical staining as in Supplemental Figure IV. Photographs from spinning disk confocal immunofluorescence microscopy are shown.

**Supplemental figure VI:** Model of adiponectin and rapamycin signaling in VSMC and EC. A, Model of adiponectin and rapamycin-induced VSMC differentiation. Adiponectin activates AMPKα2, which in turn inhibits the mTORC1/S6K1 pathway, relieving S6K1-dependent feedback inhibition of IRS-1. The resulting stabilization of IRS-1 activates PI3K and Akt2 signaling, which promotes VSMC differentiation via inhibition of FoxO4 transcription factor. Rapamycin induces a similar signaling cascade through direct inhibition of mTORC1. B, Model of adiponectin and rapamycin signaling in EC. Rapamycin significantly while adiponectin only slightly inhibits mTORC1. Also, chronic rapamycin treatment inhibits while adiponectin may preserve mTORC2 assembly, a PDK2 which phosphorylates Akt at Ser 473, therefore rapamycin significantly inhibits while adiponectin preserves Akt phosphorylation at Ser 473 in EC. Both mTORC1 and Akt are very important in promoting EC growth and survival.
References


Supplemental Fig. 1  Adiponectin Induces VSMC Differentiation

A. Dose-Effects

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| SM2-MHC | SM-α-actin | Calponin | P-AMPK (T172) | AMPK-α | P-ACC (S79) | T-ACC | P-S6K1(T389) | T-S6K1 | P-Akt (S473) | T-Akt | GAPDH | P-S6 (S240/244) | T-IRS-1 | β-tubulin |

B. Quantitations of the Time-Course

<table>
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<tr>
<th>0h</th>
<th>4h</th>
<th>8h</th>
<th>24h</th>
</tr>
</thead>
</table>

| SM2-MHC/GAPDH (Fold Change) |
| 0.0 | 2.5 | 5.0 | 7.5 | 10.0 |

| h-Caldesmon/GAPDH (Fold Change) |
| 0.0 | 2.5 | 5.0 | 7.5 | 10.0 |

| Calponin/GAPDH (Fold Change) |
| 0.0 | 2.5 | 5.0 | 7.5 | 10.0 |

| SM-α-actin/GAPDH (Fold Change) |
| 0.0 | 10 | 20 | 30 |

* p<0.05, ** p<0.01, *** p<0.001
Supplemental Fig. II Adiponectin-Induced VSMC Differentiation Is Mediated by AMPK Activation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
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</thead>
<tbody>
<tr>
<td>h-Caldesmon</td>
<td>-  +  -  +  Adpn 5µg/ml 24h</td>
</tr>
<tr>
<td>SM-α-actin</td>
<td>-  -  +  +  Compound C 20 µM</td>
</tr>
<tr>
<td>Calponin</td>
<td></td>
</tr>
<tr>
<td>P-AMPK (T172)</td>
<td></td>
</tr>
<tr>
<td>T-AMPK</td>
<td></td>
</tr>
<tr>
<td>P-ACC (S79)</td>
<td></td>
</tr>
<tr>
<td>T-ACC</td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Fig. III  Akt isoform effects

A.

- P-Akt (S473)
- T-Akt2
- P-Akt (S473)
- T-Akt1

Vehicle  Adiponectin

B.

- *p<0.01
- **p<0.05

- Vehicle
- Rapamycin
- Adiponectin

MHC/GAPDH

- pcDNA3
- wt-Akt1

h-caldesmon/GAPDH

- pcDNA3
- wt-Akt1
Supplemental Fig. III  Akt Isoform Effects

C.

Akt2/GAPDH (Fold Change)

N=9, ***p<0.001

P-Akt/T-Akt (Fold Change)

N=5, *p<0.05
Supplemental Fig. IV  Adiponectin regulates FoxO4 phosphorylation and nuclear exclusion

Control

DAPI  p-FoxO4  Merge

0 h

2 h

4 h

7 h
Supplemental Fig. V  Adiponectin and Rapamycin Have Similar Beneficial Effects on VSMC Phenotype

A.

B.
Supplemental Fig. V  Rapamycin regulates FoxO4 phosphorylation and nuclear exclusion

C.  

<table>
<thead>
<tr>
<th>Time</th>
<th>DAPI</th>
<th>p-FoxO4</th>
<th>Merge</th>
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<tr>
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<tr>
<td>Control</td>
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<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
</tbody>
</table>
Supplemental Fig. VI  Model of Adiponectin and Rapamycin Signaling in VSMC and EC

A. VSMC

Adiponectin → Adiponectin Receptors → AMPK α2 → mTOR C1 → S6K1 → S6

Rapamycin

IGF-1 → IGF-R → IRS-1 → PI3K → Akt2 → Foxo4 → Contractile Proteins
B. EC

Supplemental Fig. VI  Model of Adiponectin and Rapamycin Signaling in VSMC and EC

Rapamycin  Adiponectin

mTORC1

mTORC2

Akt p-Ser 473

EC Growth and Survival

Rapamycin (assembly)

Adiponectin