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Serum and Glucocorticoid-Regulated Kinase 1 Promotes Vascular Smooth Muscle Cell Proliferation via Regulation of β -catenin Dynamics

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Abstract

In response to arterial intimal injury vascular smooth muscle cells (VSMC) within the vessel wall proliferate upon exposure to growth factors accumulate and form a neointima that can occlude the vessel lumen. Serum and glucocorticoid inducible kinase 1 (SGK1) is a growth factor-responsive kinase; however its role in VSMC proliferation is not fully understood. Here, we examined growth factor-dependent regulation of SGK1 and defined a molecular role for SGK1 in stimulation of VSMC proliferation. We found that stimulation of VSMC with the pro-proliferative growth factor, platelet-derived growth factor BB (PDGF) significantly increased SGK1 mRNA, protein, and kinase activity in aortic VSMCs *in vitro*. To test the hypothesis that activation of SGK1 activity promotes VSMC proliferation, we examined the effects of stable expression of constitutively active (S422D) and kinase-defective (S422A) mutants of SGK1 on VSMC growth. We found that activation of SGK1 increased, whereas interference of SGK1 signaling inhibited VSMC growth *in vitro*. Consistent with these findings, expression of the S422D mutant augmented both basal and PDGF-induced BrdU uptake in VSMC. Conversely, PDGF-induced BrdU uptake was attenuated in VSMC expressing S422A. Furthermore, we determined that activated SGK1 enhanced basal and PDGF-dependent G₁→S cell cycle transition, whereas dominant-negative SGK1 abrogated G₁→S cell cycle transition under similar conditions. Downstream signaling by active SGK1 induced basal and PDGF-induced phosphorylation of glycogen synthase kinase 3 β , an effect which was attenuated when SGK1 activity was blocked by expression of the kinase-defective mutant, S422A. We also found that transfection of S422D enhanced β -catenin-nuclear localization and activation of the TOP/Flash and cyclin D1 transcriptional reporters. These effects were

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significantly blunted in VSMC transfected with the S422A mutant. Our results provide compelling evidence of a role for SGK1 in stimulation of arterial VSMC growth via regulation of β -catenin dynamics and implicate SGK1 in the progression of intimal narrowing following arterial injury. Hence, the findings presented here point to inhibition of SGK1 activity as a novel therapeutic approach for the treatment of occlusive vascular diseases.

1. Introduction

In response to arterial injury accompanying percutaneous intervention and atherosclerosis the endothelium becomes damaged exposing the underlying medial vascular smooth muscle cells (VSMC) to blood-borne components. These components such as cytokines, chemokines and growth factors induce a phenotypic switch in VSMC causing them to transition from a quiescent to an activated phenotype which undergoes dysregulated proliferation. Eventually the VSMC accumulate and migrate into the intima causing luminal occlusion and reduces blood flow to the supplying tissue [1] – [4]. Thus, VSMC hyperplasia is a critical determinant of proliferative vascular diseases that underlie the clinical sequelae of cardiac and cerebral infarcts.

Significant progress in vascular biology research has pointed to a crucial role for serine/threonine kinase signaling pathways in the cellular processes underpinning cardiovascular diseases. In particular, the protein kinase A, G, and C (AGC) family of serine/threonine kinases, which include PKA, PKC, and PKB/Akt, have been shown to produce fundamental effects on vascular cell biology through regulation of cellular growth processes essential to normal vascular function and to disease development [5] – [7]. Among the members of the AGC family, serum and glucocorticoid inducible kinase 1 (SGK1) is increasingly being implicated as a protein kinase with important roles in the vasculature.

SGK1 was initially identified in a differential screen for glucocorticoid-responsive genes in a mammary tumor cell line, and is the only family member to undergo both transcriptional and posttranslational regulation dynamically in response to signals such as growth factors, glucocorticoids, hormones, and cell volume [8]. SGK1 is activated by phosphatidylinositol-3-kinase-dependent activation of phosphoinositide-dependent protein kinase 1 (PDK1) and mammalian target of rapamycin complex C2, which leads to phosphorylation of serine 422 (S422) and threonine 256 and subsequent activation of its intrinsic kinase activity [9] – [12]. More recently, studies have emerged supporting a role for SGK1 in diseased arteries. SGK1 expression is elevated in pulmonary artery SMC during pulmonary hypertension and regulates the expression of factors that induce fibrosis [13]. Additionally, it has been shown that SGK1 is increased in the neointimal lesion of vein grafts [14] and stimulates mechanical stretch-induced proliferation in SMC from veins. However, the role of SGK1 in growth factor-dependent proliferation of arterial SMC is unknown.

To define the mechanism by which SGK1 stimulates arterial SMC proliferation, we investigated PDGF-induced regulation of SGK1 expression and activity and examined the role of SGK1 on the cell cycle in aortic SMCs *in vitro*. Our data indicate that PDGF augments SGK1 mRNA expression, protein levels and Ser422 phosphorylation in aortic

SMCs. Furthermore, we demonstrate that SGK1 activity promotes cell cycle progression and activation of β -catenin transcriptional activity. Thus, one novel therapeutic approach for the treatment of occlusive vascular diseases, therefore, may be inhibition of SGK1 activity.

2. Materials and methods

2.1. Cell culture

A7r5 embryonic aortic smooth muscle cells (ATCC) were used throughout the study at passage 4 to 15. Cells were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C and 5% CO₂. Prior to stimulation with PDGF-BB (Sigma) A7r5 cells were serum-deprived for 24 hours with DMEM/F12 supplemented with 1% FBS and 1% penicillin/streptomycin.

2.2. Western blotting

Total protein was extracted with M-PER Mammalian Protein Extraction Reagent (ThermoScientific). Cellular protein lysates (25 μ g) were separated by SDS polyacrylamide gel electrophoresis, transferred to PDVF membranes and incubated with appropriate primary antibody: SGK1, glycogen synthase kinase (GSK) GSK3 β , pGSK3 α/β , β -catenin, Tata binding protein (TBP) and cyclin D1 (Cell Signaling), pSGK1-S422 (Abcam), GAPDH and β -actin (Santa Cruz Biotechnology) overnight. The following day the PVDF membranes were washed incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed using SuperSignal West Pico Chemiluminescence Substrate (ThermoScientific). Signal was quantified by densitometry using the NIH software Image J. GAPDH, β -actin or TBP was used to verify equal loading. Normalization of phosphorylated protein was determined by its total protein levels.

2.3. Plasmid preparation and cloning

Human SGK1 cDNA (Origene) was cloned into the pDEST26 destination vector into suitable restriction sites using the Gateway Technology (Invitrogen). Site-specific mutagenesis of human SGK1 was performed according to previously published reports [12] and sub-cloned into the same vector.

2.4. RNA extraction, cDNA preparation, and quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the RNeasy kit (Qiagen) and cDNA was prepared using the SuperScript cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Expression level of SGK1 was performed utilizing forward primer: CTGCAATGTGCCTTTTCTGA and reverse primer: CTGCACACATGGGAAATACG in the Light Cycler (Roche) with SYBR Green chemistry. SGK1 mRNA expression was normalized to GAPDH.

2.5. Transfection of aortic SMC

A7r5 aortic SMC were transfected using the Nucleofector technology (Amaxa) according to the manufacturer's instructions. To generate stable cell lines, A7r5 were cultured in DMEM–F/12 growth medium supplemented with 400 μ mol/L G418 (Sigma) for 10 days.

G418-resistant cells were propagated and maintained in G418 (400 μ mol/L) selection medium. Passages 3-15 were used in experimental studies.

2.6. Determination of cell growth

An equal number of A7r5 cells (3×10^4) expressing an empty vector or SGK1 kinase active or kinase-inactive mutants were plated in each well of a 6-well dish. At the times indicated from 1 to 4 days post seeding, cells were trypsinized and counted using Guava ViaCount to obtain the number of viable cells.

2.7. BrdU Cell Proliferation assay

A colorimetric BrdU Cell Proliferation Assay (Roche) was used to measure DNA synthesis. Briefly, 1.0×10^4 A7r5 cells expressing empty vector or SGK1 activity mutants were cultured in a flat bottom 96-well plate. After 24 hours, serum containing medium was removed and replaced with DMEM/F12 growth medium with 1% FBS. Cells were then stimulated with 25ng/ml PDGF-BB or vehicle for 18 hours. BrdU labeling, cell fixation, BrdU antibody conjugation, and color development were performed according to manufacturer's instructions. Signal detection was performed on a SpectraMax (Roche) ELISA plate reader.

2.8. Cell cycle assay

Cell cycle analysis was performed using the Guava Cell Cycle Reagent. A7r5 were cultured in 6-well dishes (5×10^5); after 24 hours, medium was replaced with 1% reduced serum medium for 24 hours. Cells were then stimulated with 25ng/ml PDGF-BB or vehicle for 18 hours. Cell harvest, fixation, and staining were performed according to manufacturer's instructions. Signal detection (10,000 counts) was performed on a Guava EasyCyte (Guava Technologies) instrument. To identify cell cycle phases, flow cytometry standard files were extracted and analyzed with ModFit LT (Verity Software).

2.10. Luciferase reporter gene assay

To assay the transcriptional activity of β -catenin, A7r5 cells were transfected with the TOP/Flash TCF-responsive firefly luciferase (Upstate Biotechnology) and the pRL *Renilla* luciferase (Promega) $\pm$ empty, constitutive active (S422D) or dominant negative (S422A) SGK1 plasmids. For cyclin D1 transcriptional studies, pGL3 Basic (Promega), containing 962 bp of the proximal 5'-flanking region of human cyclin D1-responsive [15] firefly luciferase (a kind gift of Dr. Osamu Tetsu, University of California, San Francisco), and pRL were co-transfected into A7r5 cells $\pm$ empty vector, S422D- or S422A-SGK1 plasmids. Luciferase activity was measured using the Dual-Luciferase assay system (Promega). The ratio of firefly/*Renilla* luciferase activity was calculated to normalize results with respect to the efficiency of transfection.

2.11. Statistical analysis

Analysis of variance and appropriate posthoc tests were used to compare more than two groups. Statistical significance was set at $p < 0.05$.

3. Results

3.1. PDGF-BB increases SGK1 mRNA expression, protein levels and phosphorylation in aortic SMC

PDGF is secreted in response to arterial injury and has long been known to induce VSMC proliferation and the subsequent development of intimal lesions [16] – [17]. Therefore, we examined the effects of this pathologically relevant growth factor on regulation of SGK1 expression and activity in cultured aortic A7r5 SMC. Dose response studies were carried out to examine whether there is a dose-dependent effect of PDGF on regulation of SGK1 mRNA expression. Stimulation of A7r5 with varying doses of PDGF for 60 minutes caused a significant and robust increase in SGK1 mRNA expression (Fig. 1). The increase in SGK1 mRNA was rapid and transient and reached a maximal level of approximately 6-fold. Peak expression occurred within two hours of PDGF stimulation which returned to near baseline levels within eight hours (Fig. 1B).

Next, we examined the effect of PDGF on SGK1 protein levels. Stimulation of A7r5 cells with 25ng/ml PDGF for up to four hours produced an immediate (within 30 minutes) and sustained (up to four hours) increase in SGK1 protein levels. Densitometric analysis indicates that the maximal level of increase was approximately 2.5-fold (Fig. 2A and B).

Activation of SGK1 activity is associated with post-translational phosphorylation of its Ser422 residue [9] – [10]. Thus, we examined the effect of PDGF stimulation on phosphorylation of residue serine 422 within SGK1, (p422^{SGK1}). For these studies, cells were stimulated with 25ng/ml PDGF at varying time points from 0 minutes to four hours and protein extracts were analyzed by western blot with a phospho-specific antibody to Ser422. Western blot analysis indicate a 4- to 5-fold significant increase in p422^{SGK1} levels in cells stimulated with PDGF for 30 to 60 minutes (Fig. 2C and D). The results of these studies show that SGK1 is a PDGF-responsive kinase whose expression and serine/threonine kinase activity can be stimulated by PDGF in aortic SMC.

3.2. SGK1 promotes proliferation of aortic SMC

Next, we tested the hypothesis that activation of SGK1 activity promotes arterial SMC proliferation. First, in line with a previously published report [11] serine 422 within wildtype human SGK1 was mutated to aspartic acid or alanine by site-directed mutagenesis to generate constitutively active, (S422D) and kinase-inactive (S422A) SGK1, respectively. These mutants were stably transfected into A7r5 cells which were monitored in growth curve studies. In S422D-expressing cells proliferation increased 2.5-fold compared to empty vector cells. Conversely, proliferation was significantly reduced in S422A-expressing A7r5 cells. Consistent with this data, we observed a dramatic increase in BrdU uptake of approximately 400% in S422D-expressing A7r5 cells in the absence of PDGF, whereas in S422A-expressing A7r5 cells, BrdU uptake was similar to that of the empty vector A7r5 cells (Fig. 3B). Following PDGF stimulation, we observed potentiation of BrdU uptake in S422D-expressing cells compared to empty vector cells. On the other hand, PDGF-mediated BrdU uptake was significantly inhibited about 300% to 500% in S422A-expressing cells compared to empty vector or S422D-expressing cells (Fig. 3B). Thus, these studies provide

strong evidence of a vital role for SGK1 signaling pathways in regulation of arterial SMC proliferation. Furthermore, they indicate that SGK1 is necessary and sufficient to regulate arterial SMC proliferation.

3.3. SGK1 promotes G₁-S cell cycle progression in aortic SMC

To examine more closely the mechanism by which SGK1 activity promotes arterial SMC proliferation, we performed flow cytometry analysis in our stable A7r5 cell lines in the absence and presence of PDGF. As shown in Fig. 4A and B, in the absence of PDGF stimulation expression of S422D significantly increased the percentage of cells in S-phase by 30%; an effect that more than doubled upon PDGF stimulation. Alternatively, expression of dominant-negative S422A significantly reduced the percentage of cells in S-phase by about 35% (Fig. 4A and B) in the absence and presence of PDGF stimulation. Overall, these studies indicate that SGK1 activation promotes G₁ to S cell cycle progression.

3.4. Overexpression of SGK1 in VSMCs is associated with increased phosphorylation of GSK3 β and β -catenin nuclear translocation

Previous studies have shown that inhibition of glycogen synthase kinase 3 (GSK3 β) signaling induces β -catenin transcriptional activity resulting in cell cycle progression [15]. Thus, given that GSK3 β is a substrate for SGK1, we examined whether modulating SGK1 activity in aortic SMC regulates phosphorylation of GSK3 β and β -catenin dynamics. As shown in Fig. 5A and B, in the absence of growth factor stimulation GSK3 β -Ser 9 phosphorylation was significantly increased by about 2.5-fold in S422D-expressing cells, but reduced approximately 2-fold in S422A-expressing cells. Furthermore, PDGF-dependent GSK3 β -Ser 9 phosphorylation was also higher in S422D-expressing cells; whereas, PDGF-induced GSK3 β phosphorylation was suppressed in S422A-expressing cells.

Phosphorylation of Ser 9 inhibits GSK3 β activity [18] and inhibits nuclear translocation of the cell cycle transcriptional activator, β -catenin [19]. Therefore, we investigated the effect of SGK1 activity on basal and PDGF-induced β -catenin nuclear accumulation. Expression of wildtype SGK1 and S422D correlated with increased nuclear translocation of β -catenin in vehicle- and PDGF-treated cells. However, nuclear translocation of β -catenin was significantly reduced in S422A-expressing cells under basal or PDGF treatment (Fig. 6A and B). Taken together, these data support a role for SGK1 in PDGF-mediated phosphorylation of GSK3 β and in regulation of β -catenin nuclear translocation.

3.5. SGK1 stimulates β -catenin transcriptional activity in VSMC

To determine the importance of SGK1 signaling on β -catenin transcriptional activity, we employed TOP/FOP t cell factor (TCF) Flash luciferase assays. Transfection of S422D in A7r5 cells led to elevated transcriptional activity compared to empty vector. In contrast, inhibition of SGK1 activity with S422A suppressed reporter activity in A7r5 cells (Fig. 7A). Previous reports have established that the β -catenin/TCF signaling pathway regulates transcription of the cell cycle regulator, cyclin D1 [20]. Consequently, we examined the effect of SGK1 on cyclin D1 transcriptional activity in luciferase reporter assays. As shown in Fig. 7B, S422D increased cyclin D1 transcriptional activity, whereas S422A suppressed cyclin D1 transcriptional activity. Further analysis by western blotting indicated that S422D

expression was accompanied by an increase in cyclin D1 protein levels. Conversely, lower cyclin D1 protein levels were observed upon expression of S422A (Fig.7C). Overall, these data indicate that the SGK1 signaling pathway promotes growth responses in aortic SMC.

Discussion

This study provides compelling evidence that activation of SGK1 activity increases arterial SMC proliferation. In addition, we demonstrate that sustained activation of SGK1 in aortic SMC was associated with increased phosphorylation of negative regulatory sites on GSK3 β and subsequent β -catenin nuclear accumulation. This study also found that SGK1 could stimulate TCF and cyclin D1 transcriptional activity and increase cyclin D1 protein levels.

Acute injury to the endothelium exposes the underlying medial VSMC to a milieu of growth factors [21]. Thus, we postulated that mitogenic signals mediated through the growth factor PDGF could alter SGK1 expression and/or activity in VSMC. Support for this notion is evident in Figs. 1 and 2, which depicts rapid and acute induction of SGK1 mRNA and protein levels in VSMC exposed to PDGF. The time course of induction is reflective of an immediate early gene response, similar to that exhibited by nuclear proto-oncogenes c-myc, c-fos, and egr-1 (early growth response-1), which are known to promote growth of VSMC [22] – [25]. PDGF stimulation was also associated with hyper-phosphorylation of SGK1 on serine S422, a prerequisite for coordinate activation of its serine/threonine kinase activity. An increase in both SGK1 protein levels and activity would facilitate high levels of active SGK1 available to phosphorylate and modulate the function of downstream substrates important for growth regulatory responses to proceed. PDGF is known to activate SMC during arterial injury and lead to neointimal development [21]. Thus, an increase in SGK1 expression and phosphorylation in response to PDGF suggests a role for this serine/threonine kinase in regulation of pathological vascular remodeling in the context of vessel injury.

In this study, we found that expression of a constitutively active mutant for SGK1, S422D, was sufficient to increase proliferation, BrdU uptake and S-phase entry of VSMC *in vitro*. Of note, antagonizing SGK1 signaling through expression of a phosphorylation-deficient dominant-negative mutant, S422A reduced all of these responses. Thus, our findings suggest that SGK1 promotes growth of VSMC by increasing cell cycle progression. SGK1 can phosphorylate and inhibit transcriptional activation of forkhead transcription factor box O-3a triggering apoptosis [26]; therefore, we cannot rule out that survival mechanisms may also be responsible for SGK1 signaling-induced regulation of VSMC growth.

This study establishes a link between SGK1 and the β -catenin/tcf/cyclin D1 pathway, which is widely accepted as a critical modulator of VSMC proliferative responses and vascular remodeling [20]. In the canonical Wnt signaling pathway, when Wnt is absent, GSK3 β is activated and complexed with axin, APC, and β -catenin. GSK3 β activation is required for a fully functional destruction complex for β -catenin [27]. Our data suggest SGK1 may destabilize this β -catenin destruction complex by inhibiting the activity of GSK3 β . Our data also supports a model in which SGK1 acts to maintain inactive GSK3 β by phosphorylation-mediated inhibition of its kinase activity, thereby causing β -catenin to escape degradation

and accumulate in the nucleus. Nuclear β -catenin can then transactivate tcf-responsive genes, such as cyclin D1 (Fig. 8) to drive proliferation of VSMC. In that SGK1 inhibits activation of GSK3 β , these results suggest SGK1 may regulate ubiquitination-mediated degradation of β -catenin and cytoskeleton arrangements involving β -catenin. Interestingly, our data also imply SGK1 may regulate its own transcription through regulation of β -catenin dynamics. It has been reported that SGK1 is a direct transcriptional target of β -catenin [28]. Thus, by enhancing β -catenin nuclear accumulation and transcriptional activity, SGK1 could in turn stimulate its own transcription through positive feedback that amplifies and perpetuates SGK1 signaling.

In summary, we show the SGK1 signaling pathway can be activated by growth factor stimulation and can promote proliferation of arterial VSMC. The mechanistic basis for this pro-proliferation occurs, in part, through transactivation of the β -catenin/tcf signaling pathway. We are actively investigating identification of novel downstream substrates for SGK1 that might also mediate its growth promoting effects in VSMC. Arterial injury is accompanied by growth factor-dependent VSMC proliferation. Our *in vitro* data support a role for SGK1 in growth factor-induced proliferation. Thus, it is tempting to speculate a similar role for SGK1 *in vivo* that leads to the development and/or progression of proliferative vascular diseases such as atherosclerosis and stroke. Hence, the findings presented here point to inhibition of SGK1 activity as a novel therapeutic approach for the treatment of these and other occlusive vascular diseases.

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Highlights

- SGK1 signaling promotes growth in aortic smooth muscle cells.
- PDGF stimulates SGK1 activity in aortic SMC.
- SGK1 activity promotes G₁-S cell cycle progression.
- SGK1 regulates β -catenin nuclear accumulation and transcriptional activity.

Figure 1. PDGF regulates SGK1 mRNA expression in a dose- and time-dependent manner

A) SGK1 mRNA expression in response to stimulation of quiescent A7r5 cells with varying doses of PDGF-BB (0-50ng/ml). B) Time response of SGK1 mRNA expression following stimulation of quiescent A7r5 cells with PDGF-BB (25ng/ml). SGK1 mRNA expression was determined by qRT-PCR and normalized to GAPDH. Data are expressed as the mean $\pm$ SD of two independent studies performed in triplicate. * $p < 0.05$ versus vehicle control.

Figure 2. PDGF regulates SGK1 protein expression and phosphorylation

A) Representative western blot of total SGK1 protein levels in response to PDGF-BB (25ng/ml) stimulation of quiescent A7r5. B) Quantification of SGK1 protein levels by densitometry and normalized to GAPDH. C) Representative western blot analysis of pS422^{SGK1} in quiescent A7r5 (25ng/ml) for varying time points (0-240 minutes). Blot was stripped and re-probed with anti-SGK1 and -GAPDH to determine total SGK1 protein levels and to verify equal loading, respectively. D) Quantification of phosphorylation status of serine 422 of SGK1 normalized to total SGK1 by densitometry. Data are expressed as the mean $\pm$ SD of three independent studies. * $p < 0.05$ versus vehicle control.

Figure 3. SGK1 increases proliferation of vascular smooth muscle cells

A) A7r5 stable cell lines over-expressing empty vector (Empty), a constitutive active mutant of SGK1, (S422D) or a kinase inactive SGK1 mutant, S422A were plated at equal density, cultured, harvested and counted using GUAVA Viacount at the times indicated. Data is expressed as the mean $\pm$ SD of n=3 samples. Study was repeated twice with similar results. B) A7r5 stable cell lines as indicated above were plated at equal density. Following quiescence, cells were stimulated with vehicle or PDGF (25ng/ml) for 18h. BrdU uptake was measured in an ELISA-based colorimetric assay. Data are expressed as mean $\pm$ SEM (n = 12 independent experiments performed in triplicate). *p<0.05, **p<0.005, #p<0.01, ##p<0.001.

Figure 4. SGK1 enhances the number of cells in S-phase of the cell cycle

A) Representative image of cell cycle distribution of stable A7r5 cells expressing wildtype or mutant SGK1. Quiescent A7r5 stable cell lines at equal density were stimulated with vehicle or PDGF (25ng/ml) for 18h. B) Quantification of S-phase distribution of stable A7r5 cells expressing wildtype or mutant SGK1. Cell cycle analysis was determined by ModFit software. Data are expressed as the mean $\pm$ SD (n = 6 independent experiments performed in triplicate). **p<0.005, #p<0.01, ##p<0.001, %p<0.00001.

Figure 5. Activation of SGK1 activity enhances basal and PDGF-mediated phosphorylation of glycogen synthase kinase 3 β (GSK3 β)

A) Representative western blot analysis of serine 9 phosphorylation of GSK3 β in quiescent A7r5 cells expressing empty vector, active (S422D) or inactive (S422A) SGK1 stimulated with PDGF-BB (25ng/ml) for 60 minutes. Blot was stripped and re-probed with anti-GSK3 β and -GAPDH to determine total GSK3 β protein levels and to verify equal loading, respectively. B) Quantification of phosphorylation status of serine 9 of GSK3 β normalized to total GSK3 β by densitometry. Data are expressed as the mean $\pm$ SD of three independent studies. * $p < 0.05$.

Figure 6. Stimulation of SGK1 activity promotes PDGF-mediated β -catenin nuclear translocation

A) Representative western blot analysis of β -catenin nuclear translocation in Empty, wildtype SGK1, S422D and S422A A7r5 stable cell lines stimulated with vehicle or PDGF-BB (25ng/ml) for 60 minutes. B) Quantification of nuclear β -catenin protein levels normalized to Tata binding protein (TBP) by densitometry. Data are expressed as the mean $\pm$ SD of n=3 independent studies. **p<0.005, #p<0.01, %p<0.00001.

Figure 7. SGK1 stimulates tcf4-dependent transcriptional activation and cyclin D1 expression

A) A7r5 cells were co-transfected with an empty, S422D or S422A and a synthetic 4x-TCF4-responsive luciferase (TOP) reporter plasmid. Luciferase activity was determined 24h after transfection. Values were normalized to renilla luciferase activity. Data are expressed as the mean $\pm$ SD of n=3 independent studies performed in triplicate. B) A7r5 cells co-transfected with the indicated plasmids and a cyclinD1-responsive luciferase reporter plasmid. Luciferase activity was determined as previously indicated and expressed as the mean $\pm$ SD, n = 3 independent studies performed in triplicate. C) Total cell lysates from A7r5 cells expressing the indicated plasmids were analyzed by western blotting with anti-cyclin D1 antibody. β -actin was used to verify equal loading. *p<0.05, **p<0.005.

Figure 8. Model illustrating a molecular role for SGK1 in stimulation of VSMC proliferation
 SGK1 becomes activated in response to downstream growth factor-dependent signaling. Activated SGK1 phosphorylates and inhibits GSK3 β and thereby blocks degradation of β -catenin such that it accumulates and translocates to the nucleus. Inside the nucleus β -catenin can stimulate the transcription of TCF4-dependent genes that regulate cell cycle progression, i.e. cyclin D1. Increased expression of cyclin D1 promotes cell cycle progression contributing to increased proliferation. Hyperplasia of VSMC contributes to neointimal formation and compromises the size of the arterial lumen.