Transforming growth factor-\(\beta_1\) regulation of C-type natriuretic peptide expression in human vascular smooth muscle cells: dependence on TSC22D1

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Mendonça MC, Koles N, Doi SQ, Sellitti DF. Transforming growth factor-\(\beta_1\), regulation of C-type natriuretic peptide expression in human vascular smooth muscle cells: dependence on TSC22D1. Am J Physiol Heart Circ Physiol 299: H2018–H2027, 2010. First published August 27, 2010; doi:10.1152/ajpheart.00656.2010.—C-type natriuretic peptide (CNP) possesses nitric oxide-like signaling mechanisms and actions in the vasculature, including the inhibition of fibrosis and vascular remodeling through counterregulation of transforming growth factor-\(\beta\) (TGF-\(\beta\)) signaling. The leucine zipper protein transforming growth factor stimulated clone domain family; transcription factor; serine threonine kinase 16; platelet-derived growth factor; transforming growth factor stimulated clone domain 1 (TGF-\(\beta\)) signaling. The leucine zipper protein transforming growth factor stimulated clone domain family; transcription factor; serine threonine kinase 16; platelet-derived growth factor; transforming growth factor stimulated clone domain 1 (TGF-\(\beta\)) cloning, the first protein to be described as a CNP transcription factor, but the lack of supporting evidence since its discovery and its lack of a classical DNA-binding site have left in question its role in the regulation of CNP by TGF-\(\beta\) and other factors. To define a specific role for TSC22D1 in CNP transcription, we have examined the effects of the profibrotic growth factors TGF-\(\beta\) and PDGF-BB on CNP mRNA expression in cultured human vascular smooth muscle cells (SMC) in which TSC22D1 expression was suppressed with small interfering RNA. Results showed that TGF-\(\beta\) and PDGF-BB significantly increased CNP expression in all three SMC types. Twenty-four-hour TGF-\(\beta\)-induced elevations in CNP were strongly correlated with changes in TSC22D1 mRNA levels, and both genes exhibited their greatest response to TGF-\(\beta\) in coronary artery SMC. Furthermore, siRNA suppression of TSC22D1 expression in coronary artery and aortic SMC by \(\sim 90\%\) resulted in 45–65% reductions of both PDGF- and TGF-\(\beta\)-stimulated CNP expression, respectively. These results support a postulated role of TSC22D1 as an enhancer of CNP transcription and suggest that TGF-\(\beta\)-induced upregulation of CNP expression in SMC may be mediated in part by increased transcription of TSC22D1.

platelet-derived growth factor; transforming growth factor stimulated clone domain family; transcription factor; serine threonine kinase 16; fibroblast

THE FAMILY OF NATRIURETIC peptides regulates cardiovascular homeostasis, in part through the endocrine actions of circulating atrial natriuretic factor (ANF) and B-type natriuretic peptide (BNP) but also through the paracrine/autocrine actions of C-type natriuretic peptide (CNP) in the vascular wall (20). Several studies have proposed that endogenous production of vascular CNP might function like nitric oxide (NO) as an endogenous vasodilator (5) and could also play a protective role in such inflammatory/fibroproliferative diseases as athero-sclerosis and restenosis (4, 15, 21, 24). A key mechanism for CNP action in vascular fibrosis is its inhibition of cellular events downstream of transforming growth factor-\(\beta\) (TGF-\(\beta\)) and other growth factors binding to vascular smooth muscle (VSM), including the suppression of plasminogen activator inhibitor-1 (PAI-1) transcription (9). Despite the potential importance of paracrine CNP secretion in vascular homeostasis, there is still a limited understanding of the transcriptional regulation of this peptide (5). The literature on the subject largely consists of studies of cytokine and hormonal effects on CNP production, primarily in cultured rodent cells, and of the subsequent identification of several putative transcription factors/enhancers, two of which have been associated with TGF-\(\beta\)-signaling (17–19, 26). The first of these to be identified was transforming growth factor \(\beta\) stimulating clone domain 1 (TSC22D1), the founding member of a small family of evolutionarily conserved leucine zipper proteins that perform a diverse array of functions in mammalian cells, including tumor suppression (17). Although initially cloned using binding to a GC-rich element in the CNP promoter as a selection criterion (16), TSC22D1 in fact lacks a classical DNA-binding domain.

Using the same methodology of cloning putative transcription factors based on their ability to bind the GC-rich element of the CNP promoter, Ohta et al. (18) identified a second putative CNP activator as a novel protein with both DNA-binding ability and serine-threonine kinase (STK) activity. This protein, designated as TSF-1 by its discoverers and later as STK16, appears to be localized to the Golgi apparatus but can translocate to the nucleus and modulate the activation of the vascular endothelial growth factor (VEGF) gene (7). Aside from the initial reports of Ohta et al. (17, 18), there have been no further studies of either TSC22D1 or STK16 as transcriptional regulators of CNP, and a recent study of CNP transcription in rat pituitary cells reported the binding of Sp-1 and Sp-3, but not TSC22D1 at the GC-rich promoter site described above, leaving the subject of TSC22D1 involvement in CNP regulation in question (27). Here we report significant roles of both TGF-\(\beta\) and PDGF-BB in the regulation of CNP in human vascular smooth muscle cells (SMC), but not in endothelial cells or fibroblasts. Furthermore, we show using small interfering RNA (siRNA) gene silencing techniques that suppression of TSC22D1, but not of STK16, significantly reduces the growth factor upregulation of CNP. These results support a critical role for TSC22D1 in controlling the expression of CNP in human SMC phenotypes.

MATERIALS AND METHODS

Reagents. TGF-\(\beta\), PDGF-BB, VEGF, EGF, basic human FGF, bovine insulin, and TNF-\(\alpha\) were obtained from Sigma-Aldrich (St. Louis, MO). CNP-22 was obtained from Bachem Americas (Torrance, CA).

Cell culture. Primary cultures of human aortic SMC (AoSMC), human aortic endothelial cells (HAEC), human coronary artery SMC (CASM), human umbilical artery SMC (UASM), and normal human dermal fibroblasts (NHDF) were purchased from Lonza...
(Walkersville, MD) at passage 3 and grown in either SmGM-2 (smooth muscle cultures), FGM-2 (fibroblasts), or EGM (endothelial cells) obtained from Lonza. These growth media consisted of basal culture medium (SmBM, FBM, and EBM, respectively) modified by the addition of cell phenotype-specific growth factors and other supplements as supplied by the manufacturer. Cells were washed and placed in basal media (either SmBM or EBM as indicated) before experiments involving treatment of cells with specific reagents.

**Immunochemistry.** AoSMCs, which received either control or TSC22D1 siRNA as described below, were seeded onto 12-mm round glass coverslips in 24-well plates and grown in SmGM-2 before switching to serum-free SmBM for 48 h. Cells then received fresh SmBM for 24 h before immunofluorescence staining. Cells were washed with PBS, fixed in cold methanol for 20 min, and incubated with either rabbit polyclonal anti-CNP (Bachem) diluted 1:200 as previously described (11) or with rabbit polyclonal antibody against human TSC22D1 (ProteinTech Group, Chicago, IL) diluted 1:1,500 in PBS. Cells fixed on coverslips were then mounted in Vectashield (Vector Laboratories, Burlingame, CA) and photographed using a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

**Western blot.** Protein was extracted from cells grown in 25 cm² culture flasks using a modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecylsulfate, and 5 mM EDTA containing Complete Mini protease inhibitor cocktail (a proprietary mixture of inhibitors of a broad spectrum of cysteine and serine proteases, calpains, and metalloproteases; Roche Diagnostics, Indianapolis, IN) and 1 mM dithiothreitol. Protein content was determined by Bradford assay (Bio-Rad, Hercules, CA), and samples were subjected to electrophoresis for 1 h at 200 V over 4–12% bis-tris polyacrylamide gels (NuPage) in MES buffer (NuPage) and transferred to 0.2 μm polyvinylidene fluoride (PVDF) membranes using iBlot (Invitrogen). Membranes were then exposed to a blocking solution containing 0.1% Surfact-amps 20 (Thermo Fisher Scientific, Waltham, MA) and 5% nonfat milk, followed by an overnight incubation in a 1:400 dilution of rabbit polyclonal anti-human TSC22D1 (Protein Tech Group) in blocking solution at 4°C. After five washes in PBS-0.05% Surfact-amps 20, membranes received a 1:10,000 dilution of goat anti-rabbit horse-radish peroxidase (HRP)-conjugated antibody for 1 h and specific proteins were detected using a chemiluminescent HRP substrate (Super signal, West Pico; Pierce). Blots were photographed, and band intensity was determined using the Gel Doc EQ System (Bio-Rad).

**Real-time PCR.** For real-time PCR, 100 ng of total RNA was used for RT and amplification of target cDNA in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). TaqMan One-Step RT-PCR master mix reagents were purchased from Applied Biosystems. Primers and 5’FAM-labeled probe for human CNP, TSC22D1 (for variants 1/2 and variant 1 only) TSC22D3, STK16, and GAPDH (control) were purchased from Ambion (Austin, TX). TSC22D1 siRNAs targeted both variants of TSC22D1, the long isoform (variant 1; GenBank accession No. NM_183422.2) and the short isoform (variant 2; GenBank accession No. NM_006022). Validated siRNAs targeting human STK16 (GenBank accession No. NM_01009010) and GAPDH were also purchased from Ambion. All siRNAs were reconstituted to 50 μM in RNAse-free water. Cells grown in 75 cm² culture flasks were trypsinized, pelleted, and suspended in siPORT electroporation buffer (Ambion) and then aliquoted (77 μl) into 1.5-ml microcentrifuge tubes at a concentration of ~6 × 10⁶ cells/tube. A volume of 2.2 μl of 50 μM validated siRNA was added to the cell suspensions and transferred into 1-cm electroporation cuvettes (Bio-Rad) for electroporation (Gene Pulse X-Cell; Bio-Rad) in three pulses of 300 V for 0.1 ms. Electroporated cells were then suspended in SmGM-2 medium and plated in 6-well culture dishes in a volume of 1.5 ml. After 24 h incubation, medium was replaced with SmBM and cells were incubated for an additional 24 h before receiving various treatments before extraction of RNA as described above.

**Statistical analysis.** Differences between treatment groups in both Western blot analyses and in real-time PCR assays were evaluated using InStat software (GraphPad Software, San Diego, CA) with one-way ANOVA followed by the Tukey-Kramer test to determine significance.

**RESULTS**

A possible autocrine role for CNP in human vascular smooth muscle (VSM) is suggested in Fig. 1A, which shows the inhibitory effect of CNP on the induction of the profibrotic gene PAI-1 by TGF-β in primary cultures of human AoSMC. A 2.5-fold upregulation of PAI-1 following a 6-h exposure to TGF-β was reduced by 30% in the presence of 1 μM CNP. PDGF-BB also increased PAI-1 in AoSMC (~2-fold) and this too was reduced by CNP, but this reduction did not quite achieve significance (Fig. 1A). Fibroblasts also play a role in TGF-β-induced fibrosis in the vasculature, but to our knowledge the effect of PAI-1 response to CNP has not been examined previously. The PAI-1 response to CNP was increased by 2.5-fold upregulation of PAI-1 following a 6-h exposure to CNP, and this was reduced by 2-fold in the presence of 1 μM CNP.
Additional 6 h incubation at 37°C, 5% CO2. NHDF in culture received an identical regimen of preincubation with either control medium or 1/1000 SmBM containing TGF-β for 30 min, followed by an additional 6 h incubation in either basal medium or SmBM. In contrast, basal CNP expression in both HAEC and AoSMC was only about 20–30% of the CNP mRNA measured in AoSMC (Fig. 3A). Type-specific differences, with NHDF and CASMC expressing other human cell types in culture revealed consistent phenotypic regulation of CNP production in human VSM, with the two most important factors being TGF-β and PDGF.

When CNP mRNA levels occurring in response to a 24-h treatment with TGF-β1 (10 ng/ml) were compared in all five cell types (Fig. 3A), only the smooth muscle phenotypes showed a significant response. Moreover, of the three vascular smooth muscle types, CASMC showed the greatest relative increase in CNP mRNA in response to TGF-β, despite having a low basal level of transcript for the peptide (Fig. 2A). Treatment of the five phenotypes with PDGF-BB for 24 h did not result in higher CNP transcript levels than untreated control (data not shown). However, an 8-h exposure to PDGF-BB resulted in significant upregulation of CNP transcript in the three SMC types, especially in UASMC and AoSMC (Fig. 3B). Neither HAEC nor NHDF were affected by PDGF-BB.

The discrepancies between TGF-β and PDGF in the time of maximal CNP transcript elevation led us to investigate the time course of CNP regulation by TGF-β in both AoSMC and CASMC (Fig. 4). TGF-β1 treatment of AoSMC clearly indicated a significant increase in PAI-1 mRNA beginning at 2 h, but did not reveal a significant time-dependent pattern of CNP response, possibly because the maximal stimulation of CNP by TGF-β we had observed at 24 h exposure was quite low (Fig. 4A). On the other hand, a CNP mRNA response to TGF-β in CASMC was very significant and was clearly not elevated until 8 h of incubation (Fig. 4B). Moreover, expression levels of CNP remained high through 24 h (Fig. 4B). As in AoSMC,
expression of the Smad-activated PAI-1 gene in CASMC was significantly elevated above control value after only 2 h of incubation with TGF-β1 (Fig. 4B).

Since an earlier study had suggested TSC22D1 as a putative transcription factor for TGF-β1-regulated CNP expression, we used Western blot analysis to determine the content of TSC22D1 protein in human cells in culture using a polyclonal antibody against TSC22D1 (Fig. 5). To confirm the detected protein as TSC22D1, we silenced TSC22D1 in three cultures of AoSMC and compared these with three cultures of cells treated with control siRNA using Western blot analysis (Fig. 5A). Surprisingly, no TSC22D1 positive bands were detected at a molecular mass of 18 kDa, as would be predicted for the short variant (variant 2) of TSC22D1. Instead, we detected a protein of ~110–120 kDa that was reduced by ~90% in cells treated with TSC22D1 siRNA (Fig. 5B). This observation suggests that most of the TSC22D1 protein is present as the long variant (variant 1) in AoSMC. A very faint band, apparently reduced by TSC22D1 siRNA, was also observed at ~55 kDa. Nonspecific proteins reacting with anti-TSC22D1, such as a band at ~92 kDa and two strong bands at ~30 kDa, were not suppressed with TSC22D1 siRNA and possibly represent cross-reaction of the polyclonal anti-TSC22D1 antibody with unrelated epitopes.

Real-time PCR of TSC22D1 mRNA in cultured human cells showed little variation in basal levels of TSC22D1 transcript among the five human phenotypes (Fig. 5C). In contrast, expression levels of the 110–120 kDa TSC22D1 protein obtained by Western blot were comparable in three phenotypes (CASMC, AoSMC, and NHDF) but markedly lower in the other two (UASMC and HAEC; Fig. 5D). We did not detect a band at 18 kDa in any of the cell types.

We also used real-time PCR to assess the response of transcript levels of TSC22D1 and CNP to TGF-β1 in the five human cell types and to determine whether a relationship exists between the two (Fig. 6). Basal TSC22D1 mRNA levels did not correlate significantly with basal levels of CNP. However, a highly significant correlation between the fold increase of TSC22D1 mRNA over basal and the fold increase of CNP mRNA after 24-h treatment with TGF-β1 was observed in two separate studies, one of which is shown in Fig. 6, top left. Linear correlations of TSC22D1 and CNP were highly signif-

Fig. 3. Change in CNP mRNA levels in 3 smooth muscle cell (SMC) cultures and in non-SMCs in response to TGF-β1 (A) or PDGF-BB (B). Cells in basal medium for 48 h (SMCs and NHDF) or for 24 h (HAEC) received either 10 ng/ml TGF-β1, for 24 h (A) or PDGF-BB (20 ng/ml) for 8 h (B) before determination of CNP transcript levels as described in MATERIALS AND METHODS. Growth factor-stimulated CNP mRNA levels were normalized to a value of 1 in corresponding control cultures receiving no growth factor. Data presented for each study are means ± positive and negative error and are representative of 2 separate experiments performed giving similar results. *P < 0.05; ***P < 0.001 vs. respective untreated controls.

Fig. 4. Time course of change in CNP and PAI-1 mRNA levels in AoSMC (A) and CASMC (B) after addition of 10 nm TGF-β1 to serum-starved cells in 6-well plates. To avoid disturbing the cells with the addition of fresh SmBM, TGF-β1 was diluted to a concentration of 1 μg/ml in RNAse-free H2O and was added in a volume of 15 μl directly to wells containing 1.5 ml medium (final concentration of 10 ng/ml). Control wells received 15 μl of RNAse-free H2O. Cells were killed immediately (0 h) and at 1, 2, 4, 6, 8, and 24 h after the addition of either TGF-β or control. CNP and PAI-1 mRNA in each well were determined by Taqman PCR as described in MATERIALS AND METHODS. Each group consisted of 3 separate wells. Data presented for each study are means ± positive and negative error at each time point. *P < 0.05; **P < 0.01; ***P < 0.001 vs. 0 h control.
Both CNP and TSC22D1 have been reported to be TGF-β-responsive genes, so the tight correlation between mRNA levels of these genes after TGF-β treatment could conceivably be incidental to a parallel upregulation of CNP and TSC22D1 rather than control of CNP expression by TSC22D1. However, STK16 mRNA, identified as being both TGF-β-responsive and a putative CNP enhancer, showed no correlation with CNP mRNA (Fig. 6, top right and bottom left). Like STK16, mRNA levels of a different TSC domain family member (TSC22D3) in response to TGF-β1 were not correlated with CNP transcript levels (Fig. 6C). In agreement with the upregulation of TSC22D1 mRNA by TGF-β in AoSMC, we found that exposure to TGF-β1 also increased the expression level of the 110–120 kDa band over control levels in a Western blot using anti-TSC22D1, but the difference was not quite significant (Fig. 6, bottom right). Intensity of the protein bands in TGF-β-treated CASMC was not significantly different from control (Fig. 6D).

Definitive evidence of a dependence of CNP expression on TSC22D1 levels was obtained from gene-silencing studies (Fig. 7). Real-time PCR was employed to assess the effects of TSC22D1-knockdown on both basal and growth-factor stimulated CNP expression in AoSMC and CASMC. Figure 7A shows that an 87–90% knockdown of TSC22D1 expression resulted in significant 39% and 45% decreases in basal CNP and PDGF-stimulated CNP, respectively. In contrast with CNP, transient knockdown of TSC22D1 had no effect on expression levels of the related gene, TSC22D3. However, TSC22D3 expression was increased, albeit modestly by PDGF-BB. Gadd45b, a gene shown to be regulated by TSC22D1 in
mouse hepatocytes, was not affected by TSC22D1 silencing. As we had observed earlier, 24-h exposure of CASMC to 10 ng/ml TGF-β1 significantly upregulated CNP transcript levels (Fig. 7B). SiRNA suppression of TSC22D1 expression by 94% in these TGF-β1-treated cells significantly reduced CNP mRNA by 65% (Fig. 7B). However, TSC22D1-silencing did not significantly reduce CNP expression under basal conditions. Gadd45b and TSC22D3 levels were unaffected by TSC22D1-silencing in CASMC (Fig. 7B).

Since Western blotting (Fig. 5) had shown most of the silenceable TSC22D1 protein migrating as a high molecular weight form (variant 1) rather than as the 18 kDa splice variant (variant 2), we designed Taqman primers and probe from the 5’-sequence unique to variant 1 (TSC22D1V.1) to assess the effects of TGF-β and of TSC22D1 knockdown on the high molecular weight variant, specifically. Results showed that TSC22D1 variant 1 was suppressed by TSC22D1-silencing (targeting a shared variant 1/variant 2 sequence) in both AoSMC (Fig. 6A) and CASMC (Fig. 7B), supporting the identification of the 110–120 kDa band as TSC22D1, variant 1.

To assess the effect of TSC22D1-silencing on the concentration and distribution of immunoreactive CNP (ir-CNP) in cultured human vascular cells, we introduced either control or TSC22D1 siRNA into AoSMC and plated the cells onto glass coverslips. The control (Fig. 8, A and C) and TSC22D1-silenced cells (Fig. 8, B and D) were then stained for either TSC22D1 (Fig. 8, A and B) or CNP (Fig. 8, C and D) as shown in Fig. 8. Results showed reduced levels of both TSC22D1- and CNP-immunoreactive staining in the TSC22D1-silenced cells, consistent with the mRNA data shown in Fig. 7A.

**DISCUSSION**

The regulation of ANF and BNP gene expression have been exceptionally well characterized (5), in part owing to their clinical importance as markers of heart failure (20). The transcriptional regulation of CNP, however, has received much less attention, with much of the work in this area having focused on endothelial CNP and its increased synthesis in the presence of certain factors, including TGF-β, and suppression by others (1, 3, 25). Nonetheless the regulation of CNP expression represents an important level of control in suspected target tissues such as the vasculature, its potential clinical importance underscored by immunohistochemical studies showing the upregulation of smooth muscle and macrophage CNP in developing human atherosclerotic (14) and restenotic (13) le-
sions. These observations suggest that in circumstances of vascular damage, CNP is secreted by SMC and that its expression is controlled by factors produced by injured endothelium, platelets, VSM, and inflammatory cells to regulate SMC function in an autocrine or paracrine fashion. We have reported the effects of several of these mediators, including PDGF and bioactive lipids and their transduction pathways on CNP expression in human SMC (11, 12).

CNP-induced inhibition of PAI-1 expression has been cited before as one mechanism for the antifibrotic action of CNP, and we show data (Fig. 1) in support of this mechanism as one of the potential functions of growth factor-induced smooth
muscle CNP. We also illustrate a potential paracrine role for endogenously produced smooth muscle CNP in suppressing the marked TGF-β-induced upregulation of PAI-1 in fibroblasts (Fig. 1B). A CNP effect on PAI-1 expression in fibroblasts could be particularly important, since PAI-1 is upregulated ~20-fold by TGF-β in fibroblasts compared with a twofold upregulation in SMC. These observations would suggest that growth factor-induced smooth muscle CNP could perform many of the functions (including vasodilatation and suppression of SMC proliferation and fibrosis) that have previously been ascribed to a constitutive production of CNP by the endothelium (20).

As for regulation of CNP at the promoter level, studies of this process have focused primarily on factors identified by their presumed ability to bind a GC-rich element in the human CNP promoter. One of these is the leucine zipper TSC22D1 (17, 18). TSC22D1, however, lacks a classical DNA-binding site (23). Moreover, the TSC22D1 identified as binding to the GC-rich element of the human CNP promoter was derived from the nuclear extract of a rat GH3 pituitary cell line and not from human cells. There have been no additional studies of TSC22D1 regulation of CNP since the initial report of Ohta et al. (17), and a recent report on the CNP promoter in mouse pituitary specifically mentions that the investigation found no evidence of TSC22D1 involvement in CNP transcription (27).

In the present study we examine the relationship between TSC22D1 and CNP in five human cell types following stimulation with the growth factors PDGF and TGF-β and provide evidence that TSC22D1 functions in the regulation of CNP expression, at least in human vascular cells. Initially, we showed that CNP mRNA levels in five human cell types exhibited a significant correlation with TSC22D1 mRNA after 24 h stimulation with TGF-β (Fig. 6). This finding does not necessarily indicate a causal effect between TSC22D1 and CNP expression, but it does suggest that the regulation of these two genes is very closely linked. In contrast, PAI-1, also a TGF-β upregulated gene (22), did not show a strong correlation with TSC22D1 (data not shown). STK16 mRNA levels also showed no significant correlation with CNP message following 24 h TGF-β1 (Fig. 6), despite the prior identification of STK16 as a TGF-β-responsive gene (18).

Definitive evidence for the importance of TSC22D1 in regulating CNP expression was seen in gene-silencing studies showing that a ~90% reduction in TSC22D1 mRNA 2 days after silencing with specific siRNA resulted in ~50% reductions in CNP levels compared with control in both basal- and PDGF-BB-stimulated AoSMC and also in TGF-β-stimulated CASMC (Fig. 7). We emphasize that like the primers used for real-time PCR (above), the duplex siRNA used in the silencing studies was specific only for the TSC domain family member 1 (TSC22D1) and showed no DNA sequence similarity with any other protein members of this family (i.e., TSC22D2, D3, or D4). The specificity of the knockdown for TSC22D1 is further emphasized by the failure of the TSC22D1-silencing to affect the expression of a closely related member of the TSC domain family, TSC22D3.

Other than CNP, only a few other genes have been identified as being subject to regulation by TSC22D1. Gadd45b, a gene previously cited as a target of transcriptional repression by TSC22D1 in mouse hepatocytes (8), was unaffected by TSC22D1 silencing in AoSMC. Expression of Gadd45b was also not significantly altered by TSC22D1 silencing in CASMC, despite a trend toward an increase. In contrast with the TSC22D1 siRNA results, efficient silencing of STK16, another proposed CNP regulatory factor identified as binding to the GC-rich region of the CNP promoter (18), did not affect CNP expression levels (data not shown). These siRNA results suggest that CNP expression can be altered by changes in TSC22D1 but not STK16 levels. This does not necessarily exclude a role for STK16 in controlling CNP expression, since alterations in cytoplasmic to nuclear transport of STK16 may be involved (7), but it does imply that altered de novo STK16 transcription is not an important mechanism for regulating CNP.

Until recently (2, 6), little attention has been paid to the relative contributions of the long and short isoforms of TSC22D1 (variant 1 and variant 2, respectively) to gene regulation. In fact, most published studies have focused on the activities of the 18-kDa short form. Both our PCR primers and the TSC22D1 siRNAs were designed from the overlap sequence of variant 1 and variant 2 and, therefore, have detected/silenced either (or both) of the two TSC22D1 isoforms, but not any other members of the TSC domain family. However, in Western blots using both anti-TSC22D1 antibody and specific siRNA silencing to confirm that the antibody-reactive protein was indeed TSC22D1, we found that most TSC22D1 is present in the five human cell phenotypes as a ~120 kDa (long) isoform and not as the short isoform of the protein. In fact, we could not detect antibody-reactive protein at 18 kDa in any of the lysates. [The identity of a very faint band at ~55 kDa reacting with antibody against TSC22D1 and suppressed with TSC22D1-silencing (e.g., whether it is an additional splice variant of TSC22D1), remains to be determined].

While it remains possible that the short isoform in low abundance could be playing a regulatory role, we think it more likely that the long isoform of TSC22D1 is serving to regulate CNP transcription in the human. We designed primers specific only for variant 1 and found that TSC22D1 siRNA significantly suppressed the expression of this isoform, although the degree of TSC22D1 mRNA suppression detected using the variant 1-specific isoform was less than that detected with primers that could amplify both variant 1 and variant 2. Future studies of CNP regulation by TSC22D1 may need to place more emphasis on the unique amino-terminus of the long TSC22D1 isoform, especially since a motif in this region has been identified in the TSC22D1 siRNAs were designed from the overlap sequence of variant 1 and variant 2 and, therefore, have detected/silenced either (or both) of the two TSC22D1 isoforms, but not any other members of the TSC domain family. However, in Western blots using both anti-TSC22D1 antibody and specific siRNA silencing to confirm that the antibody-reactive protein was indeed TSC22D1, we found that most TSC22D1 is present in the five human cell phenotypes as a ~120 kDa (long) isoform and not as the short isoform of the protein. In fact, we could not detect antibody-reactive protein at 18 kDa in any of the lysates. [The identity of a very faint band at ~55 kDa reacting with antibody against TSC22D1 and suppressed with TSC22D1-silencing (e.g., whether it is an additional splice variant of TSC22D1), remains to be determined].

It is of interest that the upstream growth factor signal resulting in the highest CNP expression level differs dramatically between SMC derived from the coronary and the aortic vascular beds, with PDGF-BB most effective in AoSMC and TGF-β most effective in CASMC. It may be too early to determine the significance of this difference, however, since AoSMC and CASMC were derived from individuals of different ages and the profile of growth factor receptors and signaling molecules in each phenotype is not available. The response in CNP mRNA to TGF-β1 did not appear to correlate with either basal TGF-βR1 or Smad 4 expression levels as determined by real-time PCR (data not shown).

The relevance of TSC22D1 to the transcriptional regulation of CNP has remained in question since it was first isolated by
Southwestern screening from a rat GH3 (pituitary cell line) cDNA library as a protein capable of binding the GC-rich element of the CNP promoter (17). Lacking a classical DNA-binding site, TSC22D1 would not be expected to bind the CNP promoter directly but could conceivably associate with other transcription factors, including Sp-1 or proteins that do have the requisite structure to bind DNA. Further uncertainty about the role of TSC22D1 in CNP transcription was introduced by a recent study in which Sp1/Sp3 complexes, but not TSC22D1, were found to bind to the GC-rich region in the rat CNP promoter (27). It is conceivable then that there is a species-specific difference in the role of TSC22D1 in CNP transcription that depends on a number of small but important differences in the sequence of the GC-rich region. One salient difference is the possession of two GC-boxes in tandem in the human GC-rich domain while the rodent only possesses a single GC-box (i.e., Sp-1 binding site) in the homologous region. Tandem GC-box motifs have been shown to be necessary for the binding of specific transcription factors (e.g., Kruppel-like zinc finger transcription factor Zf9) to activate transcription in certain cellular contexts (e.g., TGF-β1 transcription in hepatic stellate cells; 10), and TSC22D1 binding to the CNP promoter may require a similar tandem GC-box motif that is absent in the rodent. However, whether the species differences in the CNP promoter, rather than differences in upstream signal pathways, can explain opposing effects of factors such as PDGF/BB on CNP expression that we have observed in human versus rat SMC (11) will have to await further analysis of the proximal promoter region of the human CNP gene.

In conclusion, this study provides evidence that TGF-β, in addition to PDGF/tyrosine kinase signaling as we had described earlier, is a potent stimulator of CNP mRNA expression in cultured human VSM. Furthermore, a strong correlation between CNP mRNA and leucine zipper TSC22D1 mRNA following TGF-β simulation of several human cell types in culture and a 50% reduction in TGF-β- and PDGF-stimulated CNP production following siRNA silencing of TSC22D1 in cultured VSM provide cogent evidence that TSC22D1 functions in the transcriptional regulation of CNP.

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DISCLOSURES
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REFERENCES


