Cthrc1 Is a Novel Inhibitor of Transforming Growth Factor-β Signaling and Neointimal Lesion Formation

Renée J. LeClair, Tahir Durmus, Qiaozeng Wang, Peter Pyagay, Aleksandra Terzic, Volkhard Lindner

Abstract—We identified collagen triple helix repeat containing-1 (Cthrc1) as a novel gene expressed in the adventitia and neointima on arterial injury and found that it functionally increases cell migration while reducing collagen deposition. To address the in vivo role of Cthrc1, we generated transgenic mouse lines that constitutively overexpress Cthrc1. An intercross of 2 transgenic lines produced offspring with brittle bones caused by a reduction in collagenous bone matrix. Hemizygous Cthrc1 transgenic mice developed normally but neointimal lesion formation and adventitial collagen deposition in response to carotid artery ligation were significantly reduced compared with wild-type littermates. In 75% of Cthrc1 transgenic mice, cartilaginous metaplasia of medial smooth muscle cells was observed as assessed by Alcian blue staining and expression of the chondrocyte marker collagen type II. Transforming growth factor-β signaling was reduced in smooth muscle cells of Cthrc1 transgenic arteries, as demonstrated by reduced phospho-Smad2/3 immunoreactivity, whereas Smad signaling related to bone morphogenetic proteins was unaffected. Similarly, primary smooth muscle cells and PAC1 smooth muscle cells overexpressing Cthrc1 had reduced levels of phospho-Smad2/3 as well as procollagen. Furthermore, Cthrc1 inhibited transforming growth factor-β–sensitive reporter constructs in smooth muscle but not endothelial cells. These data indicate that Cthrc1 is a cell-type–specific inhibitor of transforming growth factor-β, which in turn impacts collagen type I and III deposition, neointimal formation, and dedifferentiation of smooth muscle cells. (***Circ Res*. 2007;100:826-833.**)

Key Words: intimal hyperplasia ■ fibrosis ■ bone morphogenetic protein ■ TGF-β ■ SMC differentiation

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We originally identified collagen triple helix repeat containing-1 (Cthrc1) in a screen for differentially expressed genes in normal versus balloon-injured arteries.[1] In the vasculature, Cthrc1 expression is limited to injured tissue, where it is induced abundantly in adventitial fibroblasts and neointimal smooth muscle cells (SMCs). Expression of Cthrc1 in the injured artery is transient, with Cthrc1 levels becoming undetectable 4 weeks following injury. Thus Cthrc1 expression coincides and colocalizes with adventitial fibrosis, which is responsible for constrictive remodeling following arterial injury and contributes to the failure of angioplasty procedures.[2] Cthrc1 was also found in the matrix of calcifying human atherosclerotic plaques and in mineralized bone of skeletal tissues.[3]

The injury response of tissues involves the activated fibroblast termed myofibroblasts based on its characteristic expression of the SMC marker smooth muscle α-actin (α-SMA).[4] As part of the general wound healing process, these myofibroblasts exhibit proliferation, migration, production of collagen types I and III, and other extracellular matrix (ECM) molecules.[5] Cthrc1 enhances migration of SMCs and fibroblasts in a scratch wound assay.[1] We also demonstrated that Cthrc1 reduces collagen type I mRNA and protein, suggesting that it may play a role in matrix deposition as well as migration. This is consistent with Cthrc1 being a secreted and glycosylated molecule that is highly conserved throughout evolution, suggesting that this gene may have an essential role.

The mechanistic switch between the retention of a contractile phenotype by vascular SMCs and the transition to a proliferative phenotype is key to understanding vascular remodeling and disease progression. Transforming growth factor (TGF)-β has been implicated in myofibroblastic transdifferentiation,[2,6] and Cthrc1 expression is regulated by TGF-β family members.[1] Furthermore, inhibition of TGF-β signaling with a soluble TGF-β receptor type II blocked the myofibroblast differentiation process and inhibition of TGF-β activity has been shown to be an effective way of reducing fibrosis in a variety of animal models.[2,7] Thus it is not surprising that the TGF-β receptor type I (TGFβRI) kinase is a major target for drug development. Aberrant bone morphogenetic protein (BMP) signaling has also been implicated in cardiovascular disorders such as pulmonary hypertension[8–10] and hereditary hemorrhagic telangiectasia, which is caused by mutations in endoglin or Alk1 (reviewed elsewhere[11]).

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The present study sought to determine the mechanism of Cthrc1 function and its role in arterial remodeling and collagen matrix synthesis in vivo. The rationale for this study was based on the facts that (1) constrictive arterial remodeling with vascular fibrosis is a major cause for failed angioplasty procedures, (2) Cthrc1 is induced on vascular injury, and (3) overexpression of Cthrc1 in vitro inhibits collagen type I. We chose a genetic approach with constitutive overexpression of Cthrc1 to analyze the vascular and bone phenotype in transgenic mice.

Materials and Methods

Animals

Generation of the transgenic mice is described in detail in the online data supplement.

Immunohistochemistry and Histochemistry

Rabbit antibodies were raised against the phosphopeptide NPIS(pS)V(pS) corresponding to the C terminus of activated Smad1/5/8 and the phosphopeptide S(pS)M(pS) corresponding to the C terminus of activated Smad2/3, as previously described.[12] High-titer antiserum that did not cross-react with the nonphosphorylated control peptide (data not shown) was obtained. For staining with anti-pSmad1/5/8 antiserum (1:1000 dilution), antigens were retrieved in 10 mmol/L citrate buffer for 15 minutes before staining using standard procedures.[13] Controls with preimmune serum (1:1000 dilution) from the same rabbit were included for all tissue sections and at this dilution nonspecific staining was negligible.

Image analysis was chosen to quantify pSmad2/3 immunoreactivity on cross-sections of 2-week ligated carotid arteries (ImageJ software, NIH; n=4 per group).

The effect of Cthrc1 on pSmad2/3 levels in response to TGF-β was examined in PAC1 cells stably transfected with a Cthrc1 expression construct or empty vector. The percentage of pSmad2/3 immunoreactive nuclei was determined for cells stimulated with 5 ng/mL of TGF-β for 60 minutes as well as for unstimulated cells.

Bone matrix was quantified on Masson's Trichrome–stained cross-sections of the midsection of the tibia using image analysis (ImageJ). The percentage of the cross-sectional area occupied by bone matrix was measured for 3 transgenic and 3 wild-type newborn littermates derived from the intercross of 2 Cthrc1 transgenic lines.

Collagen content in the adventitia was quantified as Sirius red stained area on sections of carotid arteries 2 weeks after ligation using image analysis (ImageJ; n=4 per group).

Cell Lines and Luciferase Assay

Stably transfected PAC1 smooth muscle and bovine endothelial (BEC) reporter cell lines were generated with the reporter plasmid $(CAGA)_{12}$-Luc, which is activated by TGF-β via ALK5,[14] BRE-Luc (activated by BMPs via ALK1[15]), and empty control pGL3 vector. Additional experimental details can be found in the online data supplement.

Northern Blotting

Northern blotting was performed as previously described,[1] and RNA from PAC1 transfectants was examined for collagen type I and III expression using rat cDNAs (col1a1, col1a2, and col3a1). Expression was normalized to GAPDH expression. Quantification of mRNA expression levels was performed with a phosphorimager and the values are shown relative to control cells (set at 100).

Western Blotting

Wild-type and Cthrc1 transgenic mice were perfused with PBS before harvesting aortae and tibia bones for immunoblotting of organ lysates with anti-Cthrc1 antiserum.[1] Equal protein loading was verified by immunoblotting of samples with anti-actin antibody (Sigma).

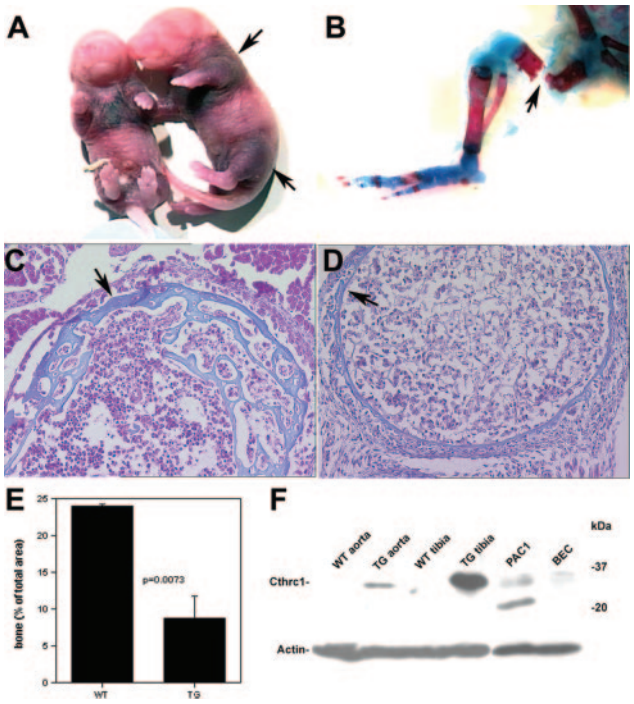

Figure 1. A, Newborn mice from a cross of 2 different hemizygous Cthrc1 transgenic lines with hemorrhaging around the upper regions of the limbs (arrows). B, Bone preparations revealed that the bleeding originated from fractured long bones such as the femur (arrow). C and D, Masson's trichrome staining was performed on cross-sectioned tibiae from wild-type (C) and Cthrc1 transgenic (D) pups, and bone matrix is seen in blue (arrow). E, A 63% reduction in tibial bone matrix was seen in the transgenic animals compared with wild-type litter mates (values represent means±SEM). F, Lysates of an aorta and a tibia from a wild-type and a Cthrc1 (Tg15) transgenic mouse were immunoblotted with anti-Cthrc1 and anti-actin antibodies. Endogenous Cthrc1 levels were also examined in BECs and PAC1 smooth muscle cells. Protein (200 μg) was loaded in each lane, with the exception of the aorta for which only 100 μg was loaded.

Results

Dose-Dependent Phenotype in Cthrc1 Transgenic Mice

Transgenic mouse lines with Cthrc1 under the control of the cytomegalovirus promoter were generated in the FVB strain. Several lines with varying levels of transgene expression were maintained. The Tg15 line expressed increased levels of Cthrc1 in arteries and bone, as assessed by immunoblotting with an anti-Cthrc1 antibody (Figure 1F), and hemizygous mice from this line were used for all vascular studies.

Breeding of hemizygous Tg15 mice led to approximately 1% embryonic lethality, as indicated by the presence of empty deciduae as early as embryonic day 10.5 (E10.5). Hemizygous Tg15 mice developed normally; however, at E16.5, transgenic embryos were visibly smaller and were smaller on average than wild-type litter mates (Table 1). This trend continued until birth. Average prenatal litter size was 9.9, which is typical for the FVB/N mouse strain[16]; however, postnatal mortality was significantly higher among transgenic pups. The increased death rate among the transgenic pups is most likely explained by parental cannibalism of those pups that were smaller before birth (Table

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TABLE 1. Breeding Statistics of Cthrc1 Transgenic and Wild-Type Mice

	Average Litter Size (Prenatal)	Postnatal Mortality	Average Weight at E16.5 (g)	Average Weight of Females at 12 Weeks (g)
Cthrc1	9.9±1.9	23.1%*	0.57±0.08*	21.7±1.77
Transgenics	n=14	n=134	n=3 litters	n=12
Wild-Type	9.5	5.5*	0.65±0.04*	22.7±0.988
FVB/N		n=134	n=3 litters	n=12

*$P<0.05$.

1). It is likely that pups carrying both the maternal and paternal transgene contributed to the increased mortality because the breeding statistics indicated that homozygous transgenic breeders were never obtained.

Intercrosses of hemizygous Tg15 and Tg32 transgenic mice produced newborns with hematoma formation around arms and legs (Figure 1A). The bleeding was caused by fractured bones (Figure 1B) and histomorphometry demonstrated a severe reduction in bone matrix (Figure 1C through 1E), which largely consists of mineralized collagen type I containing matrix.

Vascular Remodeling and Intimal Hyperplasia in Cthrc1 Transgenic Mice

Because we originally cloned Cthrc1 from injured arteries undergoing remodeling, we sought to determine the effects of elevated Cthrc1 levels on intimal lesion formation and arterial remodeling. We applied the mouse carotid artery flow cessation model[17] to assess the effects of elevated Cthrc1 levels on intimal hyperplasia and vascular remodeling 2 weeks after carotid artery ligation. The morphometric analysis of groups of Tg15 Cthrc1 transgenic mice and their corresponding wild-type littermates revealed that neointimal lesions were approximately 4-fold smaller in Cthrc1 mice, although, at the same time, there was no difference in lumen area (Table 2). Consistent with the decrease in neointimal area, a significantly lower percentage of proliferating intimal SMCs were seen in the transgenic mice (Table 2). Morphometric analyses of normal carotid arteries revealed no significant differences in lumen area or medial area between transgenic and wild-type mice (data not shown).

Inhibition of Collagen Expression in Cthrc1 Transgenic Mice

The reduction of collagen in previous in vitro assays prompted us to examine the levels of collagen expression in carotid arteries of wild-type and Cthrc1 transgenic mice. We used Sirius red staining to detect fibrillar collagens in carotid arteries. Cthrc1 transgenic carotid arteries demonstrated an approximate 50% reduction in Sirius red staining in the adventitia of arteries 2 weeks after ligation (Figure 2R and 2C and 2D) as well as decreased staining in normal arteries compared with wild-type mice (Figure 2A and 2B). Interestingly, 75% of the ligated carotid arteries of Cthrc1 transgenic mice revealed extensive areas in the media that stained positive for Alcian blue, which identifies proteoglycan-rich tissues typically associated with cartilage (Figure 2M). This apparent transdifferentiation of medial SMCs toward cartilage was further verified by demonstrating that these Alcian blue–positive cells expressed collagen type II, a chondrocyte specific marker (Figure 2N). Transdifferentiation of medial SMCs into chondrocytes was not observed in the wild-type mice.

TABLE 2. Morphometry and SMC Proliferation Data Obtained From Cthrc1 Transgenic and Wild-Type Litter Mates

	Transgenic	Wild-Type	*P*
Lumen area, μm^2	49646±9333	47027±5305	NS
Intimal area, μm^2	6434±2527	26586±6774	0.03
Medial area, μm^2	31570±1821	46286±7934	0.12
Medial SMC proliferation, BrdUrd Index, %	1.17±0.73	1.22±1.12	NS
Intimal SMC proliferation, BrdUrd Index, %	5.90±1.36	14.20±3.60	0.05

BrdUrd indicates bromodeoxyuridine.

Cthrc1 and Smad Signaling in Carotid Arteries

TGF-β has well-described profibrotic properties,[18,19] and downstream signaling of the activated TGFβRI kinase involves phosphorylation of Smad2 and Smad3 (reviewed elsewhere[20,21]). To examine the mechanism leading to the reduction in collagen we examined the levels of phosphorylated Smad2 and Smad3 (pSmad2/3) in both transgenic and wild-type mice. In normal vessels from wild-type animals, pSmad2/3 localized prominently to the nuclei of endothelial cells and the adventitia, where staining was seen in the adventitial fibroblasts and only little immunoreactivity was observed in the media (Figure 2E). In remodeling carotid arteries after ligation, wild-type vessels showed an increase in pSmad2/3 in the neointimal SMCs and adventitial fibroblasts (Figure 2K). Transgenic vessels showed a similar staining pattern; however, pSmad2/3 staining was reduced in both the endothelial cells and fibroblasts of the adventitia and no pSmad2/3 immunoreactivity was observed in medial SMCs (Figure 2F and 2H). Quantification of the percentage of the cross-sectioned vessel wall area immunoreactive for pSmad2/3 was significantly reduced in Cthrc1 transgenic mice (Figure 2S).

We also assessed BMP signaling in carotid arteries by examining the levels of phosphorylated Smad1, Smad5, and Smad8 (pSmad1/5/8) by immunohistochemistry. pSmad1/5/8 staining was similar among wild-type and transgenic arteries with endothelial cell nuclei staining uniformly positive and medial SMCs showing strikingly heterogeneous pSmad1/5/8 staining in both wild-type and Tg15 mice (Figure 2I through 2L). In contrast, pSmad1/5/8 immunoreactivity in remodeling arteries was observed in all neointimal SMCs (Figure 2K and 2P). pSmad1/5/8 staining was heterogeneous within the chondrocytic area of Tg15 carotid arteries, and these chondrocyte-

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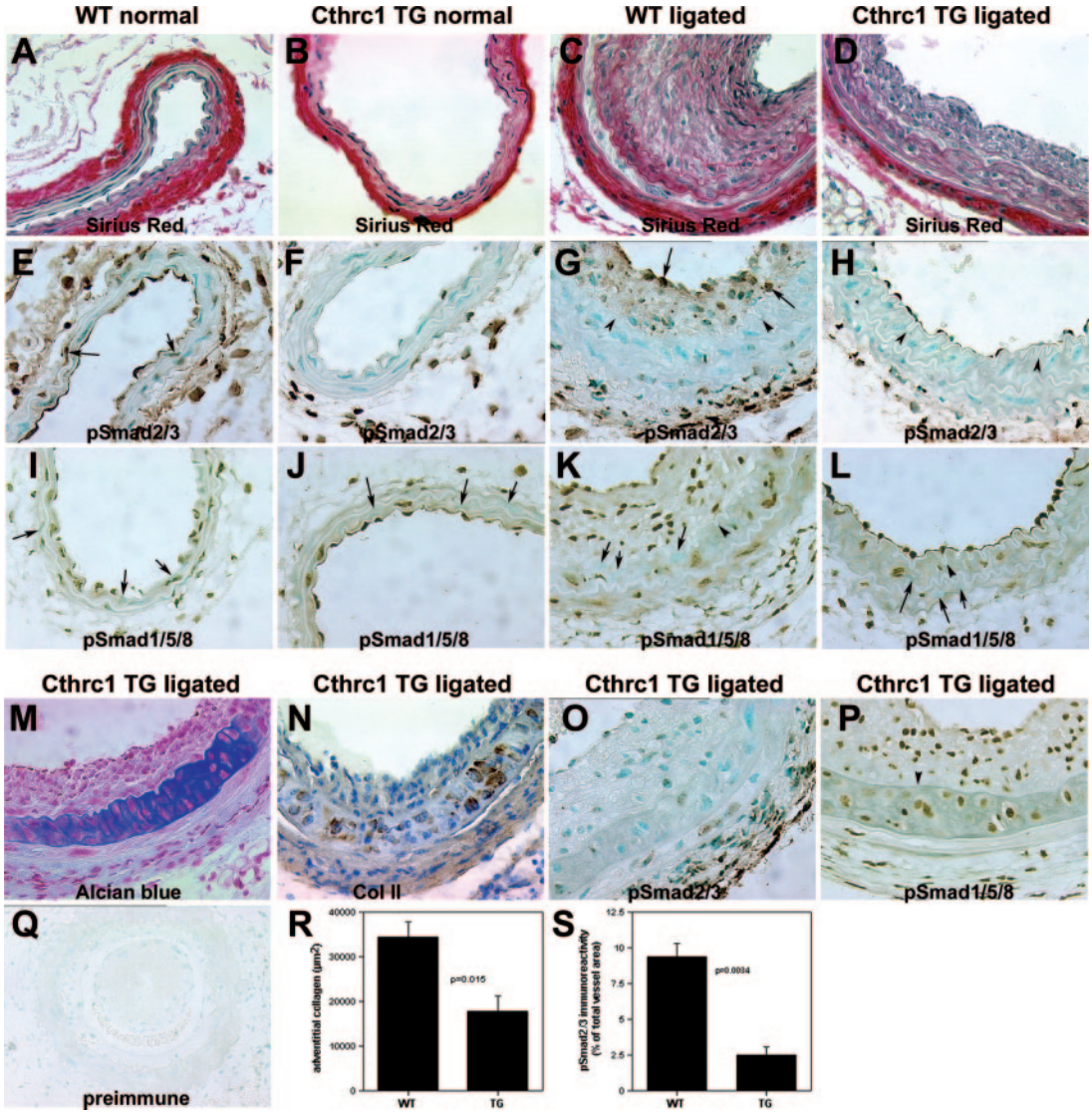

Figure 2. Expression of collagen in wild-type (A, C, E, G, I, and K) and Cthrc1 transgenic (B, D, F, H, J, and L through P) mice. A and B, Staining for fibrillar collagens with Sirius red showed more abundant adventitial collagen in normal carotid arteries from wild-type (A) vs Cthrc1 transgenic (B) mice. C and D, Sirius red staining also showed more staining in the 2-week neointima of wild-type (C) compared with transgenic (D) mice. E and F, Immunoreactivity for pSmad2/3 was present in endothelium and adventitia of normal carotid arteries from wild-type and transgenic animals. Unlike wild-type arteries (arrow) (E), pSmad2/3 was not seen in medial SMCs of the transgenics (F). G and H, In ligated arteries, staining for pSmad2/3 was pronounced in neointimal SMCs of wild-type vessels (arrows) (G) and reduced in the transgenic arteries (H). I through L, Immunohistochemistry for pSmad1/5/8 in normal vessels from both animals show strong staining in the endothelial cells and variability within the media (I and K), with many SMCs lacking pSmad1/5/8 (arrows). Neointimal SMCs in ligated arteries are more uniformly positive for pSmad1/5/8 (K), where as many medial SMCs remain negative (arrows in K and L). M and N, Large areas within the tunica media of 2-week ligated Cthrc1 transgenic vessels stained with Alcian blue (M), and cells within these areas expressed the chondrocyte marker collagen type II (N). O and P, Few cells stained positive for pSmad2/3 in the cartilaginous lesion (O), and staining for pSmad1/5/8 remained heterogeneous in chondrocytic lesion of transgenic vessels (P). Q, Preimmune vessels shown as a negative control. R, Quantification of adventitial collagen in 2-week ligated carotid arteries by image analysis revealed a 48% reduction in collagen in transgenic bones (data are means±SEM). S, Quantification of pSmad2/3 staining in 2-week ligated carotid arteries by image analysis revealed a 75% reduction in immunoreactive area in transgenic vessels (data are means±SEM). Original magnification: ×200 (A through D); ×400 (E through J). Nuclear stain with methyl green and hematoxylin; immunoreactivity is seen in brown. Arrowheads mark the internal elastic lamina.

like cells were devoid of pSmad2/3 immunoreactivity (Figure 2O and 2P).

Overexpression of Cthrc1 in PAC1 and Primary SMCs Causes Loss of Collagen Expression and Inhibition of TGF-β Signaling

To determine the consequences of increased Cthrc1 expression in vitro, we established stably transfected PAC1 cell lines. This is a well-differentiated SMC line that expresses high levels of the major interstitial collagens type I and III. In addition to the inhibition of col1a1 and col1a2, col3a1 mRNA expression was also dramatically suppressed (Figure 3A). Primary rat aortic SMCs expressed detectable levels of procollagen and this expression was increased by treatment of these cells with TGF-β (Figure 3B). Treatment of the same cells with TGF-βR:Fc, a TGF-β antagonist,[2] led to a reduc-

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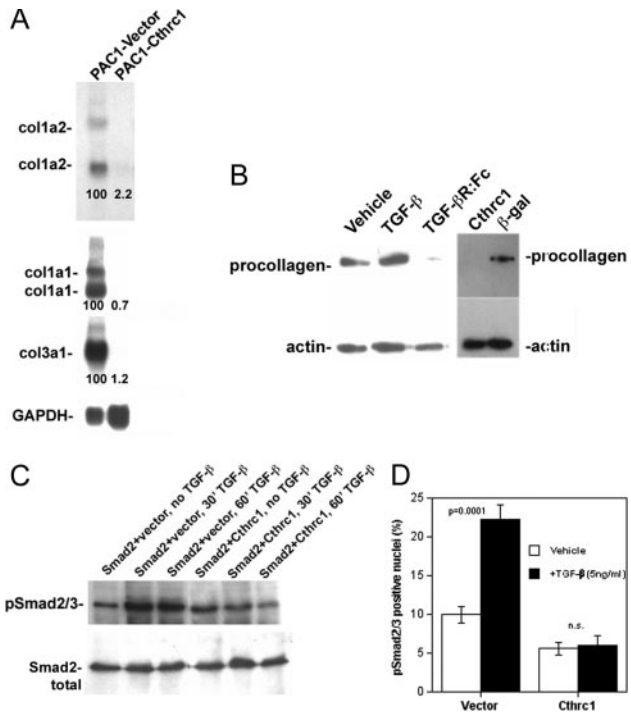

Figure 3. A, Collagen type I and III mRNA expression in PAC1 smooth muscle cells overexpressing Cthrc1 (PAC1-Cthrc1) is inhibited compared with controls (PAC1-Vector). Col1a1, col1a2, and col3a1 mRNA transcript levels were reduced to 0.7% to 2.2% of control values in Cthrc1 overexpressing cells. B, Immunoblotting of primary rat smooth muscle cells treated with control, TGF-β (5 ng/mL), TGF-βR:Fc, β-galactosidase virus, or Cthrc1 virus. The addition of either Cthrc1 virus or TGF-βR:Fc reduces collagen synthesis, whereas addition of TGF-β increases collagen production, as shown by blotting with the anti-procollagen antibody. Actin was used as a loading control. C, Immunoblotting of lysates from HEK293 cells cotransfected with a Smad2 and a Cthrc1 expression construct. Immunoblotting was performed with anti-pSmad2/3–specific and anti-(pan)Smad2 antibodies. TGF-β increased pSmad2/3 levels in empty vector transfected cells but not in the presence of overexpressed Cthrc1. D, Quantification of pSmad2/3-positive nuclei in Cthrc1 overexpressing (Cthrc1) and empty vector transfected PAC1 cells (vector). PAC1 cells were stained for pSmad2/3 before and after stimulation by TGF-β, and the percentage of positive nuclei was determined for each field of vision (n=10, data are means±SEM). Note the absence of the TGF-β response in the Cthrc1-transfected cells.

tion of procollagen demonstrating that collagen type I expression in these cells is driven by endogenous TGF-β (Figure 3B). In addition, transduction of primary rat SMCs with a Cthrc1-expressing adenovirus abolished procollagen expression, whereas a control adenovirus had no effect (Figure 3B, lanes 4 and 5).

Because the profibrotic effects of TGF-β are typically mediated through a Smad-dependent pathway,[18,19] we determined the effects of Cthrc1 on Smad2/3 phosphorylation in HEK293 cells and PAC1 cells. Cotransfection of Cthrc1 blocked the TGF-β–induced increase in pSmad2/3 levels (Figure 3C). In addition, PAC1 cells stably transfected with a Cthrc1 expression construct failed to respond to TGF-β with an increase in the number of pSmad2/3 positive nuclei (Figure 3D). Furthermore, the percentage of pSmad2/3 positive nuclei was also higher in unstimulated control transfectants (Figure 3D).

To examine the mechanisms by which Cthrc1 inhibits TGF-β signaling further, we studied the induction of several TGF-β–sensitive luciferase reporter constructs in PAC1 cells and BECs. When cells were incubated with Cthrc1 or transduced with a Cthrc1-expressing adenovirus, TGF-β was no longer capable of activating the $(CAGA)_{12}$-Luc reporter construct (Figure 4). This result was only observed in PAC1 cells; BECs were not responsive to Cthrc1 inhibition of TGF-β (Figure 4). Cthrc1 had no effect on the BMP-sensitive BRE-Luc reporter in either cell type (Figure 4). Endogenous levels of Cthrc1 are much higher in PAC1 cells than in BECs (Figure 1F).

Discussion

Arterial remodeling involves a balance of cell migration, proliferation, and matrix deposition. Previous work has demonstrated that increased Cthrc1 expression in embryonic fibroblasts and PAC1 cells increased migration, while decreasing collagen type I mRNA and protein expression without altering cell proliferation.[1] Neointimal lesion formation is dependent on both SMC proliferation as well as cell migration[22]; therefore, we sought to address the role of Cthrc1 on this process by examining arterial remodeling in a transgenic mouse line overexpressing Cthrc1 under control of the cytomegalovirus promoter. This promoter has been found to be very suitable for overexpression of transgenes in arterial SMCs.[23] We were surprised to observe that Cthrc1 transgenic mice developed much smaller neointimal lesions in a mouse carotid artery model of intimal hyperplasia despite the reported increase of cell migration attributable to Cthrc1.[1] Even though neointimal lesions were much smaller in the transgenic mice, the lumen area was not different between groups, indicating that constrictive remodeling was increased in the transgenic vessels. There was a significant decrease in fibrillar collagens in transgenic arteries following carotid ligation, as determined by quantification of Sirius red staining. With respect to the neointimal lesion formation, there are several considerations to be made when interpreting these data. For example, neointimal lesion formation is a complex 3D process involving SMC proliferation and migration, which is likely to be influenced by additional variables compared with the 2D scratch wound migration assay. Furthermore, we cannot rule out that Cthrc1 controls additional factors that have not been identified but could influence the arterial remodeling response.

The combined in vitro data and immunohistochemical analyses suggest that Cthrc1 is likely to inhibit collagen deposition by inhibiting TGF-β signaling, as shown by a reduction in activated Smad2/3. Staining of both ligated and normal vessels from transgenic animals showed a reduction in cells positive for pSmad2/3, but there was no change in activated Smad1/5/8 when compared with wild-type vessels. Smad1/5/8 activation occurs downstream of BMP signaling, and there are currently no data to suggest that Cthrc1 interferes with these pathways. There are several mechanisms by which Cthrc1 could disrupt TGF-β signaling, leading to the reduction in collagen. Because Cthrc1 is a secreted protein it would not be able to affect collagen promoter activity directly, however, it could function as a ligand of a

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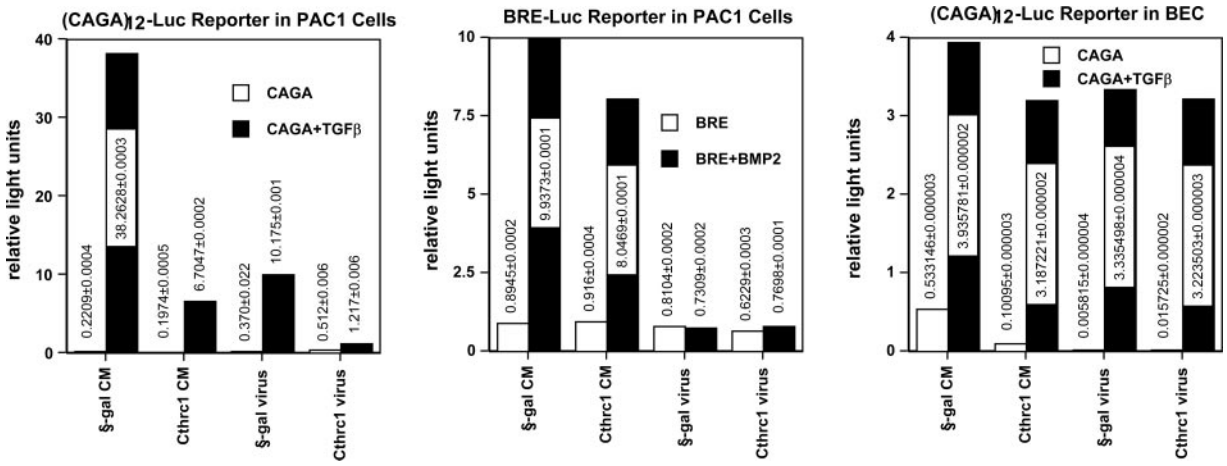

Figure 4. Luciferase reporter assays were performed in PAC1 SMCs and BECs stably transfected with the TGF-β sensitive $(CAGA)_{12}$-Luc and the BMP-sensitive BRE-Luc reporter construct. Conditioned media from CHO cells infected with an adenovirus expressing full-length Cthrc1 (Cthrc1-FL CM) inhibited the activation of the $(CAGA)_{12}$-Luc reporter by TGF-β but not the BRE-Luc reporter in PAC1 cells. Similar results were obtained when the reporter cell lines were directly transduced with a Cthrc1-expressing or β-galactosidase (β-gal) adenovirus. No inhibition of the $(CAGA)_{12}$-Luc reporter was seen in BECs in the presence of Cthrc1-FL. Data represent means±SD of triplicates; numbers were too close for error bars to appear.

signaling pathway with downstream effects on collagen promoter activity. A direct interaction of Cthrc1 with the TGF-β ligand in the form of a ligand trap is also unlikely because Cthrc1 did not inhibit the TGF-β response in endothelial cells. One possibility is that Cthrc1 signals through its own signaling pathway, which is functional in specific cell types, such as SMCs, where it prevents activation of transcription factor complexes containing Smad2 and/or Smad3. Additional studies are required to further address the mechanism of Cthrc1 function.

The widespread finding of cartilaginous metaplasia of the tunica media in Cthrc1 transgenic vessels suggests a potential role of Cthrc1 in shifting the collagen expression profile from a type I and III pattern toward a collagen type II pattern typically found in cartilage. This notion is supported by the fact that chondrocytes of the growth plate of developing bones show abundant expression of Cthrc1.[3] By inhibiting TGF-β signaling in the vessel wall through overexpression of Cthrc1, it is possible that a shift toward BMP-mediated Smad signaling occurs as more Smad4 (coSmad) might be available for complex formation with BMP-related R-Smads, which in turn would favor cartilage formation.[24] This could explain the increased tendency to undergo cartilaginous metaplasia observed in the tunica media of Cthrc1 transgenic arteries. Different from our data, we acknowledge that Schulick et al[25] have reported cartilaginous metaplasia in response to adenoviral delivery of TGF-β to rat carotid arteries. No such transdifferentiation was seen by Nabel et al[26] using a similar adenoviral approach in pig arteries. The role of BMPs in chondrocyte differentiation has been well documented,[27,28] and our data cannot rule out the possibility that Cthrc1 indirectly also affects BMP signaling. The BMP signaling pathway is a very finely tuned system, and subtle changes may not be detectable by immunohistochemistry for pSmad1/5/8 as performed here.

Medial SMCs in normal arteries represent the differentiated, quiescent phenotype. Both TGF-β and BMP signaling have been implicated in either the maintenance of a contractile phenotype or transition to a proliferative myofibroblast.[29,30] In the present study, we found that neointimal SMCs, which represent the dedifferentiated SMC phenotype, were uniformly positive for pSmad1/5/8, unlike SMCs in the media. This observation could suggest that SMC dedifferentiation may require BMP signaling and that the absence of BMP/Smad signaling in some medial SMCs is not incompatible with the differentiated SMC phenotype. Arterial injury studies have demonstrated that the presence of the endothelium plays an important role in the maintenance of the nonproliferative SMC phenotype.[31] The fact that deficient BMP receptor signaling is involved in pulmonary artery hypertension and the prominent pSmad1/5/8 immunoreactivity reported here in all of the endothelial cells raises the question of whether altered BMP/Smad signaling in the endothelium might play a more important role in vascular pathology than BMP/Smad signaling in SMCs. Limitations of the present study are that we do not know which specific BMP ligand(s) and associated R-Smads are involved because our antibody does not discriminate between pSmad1,-5, or -8. It has been reported that the SMC phenotype may be influenced differentially depending on the BMP signal involved.[29] Both BMP2 and BMP6 are able to decrease expression of SMC differentiation markers in vitro, whereas BMP4 has been shown to increase α-SMA, SM22α, and calponin.[29]

TGF-β is an important SMC differentiation factor and transcriptional control of SMC markers, such as SM22α and α-SMA, occurs through the TGF-β–controlling element TCE, which is present in the promoter region of many of these genes.[32] This regulation is thought to be at least partially controlled by TGF-β–dependent signaling via RhoA and subsequent activation of protein kinase C and p38, which is sufficient to enhance SMC marker gene expression.[33] Depending on the conditions and concentrations used, TGF-β can both stimulate or inhibit SMC proliferation in vitro.[34,35]

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Our own in vivo studies demonstrated an increase in SMC proliferation in response to this cytokine.[36] The present study provides additional insight with respect to TGF-β–mediated Smad signaling in arteries. There was robust pSmad2/3 immunoreactivity in normal vessels in adventitial fibroblasts as well as endothelial cells and very little staining in SMCs, whereas neointimal SMCs, especially those still undergoing replication in the more luminal layers, stained strongly with the pSmad2/3 antibody (Figure 2G). This observation suggests a correlation between the level of TGF-β signaling and the degree of SMC dedifferentiation or proliferation. This would be consistent with earlier studies demonstrating increased expression of TGF-β in injured arteries and increased SMC proliferation in response to exogenous TGF-β.[36] The finding of decreased SMC proliferation in arteries exhibiting reduced levels of pSmad2/3 as a result of elevated Cthrc1 expression are also consistent with our earlier findings that increased stimulation by TGF-β promotes dedifferentiation of SMCs in vivo. The prominent pSmad2/3 immunoreactivity in fibroblasts of the adventitia from normal arteries is surprising as these cells will undergo transdifferentiation into myofibroblasts when stimulated further with TGF-β.[2,37] Considering that both BMP/Smad and TGF-β/Smad signaling regulates the SMC phenotype, the present study indicates that an increased ratio of pSmad2/3 to pSmad1/5/8 correlates with SMC proliferation and thus dedifferentiation.

There are still many unresolved questions as to the identity of the lower molecular weight Cthrc1 immunoreactive bands observed on immunoblots of PAC1 cell lysates (Figure 1F) among many other in vivo and vitro specimens (data not shown).[1] We have reported earlier that endogenous Cthrc1 exists in multiple molecular-weight forms,[1] and the identity of these fragments is verified by the fact that they are recognized by more than one anti-Cthrc1 antibody (not shown). Further investigations are required to study the significance of these lower-molecular-weight forms of Cthrc1.

In summary, the present study identifies Cthrc1 as a factor that can inhibit TGF-β signaling in a cell-specific manner. This in turn is likely the mechanism by which Cthrc1 inhibits collagen type I and III expression. These antifibrotic properties of Cthrc1 may be of therapeutic value, as organ fibrosis presents a major clinical problem.

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Disclosures

None.

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