Brain cytoplasmic RNA 1 suppresses smooth muscle differentiation and vascular development in mice

Yung-Chun Wang‡, Ya-Hui Chuang‡, Qiang Shao†, Jian-Fu Chen§, and Shi-You Chen†*†

From the ‡ Department of Physiology and Pharmacology, University of Georgia, Athens, Georgia 30602, the ¶ Institute of Clinical Medicine and Department of Cardiology, Renmin Hospital, Hubei University of Medicine, Shiyuan, Hubei 442000, China, and the § Ostrow School of Dentistry, University of Southern California, Los Angeles, California 90089

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The cardiovascular system develops during the early stages of embryogenesis, and differentiation of smooth muscle cells (SMCs) is essential for that process. SMC differentiation is critically regulated by transforming growth factor (TGF)-β/SMAD family member 3 (SMAD3) signaling, but other regulators may also play a role. For example, long noncoding RNAs (lncRNAs) regulate various cellular activities and events, such as proliferation, differentiation, and apoptosis. However, whether long noncoding RNAs also regulate SMC differentiation remains largely unknown. Here, using the murine cell line C3H10T1/2, we found that brain cytoplasmic RNA 1 (BC1) is an important regulator of SMC differentiation. BC1 overexpression suppressed, whereas BC1 knockdown promoted, TGF-β-induced SMC differentiation, as indicated by altered cell morphology and expression of multiple SMC markers, including smooth muscle α-actin (αSMA), calponin, and smooth muscle 22α (SM22α). BC1 appeared to block SMAD3 activity and inhibit SMC marker gene transcription. Mechanistically, BC1 bound to SMAD3 via RNA SMAD-binding elements (rSBEs) and thus impeded TGF-β–induced SMAD3 translocation to the nucleus. This prevented SMAD3 from binding to SBEs in SMC marker gene promoters, an essential event in SMC marker transcription. In vivo, BC1 overexpression in mouse embryos impaired vascular SMC differentiation, leading to structural defects in the artery wall, such as random breaks in the elastic lamina, abnormal collagen deposition on SM fibers, and disorganized extracellular matrix proteins in the media of the neonatal aorta. Our results suggest that BC1 is a suppressor of SMC differentiation during vascular development.

The cardiovascular system is developed in the early stage of embryogenesis. Differentiation of smooth muscle cells (SMCs) is an essential process for formation of the cardiovascular system in embryos (1). SMC progenitors are recruited and then differentiated to SMCs to ensheathe the endothelial vasculature (2). Disruption of this process during embryonic development causes vascular abnormalities such as thoracic aortic aneurysms and vascular anomalies or leads to embryonic lethality (3–5). SMC differentiation is regulated elaborately in embryos at the transcriptional and translational levels (6). In addition to protein factors, non-coding RNAs (ncRNAs), such as microRNAs and long ncRNAs (lncRNAs), also play important roles in SMC differentiation (7–11). Investigating the molecular mechanisms underlying lncRNA function in SMC differentiation will advance our understanding of vascular development.

During embryogenesis, SMCs are derived from multiple origins, including the neural crest, mesoangioblasts, secondary heart field, and proepicardium (12). Thus, various in vitro cell models, such as C3H/10T1/2 (10T1/2) cells, Monc-1 cells, and JoMa1 cells, have been developed to study SMC differentiation (13). Transforming growth factor β (TGF-β) is one of the essential growth factors inducing SMC differentiation during vascular development (14). Smad proteins serve as core intracellular mediators for transducing TGF-β signaling from transmembrane receptors to the nucleus and further modulating the expression of targeted genes via binding to Smad-binding elements (SBEs) in gene promoters to initiate SMC differentiation (15). Although a few lncRNAs are regulated by TGF-β and involved in TGF-β–induced gene expression in disease states (10, 16, 17), the role of lncRNAs in TGF-β–induced SMC differentiation remains largely unknown.

Brain cytoplasmic RNA 1 (BC1) is a cytoplasmic lncRNA derived from the tRNAAla molecule and mainly presents in specific subset of neurons of the central and peripheral nervous system in rodents (18). BC200 RNA is the analog of BC1 in primates, with a similar function and expression pattern (19). Both BC1 and BC200 regulate protein biosynthesis in dendrites of neurons by interacting with eukaryotic initiation factor 4A (eIF4A), poly(A)-binding protein (PABP), and fragile X mental...
retardation protein (FMRP) (20–22). BC1-deficient mice show reduced exploratory activity along with increased anxiety and increased seizure susceptibility, although there is no observed anatomical or neurological abnormality (23–25). In humans, BC200 plays a role in tumorigenesis and neurodegeneration (26). The elevated RNA expression level of BC200 has been detected in different cancer tissues (27). In breast cancer, BC200 contributes to the progression of tumorigenesis by regulating the survival of tumor cells (28). In addition to cancer, BC200 expression is increased in brains with Alzheimer’s disease and presents a correlation with Alzheimer’s disease progression (29). Because vascular and neural systems share a similar anatomic localization, structural formation process, and signaling molecules for developmental regulation (30, 31), and because BC1 serves as an important regulator for neural plasticity (32), we sought to determine whether BC1 plays a role in vascular development.

In this study, we found that BC1 negatively regulates TGF-β–induced SMC differentiation and vascular development in mouse embryos. Ectopic expression of BC1 suppressed TGF-β–induced SMC differentiation by impeding TGF-β–induced Smad3 nuclear translocation in 10T1/2 cells. Mechanistically, BC1 binds to Smad3 via its RNA SBE (rSBE), which inhibits Smad3 nuclear translocation and subsequent activation of SMC genes. Importantly, ectopic expression of BC1 in mouse embryos caused abnormalities in the aorta because of impaired SMC differentiation.

Results

BC1 inhibited TGF-β–induced SMC differentiation

TGF-β is a central regulator for SMC fate determination during vascular development (14). To determine whether BC1 is involved in SMC differentiation, we treated 10T1/2 cells with TGF-β to induce SMC differentiation in vitro (13, 14). TGF-β induced expression of the SMC markers αSMA, CNN1, and SM22α (Fig. 1, A and C), indicative of SMC differentiation. Interestingly, BC1 expression was time-dependently decreased along with the increase in SMC markers, suggesting that BC1 may negatively regulate TGF-β–induced SMC differentiation. To confirm the specific role of TGF-β in BC-1 expression, we used the TGF-β receptor kinase inhibitor SB431542 to block TGF-β function. As shown in Fig. S1, SB431542 reversed the BC1 expression that was inhibited by TGF-β, verifying that TGF-β down-regulated BC1 expression.

To determine whether BC1 regulates TGF-β–induced SMC differentiation, we used an adenoviral vector to express BC1 cDNA (Ad-BC1) or its short hairpin RNA (shRNA, Ad-shBC1) to alter BC1 expression in 10T1/2 cells (Fig. S2). As shown in Fig. 1, D and E, ectopic expression of BC1 suppressed TGF-β–induced expression of the SMC marker proteins αSMA, CNN1, and SM22α. Conversely, knockdown of BC1 enhanced the expression of SMC markers (Fig. 1, F and G). Morphological change from a polygonal to a spindled-shaped phenotype with TGF-β induction has been observed in 10T1/2 cells (13). Consistent with marker gene expression, ectopic expression of BC1 suppressed TGF-β–induced morphological change, whereas knockdown of BC1 induced a spindled-shaped morphology even without TGF-β stimulation (Fig. 1H). These results indicated that BC1 was a negative regulator for TGF-β–induced SMC differentiation.

BC1 function in SMC differentiation is associated with Smad3 activity

Smad3 is essential for transducing TGF-β signaling and is important in TGF-β–induced SMC differentiation (33). Thus, we sought to determine whether BC1 affects Smad3 activity in 10T1/2 cells. Ectopic expression of BC1 suppressed, whereas knockdown of BC1 enhanced, Smad3 phosphorylation/expression along with the alteration of SMC marker expression (Fig. 2, A–D). However, overexpression of Smad3 (pcDNA-Smad3) in BC1-transfected 10T1/2 cells rescued the expression of SMC marker genes that was suppressed by BC1 (Fig. 2, A and B and Fig. S3A). Conversely, knockdown of Smad3 via its shRNA (Ad-shSmad3) or blockade of Smad3 activity via its selective inhibitor SIS3 impeded the expression of SMC markers that was enhanced because of knockdown of BC1 (Fig. 2, C–F, and Fig. S3, B and C). These data suggested that BC1 inhibited TGF-β–induced SMC differentiation by suppressing Smad3 signaling.

In addition to 10T1/2 cells, TGF-β stimulation of dedifferentiated SMCs have been used to study SMC differentiation (34, 35). Thus, we determined whether BC1 regulates SMC marker...
expression in TGF-β-treated SMCs. As shown in Fig. S4, ectopic expression of BC1 suppressed TGF-β-induced SMC marker expression along with inhibition in Smad3 activity, whereas knockdown of BC1 enhanced TGF-β-induced SMC marker expression along with increased Smad3 activity. These data suggest that BC1 may have a general function in regulating SMC differentiation.

**BC1 inhibited Smad3 transcription activity by suppressing its nuclear translocation**

Because Smad3 regulates SMC marker gene transcription, we tested whether BC1 regulates SMC marker gene promoter activity by performing a luciferase reporter assay. As shown in Fig. 3A and Fig. S5, forced expression of BC1 suppressed, whereas knockdown of BC1 enhanced, TGF-β–induced promoter activity of αSMA and SM22α genes. Because TGF-β–induced gene transcription is mediated by Smad3 binding to the SBE in the promoter of targeted genes (36), we tested whether BC1 regulates the activity of a promoter mainly composed of SBE elements (SBE-Luc) and assessed whether BC1 affects Smad3 binding to the SBEs in αSMA and SM22α promoters in a chromatin setting using a ChIP assay. As shown in Fig. 3A and Fig. S5, forced BC1 expression suppressed, whereas knockdown of BC1 enhanced, SBE promoter activity. The ChIP assay showed that TGF-β induction for 2 h caused strong Smad3 binding to αSMA and SM22α promoters. However, BC1 impeded Smad3 binding to both promoters (Fig. 3B).

Smad3 nuclear translocation is required for its transcriptional regulation (37). Therefore, we sought to determine whether BC1 is involved in TGF-β–induced Smad3 nuclear translocation. As shown in Fig. 3C, BC1 expression in 10T1/2 cells caused more Smad3 to be presented in the cytoplasm within 1 h of TGF-β induction compared with cells without BC1 expression. These results indicated that Smad3 nuclear translocation was inhibited by BC1. To further confirm this phenomenon, the cytoplasmic and nuclear fractions of BC1 and Smad3 were quantified by real-time quantitative PCR (for BC1) and Western blotting (for Smad3), respectively. As shown in Fig. 3, D–F, forced expression of BC1 blocked Smad3 nuclear translocation by nearly 40% at 1 h of TGF-β treatment. These results demonstrated that BC1 suppressed Smad3 transcriptional activity by inhibiting its nuclear translocation.

**BC1 physically interacted with Smad3**

Because BC1 physically interacts with eIF4A, PABP, and FMRP (20–22), BC1 may affect Smad3 nuclear translocation through a physical interaction. We therefore first detected whether BC1 co-localizes with Smad3 in 10T1/2 cells. As shown in Fig. 4A, fluorescence in situ hybridization (FISH) for BC1 and immunofluorescent (IF) staining for Smad3 showed that they were co-localized in the cytoplasm of 10T1/2 cells.
prior to TGF-β induction. Interestingly, TGF-β induced nuclear translocation of Smad3 but not BC1, indicating that Smad3 nuclear translocation may require the dissociation of Smad3 from BC1. To determine whether BC1 specifically binds to Smad3, we performed an RNA immunoprecipitation (RIP) assay to pull down the BC1–Smad3 complex using Smad3 antibody and detected BC1 RNA enrichment in 10T1/2 cells. As shown in Fig. 4B, BC1 was present in the Smad3 complex, and TGF-β significantly reduced BC1 binding to Smad3. To further confirm the interaction between BC1 and Smad3, we transfected biotin-labeled BC1 into 10T1/2 cells and then detected the presence of Smad3 in the avidin-precipitated complex. Indeed, Smad3 was present in the BC1 complex pulled down with avidin beads in vehicle-treated cells (Fig. 4, C and D). However, the presence of Smad3 in the avidin-precipitated complex was decreased with TGF-β induction (Fig. 4, C and D). To further test whether BC1 directly binds to Smad3, we performed in vitro binding assays by incubating biotin-labeled BC1 with recombinant Smad3. Smad3 was detected in the BC1–Smad3 complex pulled down with avidin beads but not in the control complex, indicating that BC1 directly and specifically bound to Smad3 (Fig. 4, E and F). These data indicated that BC1 regulated Smad3 nuclear translocation/activity via physical binding to its protein.

**BC1 interacted with Smad3 and regulated TGF-β/Smad3 signaling via its rSBE**

Previous studies have shown that Smad3 binds to microRNA and lncRNA via a conserved rSBE (10, 38). We sought to determine whether BC1 interacted with Smad3 via its rSBE. As shown in Fig. S6, two tentative rSBEs were observed in the first stem structure of BC1 RNA. To test whether BC1 interacted with Smad3 via the rSBE, we mutated these two tentative rSBEs and then performed a biotin-avidin pulldown assay in 10T1/2 cells. As shown in Fig. 5, A–D, BC1 fragment 1 (F1), with the first stem structure containing two rSBEs bound Smad3 with an abundance similar to full-length BC1. However, the F1 fragment with mutations in either rSBE bound significantly less Smad3. Importantly, mutations at both rSBEs almost abolished the Smad3–BC1 interaction. These results demonstrated that the full interaction between BC1 and Smad3 required both rSBEs in the first stem of BC1 RNA.

To determine whether BC1 regulates SMC differentiation via the rSBEs, we tested whether the BC1 fragment with mutant rSBEs affects the function of BC1 regarding SMC gene expression. As shown in Fig. 6A, both the full-length (FL) and wildtype F1 fragment of BC1 inhibited the TGF-β–induced αSMA, SM22α, and SBE promoter activities. However, BC1 fragments with mutations at either one or both rSBE sites abolished the BC1 function in suppressing promoter activity. Moreover, rSBE mutations also restored the protein expression of SMC markers (αSMA, CNN1, and SM22α) that was attenuated by BC1 (Fig. 6, B and C). These data indicated that the rSBEs were essential for BC1 to inhibit TGF-β–induced SMC differentiation of 10T1/2 cells.

**BC1 altered the SMC phenotype during vascular development**

Impaired differentiation of SMCs during embryonic development leads to abnormal formation of vasculature (39). To determine whether BC1 affects SMC differentiation in vivo, we
BC1 in SMC differentiation

Figure 5. BC1 interacted with Smad3 protein via rSBEs. A and C, mutations at either one (BC1-F1-mt1 or BC1-F1-mt2) or both rSBEs (BC1-F1-mt12) in the first stem structure of BC1 suppressed the interaction of BC1 with endogenous Smad3 in 10T1/2 cells (A, B); and recombining BC1 with Smad3 in vitro (C). B and D, quantification of Smad3 in the BC1–Smad3 complex in A and C by normalizing to GAPDH (A) and Input Smad3 (C) level, respectively. *, p < 0.05 versus vehicle-treated cells (Ctrl); #, p < 0.05 versus BC1-FL-transfected cells, n = 3.

Figure 6. BC1 attenuated TGF-β–induced SMC marker expression via the rSBE. A, mutations at either one (mt1 or mt2) or both rSBEs (mt12) in the first stem structure of BC1 reversed the inhibitory effect of BC1 on the activities of SMC marker promoters and the promoter mainly containing SBEs (SBE-Luc), as measured by luciferase assay. *, p < 0.05 versus vehicle-treated cells (Ctrl); #, p < 0.05 versus TGF-β–treated cells (TGF-β); $, p < 0.05 versus TGF-β–treated cells with full-length BC1 (FL + TGF-β); n = 3. B, mutations at either one (BC1-F1-mt1 or BC1-F1-mt2) or both rSBEs (BC1-F1-mt12) in the first stem structure of BC1 reversed the inhibitory effect of BC1 on SMC marker protein expression. C, quantification of αSMA, CNN1, and SM22α protein levels in B by normalizing to α-tubulin. *, p < 0.05 versus vehicle-treated cells; #, p < 0.05 versus TGF-β–treated cells; $, p < 0.05 versus TGF-β–treated cells with full-length BC1, n = 3.

delivered an adenoviral vector expressing BC1 into mouse embryos via intraplacental injection on embryonic day 12.5 (E12.5) (40). Thoracic aortae of pups were then collected on postnatal day 0 (P0) to analyze vascular development. As shown in Fig. 7A, BC1 was expressed nearly 2.3-fold more in aortae with the BC1 adenoviral vector, which caused a reduction in mRNA expression of the SMC markers αSMA, CNN1, and SM22α. To verify whether BC1 suppressed SMC marker gene expression in the media of the mouse aorta, we performed immunostaining of αSMA and FISH of BC1 on the aorta sections. As shown in Fig. 7, B and C, BC1 was expressed in medial SMCs where a lower level of αSMA was observed, indicating that BC1 inversely correlated with αSMA expression. In addition to αSMA, CNN1 expression was also suppressed by BC1 (Fig. S7A). These results showed that BC1 suppressed SMC marker gene expression in vivo.

SMCs exhibit a high rate of proliferation during embryonic development, although, in mature vessels, there is an extremely low rate of SMC proliferation (6). By quantifying the SMCs in the media, we found that BC1 overexpression increased the medial SMC numbers in the artery (Fig. 7D). In addition, artery media with BC1 expression exhibited an increased number of proliferating cell nuclear antigen (PCNA)–positive cells (Fig. 7E). However, the endothelium appeared not to be affected (Fig. 7F). To determine whether the proliferative cells are SMCs, we co-stained PCNA with αSMA and found that the PCNA+ cells expressed αSMA (Fig. S7B), indicating that BC1–expressing medial SMCs displayed less contractile but more proliferative properties.

Impaired SMC differentiation and maturation often disrupt vascular integrity, such as defective elastic lamina, abnormal
SMC investment, or disorganized extracellular matrix (ECM) (41–43). Indeed, arteries with BC1 expression exhibited random breakage and irregular distribution of elastic lamina (Fig. 7G). There was also an inordinate stack of SMCs (Fig. S8A), disorganized ECM, and excessive collagen accumulated around SMCs in the artery media, although the collagen deposition on the elastic lamina was reduced (Fig. S8B). These data indicated that BC1 adversely impacted the normal development of the vascular system.

Discussion

We have identified BC1 as a novel regulator for TGF-β-induced SMC differentiation. BC1 suppresses SMC marker gene expression via binding to Smad3, which prevents Smad3 binding to SMC marker gene promoters and thus inhibiting SMC gene transcription, leading to decreased expression of SMC contractile genes. Importantly, BC1 expression in mouse embryos via adenoviral delivery impairs SMC marker gene expression in the neonatal aorta while increasing SMC proliferation, indicating that BC1 suppresses SMC differentiation and leads to the proliferative phenotype. The impaired SMC differentiation appears to have a detrimental effect on artery structure. Mechanistically, BC1 inhibits Smad3 nuclear function by attenuating its phosphorylation and nuclear translocation.

BC1 may inhibit Smad3 nuclear translocation through different mechanisms. Smad3 phosphorylation is required for Smad3 nuclear translocation (44). Forced expression of BC1 suppresses the phosphorylation of Smad3 (Fig. 2A), indicating that Smad3 nuclear translocation is regulated, at least in part, by BC1. BC1 binds to Smad3 in the cytoplasm at the basal state to prevent Smad3 nuclear translocation (Fig. 4, A, top panels, and B). TGF-β stimulation releases Smad3 from BC1, resulting in its translocation into nuclei to activate downstream genes (Fig. 4, A, bottom panels, and B).

BC1 binds to Smad3 via its rSBEs. It is known that nuclear Smad3 binds to the SBE in target gene promoters to initiate their transcription (36). RNA molecules appear to interact with Smad3 in the same manner. rSBEs have been identified in microRNA and IncRNA and have been reported to regulate TGF-β signaling via binding to Smad3 (10, 38). Our previous studies have shown that IncRNA GAS5 binds to Smad3 via rSBEs to regulate TGF-β/Smad3 signaling. However, GAS5 does not affect Smad3 phosphorylation (10). Therefore, BC1 function represents a new mechanism by which IncRNA regulates TGF-β/Smad3 signaling by modulating Smad3 phosphorylation and nuclear translocation. Although BC1 binds Smad3 mainly through the two rSBEs in the first stem structure, it may also bind to Smad3 via other unknown sequences because mutation of both rSBE does not completely abrogate Smad3 binding to BC1 (Fig. 5, C and D). Further studies are required to test this possibility or identify these sequences.

In addition to Smad3 phosphorylation and nuclear translocation, BC1 appears to also regulate Smad3 expression because less Smad3 is observed in BC1-expressing cells (Fig. 2, A and B). Conversely, more Smad3 is present in cells with BC1 knockdown (Fig. 2, C–F). BC1 is reported to serve as a translational repressor to block the assembly of the 48S preinitiation complex via binding to eIF4A, PABP, and FMRP (21, 45). Therefore, it is possible that BC1 suppresses Smad3 expression by serving as translational repressor through a similar mechanism. Likewise, the decreased SMC marker expression may also partially be due to the BC1-mediated translational suppression. Moreover, BC1 may affect the Smad3 or SMC marker protein stability. Indeed, TGF-β signaling is regulated and terminated by the ubiquitin–proteasome system (46). IncRNAs have also been shown to either enhance or suppress targeted protein degradation via interacting with certain E3 ligases (47, 48). These potential interesting mechanisms are excellent subjects for future studies. Nevertheless, our data indicate that BC1 not only regulates the nuclear trafficking of Smad3 but also affects Smad3 gene expression.

Collectively, this is the first study to demonstrate the role of BC1 in SMC differentiation and vascular development. BC1 binds directly to Smad3 via rSBE in its 5′ stem structure and thus impedes the TGF-β/Smad3 signaling required for SMC differentiation. Blockage of SMC differentiation by BC1 in vascular development leads to abnormalities of vasculature characterized by random breaks of elastic lamina, inordinate stack of SMCs, and disorganized ECM in the artery media. Our data are consistent with the absence of vascular abnormality in BC1-deficient mice (23) because normal vascular development requires the absence of BC1 in vascular SMCs.

Experimental procedures

Reagents and cell culture

Human aortic SMCs and endothelial cells were purchased from Lonza. C3H10T1/2 (10T1/2) cells were purchased from the ATCC. Mouse aortic SMCs and endothelial cells were cultured from thoracic aorta of C57BL/6j mice. Cells were maintained in Dulbecco’s modification of Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 1% l-glutamine (Corning) at 37 °C in a humidified atmosphere with 5% CO2. Growth factors and chemicals were obtained from the following sources: TGF-β1 (R&D Systems, 240-B), Smad3 inhibitor SIS3 (Sigma-Aldrich, S0447), and recombinant human Smad3 protein (Sigma-Aldrich, SRP5132). Antibodies were purchased from various vendors: α-SMA (Abcam, ab5694), Calponin (Abcam, ab46794), SM22α (Abcam, ab10135), α-tubulin (Cell Signaling Technology, 2125), Smad3 (Cell Signaling Technology, 9523S), phospho-Smad3 (Cell Signaling Technology, 9520S), GAPDH (Sigma-Aldrich, G8795), and PCNA (Santa Cruz Biotechnology, sc-56).

Construction of adenovirus

The cDNA fragment encoding the full length of mouse BC1 was amplified from 10T1/2 cells by PCR and then inserted into the pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and HindIII sites. The BC1 RNA (shBC1) was inserted into the pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and HindIII site. The primers used to amplify mouse BC1 were the pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and HindIII site. The BC1 RNA (shBC1) was inserted into the pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and HindIII site. The BC1 RNA (shBC1) was inserted into the pRNAT-H1.1/Adeno vector (Genscript) between the Mlu I and HindIII site.

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Amine without FBS for 24 h and then treated with TGF-

The adenoviral vector of BC1 and shBC1 was

ng/ml) or vehicle, as indicated, for 1 h to detect phospho-

(H11032)

expression was normalized to U6. The primer sequences were

sion was normalized to cyclophilin (CYP), and LncRNA BC1

master mix (Agilent Technologies). Each sample, including the

on a Stratagene Mx3005P qPCR instrument using SYBR Green

The adenovirus was purified by gradient density ultracentrifugation of cesium chloride followed by dialysis in dialysis buffer

The ChIP assay was performed with modifications using a ChIP assay kit (EMD Millipore, 17-295). 10T1/2 cells were fixed with 1% formaldehyde at room temperature for 15 min and then incubated with 0.2 m glycine at room temperature for additional 5 min. Cells were scraped off and then lysed in SDS lysis buffer containing protease and phosphatase inhibitors at 4 °C for 10 min. Cell lysates were sonicated for 5 rounds of 50% protein levels were detected with enhanced chemiluminescence (Millipore).

Real-time quantitative PCR (qPCR)

Total RNA of cultured cells was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). qPCR was performed on a Stratagene Mx3005P qPCR instrument using SYBR Green master mix (Agilent Technologies). Each sample, including the no-template control, was amplified in triplicate. mRNA expression was normalized to cyclophilin (CYP), and LncRNA BC1 expression was normalized to U6. The primer sequences were

10T1/2 cells were seeded on gelatin-coated coverglasses using gelatin-based coating solution (Cell Biologics, 6950). Cells were transduced with either Ad-Ctrl, Ad-BC1, or Ad-shBC1 for 2 days. The culture medium was then replaced with starving DMEM (no FBS, 5% L-glutamine) when the cell density reached 50% confluence. After starvation for 24 h, cells were treated with vehicle or TGF-β (5 ng/ml) for an additional 48 h. Cells were then fixed with 1% formaldehyde, and cell morphology was observed using a Nikon Eclipse 90i microscope.

Cell fractionation

Cell fractionation was performed using a nuclear extraction kit (EMD Millipore, 2900) by following the procedure recommended by the manufacturer. Briefly, 10T1/2 cells were washed with cold PBS and then scraped off. Cell pellets were collected through centrifugation at 250 × g for 5 min. The cell pellets were resuspended with ice-cold cytoplasmic lysis buffer and incubated on ice for 15 min. Cell lysates were passed through a syringe with a 27-gauge needle 10 times, followed by centrifugation at 8000 × g at 4 °C for 20 min to separate the cytoplasmic fraction (supernatant) and nuclear fraction (pellet). The pellet containing the nuclear fraction was then lysed with RIPA buffer containing protease and phosphatase inhibitors. The protein concentrations of both cytoplasmic and nuclear fractions were measured using BCA protein assay reagent (Thermo Scientific).

Western blot analysis

10T1/2 cells were starved in DMEM containing 5% L-glutamine without FBS for 24 h and then treated with TGF-β (5 ng/ml) or vehicle, as indicated, for 1 h to detect phospho-Smad3 or for 24 h to detect Smad3, αSMA, CNN1, and SM22α protein levels. Cells were infected with adenovirus or transfected with plasmid before starvation and further treatment. The cells were then washed with PBS twice, followed by RIPA protein extraction using RIPA buffer (50 mmol/liter Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% (w/v) sodium deoxycholate, 150 mmol/liter NaCl, 1 mmol/liter EGTA, protease inhibitors (Thermo Scientific), phosphatase inhibitors (Thermo Scientific), and 0.1% SDS). Protein concentration was measured using BCA protein assay reagent (Thermo Scientific). Equal amounts of proteins were resolved on SDS-PAGE and then transferred to polyvinylidene fluoride (Bio-Rad) or nitrocellulose membranes (Bio-Rad). Nonspecific bindings were blocked with 5% BSA in Tris-buffered saline (TBS) plus 0.1% Tween 20 (TBST) at 4 °C for 1 h and then incubated with primary antibodies in blocking buffer at 4 °C for 16 h. After washing three times with TBST, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody (Sigma) at room temperature for 1 h. Following three washes with TBST, the

IF

10T1/2 cells or artery cryosections (10 μm) were fixed with methanol/acetone (1:1) at −20 °C for 15 min. Fixed cells or sections were then washed with PBS three times, followed by blocking with 10% goat serum at room temperature for 30 min. After blocking, cells or sections were incubated with primary antibody (Smad3, αSMA, or PCNA) at 4 °C overnight. The next day, cells or sections were washed with PBST three times, followed by secondary antibody incubation at 37 °C for 30 min. Then sections were washed with PBST three times followed by counterstaining with DAPI. The cell and cross-sectional images were captured using a Nikon Eclipse 90i microscope.

ChIP assay

The ChIP assay was performed with modifications using a ChIP assay kit (EMD Millipore, 17-295). 10T1/2 cells were fixed with 1% formaldehyde at room temperature for 15 min and then incubated with 0.2 m glycine at room temperature for additional 5 min. Cells were scraped off and then lysed in SDS lysis buffer containing protease and phosphatase inhibitors at 4 °C for 10 min. Cell lysates were sonicated for 5 rounds of 50% power to shear the nucleic acids, followed by collecting the supernatant. Cell supernatants were diluted with ChIP dilution buffer containing protease and phosphatase inhibitors. After preclearing cell supernatants with 50 μl of protein A/G–
agarose beads (Santa Cruz Biotechnology, sc-2003) at 4 °C for 4 h, cell supernatants were incubated with rabbit IgG (negative control) or Smad3 antibodies at 4 °C overnight. The input sample (10%) was collected after the pre-clearing step. The next day, the DNA–protein complex was captured by 50 μl of protein A/G–agarose beads at 4 °C for 2 h, followed by serial wash steps with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer, and Tris borate-EDTA buffer. Each wash step was repeated three times. After the washing, protein A/G–agarose beads were incubated with freshly prepared elution buffer (1% SDS and 0.1 M NaHCO3) at 37 °C for 15 min to elute the DNA–protein complex. Elutes containing DNA–protein complex were added to 5 M NaCl and incubated at 65 °C for 4 h. Then, elutes were added to EDTA, 1 M Tris-HCl (pH 6.5), and Proteinase K and incubated at 45 °C for 1 h. DNA was recovered via phenol/chloroform extraction and ethanol precipitation. Purified DNA was then examined via PCR to measure the enrichment of αSMA and SM22α gene promoter regions containing the SBE. Primer sequences used were as follows: αSMA-SBE, 5'-GTT CCA AGC AGA C-3' (forward) and 5'-CCA GTA AAT CAA GCG TTG TT-3' (reverse); SM22α-SBE, 5'-GTT CCA GCG TTG TT-3' (forward) and 5'-CGA GTA AAT CAA GCG TTG TT-3' (reverse); and BC1-T3-FL, 5'-GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A-3' (forward); BC1-T3-F1-mt2, 5'-GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A-3' (reverse). Different DNA templates were amplified by using different combinations of primer sets: BC1-FL template, BC1-T7-FL, and BC1-T3-FL; BC1-F1 template, BC1-T7-FL, and BC1-T3-F1; BC1-F1-mt1 template, BC1-T7-F1-mt1 and BC1-T3-F1; BC1-F1-mt2 template, BC1-T7-FL and BC1-T3-F1-mt2; BC1-F1-mt12 template, BC1-T7-F1-mt1 and BC1-T3-F1-mt2.

Luciferase reporter assay

10T1/2 cells were transduced with either Ad-Ctrl, Ad-BC1, or Ad-shBC1 for 2 days. Then the cells were reseeded into a 12-well cell culture plate. The next day, the cells were transfected with plasmids containing a firefly luciferase reporter driven by either an αSMA, SM22α, or SBE (SBE-Luc) promoter via Lipo-fectamine LTX (Thermo Fisher Scientific, 15338100) for 6 h. Then cells were starved overnight, followed by TGF-β (5 ng/ml) treatment for an additional 16 h. Luciferase activity was measured by Dual-Luciferase reporter assay system (Promega, E1910).

In vitro transcription

DNA templates of BC1 FL, BC1 F1, and the F1 fragment with mutated rSBE (mt1, mt2, and mt12) driven by the T7 (sense strand) or T3 (antisense strand) promoter were amplified by PCR. In vitro transcription was performed using the T7 or T3 RNA polymerase kit (Roche) and following the protocol provided by the manufacturer. Briefly, 1 μg of DNA template was used in the reaction containing 40 units/ml RNase inhibitor (RNasin) (Sigma-Aldrich, R1158). The reaction was then incubated at 37 °C for 1 h, followed by DNase I treatment. Synthesized RNA was purified using the RNeasy Mini Kit (Qiagen, 74104). BC1 synthesized from the in vitro transcription was labeled with biotin (Thermo Fisher Scientific, AM8452) for a pulldown assay or fluorescein (Roche, 11427857910) for a FISH assay. The primer sequences used in this experiment were as follows: BC1-T7-FL, 5'-GAG TAA TAC GAC TCA CTA TAG GGA AGG GGT TGG GGA TTT AGC T-3' (forward); BC1-T3-FL, 5'-GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A-3' (reverse); BC1-T3-F1, 5'-GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A-3' (reverse); and BC1-T7-F1-mt1, 5'-GAG TAA TAC GCA TCT TAG GGA AGG TTT GGG ATT TAG CTT GGT GGT A-3' (forward); BC1-T3-F1-mt2, 5'-GAG ATT AAC CCT CAC TAA AGG GAT TAG AGC TAA TAC GAC TCA CTA TAG GGA AGG TTG GGG ATT TAG CTT GGT GGT A-3' (reverse). Different DNA templates were amplified by using different combinations of primer sets: BC1-FL template, BC1-T7-FL, and BC1-T3-FL; BC1-F1 template, BC1-T7-FL, and BC1-T3-F1; BC1-F1-mt1 template, BC1-T7-F1-mt1 and BC1-T3-F1; BC1-F1-mt2 template, BC1-T7-FL and BC1-T3-F1-mt2; BC1-F1-mt12 template, BC1-T7-F1-mt1 and BC1-T3-F1-mt2.

FISH

FISH was performed as described previously (50). 10T1/2 cells or artery cryosections (10 μm) were fixed with methanol/acetone (1:1) at −20 °C for 15 min. Fixed cells or sections were then washed four times with PBST containing 40 units/ml RNasin, followed by washing with 1× PBST/hybridization buffer (50% formamide, 5× standard saline citrate, 100 μg/ml fragmented salmon testis DNA, 50 μg/ml heparin, 0.1% Tween 20, and 40 units/ml RNasin) for 10 min. Then cells or sections were incubated with hybridization buffer in serial steps at 50 °C for 5, 30, and 30 min. The BC1 RNA probe in hybridization buffer was prepared at a final concentration of 1 μg/ml. The BC1 RNA probe labeled with fluorescein was synthesized via in vitro transcription as described above. The BC1 RNA probe was denatured at 80 °C for 2 min and then placed on ice before use. Fixed cells and sections were incubated with the denatured BC1 RNA probe in a dark and humid environment at 50 °C overnight. Negative control was performed with the same procedure without the BC1 RNA probe in hybridization buffer. The next day, cells or sections were incubated with hybridization buffer in serial steps at 50 °C for 5, 30, and 30 min, followed by washing with 1× PBST/hybridization buffer for an additional 10 min. Cells or sections were then washed four times with PBST containing 40 units/ml RNasin. To perform the co-staining procedure, cells or sections were blocked with 10% goat serum at room temperature for 30 min. After blocking, cells or sections were incubated with Smad3 primary antibody containing 40 units/ml RNasin at 4 °C overnight. The next day, cells or sections were washed three times with PBST containing 40 units/ml RNasin, followed by incubation for 30 min with secondary antibody containing 40 units/ml RNasin at 37 °C. Then cells or sections were washed three times with PBST containing 40 units/ml RNasin, followed by counterstaining with DAPI. The cell and cross-sectional images were captured using a Nikon Eclipse 90i microscope.

RIP assay

The RIP assay was performed with modifications as described previously (10). Briefly, 10T1/2 cells were fixed with 1% formaldehyde at room temperature for 15 min and then incubated with 0.2 mM glycine at room temperature for an additional 5 min. Cells were scraped off and then lysed in FA lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, and 0.1% (w/v) sodium deoxycholate) containing 40 units/ml RNase inhibitor (RNasin, Sigma-Aldrich, R1158) and 1× Halt™ protease inhibitor mixture (Thermo Scientific). After incubation at 4 °C for 1 h, cell lysates were passed through
The RNA–protein complex was incubated with 5M NaCl for 10 min to elute the RNA–protein complex from beads. After preclearing cell supernatants with 50 μl of protein A/G–agarose beads (Santa Cruz Biotechnology, sc-2003) at 4 °C for 4 h, cell supernatants were incubated with rabbit IgG (negative control) or Smad3 antibodies at 4 °C overnight. The input sample (10%) was collected after the preclearing step. The next day, the RNA–protein complex was captured by 50 μl of protein A/G–agarose beads at 4 °C for 2 h, followed by serial washing with FA lysis buffer, FA500 (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, and 0.1% (w/v) sodium deoxycholate), LiCl washing buffer (10 mM Tris/Cl, 250 mM LiCl, 0.5% (v/v) Nonidet P-40, 0.1% (w/v) sodium deoxycholate, and 1 mM EDTA), and TE/100 mM NaCl (10 mM Tris/Cl, 1 mM EDTA, and 100 mM NaCl). Each washing buffer was added to 40 units/ml RNasin before use, and each wash step was repeated three times. After washings, protein A/G–agarose beads were incubated with RIP elution buffer (100 mM Tris/Cl, 10 mM EDTA, and 1% (w/v) SDS) at 37 °C for 10 min to elute the RNA–protein complex from beads. The RNA–protein complex was incubated with 5 M NaCl together with Proteinase K at 42 °C for 1 h and then at 65 °C for an additional 1 h. The immunoprecipitated RNA was purified using a RNeasy Mini Kit (Qiagen, 74104). Purified RNA was analyzed by qRT-PCR for measuring IncRNA enrichment.

**Biotin-avidin pulldown assay**

The procedure was performed as in a previous study (10). Biotin-labeled FL BC1, F1, or F1 with mutation at either one (mt1, mt2) or both RsBE sites (mt12) was synthesized via in vitro transcription as described above. For the in vitro binding assay, different amounts of synthesized RNA (0, 100, 200, or 400 ng) were incubated with 400 ng of recombinant human Smad3 (rhSmad3) in 50 μl of wash/binding buffer (PBS with 0.1% SDS, 1% Nonidet P-40, and 40 units/ml RNasin) at 4 °C overnight, followed by the pulldown procedure. For the in-cell binding assay, 10T1/2 cells were transfected with 1 μg of synthesized RNA via Lipofectamine LTX overnight, followed by fixation with 1% formaldehyde. Negative control for the binding between nucleotides and Smad3 was performed with the same procedure, using a biotin-labeled random nucleotide sequence that was not overlapped with the mouse genome, as examined by the Basic Local Alignment Search Tool provided by the National Center for Biotechnology Information. The random nucleotide fragment sequence was 5’-Bio-ATC GTT TCC GCT TAA CGG CG-biotin-3’. Cells were then lysed with FA lysis buffer containing protease inhibitor and 40 units/ml RNasin, followed by DNase I treatment and then the pulldown procedure. For the pulldown procedure, the BC1–Smad3 mixture and cell lysates were precleared with Pierce control agarose resin (Thermo Fisher Scientific, 26150) at 4 °C for 2 h, followed by incubation with Pierce streptavidin–agarose (Thermo Fisher Scientific, 20347) for BC1 pulldown or Pierce control agarose resin for negative control at 4 °C for an additional 2 h. The input sample (10%) was collected after the preclearing step. After washing four times with washing/binding buffer and twice with RIPA buffer containing 40 units/ml RNasin, the bead–RNA–protein complex was collected and lysed in RIPA buffer containing protease inhibitors. Protein concentration was measured using BCA protein assay reagent (Thermo Scientific), and the abundance of Smad3 was measured via Western blot analysis.

**Viral inoculation of mouse embryos**

C57BL/6J mice were housed under conventional conditions in animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Two-month-old C57BL/6J mice were mated to produce embryos for viral inoculation. Females were checked every morning to see whether there was a vaginal plug. The day when a plug was found was considered E0.5. Pregnant C57BL/6J mice with E12.5 embryos were anesthetized by isoflurane inhalation. Adenovirus administration to mouse embryos was performed with modifications as in a previous study (40). A glass micropipette needle (diameter, 50–100 μm) was used to inject adenovirus into the embryo. The labyrinth zone of the embryo placenta was injected with 5 μl of Ad-Ctrl or Ad-BC1 (1 × 10^9 pfu). To prevent unnatural abortion, we avoided viral inoculation into the embryos next to ovaries and the upper vagina. Pups were euthanized on the day of birth by CO2 inhalation (~2 liters/min). Thoracic aortae of pups were then perfused with saline, fixed with 4% formaldehyde, dehydrated with 15% and 30% sucrose prepared in PBS, and embedded in optimal cutting temperature (OCT) compound for further sectioning and subsequent morphometric analyses.

**Histomorphometric analysis**

Newborn mouse aorta cryosections (10 μm) were fixed with 1% formaldehyde and hydrated with PBS. The sections were then stained with either modified hematoxylin and eosin (American MasterTech, KTHNEPT), Elastica van Gieson (Electron Microscopy Sciences, 26350), or Masson’s trichrome staining reagents (American MasterTech, KTMR2). The procedure was performed by following protocols provided in each kit. The cross-sectional images were captured using a Nikon Eclipse 90i microscope.

**Predicted secondary structure of BC1**

The *Mus musculus* BC1 secondary structure was predicted based on minimum free energy structure (51). The schematic secondary structure was then illustrated and modified via the RNAViz2 program (52).

**Statistical analysis**

Results are presented as mean ± S.D. Comparison between two groups was evaluated with two-tailed independent Student’s *t* test. Comparison among more than two groups was evaluated by one-way analysis of variance followed by Fisher’s least significant difference test. *p* < 0.05 was considered statistically significant.
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