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Original Article

Identification of Long Noncoding RNAs Involved in Differentiation and Survival of Vascular Smooth Muscle Cells

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Long noncoding RNAs (lncRNAs) have recently been implicated in many pathophysiological cardiovascular processes, including vascular remodeling and atherosclerosis. However, the functional role of lncRNAs in the differentiation, proliferation, and apoptosis of vascular smooth muscle cells (VSMCs) is largely unknown. In this study, differentially expressed lncRNAs in synthetic and contractile human VSMCs were screened using RNA sequencing. Among the seven selected lncRNAs, the expression of *MSC-AS1*, *MBNL1-AS1*, and *GAS6-AS2* was upregulated, whereas the expression of *NR2F1-AS1*, *FUT8-AS1*, *FOXC2-AS1*, and *CTD-2207P18.2* was reduced upon VSMC differentiation. We focused on the *NR2F1-AS1* and *FOXC2-AS1* lncRNAs and showed that their knockdown significantly reduced the expression of smooth muscle contractile marker genes (*ACTA2*, *CNN1*, and *TAGLN*). Furthermore, *FOXC2-AS1* was found to regulate cell proliferation and apoptosis through Akt/mTOR signaling, and affect Notch signaling, which is a key regulator of the contractile phenotype of VSMCs. Taken together, we identified novel lncRNAs involved in VSMC proliferation and differentiation and *FOXC2-AS1* as a multifunctional regulator for vascular homeostasis and associated diseases.

INTRODUCTION

Cardiovascular diseases (CVDs), including hypertension, arteriosclerosis, and vascular stenosis, are closely related to vascular smooth muscle cell (VSMC) dysfunction.[1–3] VSMCs can change their phenotype in response to various stimuli such as growth factors (platelet-derived growth factor [PDGF], transforming growth factor β [TGF-β], basic fibroblast growth factor), cytokines (interleukin-10, tumor necrosis factor alpha), and other factors such as serum depletion and vascular injury.[4] When these stimuli are applied, smooth muscle cells (SMCs) convert between the synthetic and contractile phenotypes. Phenotypic modulation of VSMCs is a key factor in understanding the mechanism of cardiovascular development and CVDs.[5]

Through the activation of SMADs, TGF-β promotes the expression of VSMC differentiation markers such as smooth muscle calponin (*CNN1*), α-smooth muscle actin (α-SMA or *ACTA2*), transgelin (*TAGLN* or smooth muscle 22-alpha), and smooth muscle myosin heavy chain (*MYH11*), which regulate smooth muscle contractility.[6,7] Similar to TGF-β, serum deprivation not only causes growth arrest but also the differentiation of cultured SMCs.[8,9] On the contrary, PDGF and vascular injury promote the proliferation and migration of cultured VSMCs.[10] Differentiation marker genes generally have various binding elements and enhancers in their promoter that can be regulated at the transcriptional level.[11–13] The Smad binding element (SBE) is the region where phosphorylated SMADs bind, and myocardin (MYOCD), the transcriptional coactivator of serum response factor (SRF), interacts with myocardin-related transcription factor A (MRTFA) and binds the serum response element (SRE) on the promoter of target genes.

Many studies have reported that noncoding RNAs play important roles in vascular biology, including vascular development and CVDs.[14–17] Long noncoding RNAs (lncRNAs) are transcripts of more than 200 nt that do not encode proteins and are known to be involved in a variety of biological processes, including the cardiovascular system. Recent studies have shown that *SMILR* (smooth muscle-induced lncRNA enhances replication) is highly expressed in unstable plaques of atherosclerosis and promotes abnormal proliferation of VSMCs by regulating the *CENPE* mRNA.[18,19] Moreover, nuclear enriched abundant transcript 1 (*NEAT1*), which is widely expressed in many cancers and other diseases, has been reported to regulate the expression of smooth muscle-specific genes by modulating histone methylation.[20] However, the lncRNAs and their molecular function in the phenotypic modulation of VSMCs is not yet fully understood.

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In this study, we screened lncRNAs involved in differentiation, proliferation, and apoptosis in a serum deprivation-induced VSMC differentiation model. We found that *NR2F1-AS1* and *FOXC2-AS1* can modulate the contractility of SMCs through the regulation of contractile gene expression. The deficiency of *FOXC2-AS1* inhibits the proliferation of VSMCs and induces apoptosis.

RESULTS

Identification of lncRNAs Involved in the Phenotypic Change of VSMCs

To select lncRNAs involved in the VSMC phenotype changes, we used a previously reported model for VSMC differentiation.[21] Most studies have used serum deprivation or growth factors such as TGF-β1 and PDGF to differentiate VSMCs.[8,9,22] Using a differentiation medium (DM) with reduced serum, we differentiated synthetic VSMCs to contractile VSMCs that are characterized by increased expression of differentiation markers such as *ACTA2*, *TAGLN*, and *CNN1*. RNA sequencing (RNA-seq) was performed on synthetic and contractile VSMCs (Figure 1A). We selected 116 lncRNAs that were differentially expressed during VSMC differentiation based on several criteria (see Materials and Methods). Finally, seven candidate lncRNAs (*NR2F1-AS1*, *FUT8-AS1*, *FOXC2-AS1*, *CTD-2207P18.2*, *MSC-AS1*, *GAS6-AS2*, and *MBNL1-AS1*) were selected by combining our previously reported data on muscle differentiation-related lncRNAs[21] with the current RNA-seq results (Figure 1B). During the VSMC differentiation, the expression of *NR2F1-AS1*, *FUT8-AS1*, *FOXC2-AS1*, and *CTD-2207P18.2* was decreased, whereas the expression of *MSC-AS1*, *GAS6-AS2*, and *MBNL1-AS1* was increased (Figure 1C).

The abnormally activated proliferation and inflammation in VSMCs are characteristic of vascular pathologies such as atherosclerosis and neointimal hyperplasia.[5] Thus, to check whether the expression of the selected lncRNAs correlates with their expression in atherosclerosis progression samples, we obtained the relevant RNA-seq data from the GEO database (GEO: GSE120521),[19] which compared the transcriptomes of stable and unstable plaques in atherosclerosis. We found that among our seven candidate lncRNAs with meaningful expression in both RNA-seq data, five lncRNAs (*NR2F1-AS1*, *FUT8-AS1*, *MSC-AS1*, *MBNL1-AS1*, and *GAS6-AS2*) showed an inverse pattern of expression change between the two datasets, similar to the expression of contractile marker genes (Figures S1A and S1B). For example, one of the lncRNAs that showed the most dramatic inverse expression pattern between VSMC differentiation and atherosclerosis, cardiac mesoderm enhancer-associated noncoding RNA (*CARMN*) has been reported to regulate smooth muscle differentiation as well as cardiac differentiation and specification (Figure S1B).[23–25] These analyses showed that our candidate lncRNAs may be involved in the VSMC differentiation and could be associated with atherosclerosis.

Characterization of lncRNAs Involved in the Phenotypic Change of VSMCs

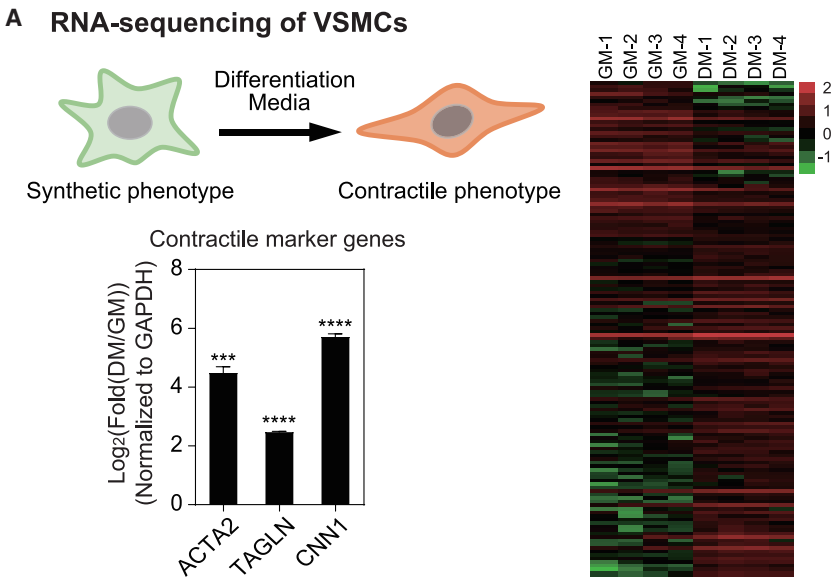
To confirm the expression pattern of our candidate lncRNAs, which were selected based on RNA-seq, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on primary human coronary artery SMCs (HCASMCs). We verified that the candidate lncRNAs were differentially expressed in the same pattern as that observed by RNA-seq (Figure 2A). In addition, when the T/G HA-VSMC cell line, derived from the human aorta, was differentiated by serum deprivation, the candidate lncRNAs except *FUT8-AS1* were differentially expressed, consistent with the above results (Figure 2B).

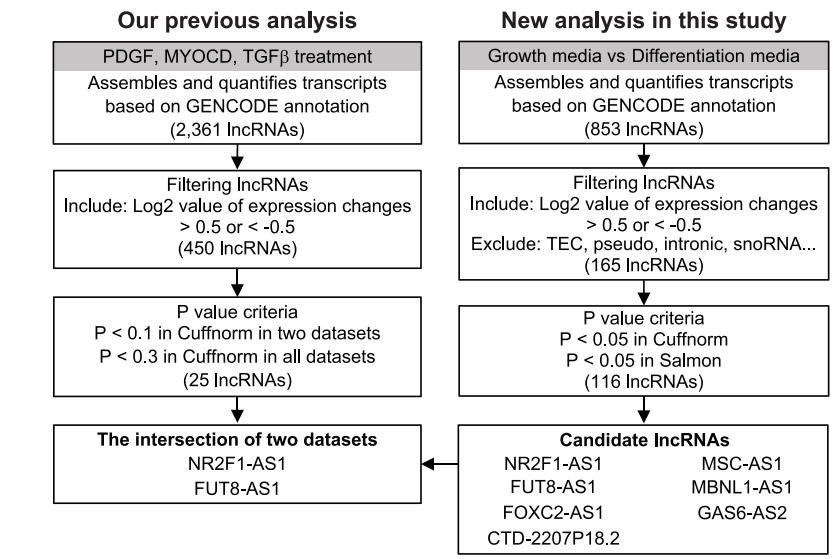
Next, tissue- and cell type-specific expression patterns of the candidate lncRNAs were examined through the GTEx portal (https://gtexportal.org/home/)[26] and measured in several cell lines (Figures S2 and S3). *FOXC2-AS1*, *GAS6-AS2*, *MBNL1-AS1*, *MSC-AS1*, and *CTD-2207P18.2* were highly expressed in blood vessels, including the aorta, coronary artery, and the tibial artery. *NR2F1-AS1* and *FUT8-AS1* were highly expressed in the nervous system and neuroblastoma cell lines, as well as in blood vessels. Recent studies have shown that *NR2F1-AS1* regulates neuronal development and that *FUT8-AS1* is highly expressed in glioblastomas,[27–29] suggesting that they have functions not only in blood vessels but also in various tissues, including the nervous system.

The mechanism of action of lncRNAs is known to depend on their subcellular localization.[30] To investigate the subcellular distribution of lncRNAs, qRT-PCR was performed after subcellular fractionation of differentiated and undifferentiated VSMCs (Figure 2C). The results revealed that *FOXC2-AS1* was mainly distributed in the cytoplasm, while *CTD-2207P18.2* localized to the nucleus. *MSC-AS1*, *MBNL1-AS1*, *GAS6-AS2*, and *NR2F1-AS1* were distributed in both the nucleus and the cytoplasm. Furthermore, the subcellular distribution of these lncRNAs did not change significantly after differentiation of HCASMCs (Figure 2C).

Some lncRNAs were known to encode micropeptides.[31,32] To exclude this possibility, we analyzed the coding potential of candidate lncRNAs based on bioinformatics analyses (Figure 2D). We verified that six candidate lncRNAs, except *MBNL1-AS1*, have no possibility of encoding protein.

We also investigated whether the candidate lncRNAs were conserved in the mouse genome. Since the genomic loci of lncRNAs are often conserved across species, we identified six noncoding transcripts that share the same gene neighborhood as our lncRNAs, and tested their coding potential (Figure S4A). Cell type-specific expression patterns of these noncoding transcripts were tested in several cell lines as shown in Figure S4B. We also observed that the expression of *Nr2f1-as1* and *Msc-as1* increased while that of *D030025P21Rik* and *Gm48709* decreased in the later stage of myoblast differentiation (Figures S4C and S4D). To analyze the differential expression of these noncoding transcripts during the differentiation of mouse SMCs, we treated a mouse aorta-derived cell line, MOVAS, with TGF-β1 and examined the expression of contractile markers and conserved lncRNA transcripts (Figure S4E). The lncRNAs showed the same pattern of expression change between TGF-β1-treated MOVAS cells and HCASMCs, respectively (Figures S4E and S4F). Finally, we selected two lncRNAs,





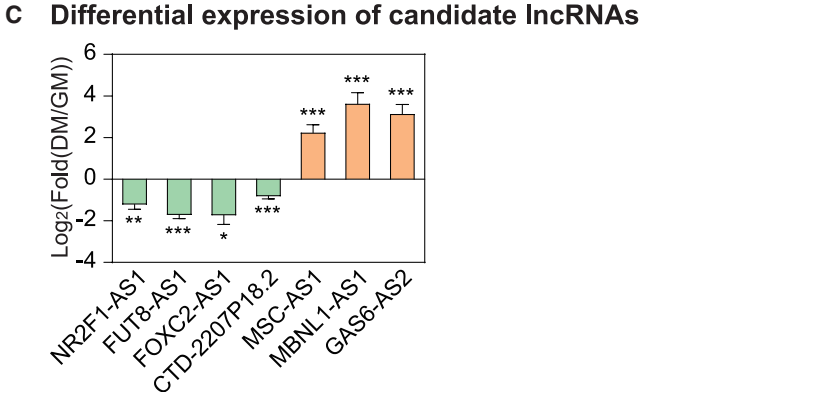


Figure 1. Identification of lncRNAs Involved in the Phenotypic Change of VSMCs

(A) RNA sequencing of VSMCs with synthetic and contractile phenotype. Quantitative RT-PCR (qRT-PCR) measurement of expression of contractile marker genes (*ACTA2*, *TAGLN*, and *CNN1*) in HCASMCs during differentiation (n = 3). The expression of contractile marker genes was normalized to *GAPDH*. RNA sequencing was performed using these samples, and the result is depicted as a heat map after hierarchical clustering. GM, growth medium; DM, differentiation medium. (B) The procedure and criteria to select candidate lncRNAs associated with the phenotypic change in VSMCs. (C) Log_2(fold change) values for the fragments per kilobase of transcript per million mapped reads (FPKM) of differentially expressed lncRNAs during VSMC differentiation (n = 4). Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

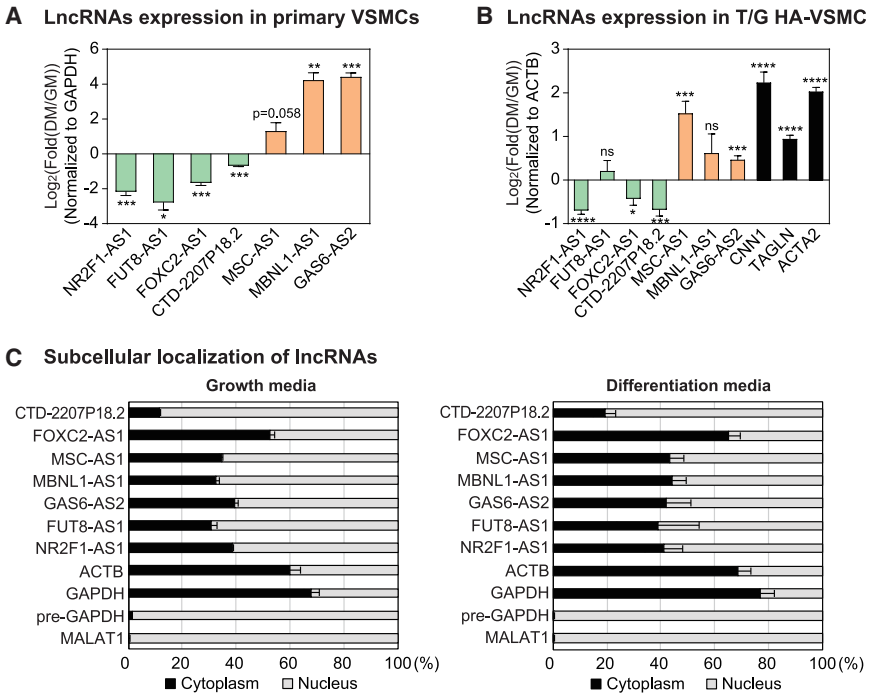


Figure 2. Characterization of lncRNAs Involved in the Phenotypic Change of VSMCs

(A) qRT-PCR measurement of the expression of candidate lncRNAs and contractile marker genes in HCASMCs differentiated for 3 days (n = 3). The expression of lncRNAs was normalized to *GAPDH*. (B) qRT-PCR measurement of the expression of candidate lncRNAs and contractile marker genes in T/G HA-VSMCs differentiated for 3 days (n = 3). The expression of lncRNAs and contractile marker genes was normalized to *ACTB*. (C) Subcellular localization of lncRNAs in HCASMCs cultured in growth media or differentiation media (n = 3). Pre-*GAPDH* and *MALAT1* were used as controls for nuclear RNA, and *GAPDH* and *ACTB* were used as controls for cytoplasmic RNA. (D) The protein-coding potential of lncRNAs. The protein-coding potential of lncRNAs was assessed by CPC 2.0 and CPAT. *GAPDH* was used as a control for the protein-coding gene, and *MALAT1* was used as a control for the noncoding gene. Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

D Protein-coding potential of lncRNAs

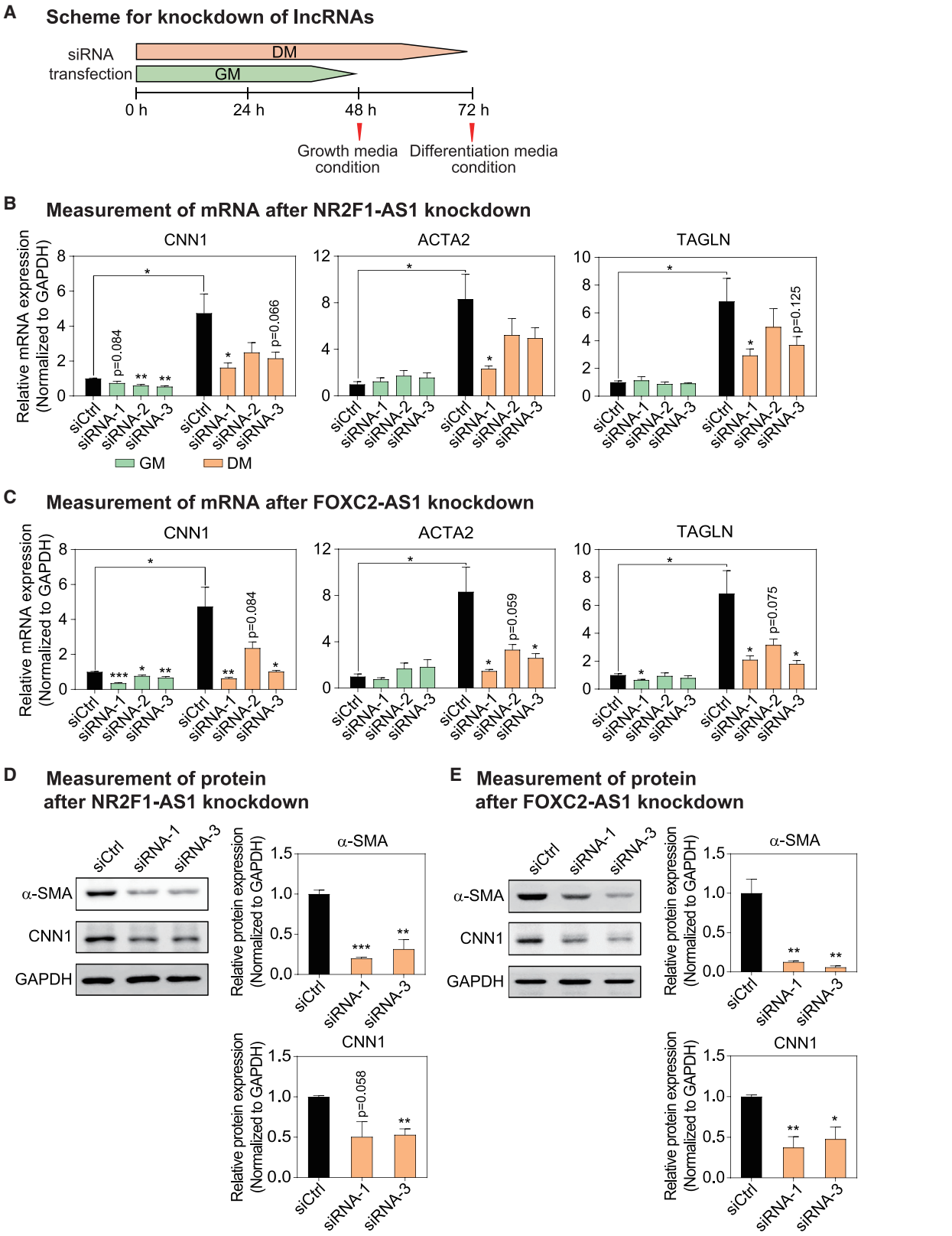
Gene Name	CPC 2.0	CPAT
NR2F1-AS1	0.13449	0.05109
FUT8-AS1	0.16685	0.32055
FOXC2-AS1	0.04192	0.04393
CTD-2207P18.2	0.05184	0.04216
MSC-AS1	0.04951	0.00252
MBNL1-AS1	0.21895	0.40595 (yes)
GAS6-AS2	0.04998	0.10537
MALAT1	0.04383	0.01426
GAPDH	0.99999 (coding)	0.99996 (yes)

NR2F1-AS1 and *FOXC2-AS1*, because they are not protein-coding genes, *NR2F1-AS1* showed high conservation among species and high expression in VSMCs, and *FOXC2-AS1* is a human-specific lncRNA with high expression in VSMCs. Moreover, in the genomic locus, *FOXC2-AS1* lncRNA has a neighboring protein-coding gene with a possible function in VSMCs (see below).

FOXC2-AS1 and *NR2F1-AS1* Regulate VSMC Differentiation by Modulating the Expression of Contractile Markers

To investigate the function of lncRNAs in VSMC differentiation, *NR2F1-AS1* and *FOXC2-AS1* were silenced using three small interfering RNAs (siRNAs) for each lncRNA. The knockdown was performed for 48 and 72 h in growth medium (GM) and DM, respectively (Figure 3A). Three different siRNAs for each of *NR2F1-AS1* and *FOXC2-AS1* were used. Since *NR2F1-AS1* has diverse isoforms, each siRNA was designed to silence all (siRNA-1) or most (siRNA-2 and siRNA-3) isoforms, respectively (Figure S5A). When the siRNAs were transfected, *NR2F1-AS1* and *FOXC2-AS1* expression decreased significantly in both GM and DM (Figures S5B and S5C). The increased mRNA levels of contractile markers during differentiation were significantly attenuated after silencing *NR2F1-AS1* (Figure 3B). The knockdown of *FOXC2-AS1* also resulted in the reduction of contractile markers during differentiation (Figure 3C). The reduction of these contractile markers was also validated at the protein level (Figures 3D and 3E). To determine the effects of *NR2F1-AS1* and *FOXC2-AS1* overexpression on the expression of contractile marker genes, we prepared plasmids containing the lncRNA sequences. To construct *NR2F1-AS1*-expressing plasmid, the isoform that contains most of the common exons among the diverse isoforms was selected (the third isoform in Figure S5A). Overexpression of *NR2F1-AS1* and *FOXC2-AS1* resulted in a significant increase of contractile marker genes in HCASMCs (Figure S6A). Additionally, we used the CRISPR (clustered regularly interspaced short palindromic repeats) activation system to confirm these results. After selecting a single guide RNA that shows high overexpression efficiency in HeLa cells, we activated the transcription of endogenous lncRNAs in HCASMCs (Figures S6B and S6C). The induction of endogenous *NR2F1-AS1* and *FOXC2-AS1* enhanced the expression of contractile marker genes in HCASMCs (Figure S6D). Thus, these results show that *NR2F1-AS1* and *FOXC2-AS1* can regulate the expression of differentiation markers in VSMCs.

The differentiation of VSMCs results in the change of cellular phenotypes such as contractility. To test whether *FOXC2-AS1* and *NR2F1-AS1* affect contractility of VSMCs, we performed



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immunofluorescence staining and a contraction assay. From the immunostaining data, we confirmed the decreased expression of CNN1, the contractility marker, in HCASMCs when *NR2F1-AS1* and *FOXC2-AS1* were inhibited (Figure 4A). For the collagen gel contraction assay, we treated siRNAs against *FOXC2-AS1* or *NR2F1-AS1* in HCASMCs for 72 h, and equal amounts of transfected cells were mixed with collagen. The size of the gel discs was measured for 3 days. Compared to cell-free gels, gel contraction occurred in all cell-containing gels and was significantly reduced when *FOXC2-AS1* or *NR2F1-AS1* was depleted (Figures 4B and 4C). Taken together, these results suggest that *FOXC2-AS1* and *NR2F1-AS1* influence the differentiation and contractility of VSMCs possibly by regulating the expression of differentiation genes.

FOXC2-AS1 Regulates Proliferation and Apoptosis of VSMCs through the Akt/mTOR Signaling Pathway

Several studies on phenotypic switching in SMCs have shown that the expression of differentiation markers reduces during cell proliferation.[17,18,33] Thus, we hypothesized that since *FOXC2-AS1* and *NR2F1-AS1* regulate the phenotypic switching of VSMCs, they may also affect cell proliferation. To test whether silencing of these lncRNAs can alter the proliferation of VSMCs, cell counting and cell viability assays were performed on HCASMCs. Unexpectedly, these experiments showed that the deficiency of *FOXC2-AS1* inhibited VSMC proliferation (Figures 5A and 5B), suggesting that *FOXC2-AS1* can also regulate the survival and maturation as well as the differentiation of VSMCs. However, knockdown of *NR2F1-AS1* did not affect the proliferation of VSMCs (Figure S7). Based on these results, we tested whether *FOXC2-AS1* can affect the survival and apoptosis of VSMCs. Interestingly, the deficiency of *FOXC2-AS1* led to the apoptosis of VSMCs during differentiation (Figure 5C). In addition, *FOXC2-AS1* deficiency inhibited the phosphorylation of AKT and mTOR, and it increased the cleavage of caspase-3 and PARP, which are markers of apoptosis (Figure 5D), suggesting that apoptosis and survival of VSMCs can be regulated by *FOXC2-AS1* via Akt/mTOR signaling.

Analysis of the Regulatory Network of lncRNAs

Many lncRNAs have been reported to act as *cis*-acting regulators of their neighboring genes.[34,35] Accordingly, we measured the expression of the genes neighboring our lncRNAs following the differentiation of SMCs to examine the regulatory network of the candidate lncRNAs. The neighboring genes of six lncRNAs showed the same expression pattern with their lncRNAs, whereas latent-transforming growth factor beta-binding protein 2 (*LTBP2*) showed the opposite expression pattern with adjacent *CTD-2207P18.2* lncRNA as measured from RNA-seq data (Figures 1C and 6A). *NR2F1-AS1* and *FOXC2-AS1* are transcribed in the divergent direction to their neighboring genes, that is, *NR2F1* (nuclear receptor subfamily 2 group F member 1) and *FOXC2* (forkhead box protein C2), respectively (Figure S5A). The silencing or overexpression of *NR2F1-AS1* did not affect the expression of *NR2F1* (Figure S8A). In contrast, the silencing of *FOXC2-AS1* lncRNA decreased the mRNA levels of *FOXC2*, suggesting that *FOXC2-AS1* can regulate neighboring gene expression in VSMCs (Figure 6B, left panel). *FOXC2* is known to modulate Akt/mTOR signaling-mediated apoptosis, which may have a functional correlation with the effect of *FOXC2-AS1* on apoptosis as seen above (Figure 5D).[36] There is a 147-nt genomic region that is complementary between the *FOXC2* mRNA and the *FOXC2-AS1* lncRNA (Figures S5A and S8B). Indeed, the potential for *FOXC2-AS1* to regulate *FOXC2* expression has been reported recently.[37] We confirmed that the silencing of *FOXC2-AS1* affects the expression of the *FOXC2* mRNA (Figure 6B, left panel). *FOXC2* is also known to regulate the differentiation and maturation of VSMCs by regulating Notch signaling (Figure S8C).[38] Therefore, we investigated whether the expression of Notch signaling-related genes is altered by silencing *FOXC2-AS1*. We found a reduction in the mRNA levels of *NOTCH1*, *NOTCH3*, *JAG1*, *HEY2*, and *HES1* (Figure 6B, right panel). In addition, we also confirmed that overexpression of *FOXC2-AS1* increases the expression of *FOXC2* and Notch signaling-related genes (Figure 6C). The regulation of the neighboring gene by endogenous lncRNA was further verified using the CRISPR activation system (Figure S8D). Taken together, we suggest that *FOXC2-AS1* plays an important role in the survival and differentiation of VSMCs, possibly through association with FOXC2, which may in turn influence the Notch and Akt/mTOR signaling pathways.

DISCUSSION

Our study identified several lncRNAs that affect the differentiation and proliferation of VSMCs that are closely related to various CVDs, including atherosclerosis, neointimal hyperplasia, and vascular calcification. We screened for lncRNAs that control VSMC differentiation and found that *NR2F1-AS1* and *FOXC2-AS1* could regulate the contractility of VSMCs by modulating the expression of differentiation markers. In particular, *FOXC2-AS1* responded to the serum deprivation-based VSMC differentiation stimuli and modulated Akt/mTOR and Notch signaling pathways by regulating the neighboring gene, *FOXC2* (Figure S9, left panel). It was reported that *FOXC2* regulates the differentiation of VSMCs through Notch signaling and regulates proliferation and apoptosis via Akt/mTOR signaling.[36,38–40] Notably, Notch and TGF-β signaling pathways cooperatively promote the differentiation of VSMCs by enhancing Smad2/3 binding to the promoter of contractile marker genes.[41] It

Figure 3. The Expression of Contractile Marker Genes after lncRNA Knockdown

(A) Experimental design for lncRNA knockdown in GM or DM. After the transfection of siRNA, RNA and protein samples were prepared after 48 h (GM) or 72 h (DM). Confirmation of lncRNA knockdown in HCASMCs is shown in Figure S5. siCtrl, negative control siRNA. (B and C) qRT-PCR measurement of the expression of contractile marker genes after knockdown of (B) *NR2F1-AS1* and (C) *FOXC2-AS1* (GM, n = 3; DM, n = 4). (D and E) Western blot analysis of the expression of contractile marker genes after knockdown of (D) *NR2F1-AS1* and (E) *FOXC2-AS1* in DM (n = 3). The expression levels of RNA and protein were normalized to GAPDH. Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

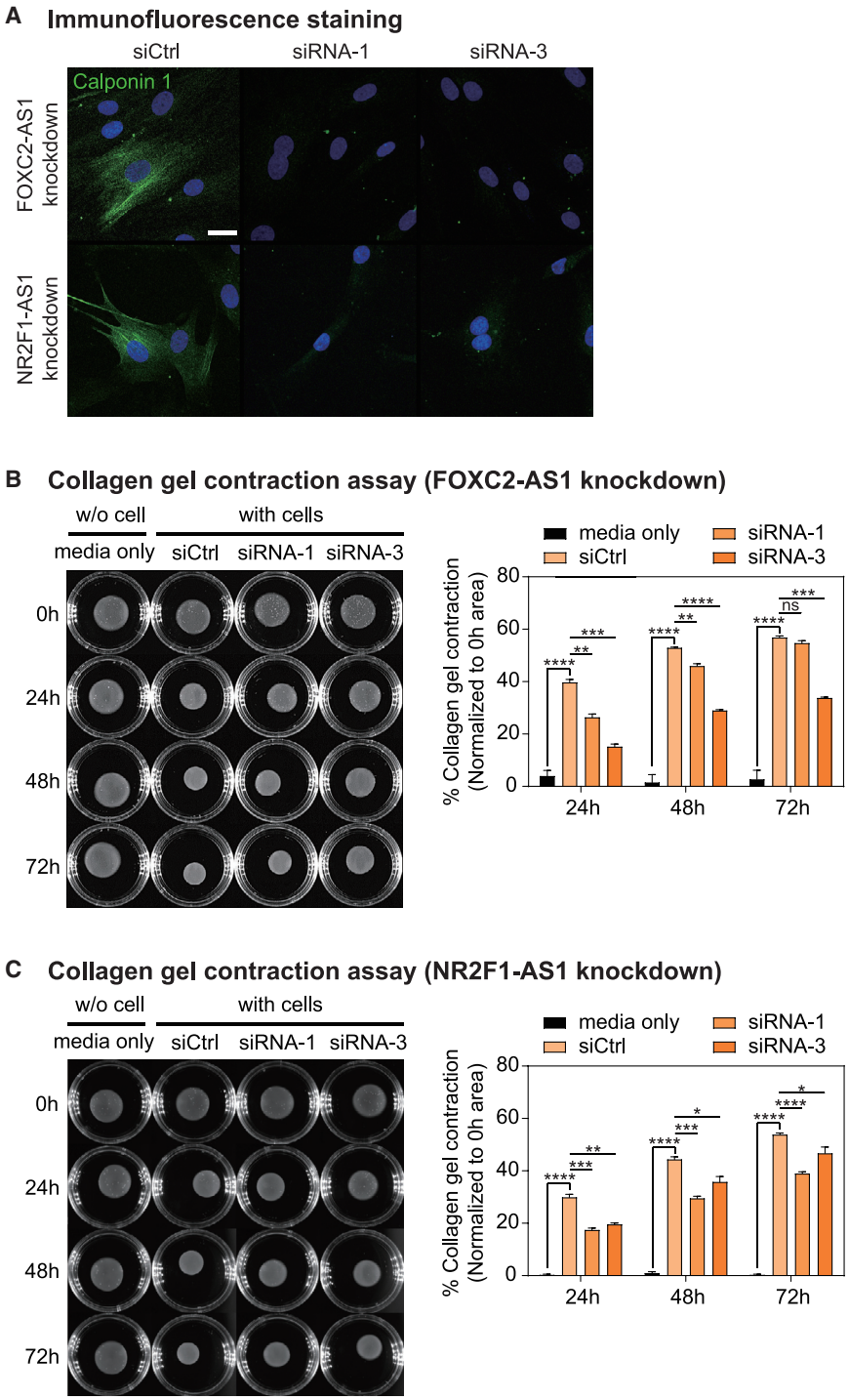

Figure 4. *FOXC2-AS1* and *NR2F1-AS1* Regulate Contractility of VSMCs

(A) Immunofluorescence analysis to measure the change in the expression of CNN1 after silencing lncRNAs in HCASMCs (72 h in DM, n = 3). The CNN1 and nuclei are indicated by green and blue (DAPI), respectively. Scale bar, 20 μm. (B and C) Collagen gel contraction assays were used to determine the contractility of (B) *FOXC2-AS1*-depleted or (C) *NR2F1-AS1*-depleted HCASMCs. The images show a collagen gel disc 0, 24, 48, and 72 h after the gelation without (w/o) cells or gelation with the transfected cells (with cells). The histogram shows the mean percentile reduction in the gel area (n = 3). Each collagen gel area was normalized to the 0 h area. Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

is also known that Notch signaling is a key mediator of the contact-dependent signaling between VSMCs and vascular endothelial cells.[42,43] Interestingly, we found that *FOXC2-AS1* regulates Notch signaling and is highly expressed in human umbilical vein endothelial cells (HUVECs) as well as VSMCs, suggesting that the Notch signaling system between VSMCs and vascular endothelial cells can be regulated by *FOXC2-AS1* (Figures 6 and S3). Thus, *FOXC2-AS1* can operate as a multifunctional regulator in vascular homeostasis and disease by regulating the biological processes involved in the differentiation and proliferation of SMCs. Further studies on the *FOXC2-AS1*/*FOXC2*/Notch signaling axis in SMCs and endothelial cells are required to elucidate the regulatory mechanisms of *FOXC2-AS1* in the vascular system.

Contrary to *FOXC2-AS1*, *NR2F1-AS1* did not affect the expression of its neighboring gene *NR2F1* (Figures S8A and S8D). Because we found that *NR2F1-AS1* regulates the contractility of VSMCs by controlling the contraction markers (Figures 3 and 4), it is expected that *NR2F1-AS1* could operate through other mechanism, including as a miRNA sponge, as suggested in our previous study (Figure S9, right panel).[21]

Our previous study demonstrated that the expression of some lncRNAs may have a correlation between smooth and skeletal muscles.[21] Interestingly, our findings showed that *NR2F1-AS1*, *MSC-AS1*, *MBNL1-AS1*, *FOXC2-AS1*, and *CTD-2207P18.2* are highly enriched in skeletal myoblasts as well as in vascular cells compared to other cell types, suggesting that they may have a role in skeletal muscle as well as smooth muscle (Figures S3 and S4).

Our analyses also showed that *CARMN*, *MIR22HG* (miR-22 host gene), and *CDR1-AS* (cerebellar degeneration-related protein 1 antisense transcript) were highly expressed during VSMC differentiation

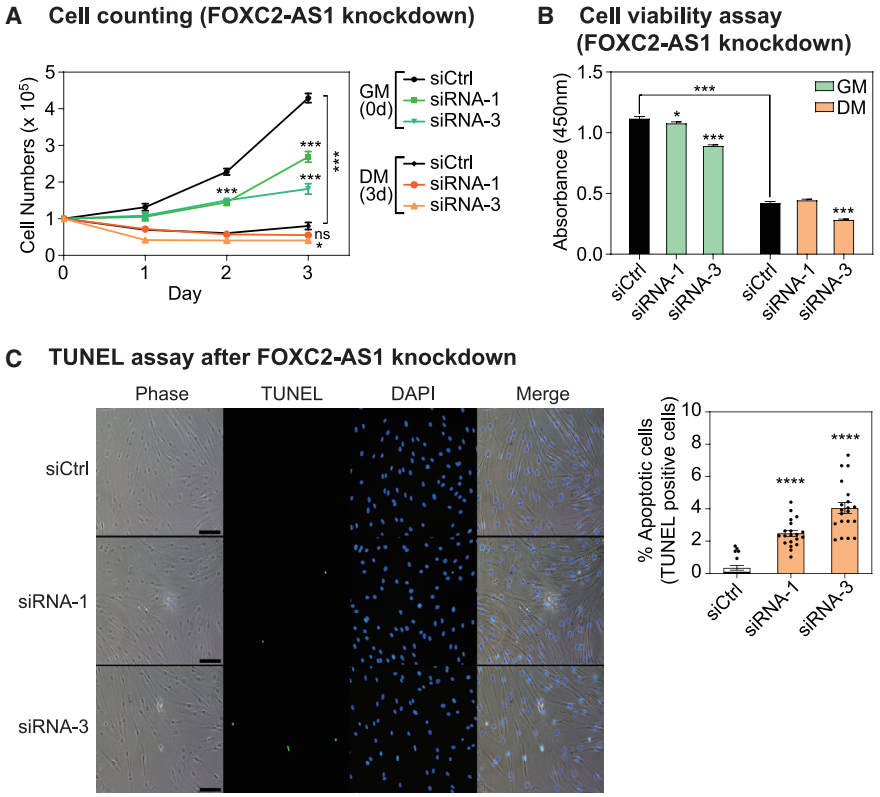

Figure 5. *FOXC2-AS1* Regulates Proliferation and Apoptosis of VSMCs by the Akt/mTOR Signaling Pathway

(A) HCASMCs were transfected with control siRNA (siCtrl) or siFOXC2-AS1 in GM or DM, and the transfected cells were counted at each time point as indicated (n = 3). (B) After the transfection with siCtrl or siFOXC2-AS1 into HCASMCs, cell proliferation was measured (n = 4). (C) Left: representative images of HCASMCs after siCtrl or siFOXC2-AS1 transfection. The cells were stained with TUNEL (green) or DAPI (blue). Scale bars, 100 μm. Right: the apoptotic index expressed as a percentile of TUNEL-positive nuclei relative to the total nuclei (n = 20 for each of siCtrl and siFOXC2-AS1-treated samples merged from three independent experiments). (D) Western blot analysis of the Akt/mTOR signaling pathway and apoptotic markers (cleaved caspase-3 and cleaved PARP) after knockdown of *FOXC2-AS1* in DM (n = 3). The expression was normalized to GAPDH. Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

and low in unstable plaques of atherosclerosis with enhanced proliferative potential (Figure S1B). Indeed, *CARMN* and *miR-22* have recently been reported to be involved in CVDs by influencing the phenotypic switching of VSMCs.[23–25,33,44] Moreover, although *CDR1-AS* has no known function in VSMCs, it could be a biomarker of acute myocardial infarction.[45–47] Therefore, our analyses suggest that the candidate lncRNAs identified in this study, in addition to the lncRNA *FOXC2-AS1*, regulate the differentiation and proliferation of VSMCs, and could be involved in the underlying pathophysiological mechanisms of CVDs.

MATERIALS AND METHODS

RNA Preparation and RNA-Seq Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. DNase I (Takara) was used to remove the residual DNA. RNA integrity and purity were confirmed by electrophoresis and using a NanoPhotometer (Implen). Four samples from each of the synthetic and contractile phenotypes were prepared for RNA-seq. RNA-seq libraries were generated using the TruSeq stranded total RNA kit (Illumina). The libraries were sequenced with HiSeq 2500 (Illumina). The raw data produced from the sequencer underwent quality control, and low-quality sequences were removed using the Trimmomatic algorithm.[48] Sequencing reads were aligned to the human genome by STAR,[49] and fragments per kilobase of transcript per million mapped reads (FPKM) were calculated using Cuffnorm.[50] We also calculated the differential expression between the two groups using Salmon and edgeR algorithms.[51,52] We combined the results obtained from the two algorithms to increase the reliability of the lncRNAs selection procedure as previously reported.[53]

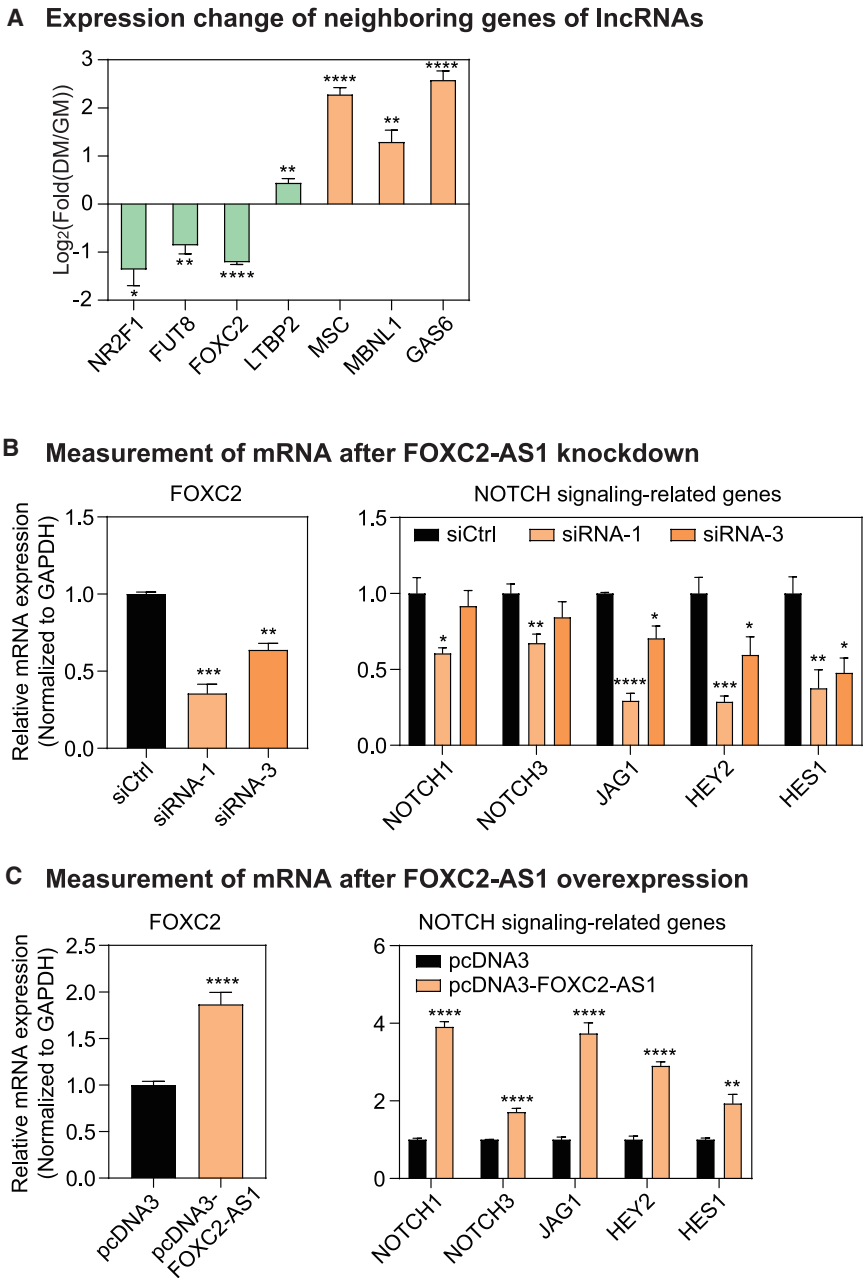

Figure 6. Analysis of the Regulatory Network of lncRNAs

(A) The fold change of neighboring genes of differentially expressed lncRNAs during VSMC differentiation (n = 4). (B) qRT-PCR analysis of the expression of *FOXC2* mRNA (n = 3) and Notch-related genes (n = 4) after knockdown of *FOXC2-AS1* in HCASMCs cultured in DM. (C) qRT-PCR analysis of the expression of *FOXC2* mRNA and Notch-related genes after overexpression of *FOXC2-AS1* in HCASMCs cultured in DM (n = 3). The expression was normalized to *GAPDH*. Data are presented as mean ± SEM. A Student's t test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

Selection of Candidate lncRNAs Involved in Smooth Muscle Differentiation

From the RNA-seq results, only lncRNAs with an average FPKM value greater than 1 and not 0 in any sample were selected. To select the candidate lncRNAs with differential expression during the differentiation of VSMCs, we only selected those lncRNAs with a $\log_2$ value of expression changes greater than 0.5 or less than −0.5 between synthetic and contractile VSMCs. To select statistically significant lncRNAs, a two-tailed t test was applied to each dataset, and the lncRNAs were selected when the p values were less than 0.05 in both the Cuffnorm results and Salmon results. Additionally, candidate lncRNAs were compared to the RNA-seq result from our previous report (RNA-seq datasets of PDGF-, MYOCD-, and TGF-β-treated VSMCs).[21]

To compare and analyze the lncRNA candidates in the differentially expressed lncRNAs during atherosclerosis progression, we used public RNA-seq data from unstable or stable atherosclerotic plaques (GEO: GSE120521).[19]

Cell Culture

HCASMCs (Gibco) were maintained in medium 231 (Gibco) supplemented with smooth muscle growth supplement (SMGS, Gibco) and 1% antibiotic/antimycotic solution (WELGENE). The HCASMCs were differentiated by culturing them in medium 231 supplemented with smooth muscle differentiation supplement (SMDS, Gibco) for 3 days. The cells between passages 4 and 8 were used throughout this study. T/G HA-VSMCs derived from human aortic SMCs (CRL-1999, ATCC) were cultured in Ham's F12K medium (Gibco) supplemented with fetal bovine serum (FBS) to a final concentration of 10%, endothelial cell growth supplement (ECGS, BD Biosciences), 1% ITS-G (insulin/transferrin/selenium, Gibco), 10 mM HEPES (WELGENE), and 1% antibiotic/antimycotic solution. To induce the differentiation of T/G HA-VSMCs, they were cultured in Ham's F12K medium supplemented with 1% FBS for 3 days. The MOVAS mouse aortic SMC line (CRL-2797, ATCC) was cultured in Dulbecco's modified Eagle's Medium (DMEM; WELGENE) supplemented with 10% FBS, 1% antibiotic/antimycotic solution, and 0.2 mg/mL Geneticin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

For differentiation by TGF-β1 (PHG9204, Gibco) treatment, 1.5×10^5 MOVAS cells per well were seeded in six-well plates. After a day of serum deprivation in DMEM supplemented with 0.5% FBS,

the cells were treated with TGF-β1 (5 ng/mL) for 2 days. For the differentiation of HCASMCs, 2×10^5 cells per well were seeded in a six-well plate and cultured in medium 231 supplemented with SMDS. One day later, the cells were treated with 5 ng/mL of TGF-β1 for 2 days. For differentiation of C2C12, the cells were transferred to the DM consisting of DMEM with 2% horse serum and 1% antibiotic/antimycotic solution.

Analysis of lncRNA Expression by qRT-PCR

To measure the expression of lncRNAs, we used HCASMCs, T/G HA-VSMCs, HUVECs (human umbilical vein endothelial cells, ATCC), skeletal myoblasts (ATCC), SH-SY5Y cells (neuroblastoma, ATCC), NCI-H460 cells (lung cancer, ATCC), HeLa cells (cervical carcinoma, ATCC), and SNU-638 cells (gastric cancer, Korean cell line bank) for human cell types, and MOVAS, C2C12 (skeletal muscle, ATCC), Neuro-2a (neuroblastoma, ATCC), mESCs (mouse embryonic stem cells, ATCC), and MEFs (mouse embryonic fibroblasts, ATCC) for mouse cell types. We cultured the cells according to the ATCC and Korean Cell Line Bank guidelines.

To measure lncRNA expression, 1 μg of total RNA, RevertAid reverse transcriptase (Thermo Scientific), and random hexamers (Thermo Scientific) were used to synthesize complementary DNA (cDNA). RNA was measured by real-time PCR based on the comparative Ct method using the Power SYBR Green PCR master mix (Applied Biosystems) and the Rotor-Gene Q real-time PCR system (QIAGEN). *GAPDH* or *ACTB* primer sets were used for normalization, and each sample was measured in triplicate. The primers for the amplification of mRNAs and lncRNAs are listed in Table S1.

Cellular Fractionation

To fractionate the HCASMCs into nuclear and cytoplasmic fractions, the cells were collected and treated with buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT). After a 25-min incubation on ice, 10% Nonidet P-40 (NP-40) was added to a final concentration of 0.25% and incubated for an additional 2 min. After centrifugation, cytoplasmic RNA was isolated from the supernatant using TRIzol LS reagent (Invitrogen). The pellet was resuspended in K100 buffer D (20 mM Tris [pH 8.0], 100 mM KCl, 0.2 mM EDTA) followed by centrifugation to obtain nuclear RNA. Precursor *GAPDH* (pre-*GAPDH*) and *MALAT1* were used as controls for the nuclear fraction, and *GAPDH* and *ACTB* were used as cytoplasmic controls.

Western Blot Analysis

HCASMCs were harvested and incubated in ice-cold radioimmunoprecipitation assay buffer (Translab) for 10 min. Protein extracts were quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Fifteen micrograms of protein was loaded on a 10%–12% SDS-polyacrylamide gel, and the protein was transferred onto a polyvinylidene fluoride membrane (Millipore) activated by methanol. The membrane was blocked with 5% skim milk (BD Biosciences) or 5% bovine serum albumin (Sigma) for 1 h at room temperature (15°C –25°C), followed by incubation with primary antibodies (1:1,000) overnight at 4°C. Primary antibodies against ACTA2 (Abcam), CNN1 (Cell Signaling Technology), AKT (Cell Signaling Technology), phosphorylated (phospho-)AKT (Cell Signaling Technology), mTOR (Cell Signaling Technology), phospho-mTOR (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), cleaved PARP (Cell Signaling Technology), and GAPDH (Santa Cruz) were used. The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at room temperature and visualized using an enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific) and Fusion Solo software (Vilber). Protein expression was normalized to the expression of GAPDH.

siRNA Design and Transfection

We used the siDESIGN Center in horizon discovery (https://horizondiscovery.com/en/products/tools/siDESIGN-Center) and i-Score Designer (https://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html)[54] to design siRNAs against lncRNAs. AccuTarget negative control siRNAs (Bioneer) were used as a negative control. The sequences of the siRNAs used are listed in Table S1. For lncRNA knockdown, 2×10^5 HCASMCs per well were seeded in a six-well plate. A day after cell seeding, siRNAs were transfected into the HCASMCs at a concentration of 30 nM using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Plasmid Construction and CRISPR Activation

The pcDNA3 was used to construct plasmids containing lncRNA sequences. The lncRNA sequences were amplified from cDNA of HCASMCs through PCR, followed by TA cloning (TOPcloner PCR cloning kit, Enzynomics) and sub-cloning into pcDNA3 using KpnI and NotI restriction enzymes sites (Thermo Scientific). The sequences of the primer sets used are listed in Table S1. For overexpression of *NR2F1-AS1* and *FOXC2-AS1*, 1 μg of pcDNA3-NR2F1-AS1 or pcDNA3-FOXC2-AS1 was transfected into HCASMCs in a six-well plate using Lipofectamine 3000.

To design a single guide RNA (sgRNA) for CRISPR activation, we used GuideScan, the CRISPR guide RNA design tool (http://www.guidescan.com/).[35,55,56] The target site of sgRNAs was selected upstream (−1 to −200 nt) of the transcription start site (TSS) of *NR2F1-AS1* or *FOXC2-AS1*, and included a site for the FastDigest Eco31I restriction enzyme (Thermo Scientific) at the ends of the oligonucleotides to facilitate cloning. The sequences of the sgRNA for the lncRNA are listed in Table S1. The CRISPR activator plasmid (SP-dCas9-VPR, Addgene #63798) and sgRNA expression plasmid (pRG2, Addgene #104174) were used for overexpression of *NR2F1-AS1* and *FOXC2-AS1*. Briefly, the oligonucleotides containing the sgRNA sequences were annealed by cooling to 25°C after 5 min of denaturation at 95°C in a T4 DNA ligase buffer. The pRG2 vector was digested by Eco31I and ligated with the annealed sgRNA using T4 DNA ligase (Takara). After cloning, the sequence was confirmed by Sanger sequencing. For overexpression of *NR2F1-AS1* and *FOXC2-AS1*, 750 ng of SP-dCas9-VPR vector and 250 ng of pRG2 vector (3:1 ratio) were transfected into HCASMCs and HeLa cells in a six-well plate using Lipofectamine 3000.

Immunofluorescence Staining

For the immunofluorescence staining, 8×10^4 HCASMCs were plated on 18-mm coverslips (SPL Life Sciences) and fixed in 4% paraformaldehyde solution for 25 min at 4°C. The cells were then rinsed with 1× phosphate-buffered saline (PBS) three times, and incubated with anti-CNN1 antibody (1:200, Cell Signaling Technology) in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate, and 450 mM NaCl [pH 7.4]) overnight at 4°C. The next day, the cells were rinsed with 1× PBS three times and incubated with the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (1:200) secondary antibody for 2 h at room temperature. Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) solution (1:100) for 15 min at room temperature and mounted using a Vectashield mounting medium (Vector Laboratories). Images were captured using the Eclipse Ts2 fluorescence microscope (Nikon).

Collagen Gel Contraction Assay

The contraction assay was used to investigate the alteration in the contractility of HCASMCs following the knockdown of lncRNAs. Collagen type I (Corning Life Sciences) was diluted to 2 mg/mL and adjusted to a pH of 7.4 with 1 M NaOH. A collagen-cell suspension was plated as 1.5×10^5 cells in 0.5 mL of media per well of a flat-bottom 24-well plate. This plate was incubated for 1 h in an incubator with a humidified atmosphere at 37°C and 5% CO_2 to complete gelation. Using a sterilized spatula, the gels were gently transferred to 35-mm cell culture dishes containing 3 mL of medium 231 supplemented with SMDS and incubated in a cell incubator. The collagen gel size was quantified after 0, 24, 48, and 72 h using ImageJ software.[57]

Cell viability assay

For cell counting, the lncRNA-depleted HCASMCs were seeded in a 12-well plate and the cell numbers were manually counted after 1, 2, and 3 days using hemocytometer C-Chips (INCYTO). The proliferation of HCASMCs was measured using the EZ-Cytox cell viability and proliferation assay kit (DoGEN) according to the manufacturer's protocol. Briefly, lncRNA-depleted HCASMCs (5×10^3) were seeded in a 96-well plate and cultured for 2 days. Cells were then incubated with 10 μL of EZ-Cytox reagent (water soluble tetrazolium salt) for 4 h and absorbance was measured at 450 nm with a microplate reader (BioTek).

TUNEL Assay

For the TUNEL (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling) assay, 8×10^4 HCASMCs were plated on 18-mm coverslips (SPL Life Sciences) and fixed in 4% paraformaldehyde solution for 25 min at 4°C, followed by permeabilization in 0.3% Triton X-100 in PBS for 30 min at room temperature. The cells were stained using the DeadEnd fluorometric TUNEL system kit (Promega) according to the manufacturer's protocol. Cell nuclei were counterstained with DAPI solution (1:100) for 15 min at room temperature and mounted using Vectashield mounting medium. Images were captured using the Eclipse Ts2 fluorescence microscope (Nikon). TUNEL assay results were represented by the apoptotic index (i.e., the number of positively stained nuclei/total number of nuclei counted × 100%).

Bioinformatics Analysis

CPC 2.0 (coding potential calculator 2, http://cpc2.gao-lab.org/)[58] and CPAT (coding potential assessment tool, http://lilab.research.bcm.edu/cpat/index.php)[59] were used to investigate the coding potential of the lncRNA transcripts. Protein interactomes of FOXC2 were predicted through STRING (https://string-db.org/).[60] The interaction between *FOXC2-AS1* and *FOXC2* mRNA was predicted using the RNA-RNA interaction prediction tool IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp).[61]

Statistical Analysis

Statistical analysis was performed using an unpaired Student's t test. All data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.001$ were considered statistically significant. Prism 8 (GraphPad) was used for statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.08.032.

AUTHOR CONTRIBUTIONS

Y.-H.L., H.K., and Y.-K.K. designed the study; Y.-H.L. and J.R. conducted the study; Y.-H.L. collected data; Y.-H.L., H.K., and Y.-K.K. analyzed and interpreted data; Y.-H.L. wrote a draft of the manuscript; Y.-H.L. and Y.-K.K. revised the manuscript content; H.K. and Y.-K.K. acquired funding for the study.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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