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 (MYOCID), 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 IncRNAs 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 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.7 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 mesodern 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) Log2(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.
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 S5 A). 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
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 SMCs, 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 lincRNA (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

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**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.
is also known that Notch signaling is a key mediator of the contact-dependent signaling between VSMCs and vascular endothelial cells. Interestingly, we found that \textit{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 \textit{FOXC2-AS1} (Figures 6 and S3). Thus, \textit{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 \textit{FOXC2-AS1}/FOXC2/Notch signaling axis in SMCs and endothelial cells are required to elucidate the regulatory mechanisms of \textit{FOXC2-AS1} in the vascular system.

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

Our previous study demonstrated that the expression of some IncRNAs may have a correlation between smooth and skeletal muscles.\textsuperscript{21} Interestingly, our findings showed that \textit{NR2F1-AS1}, \textit{MSC-AS1}, \textit{MBNL1-AS1}, \textit{FOXC2-AS1}, and \textit{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 \textit{CARMN}, \textit{MIR22HG} (miR-22 host gene), and \textit{CDR1-AS} (cerebellar degeneration-related protein 1 antisense transcript) were highly expressed during VSMC differentiation.
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. 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. Sequencing reads were aligned to the human genome by STAR, and fragments per kilobase of transcript per million mapped reads (FPKM) were calculated using Cuffnorm. We also calculated the differential expression between the two groups using Salmon and edgeR algorithms. We combined the results obtained from the two algorithms to increase the reliability of the lncRNAs selection procedure as previously reported.
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 log2 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/antimycoptic 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% CO2.

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 IncRNA Expression by qRT-PCR
To measure the expression of IncRNAs, 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 IncRNA 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 IncRNAs 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 radioimmuno-precipitation assay buffer (TranSalb) 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) to design siRNAs against IncRNAs. AccuTarget negative control siRNAs (Bioneer) were used as a negative control. The sequences of the siRNAs used are listed in Table S1. For IncRNA 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 IncRNA sequences. The IncRNA sequences were amplified from cDNA of HCASMCs through PCR, followed by TA cloning (TOPcloner PCR cloning kit, Enzymonics) 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/). The target site of sgRNAs was selected up-stream (~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 IncRNA 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, $8 \times 10^3$ 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 DAPI solution (1:100) for 15 min at room temperature and mounted using 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 $1.5 \times 10^5$ 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% CO2 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 10% FBS and 450 mM NaCl [pH 7.4] overnight at 4°C. The contraction assay was used to investigate the alteration in the contractility of HCASMCs following the knockdown of lncRNAs. The cells were then 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 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).

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 ($5 \times 10^4$) 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, $8 \times 10^4$ 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/) and CPAT (coding potential assessment tool, http://filab.research.bcm.edu/cpat/index.php) were used to investigate the coding potential of the lncRNA transcripts. Protein interactomes of FOXC2 were predicted through STRING (https://string-db.org/). The interaction between FOXC2-ASI and FOXC2 mRNA was predicted using the RNA-RNA interaction prediction tool IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/InPut.jsp).

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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REFERENCES

1. Spyridopoulos, I., and Andrés, V. (1998). Control of vascular smooth muscle and endothelial cell proliferation and its implication in cardiovascular disease. Front. Biosci. 3, d269–d287.
2. Frismantiene, A., Philippova, M., Erne, P., and Resink, T.J. (2018). Smooth muscle cell-driven vascular diseases and molecular mechanisms of VSMC plasticity. Cell. Signal. 52, 48–64.
3. Bennett, M.R., Sinha, S., and Owens, G.K. (2016). Vascular smooth muscle cells in atherosclerosis. Circ. Res. 118, 692–702.
4. Gerthoffer, W.T. (2007). Mechanisms of vascular smooth muscle cell migration. Circ. Res. 100, 607–621.
5. Gomez, D., and Owens, G.K. (2012). Smooth muscle cell phenotypic switching in atherosclerosis. Cardiovasc. Res. 95, 156–164.
24. Plaisance, I., Sánchez-Galán, E., Santamaría, B., Sánchez-López, E., Rodríguez-Diez, R., Blanco-Colio, L.M., Egido, J., Ortiz, A., and Ruiz-Ortega, M. (2008). Essential role of TGF-β/Smad pathway on statin dependent vascular smooth muscle cell regulation. PLoS ONE 3, e3959.

25. Vacante, F., Denby, L., Shuiter, J.C., and Baker, A.H. (2019). The function of miR-143, miR-145 and the miR-143 host gene in cardiovascular development and disease. Vascul. Pharmacol. 112, 24–30.

26. GTEx Consortium (2013). The Genotype-Tissue Expression (GTex) project. Nat. Genet. 45, 580–585.

27. Bergeron, K.F., Nguyen, C.M., Cardinal, T., Charrier, B., Silversides, D.W., and Pilon, N. (2016). Upregulation of the NR2F1-A830082KRik gene pair in murine neural crest cells results in a complex phenotype reminiscent of Waardenburg syndrome type 4. Dis. Model. Mech. 9, 1283–1293.

28. Ang, C.E., Ma, Q., Wapinski, O.L., Fan, S., Flynn, R.A., Lee, Q.Y., Coe, B., Nomaguchi, M., Ohmos, V.H., Do, B.T., et al. (2019). The novel IncRNA Inc-NR2F1 is pro-neurogenic and mutated in human neurodevelopmental disorders. Cell 18, e41770.

29. Rodriguez-Vita, J., Sánchez-Galán, E., Santamaría, B., Sánchez-López, E., Rodrigues-Sanjuan, F., Alexanian, M., Gonzales, C., Ng, S.Y., et al. (2015).}

30. Sjölund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C.H., and Thyberg, J. (1990). Expression of PDGF α- and β-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors 3, 191–203.

31. Kumar, M.S., and Owens, G.K. (2003). Combinatorial control of smooth muscle-specific gene expression. Arterioscler. Thromb. Vasc. Biol. 23, 737–747.

32. Jin, L., Lin, X., Yang, L., Fan, X., Wang, W., Li, S., Li, J., Liu, X., Bao, M., Cui, X., et al. (2018). AK098656, a novel vascular smooth muscle cell dominant long noncoding RNA, promotes hypertrophy. Hypertension 72, 262–272.

33. Bell, R.D., Long, X., Lin, M., Bergmann, J.H., Nanda, V., Cowan, S.L., Zhou, Q., Han, Y., Spector, D.L., Zheng, D., and Miano, J.M. (2014). Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler. Thromb. Vasc. Biol. 34, 1249–1259.

34. Ballantyne, M.D., Pinel, K., Dakin, R., Vesse, A.T., Diver, L., Mackenzie, R., Garcia, S., Sjölund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C.H., and Thyberg, J. (2009). Expression of PDGF α- and β-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors 3, 191–203.

35. Liu, N., and Olson, E.N. (2010). MicroRNA regulatory networks in cardiovascular development. Dev. Cell 18, 510–525.

36. Jin, L., Lin, X., Yang, L., Fan, X., Wang, W., Li, S., Li, J., Liu, X., Bao, M., Cui, X., et al. (2018). AK098656, a novel vascular smooth muscle cell dominant long noncoding RNA, promotes hypertrophy. Hypertension 72, 262–272.

37. Bell, R.D., Long, X., Lin, M., Bergmann, J.H., Nanda, V., Cowan, S.L., Zhou, Q., Han, Y., Spector, D.L., Zheng, D., and Miano, J.M. (2014). Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler. Thromb. Vasc. Biol. 34, 1249–1259.

38. Ballantyne, M.D., Pinel, K., Dakin, R., Vesse, A.T., Diver, L., Mackenzie, R., Garcia, S., Sjölund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C.H., and Thyberg, J. (2009). Expression of PDGF α- and β-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors 3, 191–203.

39. Liu, N., and Olson, E.N. (2010). MicroRNA regulatory networks in cardiovascular development. Dev. Cell 18, 510–525.

40. Jin, L., Lin, X., Yang, L., Fan, X., Wang, W., Li, S., Li, J., Liu, X., Bao, M., Cui, X., et al. (2018). AK098656, a novel vascular smooth muscle cell dominant long noncoding RNA, promotes hypertrophy. Hypertension 72, 262–272.

41. Bell, R.D., Long, X., Lin, M., Bergmann, J.H., Nanda, V., Cowan, S.L., Zhou, Q., Han, Y., Spector, D.L., Zheng, D., and Miano, J.M. (2014). Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler. Thromb. Vasc. Biol. 34, 1249–1259.

42. Ballantyne, M.D., Pinel, K., Dakin, R., Vesse, A.T., Diver, L., Mackenzie, R., Garcia, S., Sjölund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C.H., and Thyberg, J. (2009). Expression of PDGF α- and β-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors 3, 191–203.

43. Liu, N., and Olson, E.N. (2010). MicroRNA regulatory networks in cardiovascular development. Dev. Cell 18, 510–525.

44. Jin, L., Lin, X., Yang, L., Fan, X., Wang, W., Li, S., Li, J., Liu, X., Bao, M., Cui, X., et al. (2018). AK098656, a novel vascular smooth muscle cell dominant long noncoding RNA, promotes hypertrophy. Hypertension 72, 262–272.

45. Bell, R.D., Long, X., Lin, M., Bergmann, J.H., Nanda, V., Cowan, S.L., Zhou, Q., Han, Y., Spector, D.L., Zheng, D., and Miano, J.M. (2014). Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler. Thromb. Vasc. Biol. 34, 1249–1259.

46. Ballantyne, M.D., Pinel, K., Dakin, R., Vesse, A.T., Diver, L., Mackenzie, R., Garcia, S., Sjölund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C.H., and Thyberg, J. (2009). Expression of PDGF α- and β-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors 3, 191–203.

47. Liu, N., and Olson, E.N. (2010). MicroRNA regulatory networks in cardiovascular development. Dev. Cell 18, 510–525.
