Angiotensin II-Induced Long Non-Coding RNA Alivec Regulates Chondrogenesis

Subjects: Biochemistry & Molecular Biology

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Angiotensin II (AngII)-regulated Long non-coding RNAs (lncRNAs) Alivec functions, at least in part, to mediate the AngII-induced chondrogenic transformation of vascular smooth muscle cells (VSMCs) implicated in vascular dysfunction and hypertension.

Angiotensin II lncRNAs cardiovascular disease vascular smooth muscle cells chondrocytes hypertension

1. Introduction

Cardiovascular diseases (CVDs), such as hypertension and atherosclerosis, are leading causes of morbidity and mortality worldwide [1]. Vascular smooth muscle cells (VSMCs) in the arterial wall maintain vascular tone and blood pressure and are under the control of the renin–angiotensin system (RAS)-Angiotensin II (AngII) system.

In CVD or vascular injury, dysregulated growth factor and AngII signaling promotes VSMCs to switch from a contractile to synthetic phenotype [2]. The synthetic phenotype manifests in increased VSMC proliferation, hypertrophy, migration, inflammation and the key processes associated with the pathogenesis of arterial stenosis/restenosis, hypertension and atherosclerosis [3][4][5][6]. Furthermore, the synthetic VSMCs tend to transform into chondrocyte-like cells, which promotes extracellular calcium deposition and vascular dysfunction associated with these pathologies [7][8][9]. Aggrecan (Acan) is an extracellular matrix protein that is prominent in chondrocytes during cartilage formation and is upregulated in aortic VSMCs after injury [9]. The transcription factor (TF) Sox9, which regulates chondrogenesis, is associated with VSMC synthetic/chondrocyte phenotype and promotes extra-cellular matrix (ECM) alterations and calcium deposition [10]. However, the mechanisms involved in AngII-mediated phenotypic transformation of VSMC to chondrocyte-like cells are not well understood.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs (ncRNAs) that are more than 200 nucleotides in size and are processed like protein coding mRNAs but lack protein-coding potential [11]. LncRNAs have diverse functions and regulate gene expression at the level of transcription through the interaction with and recruitment of TFs, chromatin modifier proteins and ribonucleoproteins to specific target gene loci, or via the post-transcriptional regulation of microRNAs and signaling proteins [12]. Genome-wide association studies (GWAS) identified several single nucleotide polymorphisms (SNPs) associated with CVDs that reside in the lncRNA loci [13]. LncRNAs

regulate various physiological and pathological processes [14]. In VSMCs they regulate cell proliferation, migration, reactive oxygen species (ROS) production and inflammation, key factors associated with CVDs [15][16]. Researchers identified the first lncRNAs regulated by AngII in rat VSMCs (RVSMCs) using integrated analysis of RNA-seq data with ChIP-seq datasets from histone H3K4me3 and H3K36me3 profiling [17]. Since then, several VSMC lncRNAs such as *SENCR*, *MYOSLID* and *SMILR* were described and found to play key roles in CVDs [18][19][20].

2. Alivec Is an AngII-Induced lncRNA Adjacent to Chondrogenic Gene Acan in RVSMCs

Here analyzed RNA-seq data previously generated in a laboratory from RVSMCs treated with AngII (100 nM, 3 h) [17] using STAR aligner and observed that a previously identified novel lncRNA (*lnc Ang26*), which named *Alivec*, was highly induced by AngII (**Figure 1**A). To further characterize the *Alivec* locus, researchers integrated the RNA-seq data with histone H3K27ac (enhancer mark) ChIP-seq data from AngII treated RVSMCs [21]. Combined RNA-seq and ChIP-seq data showed that the lncRNA *Alivec* locus overlaps with an AngII-induced H3K27ac enriched region (**Figure 1**B). *Alivec* has 3 exons and the gene is located on rat chromosome 1 adjacent (117 kb distance) to the protein-coding gene *Acan* (**Figure 1**B). RNA-seq analyses also showed that the expression of the nearby gene *Acan*, was likewise increased by AngII. Furthermore, RT-qPCR validation showed that RVSMCs exposed to AngII displayed marked induction of *Alivec* expression (up to 30-fold) within 3 h of treatment; this persisted even at 6 h compared to the control cells (**Figure 1**C). Under the same conditions, the induction of *Acan* was also observed (**Figure 1**D), suggesting a potential role for *Alivec* in the regulation of *Acan* expression by AngII. This was interesting, as *Acan* codes for the protein aggrecan, which is known to be induced by growth factors and cytokines and is also a key biomarker of chondrogenesis associated with VSMC dysfunction in CVDs [22].

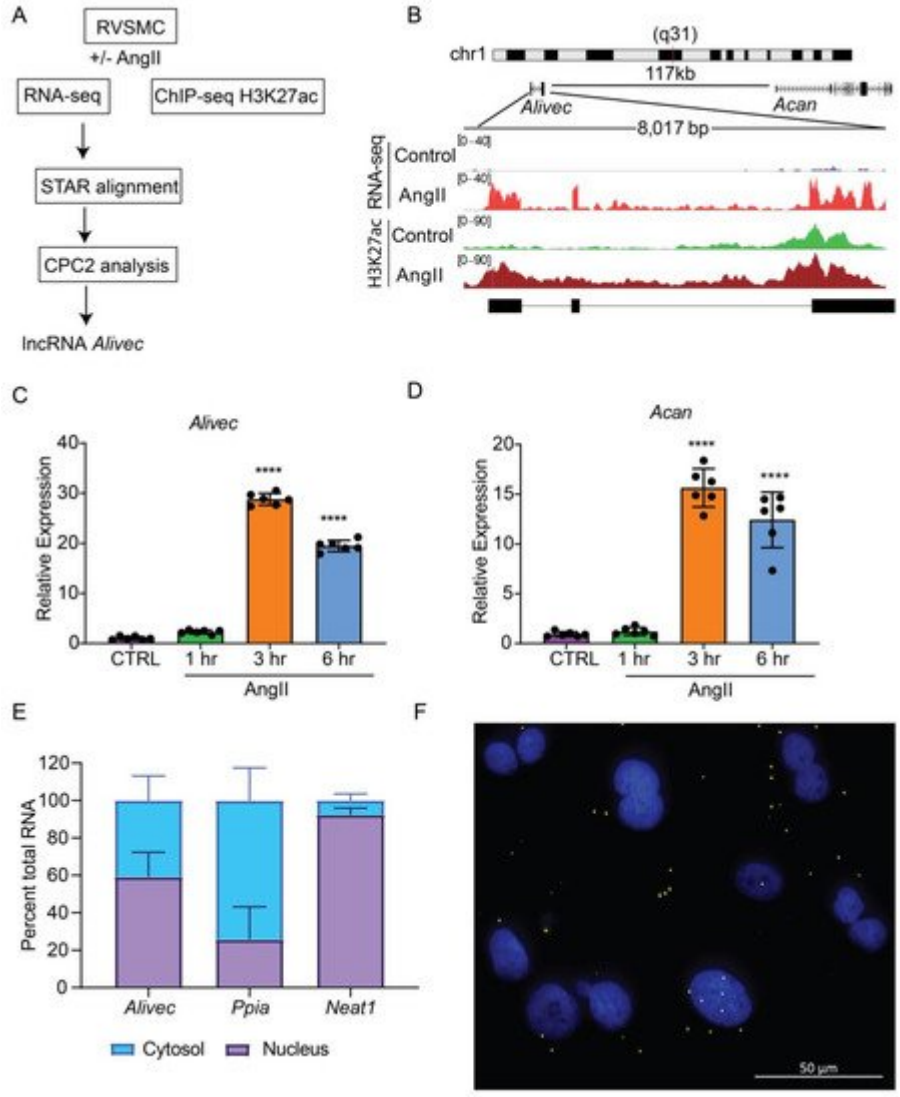

Figure 1. *Alivec* is an AngII-induced enhancer-associated lncRNA adjacent to chondrogenic gene *Acan* in RVSMCs. (**A**) Schematic diagram depicting RNA-seq and H3K27ac ChIP-seq alignment pipeline for the identification of lncRNA *Alivec* (AngII-induced lncRNA in vascular smooth muscle cells eliciting chondrogenic phenotype) exons, overlapping H3 lysine 27 acetylation (H3K27ac) enrichment and *Alivec*'s coding potential, which was determined using the software CPC2 (coding potential calculator 2). (**B**) Schematic showing genomic organization of *Alivec* and the neighboring gene *Acan* in the rat genome. Integrative Genomics Viewer (IGV) tracks showing *Alivec* locus with representative RNA-seq tracks (RNA-Seq) and H3K27ac ChIP-seq tracks (H3K27ac) from control- and AngII-treated RVSMCs. (**C**,**D**) RT-qPCR analysis of *Alivec* and *Acan* expression in RVSMCs treated ± AngII (100 nM) for the indicated time periods. Data presented as mean ± SD, n = 6 biological replicates, one-way ANOVA followed by Dunnett's multiple comparisons test and **** $p < 0.0001$ vs. control untreated cells (CTRL (**E**) RT-qPCR analysis of *Alivec*, *Ppia* and *Neat1* showing their relative enrichment in cytosolic and nuclear fractions of AngII-treated RVSMCs. (**F**) Subcellular localization of *Alivec* in AngII-treated RVSMCs, determined by RNA–FISH analysis. *Alivec* is shown as distinct yellow spots and nuclei are stained with DAPI (blue). Scale bar 50 µm.

Next, researchers performed experiments to further characterize *Alivec*. Rapid amplification of cDNA end (RACE)-PCR experiments verified the 5′ and 3′ ends of *Alivec* and defined the total transcript size to be 2275 nucleotides. Considering the localization of lncRNAs in the nucleus or cytoplasm can determine their functions, [23] researchers examined the cellular localization of lncRNA *Alivec*. In AngII-treated RVSMCs, sub-cellular fractionation followed by RT-qPCR showed that *Alivec* is distributed in the nucleus and cytosol (**Figure 1**E). *Ppia* and a lncRNA *Neat1* served as controls for cytoplasmic and nuclear fractions, respectively (**Figure 1**E). RNA–FISH experiments with branched DNA probes, further confirming nuclear and cytoplasmic localization of *Alivec*, as indicated by the presence of distinct spots/foci distributed in both compartments (**Figure 1**F). These spots were not visible in the absence of the probes. The protein-coding potential analysis of *Alivec* (coding potential calculator version 2.0, CPC2) showed that it had a coding probability of 0.31, classifying it as a non-coding transcript. The lack of coding potential was confirmed by in vitro transcription/translation assays using pcDNA *Alivec* plasmids, which showed no detectable peptide product from *Alivec*, as compared to the positive luciferase control. Together, these results indicate that *Alivec* is an AngII-induced lncRNA in RVSMCs.

3. AngII-induces Alivec and Acan Expression via Activation of AT1R and Src Kinase

AngII, through the activation of AT1R, induces multiple signaling pathways to regulate downstream gene expression and alter VSMC function [24]. To elucidate the role of AT1R signaling in regulating *Alivec* and *Acan* expression, researchers pre-treated RVSMCs with the AT1R antagonist, losartan (10 µM, 30 min), followed by AngII (100 nM). Losartan abrogated the AngII-mediated induction of *Alivec* (**Figure 2**A). *Acan* expression was also attenuated by losartan (**Figure 2**B). Pre-treatment of RVSMCs for 30 min with specific inhibitors of key signaling kinases, ERK1/2 (U0126, 10 µM) and Src (PP1,10 µM), significantly reduced AngII-induced *Alivec* expression. Conversely, inhibitors of the p38 MAP kinase (SB202190, 5 µM) and JAK (Inhibitor I, 10 µM), had minimal effect on *Alivec* expression (**Figure 2**C). Interestingly, the AngII-mediated induction of *Acan* was significantly suppressed by inhibition of the Src kinase, and to a lesser extent, by inhibitors of ERK1/2 and JAK (**Figure 2**D). These results demonstrate key roles of AT1R signaling and downstream kinases in the regulation of *Alivec* and *Acan* in RVSMCs.

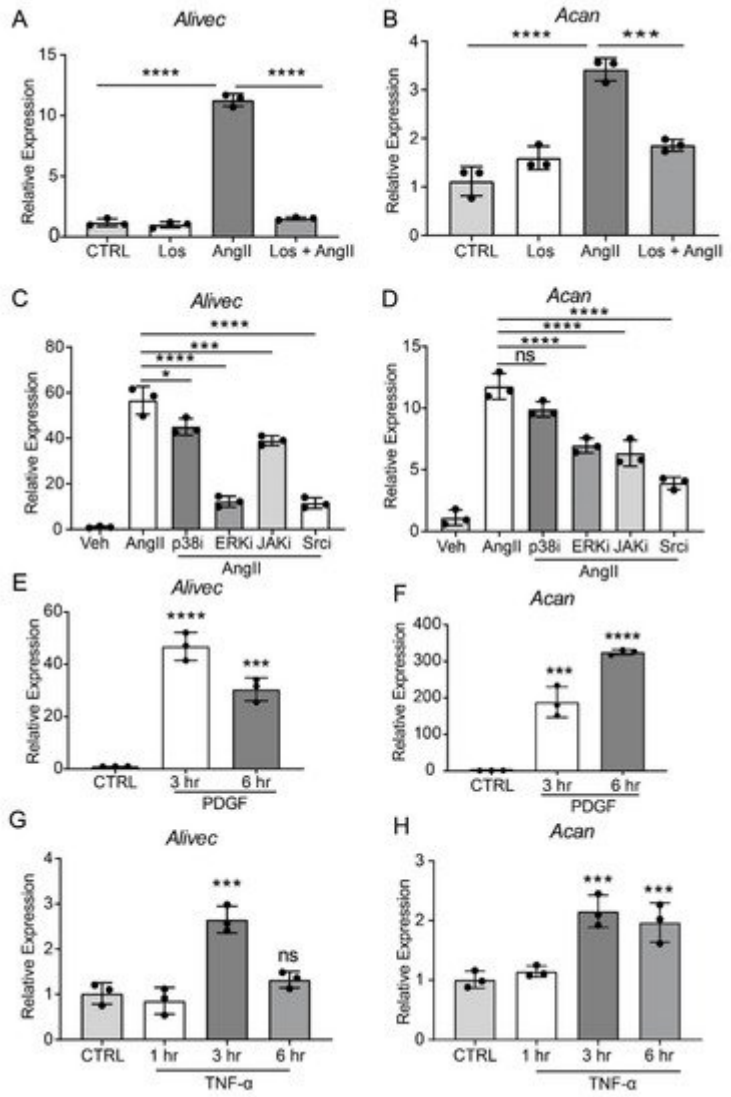

Figure 2. AngII-induced *Alivec* expression is regulated by AT1R and downstream kinases Src and ERK1/2. (**A**,**B**) RT-qPCR analysis of *Alivec* and *Acan* expression in RVSMCs pre-treated with the AT1R inhibitor Losartan (Los, 10 µM) for 30 min, followed by AngII treatment (100 nM, 3 h). (**C**,**D**) RVSMCs were pre-treated with vehicle DMSO (Veh) or inhibitors (i) of p38, ERK1/2, JAK and Src kinases for 30 min, followed by AngII treatment (100 nM, 3 h). (**E**–**H**) RT-qPCR analysis of *Alivec* and *Acan* expression in RVSMCs, treated with PDGF (10 ng/mL) and TNF-α (10 ng/mL). Data presented as mean ± SD. Comparisons were performed by one-way ANOVA with Tukey's post-hoc test. (**A**–**D**) Dunnett's multiple comparisons test (**E**–**H**), * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ vs. CTRL or AngII.

It also was determined if *Alivec* is induced by other VSMC growth factors, such as PDGF, and the inflammatory cytokine TNF-α. RVSMCs treated with PDGF (10 ng/mL) and TNF-α (10 ng/ml, 3 and 6 h) showed increased *Alivec* and *Acan* mRNA levels, although with varying kinetics (**Figure 2**E–H) suggesting a general effect. Together, these results show that *Alivec* and *Acan* are upregulated by not only AngII, but by other growth factors and inflammatory cytokines that might co-operate to augment VSMC dysfunction upon vascular injury.

4. Alivec Knockdown Attenuates AngII-Induced Upregulation of Genes Associated with Chondrogenesis

AngII regulates the expression of inflammatory, fibrotic and calcification-related genes in VSMC [24]. To further characterize the role of *Alivec* in the regulation of AngII-induced gene expression in RVSMCs, a locked nucleic acid (LNA)-modified anti-sense oligonucleotide (ASO) GapmeRs-mediated knockdown of *Alivec* was performed. Three GapmeRs targeting *Alivec* were tested to assess their efficacy in RVSMCs (Supplementary Figure S2A). Results showed that GapmeR3 (denoted as *Alivec*Gap) achieved maximum reduction (~60%) in AngII-induced *Alivec* expression, as compared to the control GapmeR (NCGap) (**Figure 3**A and Supplementary Figure S2B). RVSMCs were transfected with *Alivec*Gap or NCGap and treated with or without AngII. RNA extracted from these cells was subjected to microarray expression profiling (Supplementary Figure S3A,B). After *Alivec* knockdown, researchers identified 1169 differentially expressed genes in untreated RVSMCs (676 downregulated and 493 upregulated), and 1294 differentially expressed genes in AngII-treated RVSMCs (664 downregulated and 630 upregulated), which included several chondrogenic genes (**Figure 3**B). Gene ontology (GO) analysis of downregulated genes showed enrichment of biological processes, such as cell adhesion and the circulatory system (**Figure 3**C), which are important functions of VSMC and the cardiovascular system. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed enrichment of pathways involved in mucin type O-glycan biosynthesis, nitric oxide second messenger cGMP signaling and vascular smooth muscle contraction (**Figure 3**D) that could be associated with VSMC functions and hypertension.

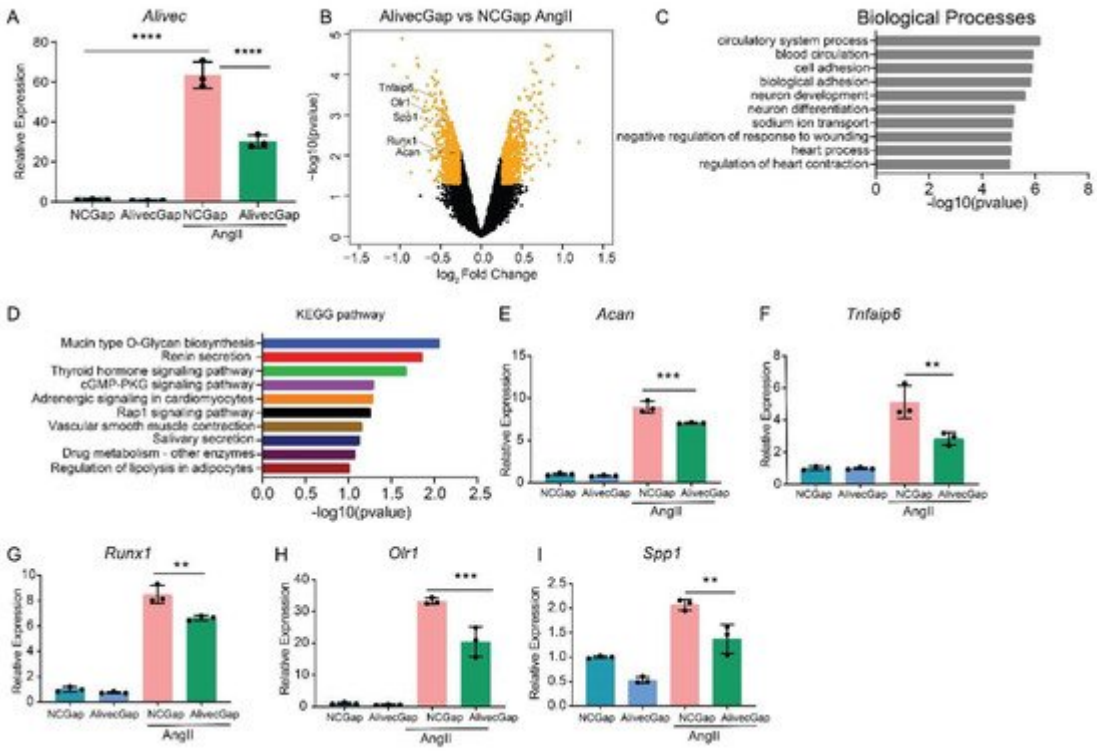

Figure 3. *Alivec* knockdown attenuates upregulation of AngII-induced chondrogenic genes in RVSMCs. (**A**) Knockdown efficiency of LNA GapmeR targeting *Alivec* (*Alivec*Gap) (100 nM) vs. non-targeting control GapmeR (NCGap) (100 nM), as determined by RT-qPCR. Data presented as mean ± SD, one-way ANOVA followed by

Tukey's post-hoc test and **** $p < 0.0001$ vs. indicated groups. (**B**) Volcano plot showing differentially expressed genes (orange color) in AngII-treated RVSMCs transfected with *Alivec*Gap vs. NCGap. Labeled dots indicate genes involved in chondrogenesis. (**C**) Gene ontology (GO) analysis by the TOPPGENE tool of differentially-expressed (DE) genes showing the top 10 biological processes enriched in downregulated genes after *Alivec* knockdown. (**D**) KEGG pathway analysis of differentially-expressed (DE) genes, showing the top 10 molecular pathways affected in downregulated genes after *Alivec* knockdown. (**E**–**I**) RT-qPCR validation of indicated chondrogenic genes after *Alivec* knockdown in RVSMCs treated ± AngII (100 nM, 3 h). Data presented as mean ± SD, one-way ANOVA followed by Tukey's post-hoc test and ** $p < 0.01$ and *** $p < 0.001$ vs. indicated groups) n = 3 biological replicates.

RT-qPCR validation of microarray data confirmed downregulation of *Acan* and several other chondrogenic genes, including *Tnfaip6*, *Runx1*, *Olr1* and *Spp1* (**Figure 3**E–I), after *Alivec* knockdown in RVSMCs. In addition, *Acan* downregulation is consistent with the known role of lncRNAs in regulating adjacent genes (**Figure 3**B).

Conversely, in gain-of-function experiments, transient overexpression of *Alivec* increased mRNA levels of *Acan*, *Runx1*, *Tnfaip6*, *Olr1* and *Runx2*, relative to the controls (**Figure 4**A–F). Together, these results demonstrate that lncRNA *Alivec* plays a key role in the regulation of AngII-induced chondrogenic genes in RVSMCs.

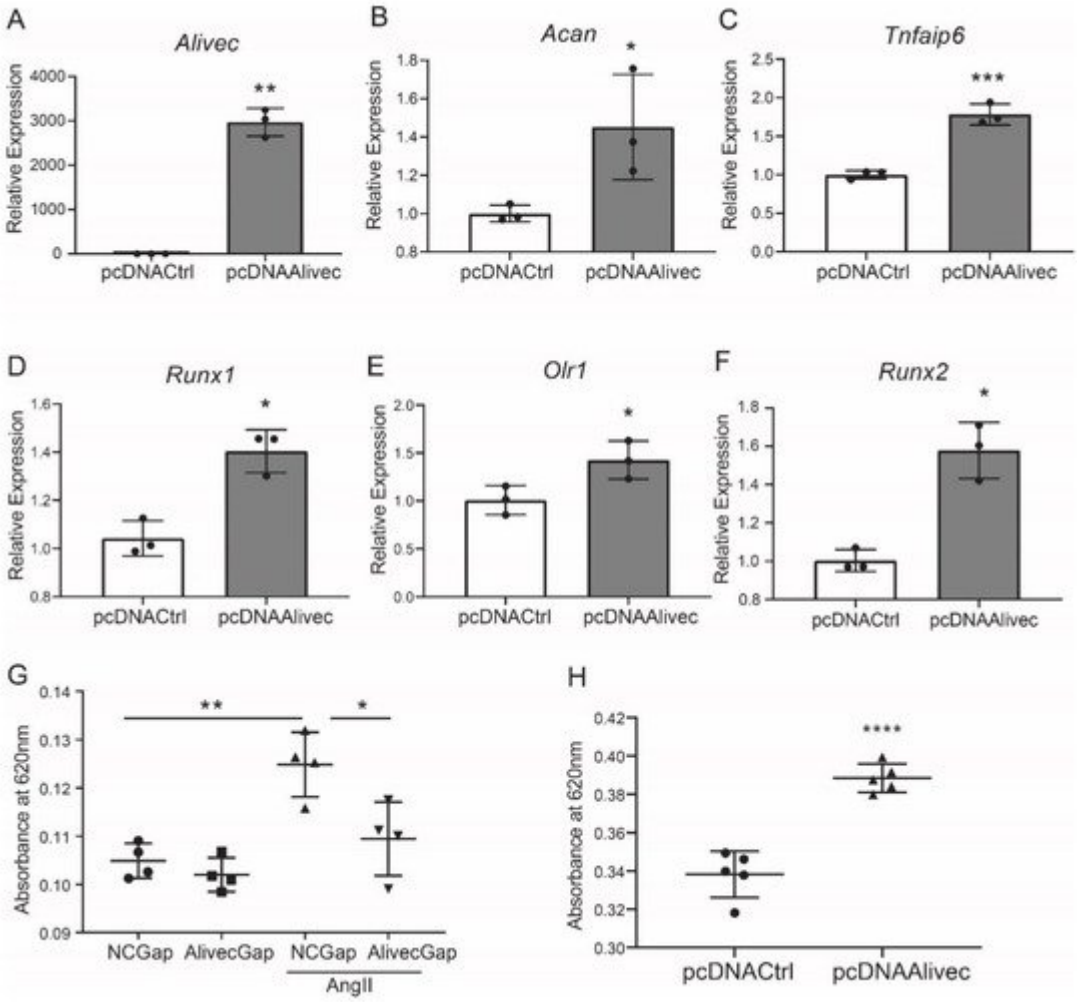

Figure 4. *Alivec* overexpression promotes and its knockdown inhibits the chondrogenic/osteogenic phenotype in RVSMCs. (**A**) RT-qPCR analysis showing expression of *Alivec* after transfection of RVSMC with pcDNAAlivec vs. empty vector (pcDNACtrl). (**B**–**F**) RT-qPCR analysis showing expression of target genes *Acan*, *Tnfaip6*, *Runx1*, *Olr1* and *Spp1* after overexpression of *Alivec* in RVSMCs. Data presented as mean ± SD, n = 3 biological replicates, unpaired two-tailed Student's *t*-test and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. pcDNACtrl. (**G**) Alcian blue staining performed on RVSMCs transfected with NCGap and *Alivec*Gap and treated ± AngII (100 nM). Data were presented as mean ± SD, $n = 4$ biological replicates, one-way ANOVA followed by Tukey's post-hoc correction and * $p < 0.05$, ** $p < 0.01$ vs. indicated groups. (**H**). Alcian blue staining after overexpression of *Alivec* in RVSMCs. Data presented as mean ± SD, $n = 5$ biological replicates, unpaired two-tailed Student's *t*-test and **** $p < 0.0001$ vs. pcDNACtrl.

5. Alivec Mediates a Chondrogenic/Osteogenic Phenotype in RVSMCs

Alcian blue stains glycosaminoglycan proteins, including aggrecan, that are associated with the ECM and chondrogenic differentiation [25]. Relative to control, AngII-treated RVSMCs showed increased alcian blue staining, and this was significantly decreased by *Alivec* knockdown with GapmeR (**Figure 4**G). Conversely, overexpression of *Alivec* increased alcian blue staining (**Figure 4**H). These data demonstrate that *Alivec* regulates expression of several AngII-induced chondrogenic genes, including nearby *Acan*, and promotes a chondrocyte phenotype in RVSMCs.

6. Transcription Factor Sox9 Controls Alivec Expression in RVSMCs

Transcription factor (TF) motif analyses of 500 bases upstream of the *Alivec* transcription start site (TSS) showed enrichment of ten TFs, including Sox9 (**Figure 5**A). Sox9 regulates chondrogenesis and osteogenesis in mesenchymal stem cells [26]. researchers examined Sox9 interaction with the *Alivec* promoter in RVSMCs transfected with a Sox9 expression plasmid (pcDNASox9) and a control vector (pcDNACtrl), using chromatin immunoprecipitation (ChIP) assays with the Sox9 antibody. ChIP-qPCR showed enrichment of Sox9 in the predicted Sox9-binding region, upstream of the *Alivec* TSS, as compared with the control pcDNACtrl plasmid-transfected cells (**Figure 5**B). Transfection of RVSMCs with the siRNAs targeting *Sox9* (siSox9), reduced the Sox9 protein and transcript levels in control- and AngII-treated cells (**Figure 5**C,D). Sox9 knockdown also decreased the AngII-induced expression of *Alivec* and *Acan* (**Figure 5**E,F). Conversely, the overexpression of Sox9 using the pcDNASox9 plasmid in RVSMCs increased *Alivec* and *Acan* vs. the control vector-transfected cells (**Figure 5**G–I). These results demonstrate that Sox9 can regulate *Alivec* and *Acan* expression in response to AngII in RVSMCs.

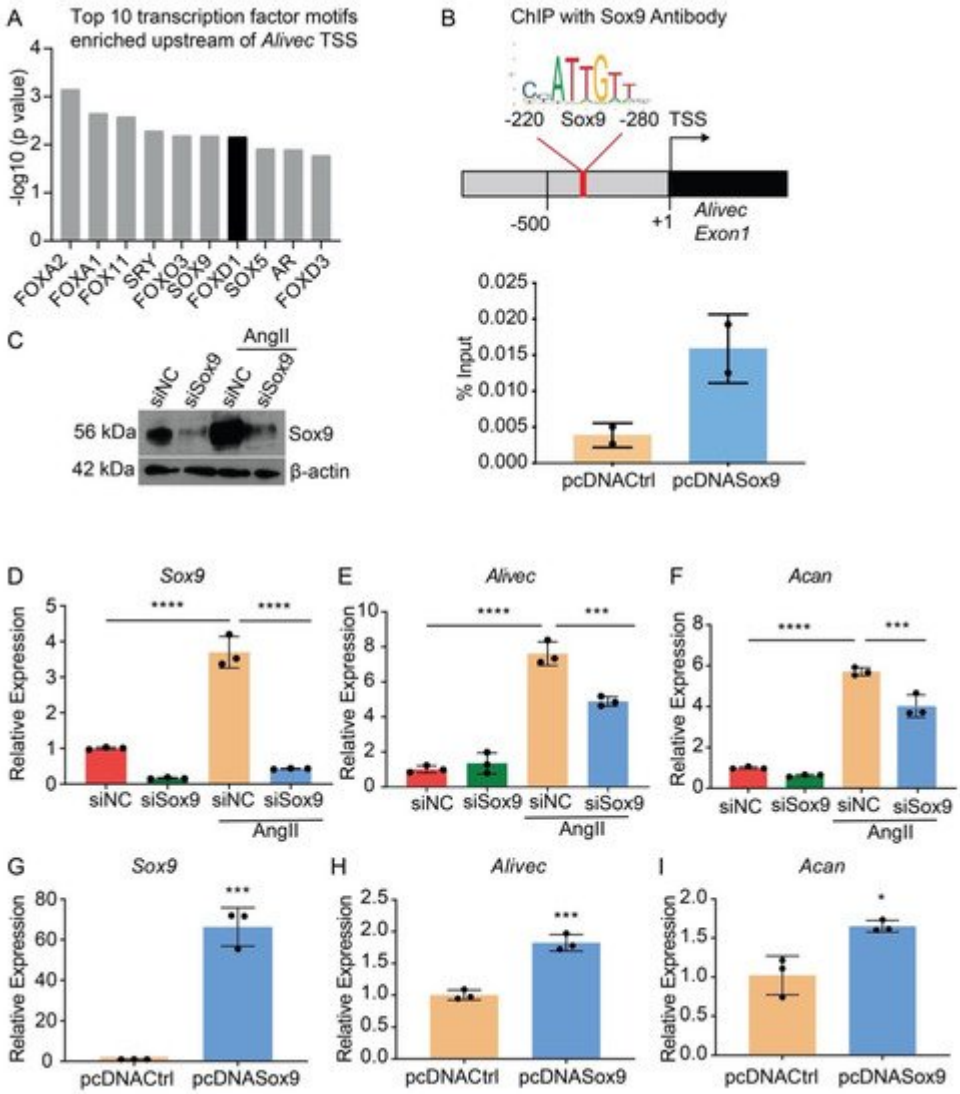

Figure 5. Transcription factor Sox9 controls *Alivec* expression in RVSMCs (**A**). Top 10 transcription factor (TF) binding motifs, enriched in the genomic region upstream of *Alivec* transcription start site (TSS). (**B**) ChIP assays with Sox9. Upper panel depicts schematic of the predicted Sox9-binding site upstream of *Alivec* TSS (arrow). Lower panel show ChIP-qPCR data with Sox9 antibody in RVSMCs transfected with pcDNACtrl and pcDNASox9 plasmids. ChIP-DNA was analyzed by qPCR with *Alivec* promoter primers overlapping Sox9-binding sites (n = 2). (**C**) Sox9 knockdown with siRNAs. Sox9 protein levels determined by Western blotting in RVSMCs transfected with siRNA targeting *Sox9* (siSox9) or negative control (siNC) oligonucleotides, and treated ± AngII (upper panel). β-actin protein levels (lower panel) were used as internal control. (**D**–**F**) RT-qPCR analysis of indicated genes in siSox9- and siNC-transfected RVSMCs at the basal level and after stimulation with AngII. Data presented as mean ± SD, n = 3 biological replicates, one-way ANOVA followed by Tukey's post-hoc test and *** $p < 0.001$, **** $p < 0.0001$ vs. indicated groups. (**G**–**I**). RT-qPCR analysis of indicated genes in RVSMCs, transfected with Sox9 expression plasmid (pcDNASox9) and pcDNACtrl control plasmid. Data represented as mean ± SD, n = 3 biological replicates, unpaired Student's t-test and * $p < 0.05$, *** $p < 0.001$ vs. control plasmid.

7. Alivec RNA Interacts with hnRNPA2B1 as well as with Tropomyosin alpha-3 Chain, a Protein with Putative Association with the Contractile Phenotype of RVSMCs

LncRNAs can regulate transcription, gene expression and cellular phenotype through interactions with proteins [27][28]. Researchers performed RNA-pulldown assays with *Alivec*, followed by mass spectrometry, and found a number of proteins associated with *Alivec*, relative to negative control. STRING analysis demonstrated that the *Alivec* interacting proteins were associated with VSMC contractile functions, nuclear membrane organization and regulation of gene expression (**Figure 6**A). One of these proteins, a tropomyosin alpha-3 chain (Tpm3) [29] was noteworthy, due to the known roles of alpha-tropomyosin isoforms in VSMC contractile functions and gene regulation [30][31]. RNA–protein interaction prediction (RPISeq) software showed that the *Alivec*-Tpm3 RNA–protein interaction had a positive interaction probability of 0.75 (>0.5 considered positive). Researchers then performed RNA-pulldown, followed by Western blot analysis, in order to validate the Tpm3 association with *Alivec* (**Figure 6**B), which confirmed the mass spectrometry results. Specific interaction of *Alivec* with Tpm3 was also supported by RNA-immunoprecipitation (RNA-IP), using an antibody against Tpm3. No interaction was seen with *Gapdh* mRNA and *H19* lncRNA (negative controls, **Figure 6**C). In addition, mass spectrometry showed that *Alivec* interacts with the RNA-binding protein, heterogeneous nuclear ribonucleoproteinA2B1 (hnRNPA2B1), which was validated by RNA-pulldown, followed by Western blotting (**Figure 6**B, lower panel). These results indicate that lncRNA *Alivec* mediates AngII-induced VSMC dysfunction via interaction with both cytoplasmic and nuclear proteins.

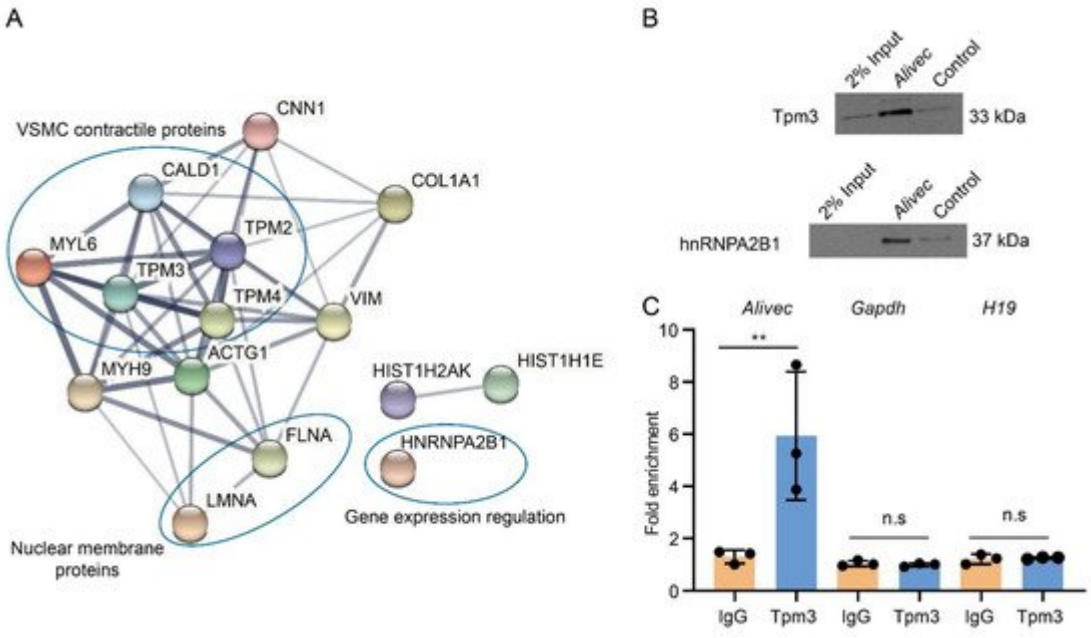

Figure 6. *Alivec* RNA interacts with hnRNPA2B1 and tropomyosin-3-alpha, a protein potentially associated with the contractile phenotype of VSMCs. (**A**) Network of protein complexes generated (using STRING database) from *Alivec*-specific interacting proteins identified by RNA-pulldown coupled to mass spectrometry. (**B**) Western blot analysis with Tpm3 antibody (upper panel) or hnRNPA2B1 antibody (lower panel) following RNA-pulldown assays

with RVSMCs extracts using biotinylated *Alivec* RNA and poly A RNA as negative control (Control). (**C**) RNA immunoprecipitation assays with UV-cross-linked RVSMC cell extracts using anti-Tpm3 antibody and IgG as negative control. RNA from Tpm3 and IgG immunoprecipitates were analyzed by RT-qPCR, using indicated primers. Results were shown as fold enrichment over IgG. Data presented as mean ± SD, n = 3 biological replicates and ** $p < 0.01$ vs. IgG, using unpaired Student's t-test. N.s. indicates not significant.

8. AngII Treatment Increases Aortic Expression of Alivec in Rats

Next, study examined whether AngII upregulates the expression of *Alivec* and *Acan* in vivo in rats. Male Sprague–Dawley rats (12-weeks-old), infused with AngII (200 ng/kg/min, four weeks), showed the expected increase in systolic blood pressure (SBP) compared to control vehicle (PBS) infused rats (**Figure 7**A). Aortic thickening was noted in AngII-infused animals relative to the controls (**Figure 7**B). Immunohistochemical staining showed marked increases in aggrecan and Runx1 proteins and decreases in the smooth muscle contractile proteins α-SMA and SM22 alpha (**Figure 7**B). Furthermore, mRNA levels of *Alivec*, *Acan* and *Runx1* were significantly increased in vessels from the AngII group (**Figure 7**C–E).

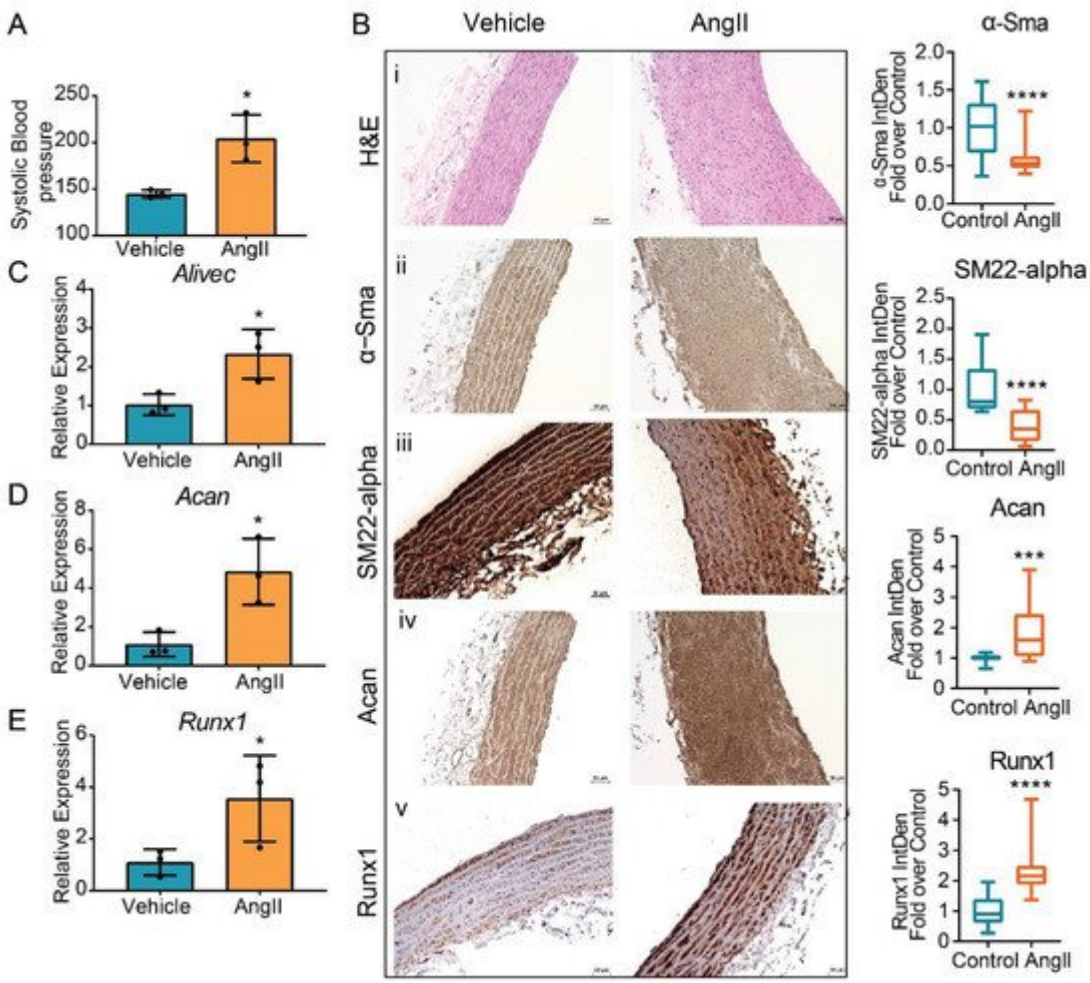

Figure 7. Regulation of *Alivec* and *Acan* in the aortas from a rat model of AngII-induced hypertension. (**A**) Systolic blood pressure (SBP) measured in male Sprague–Dawley rats infused with AngII or vehicle for 4 weeks. (**B**) Representative images of hematoxylin and eosin (H&E) staining (i) and IHC staining for α-Sma (ii), SM22-alpha (iii), Acan (iv) and Runx1 (v) proteins on aortic tissue sections from vehicle or AngII-infused rats, scale bar: 50 µM. Box plots on the right show the quantification of aortic staining of indicated proteins shown in panels (ii) to (v). Box plots on the right show integrated density (IntDen) expressed as fold-over control. Staining was quantified using ImageJ software in 20 different areas for each group (3 aortas in control and 3 in AngII group. Data represented as mean and minimum/maximum, unpaired Student's *t*-test and *** $p < 0.001$ and **** $p < 0.0001$). (**C**–**E**) RT-qPCR analysis showing gene expression of *Alivec*, *Acan* and *Runx1* in aortas from AngII-infused rats in comparison to vehicle-treated rats. Data presented as mean ± SD, $n = 3$ biologic replicates and unpaired Student's *t*-test. * $p < 0.05$ vs. vehicle.

9. Conclusion

A novel AngII induced lncRNA *Alivec* regulates genes associated with chondrogenic transformation of VSMCs implicated in vascular dysfunction, which could lead to the identification of non-coding RNA based biomarkers and therapeutic targets for CVDs.

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