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Novel Long Non-Coding RNAs Are Regulated by Angiotensin II in Vascular Smooth Muscle Cells

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Abstract

Rationale—Misregulation of angiotensin II (Ang II) actions can lead to atherosclerosis and hypertension. Evaluating transcriptomic responses to Ang II in vascular smooth muscle cells (VSMCs) is important to understand the gene networks regulated by Ang II which might uncover previously unidentified mechanisms and new therapeutic targets.

Objective—To identify all transcripts, including novel protein-coding and long non-coding RNAs, differentially expressed in response to Ang II in rat VSMCs using transcriptome and epigenome profiling.

Methods and Results—De novo assembly of transcripts from RNA-seq revealed novel protein-coding and long non-coding RNAs (lncRNAs). The majority of the genomic loci of these novel transcripts are enriched for histone H3 lysine-4-trimethylation and histone H3 lysine-36-trimethylation, two chromatin modifications found at actively transcribed regions, providing further evidence that these are bonafide transcripts. Analysis of transcript abundance identified all protein-coding and lncRNAs regulated by Ang II. We further discovered that one Ang II-regulated lncRNA functions as the host transcript for *miR-221* and *miR-222*, two miRNAs implicated in cell proliferation. Additionally, siRNA-mediated knockdown of *Lnc-Ang362* reduced proliferation of VSMCs.

Conclusions—These data provide novel insights into the epigenomic and transcriptomic effects of Ang II in VSMCs. They provide the first identification of Ang II-regulated lncRNAs, which suggests functional roles for these lncRNAs in mediating cellular responses to Ang II. Furthermore, we identify one Ang IIregulated lncRNA that is responsible for the production of two miRNAs implicated in VSMC proliferation. These newly identified non-coding transcripts could be exploited as novel therapeutic targets for Ang II-associated cardiovascular diseases.

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DISCLOSURES
None

Keywords

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INTRODUCTION

Cardiovascular diseases (CVDs) such as hypertension and atherosclerosis are associated with several pathophysiological changes which include hyperproliferation and hypertrophy of VSMCs within the blood vessel wall[1]. Fundamental to the development of these pathologies is angiotensin II (Ang II), a small polypeptide hormone which promotes many processes including vasoconstriction, inflammation, fibrosis, and cellular growth[2]. The Type 1 receptor (AT1R) and the Type 2 receptor (AT2R) mediate Ang II-signaling. Ang II-binding to AT1R results in most of its pathological effects[1–6]. Angiotensin converting enzyme inhibitors, which reduce Ang II availability, and AT1R blockers have been effective in reducing blood pressure and renal disease in patients and animal models, highlighting the importance of Ang II in the pathogenesis of cardiovascular and renal complications[7, 8]. At the cellular level, VSMCs treated with Ang II increases expression of pro-inflammatory and pro-fibrotic genes, including monocyte chemoattractant protein-1 (*Mcp-1*, also known as *Ccl2*), interleukin-6 (*Il-6*), and plasminogen activator inhibitor-1 (*Pai-1*). Additionally, extracellular matrix genes (ECM) such as collagen and fibronectin are also induced by Ang II[1, 4, 6, 9–11], however, the molecular mechanisms that mediate the cellular response to Ang II and ultimately the pathogenesis of CVDs are not fully clear.

Recent studies have begun to elucidate the role of non-coding RNAs in VSMC biology. MicroRNAs (miRNAs) are short non-coding RNAs that have been shown to be important for VSMC proliferation, differentiation, and contractility[12–14]. We recently characterized specific miRNAs that are involved in mediating inflammatory and fibrotic responses of VSMCs to Ang II[15]. It has recently become apparent that, in addition to small RNAs, many long non-coding transcripts (lncRNAs) function in a variety of responses which include differentiation, cell cycle, and maintenance of stem-cell like phenotypes, and are cell-type specific in their expression[16–23]. Yet, very little is known about their regulation or roles in disease states. RNA-sequencing (RNA-seq) has recently been applied to assess the transcriptome of normal and failing murine hearts of mice to discover lncRNAs that may be involved[24]. However, it is not known whether lncRNAs have a role in VSMCs or in the actions of Ang II since a comprehensive analysis of the VSMC transcriptome in response to Ang II has not yet been performed. Therefore, a thorough investigation of transcripts regulated by Ang II is important to further understand the molecular mechanisms that drive CVDs.

In this study, we sought to identify novel lncRNAs in the rat genome that may play a role in the response of VSMCs to Ang II. We performed RNA-seq followed by *de novo* assembly of transcripts from rat VSMCs and identified previously unannotated transcripts. A large number of these novel transcripts have limited protein-coding potential which we classified as lncRNAs. We profiled histone H3-lysine-4 trimethylation (H3K4me3) and histone H3-lysine-36 (H3K36me3), two epigenetic chromatin modifications associated with active transcription[21], genome-wide with ChIP-seq and discovered that the majority of genomic regions with novel transcripts, both protein-coding and non-coding RNAs, are enriched for these modifications. This suggests that the novel transcripts identified are *bona fide* transcripts.

We next identified 491 transcripts that are differentially expressed in response to Ang II including 14 novel protein-coding transcripts and 24 novel lncRNAs. Many of the lncRNAs are located proximal to other Ang II-regulated genes suggesting that they may be co-regulated in response to Ang II. One lncRNA, *Lnc-Ang362*, is proximal to two miRNAs, *miR-222* and *miR-221*, which have been previously associated with regulating VSMC proliferation and Ang II actions in endothelial cells[25, 26]. *Lnc-Ang362* and the two mature miRNAs are similarly upregulated in response to Ang II in VSMCs. Knockdown of *Lnc-Ang362* reduces the expression of the miRNAs, indicating that miRNAs are co-regulated with the lncRNA. We furthermore observed that a reduction of *Lnc-Ang362* transcript level is associated with a decrease in cell proliferation, suggesting that it plays a role in cell growth. The differential expression of *Lnc-Ang362* and the mature miRNAs with Ang II treatment is also supported by *ex vivo* experiments.

Taken together, our studies reveal genome-wide changes in gene expression and key histone modifications in Ang II-treated VSMCs that have led to the first identification of novel protein-coding transcripts and lncRNAs that may function in Ang II-mediated cellular responses. The data also reveals that one lncRNA functionally acts as a host transcript for two miRNAs with key pathological effects in VSMCs. Given that the rat genome is much less annotated than the human or mouse, our data also provides new insights into this valuable animal model.

METHODS

Cell culture and Ang II treatment of VSMCs

Animal studies were approved by the Institutional Animal Care and Use Committee and performed according to approved protocols. VSMCs were isolated and cultured as previously described[3, 9]. Ang II(0.1uM, Bachem) treated cells were processed for RNA extraction or chromatin-immunoprecipitation experiments. For *ex vivo* analyses, aortas were isolated from male rats, the adventitial layers removed, and split for control and Ang II treatment. For RNA analysis, aortas were homogenized with bead beating.

RNA sequencing

RNA was extracted from cells using Trizol(Invitrogen). RNA(2ug) was depleted of ribosomal RNA(Ribominus, Invitrogen). Eluted RNA was prepared for sequencing using Illumina protocols, and then sequenced on the HiSeq 2000(Illumina) to generate 2×80bp paired-end reads. We obtained 86M and 80M reads for two controls(independent biological replicates) and 88.9M and 89M reads for two Ang IItreated VSMCs. The reads were aligned to the rat genome [version rn4 (Nov. 2004), from UCSC Genome Browser] using Bowtie(0.12.7)[52] with Tophat(1.3.0)[53]. We used Cufflinks[53] to first assemble transcripts from all datasets using the RefSeq gene annotation as a reference guide. All mouse and human XenoRefSeq annotations that overlapped with the unannotated transcripts were identified. Nonoverlapping transcripts were assessed for protein-coding potential using PhyloCSF[27](Dr. Michael F. Lin, Massachusetts Institute of Technology, Boston). Using a threshold previously used to identify non-coding RNAs, all multi-exonic transcripts with a PhyloCSF score>20 were called novel protein-coding transcripts and <20 as lncRNAs[27, 28]. To assess the biological reproducibility between the replicates for each condition, FPKM was determined for all genes and Pearson's Correlation value was determined for each gene (Online Figure III). Cuffdiff was used to identify differentially expressed transcripts with the reference annotation containing rat RefSeq genes, homologous mouse and human XenoRefSeq genes, novel protein-coding transcripts, and lncRNAs with an FPKM>0.5. RT-QPCR was performed with at least three biological replicates of control and Ang II-treated cells to validate the changes in gene expression identified for twenty genes. Specifically,

RNA was extracted, converted to cDNA(Taqman Kit, Applied Biosystems), and analyzed for transcript abundance using QPCR(Kapa SYBR Green, Kapa Systems; AB7500, Applied Biosystems; primers (Online Table VII)). To compare RT-QPCR values and RNA-seq, each transcript was normalized to *CYPA* and assessed with Pearson's correlation. For analysis of transcript size, genomic coordinates were obtained for each rat RefSeq gene, non-coding transcript, and protein-coding transcript. Transcript size was determined from the start and stop of each coordinate. For expression level analysis comparing rat RefSeq transcripts, novel protein-coding, and lncRNAs, FPKM of each transcript in control and Ang II-treated cells were averaged and the frequency was determined.

To assess the significance of the proximity of co-regulated lncRNAs and protein-coding genes, we carried out 100,000 simulations of proximity measurement on randomly placed co-regulated lncRNAs and protein-coding genes in the rat genome. Specifically, we first placed 30,680 "genes" and 466 "lncRNAs" randomly across the 2,718,881,021bp genome. We then randomly selected 467 of the "genes" and 24 of the "lncRNAs" to be co-regulated and counted the number of simulated Ang II-regulated "lncRNAs" were within 500kb of a simulated Ang II-regulated "gene." From this analysis, the empirical *p*-value for 7 co-regulated lncRNAs and protein-coding genes was 0.02734.

miRNA expression and transcript analysis

Total RNA was extracted at the specified time points after Ang II treatment. miRNA cDNA was generated (qscript (Quanta Biosciences)) for QPCR. miRNA levels were normalized to small RNA *U6*. For the analysis of *miR-222* and *miR-221* host transcript, cDNA was generated from total RNA using GeneRacer Kit (Life Technologies). Primers were generated to amplify transcripts containing *miR-222- miR-221* in PCR reaction (Phusion Polymerase, Invitrogen). As a control, cDNA reaction was carried out without reverse transcriptase (−RT) and genomic DNA was amplified as positive control.

Chromatin Immunoprecipitation (ChIP) –sequencing

ChIPs were carried out with an anti-H3K4me3 antibody (04–745, Millipore) and anti-H3K36me3(ab9060, Abcam) using standard ChIP protocols. 20ng of ChIP DNA was prepared for sequencing on the Illumina GAII machine which produced 42bp reads. We obtained 44.5M reads for Control-H3K4me3, 42.9M reads for Control-H3K36me3, 48.7M reads for Control-Input, 41.7M reads for Ang II-H3K4me3, 42.9M reads for Ang II-H3K36me3, and 48.2M reads for Ang II-Input. Bowtie was used to map the reads to the rat genome(rn4, UCSC Genome Browser) resulting in 33.2M reads for Control-H3K4me3, 34.9M reads for Control-H3K36me3, 37.3M reads for Control-Input, 32.2M reads for Ang II-H3K4me3, 31.2M reads for Ang II-H3K36me3, and 36.4M reads for Ang II-Input – roughly 70% of the reads aligned to the genome. Normalized profiles of histone modifications in promoter regions were performed as described previously[54].

RSEG was used to identify regions of the genome that are enriched for H3K4me3 and H3K36me3 compared to input samples using default parameters(bins with $p<0.05$ were defined as boundaries)[55]. Genomic regions enriched for H3K4me3 and H3K36me3 in control and Ang II-treated samples were compared to the genomic coordinates for rat RefSeq genes, mouse and human XenoRefSeq genes, novel protein-coding transcripts, and lncRNAs. Any overlap between the modification enriched regions and the genes were determined to be positive enrichment of the modification at the gene.

Transfections

siRNA knockdown of *Lnc-Ang362* performed using 1million cells were transfected according to manufacturer's protocol(Amaxa) using 50nM of a pool of siRNAs targeting

Lnc-Ang362 or 50nM of control siRNAs(Non-Targeting Control, Thermo Scientific). The algorithm GPboost was used to design siRNAs for targeting *Lnc-Ang362*[56].

The pool of siRNA oligos are the following: 5' –TTTCTCTGTACAAATGTCAUU-3'/5' TGACATTTGTACAGAGAAAUU-3', 5'- UUUCUGCCAUGACAUUUACUU-3'/5' GUAAAUGUCAUGGCAGAAAUU-3', 5'- UAAGCAGAUGAAUUCUAACUU-3'/5'- GUUAGAAUUCAUCUGCUUAUU-3', 5'- TTTATTTGTCAGAACCTTTUU-3'/5'- AAAGGTTCTGACAAATAAAUU-3', and 5'- TATTTCTCTGATAAAGCATUU-3'/5' ATGCTTTATCAGAGAAATAUU-3'. 48 hours after transfection, the cells were treated with Ang II for three hours and RNA was collected by Trizol for standard RT-QPCR analysis.

Cell proliferation assay

VSMCs were plated subconfluent for Lipofectamine(Invitrogen) transfections with siRNAs (NTC or siRNAs targeting *Lnc-Ang362*). After five days, methyl thiazole tetrazolium assay was performed. Briefly, 5mg/ml of MTT dissolved in PBS was added to the media of the cells, cells were incubated for 3 hours to allow uptake of MTT, cells were then solubilized with 95% DMSO, and absorbance of the solution was measured.

Data access

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zvsnxeuosqymani&acc=GSE38056

RESULTS

Identification and characterization of novel lncRNAs in rat VSMCs

To uncover the transcriptomic effects of Ang II, we treated rat VSMCs for three hours with Ang II and then performed RNA-seq of RNA isolated from control and Ang II-treated cells from two independently isolated biological replicates. Initial assessment of the data revealed that many sequencing reads, from both control and Ang II-treated cells, align to regions outside of gene annotated regions (Online Table I). This was reminiscent of recent studies of the mouse and human genomes which discovered many lowly expressed intergenic transcripts, many of which are lncRNAs[16, 21, 22]. We therefore identified all intergenic transcripts that are expressed in rat VSMCs. Transcripts were first assembled *de novo*, and multi-exonic transcripts that did not overlap with known rat genes or homologous mouse and human genes were classified as novel (Fig. 1A). We discovered 833 transcripts using this approach. We further classified them as protein-coding or non-coding by assessing their protein-coding potential[27, 28]. We classified 466 transcripts with limited protein-coding potential as lncRNAs and 367 as protein-coding transcripts (Fig. 1A, OnlineTables II–III).

A number of these transcripts are proximal (< 500kb)[29] to previously annotated genes. For example, *RpcRNA-297* (rat protein-coding-RNA-297), a novel protein-coding RNA, and *Lnc-Ang52* (Lnc-Ang52) are within 100kb of *Cpe* and *Fzd2*, respectively (Fig. 1B–C). In comparison to previously annotated rat genes, both novel protein-coding and lncRNAs are shorter in length and are less abundant (Fig. 2A–C). The low abundance of lncRNAs is consistent with previously reported human and mouse lncRNA expression data[21, 28].

Evidence shows that certain chromatin modifications can be used to identify functional regions of the genome[30]. For example, studies have used histone H3K4me3, which is associated with transcriptional start sites, and histone H3K36me3, which is associated with gene bodies, to identify known as well as novel transcripts[21]. We performed, in parallel with the RNA-seq analysis, ChIP-sequencing to identify regions of the genome that were enriched with H3K4me3 and H3K36me3[31, 32]. As expected, in both control and Ang II-

treated VSMCs, H3K4me3 is enriched near transcriptional start sites (TSS) while H3K36me3 is enriched in gene bodies of annotated rat genes (Fig. 1B–C, Online Figure I). We found that the majority of the novel loci are also enriched for both chromatin modifications at levels similar to previously annotated rat gene loci (Fig. 2D). Eight percent of the novel transcripts do not show enrichment of either modification. We hypothesized that these loci are transcribed at very low rates and correspondingly may be enriched for the modifications below our detection threshold. To test this, we separated annotated rat genes by their expression levels and assessed the enrichment of the two modifications. Genes with low expression levels are more likely to be devoid of these two modifications in line with our hypothesis (Online Figure II). These data reveal that the novel loci have similar enrichment of chromatin modifications as previously annotated genes. Altogether, our analysis has identified novel protein-coding transcripts as well as lncRNAs which are expressed in rat VSMCs.

Ang II-regulated transcript expression in VSMCs

With the expanded list of rat gene annotation which includes homologous human and mouse genes, and novel protein-coding and lncRNAs, we next identified genes that are regulated in an Ang IIdependent manner. For this analysis, we retained only those transcripts that were transcribed higher than 0.5 fragments per kilobases per million reads (FPKM) in either control or Ang II-treated cells. Previous studies have used this cutoff and successfully identified lncRNAs associated with type 2 diabetes[33]. In total, from RNA-seq of two biological replicates of each condition, for which we found high correlation (Online Figure III), 491 genes are differentially expressed in the Ang II-treated VSMCs with 331 that are Ang II-upregulated and 160 transcripts that are downregulated ($p<0.05$, FDR adjusted with Benjamini-Hochberg) (Fig. 3A, Online Table IV). Of those, five previously annotated lncRNAs and 24 novel lncRNAs are Ang II-regulated (19 upregulated, 5 downregulated). We also find 14 novel protein-coding transcripts are Ang II-regulated. To validate the expression profiles generated from the RNA-seq, we performed RT-QPCR analysis on twenty differentially expressed genes and found high correlation between the two datasets (all $p<0.05$ comparing means of Ang II-treated to control VSMCs) (Fig. 3B, Online Figure IV). Within the list of genes defined as differentially expressed, a number of proinflammatory and pro-fibrotic genes (e.g. *Mcp1* and *Serpine1 (Pai1)*) were identified consistent with previous reports[3, 34] (Fig. 3C, Online Table IV). Our combined RNA-seq/ChIP-seq analyses also revealed previously unknown Ang II-regulated genes such as *Adamts15*, proximal to the rat homolog of human *ADAMTS8* (Fig. 3D), and *Hivep3* (Fig. 3E). Expression of *Adamts15* is downregulated while *Hivep3* is dramatically upregulated. Interestingly, it has previously been shown that *Hivep3*, contains many alternative isoforms, and utilizes different promoters for transcription of its different isoforms[35]. In our H3K4me3 data, we observed two distinct regions that are enriched for H3K4me3 suggesting that those are the promoters that are utilized in VSMCs.

Thus, RNA-seq identified previously known and unknown transcripts regulated by Ang II. Interestingly, upon close inspection, the RNA-seq data contained many intronic reads such as those observed at the *Serpine1* (*Pai1*) locus which potentially represent nascent transcripts yet to be cotranscriptionally spliced, as described recently[36].

Ang II-upregulated gene loci exhibit increased enrichment of H3K4me3 and H3K36me3

To characterize the changes occurring at the chromatin level induced by Ang II, we assessed the enrichment of H3K4me3 and H3K36me3 in both control and Ang II-treated VSMCs. As noted above, H3K4me3 is enriched near the TSS while H3K36me3 is enriched in gene bodies (Online Figure I). We analyzed the enrichment of these marks at loci of genes with increased transcript abundance identified by the gene expression profiling. We first ranked

the genes by their fold change in cells treated with Ang II relative to control cells, and then compared the enrichment of H3K4me3 surrounding the TSS, and H3K36me3 across the gene body for each differentially expressed gene (Fig. 4A). Compared to the Ang II-downregulated loci, Ang II-upregulated gene loci exhibit increased enrichment of H3K4me3 and H3K36me3 (Fig. 4B–C). The ChIP-seq data was validated by follow up ChIP-QPCRs on a set of differentially modified regions (Online Figure V). These data indicate that methylation of H3K4 and H3K6 accompany most increases in transcript abundance, indicating changes in transcriptional rate in response to Ang II.

lncRNA expression is dynamic with Ang II treatment, and effects are recapitulated ex vivo

To further validate the regulation of key lncRNAs by Ang II, we analyzed the time course of expression of two downregulated lncRNAs, *Lnc-Ang219* and *lncRNA-Ang249*, and three upregulated lncRNAs, *Lnc-Ang362, Lnc-Ang162*, and *Lnc-Ang112* (Fig. 5A–B, $N = 5$). The abundance of both downregulated lncRNAs is reduced by Ang II at 3hr and 6hr time points ($p < 0.05$), but return to basal level by 24 hours. Similarly, the upregulated lncRNAs are increased during the early timepoints, but return to basal level by 24 hours (*Lnc-Ang362*, 3hr and 6hr, *Lnc-Ang162*, 3hr, *Lnc-Ang112*, 3hr and 6hr ($ps < 0.05$)). These data indicate that the expression of these lncRNAs in response to Ang II is dynamic. We next examined whether these observations in vitro could be recapitulated *ex vivo*. Aortas were isolated and treated with Ang II and expression of the lncRNAs was assessed ($N = 6$). Indeed, with Ang II treatment, *Lnc-Ang362* (6hr), *Lnc-Ang112* (3hr, 6hr, and 24hr) and *Lnc-Ang162* (6hr) are upregulated, while *Lnc-Ang219* (3hr) and *Lnc-Ang249* (3hr, 6hr, and 24hr) are downregulated (Fig. 5C–D, $ps < 0.05$).

A key biological function of an Ang II-regulated lncRNA is co-transcription with miRNAs and alteration of VMSC proliferation

Previous analyses of lncRNAs have indicated that they are often co-regulated with neighboring genes and can have enhancer-like activities[22, 29]. We found that seven of our differentially expressed lncRNAs are proximal (< 500kb) to differentially expressed genes (Table 1, $p < 0.05$, all adjacent genes can be found in Online Table V). Six of these lncRNAs are regulated by Ang II in the same direction as the proximal gene in our RNA-seq dataset.

Within this list is *Lnc-Ang362*, an Ang II-upregulated lncRNA proximal to *miR-221* and *miR-222* (Fig. 6A). Previous studies have shown these two miRNAs are involved in VSMC proliferation and are upregulated in response to Ang II in endothelial cells to promote inflammation and migration[25, 26]. Our RNA-seq analysis did not identify *miR-221* or *miR-222* because our method of RNA-sequencing does not assess small RNAs. To verify whether these miRNAs are upregulated in response to Ang II, we assessed the expression of mature miR-222 and miR-221 by RT-QPCR. We found that these miRNAs are indeed upregulated (Fig. 6B, *miR-222*, 3hr and 6hr, and *miR-221*, 6hr, $ps < 0.05$ $N = 5$). Furthermore, we found that the mature miRNAs are also upregulated with Ang II treatment of aortas *ex vivo* (Fig. 6C, *miR-222* and *miR-221* 3hr, 6hr, and 24hr, $N = 6$, $ps < 0.05$). These data indicate that the regulation of the mature miRNAs by Ang II in vitro is recapitulated *ex vivo*. Interestingly, *miR-221*/*miR-222* locus is not enriched for H3K4me3 suggesting that the initiation of transcription for these two miRNAs is further upstream (Fig. 6A). The closest region enriched for H3K4me3 upstream from the miRNAs is located at the transcriptional start site for *Lnc-Ang362*. LncRNAs, including the *H19* RNA that is involved in imprinting and X chromosome inactivation [37], have been shown to encode miRNAs. We therefore hypothesized that the two miRNAs are co-transcribed with *Lnc-Ang362* and we decided to test whether the miRNAs are transcribed as part of a larger transcript. PCR amplification of cDNA from VSMCs utilizing primers surrounding the miRNA locus (Fig. 6D) shows that the miRNAs are indeed transcribed as part of a larger

transcript (Fig. 6E). Furthermore, the abundance of this transcript is, as posited, increased by Ang II and in a time-dependent manner. We were further able to amplify a ~6kb amplicon from cDNA containing the two miRNAs and the 3' end of *Lnc-Ang362* (Online Figure VI). This suggests that the *miR-221* and *miR-222* are co-transcribed with *Lnc-Ang362*, the host transcript from which these miRNAs are excised, and that the transcription initiation site to produce the two miRNAs is further upstream.

We next assessed whether the expression of *Lnc-Ang362* is necessary for the expression of the mature miRNAs during Ang II treatment by evaluating the effects of siRNA knockdown of *Lnc-Ang362*. Transfection of VSMCs with specific siRNAs designed using the GPboost algorithm[38] targeting *Lnc- Ang362* was able to significantly attenuate the expression of the mature miRNAs, miR-221 and miR-222 (Fig. 7A, $p < 0.05$, $N = 3$). Furthermore, we evaluated the functional consequences of this knockdown by assessing the rates of proliferation of VSMCs transfected with siRNAs targeting *Lnc-Ang362*. Results showed that these cells proliferate to a lesser extent than VSMCs transfected with control siRNAs (Fig. 7B, $p < 0.05$, $N = 6$), suggesting that the lncRNA plays a role in VSMC growth. Furthermore, knockdown of *Lnc-Ang362* produced a significant reduction in the expression of *Mcm7* (Fig. 7C, $p < 0.05$, $N = 3$), a member of the minichromosome maintenance (MCM) protein complex which contains DNA helicase activity required for the initiation of DNA replication and cell cycle progression[39]. Expression of MCMs have previously been shown to be induced by Ang II in human VSMCs, and we also observed this in rat VSMCs (not shown)[40]. Since *miR-222* and *miR-221* are known to play a role in proliferation of VSMCs, the effects of *Lnc-Ang362* on proliferation may likely be through its role as the host transcript for the two miRNAs. Interestingly, maps of H3K4me3 from various mouse and human cells show that this chromatin modification is not enriched at the locus encoding for *miR-222/miR-221* but is enriched ~25kb upstream from them even in these cells (Online Figure VII)[41,42]. This suggests that the function of the lncRNA may be conserved in human and mice.

DISCUSSION

The profiling approaches used in this study have led to several novel discoveries in rat genomics, VSMC and Ang II-mediated gene regulation. Since rats are a widely used model organism for research related to cardiovascular disease, diabetes and other pathologies, it is imperative to analyze their transcriptome in-depth. Our annotation of novel transcripts significantly expands the existing number of known transcripts expressed in the rat genome, which, compared to the human and mouse genomes, remains less well-annotated. We found that, similar to mouse and human, the rat genome contains lncRNAs which are less abundantly expressed than previously annotated rat transcripts[22]. Notably, our studies revealed that the identified lncRNAs may function as host transcripts for small RNAs such as miRNAs. Our analysis of the transcriptome and epigenome associated with H3K4me3 and H3K36me3 uncovered that *Lnc-Ang362* is proximal to *miR-221* and *miR-222*. Based on the enrichment of H3K4me3, which is at the TSS of *Lnc-Ang362* but not at the locus containing the two miRNAs, we speculated that the lncRNA and miRNAs are co-regulated and that *Lnc-Ang362* is the host transcript for the two miRNAs. Indeed with knockdown studies, we found that the miRNAs are transcribed as a large transcript that is Ang II-regulated and that *Lnc-Ang362* is required for the expression of the two miRNAs. We also found that *Lnc-Ang362* affects cell proliferation and expression of a critical component of cell cycle progression, *Mcm7*, presumably through its effects on the miRNAs. Additionally, this reveals that the transcription initiation site for the production of the two miRNAs (*miR-221* and *miR-222*) is further upstream than would be expected and that this may be conserved in human and mice based on enrichment profiles of H3K4me3. It is worth noting that the H3K4me3 domain related to *miR-132* and *miR-212*, which we had previously

discovered to be regulated by Ang II[15], is on the other hand, very proximal to the locus containing the miRNAs (Online Figure VIII). Further analysis in the regulation of transcription initiation at the TSS of *Lnc-Ang362* will provide pertinent information on how the two miRNAs and *Lnc- Ang362* are specifically regulated during VSMC proliferation. It is also possible that the host *Lnc-Ang362* has miRNA-independent affects. To-date, only few studies have reported functional roles of lncRNAs due to their long size and secondary structure which makes it more difficult to target with siRNAs or generate expression vectors[43].

In addition to functioning as host transcripts for miRNAs, lncRNAs may function as enhancers and regulate the expression of proximal genes[29, 44].We discovered many novel lncRNAs that are differentially expressed by Ang II and are proximal to other transcripts that are also Ang II-regulated. Studies investigating co-regulation of lncRNAs with their neighbors and whether this occurs via alterations of enhancer chromatin modifications will be informative in understanding the role of the lncRNAs in Ang II effects.

We also provide genome-wide enrichment profiles of H3K4me3 and H3K36me3 in control and Ang II-treated VSMCs. We found that genomic regions with upregulated transcripts are more enriched for these two modifications with Ang II than genomic regions with downregulated transcripts. This is consistent with the idea that the increase in transcript abundance is generally regulated at the transcriptional level which in turn is associated with increase in H3K4me3 and H3K36me3. By comparison, downregulation of genes with Ang II is not accompanied by a marked decrease of H3K4me3 or H3K36me3. This could be due to a number of scenarios: 1) if the downregulation of transcript abundance is at the transcriptional level then these results would indicate that the removal of H3K4me3 and H3K36me3 is not as dynamic as their addition at 3 hours, or 2) the transcript abundance is regulated at a post-transcriptional level such as miRNA-directed degradation or mRNA stability. Similar lack of decrease in histone methylation has previously been observed in other systems as well[45]. It is likely that a combination of these scenarios is functioning in response to Ang II.

Notably, this is the first study to profile the expression of transcripts by high-throughput sequencing methods in VSMCs and their response to Ang II. We were able to identify new genes regulated by Ang II. One of these is *Adamts15*, which encodes for an ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein. ADAMTS family members have been found to play a role in VSMC migration and invasion[46]. Low expression of *ADAMTS15* has been suggested to be involved in colorectal, breast, and prostate cancer perhaps through a TGF-β signaling mechanism[47, 48]. Interestingly, while *Adamts15* is proximal to *Adamts8*, Ang II only reduces the expression of *Adamts15* and not *Adamts8*. Comparing the regulation of *Adamts15* to its family members may identify key features such as transcription factor networks that regulate ADAMTS proteins in response to Ang II. Our dataset also revealed that one of the novel genes highly induced by Ang II is *Hivep3* (also known as *Zas3/KRC/Shn-3*), a member of the Shnurri family of proteins which encodes for a transcription factor known to bind to NF-κB motifs and modulate the effects of NF-κB on gene expression[49]. This gene plays a key role in osteogenesis, vascular calcification and bone resorption[50, 51], but the precise role of *Hivep3* in VSMCs and on Ang II effects has not been explored. Investigations into the role of *Adamts15* and *Hivep3* have the potential to elucidate novel gene targets and molecular mechanisms that mediate Ang II effects. Furthermore, we cross-referenced human genes previously associated with cardiovascular disease and/or traits and found that a number of them are among the genes that we identified to be regulated by Ang II (Online Table VI). This suggests that the genes may potentially be involved in the vascular effects of Ang II. Further investigations will be

necessary to examine these interesting correlations and the role of these genes in Ang II biology.

Taken together, this study provides an in-depth view into the transcriptome of rat cells that has led to the first elucidation of novel genes and lncRNAs regulated by Ang II. These transcripts represent potential new avenues of research into how VSMCs respond to vasoactive stimuli like Ang II and ultimately promote CVDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

Ang II	angiotensin II
H3K4me3	Histone H3 Lysine 4 trimethylation
H3K36me3	Histone H3 Lysine 36 trimethylation
lncRNA	long non-coding RNA
RNA-seq	RNA-sequencing
ChIP-seq	Chromatin immunoprecipitation sequencing

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Novelty and Significance

What Is Known?

- Angiotensin II (Ang II), a major player in cardiovascular disease (CVD), promotes proliferation, hypertrophy, and inflammatory responses in vascular smooth muscle cells (VSMCs).
- Several downstream signaling pathways, transcription factors and protein coding genes mediate the effects of Ang II in VSMCs.
- Ang II regulates microRNAs, small non-coding RNAs that regulate translation and stability of messenger RNAs. These microRNAs contribute to inflammatory and growth promoting effects of Ang II.

What New Information Does This Article Contribute?

- Genome-wide transcriptome and epigenome profiling led to annotation of several novel transcripts in VSMCs, including those that are regulated by Ang II.
- Transcriptome profiling further identified, for the first time, long non-coding RNAs (lncRNAs) that might contribute to the effects of Ang II in VSMCs.
- Ang II regulated lncRNAs serve as hosts to specific microRNAs that are involved in VSMC proliferation.

Understanding the molecular mechanisms regulated by Ang II might lead to the identification of new therapeutic targets for the treatment of common CVDs such as hypertension and atherosclerosis. While recent studies have elucidated the role of small non-coding RNAs in regulating the biological effects of Ang II, the roles of lncRNAs have been largely unexplored. In the present study, we utilized a combination of transcriptome and epigenome profiling to identify lncRNAs that are expressed in VSMCs including those that are regulated by Ang II. We found that several lncRNAs are expressed dynamically in response to Ang II both in vitro and ex vivo. We also discovered that *Lnc-Ang362* is required for the expression of two microRNAs known to be involved in cell proliferation induced by Ang II. Knock down of *Lnc-Ang362* was associated with reduced proliferation of the VSMCs. These findings illustrate how newer genomic approaches could be used to identify novel factors and mechanisms that may drive CVD development and could be targeted by therapeutic interventions.

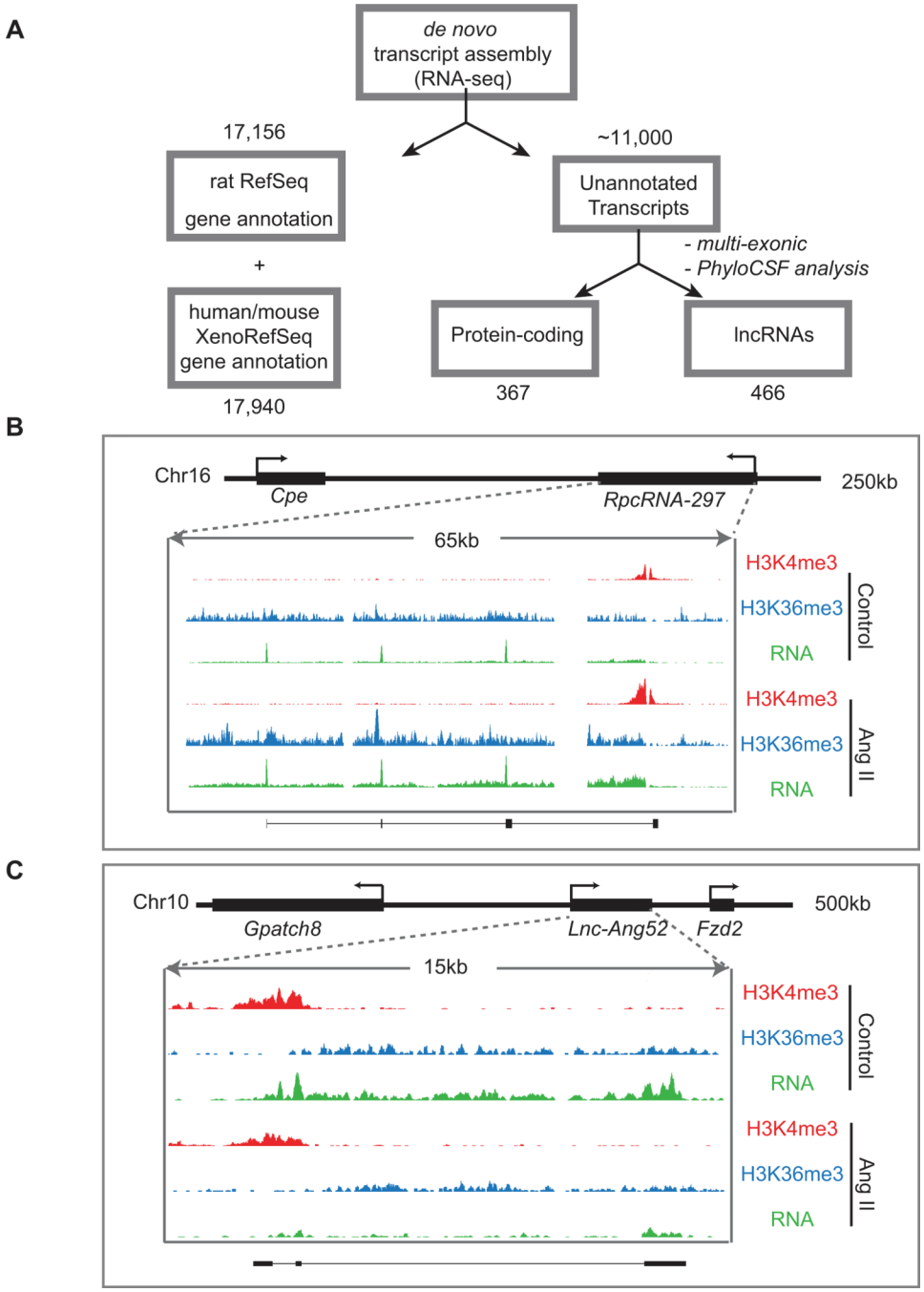

Figure 1. Identification of Novel Transcripts

A) Scheme for identifying novel transcripts. After de novo assembly of transcripts from RNA-seq, overlapping rat RefSeq genes and human/mice XenoRefSeq genes were removed. Multi-exonic transcripts were then assessed for protein-coding potential using PhyloCSF. (Numbers indicate the number of transcripts) **B**) Examples of novel protein-coding transcripts (*RpcRNA-297*) and **C**) lncRNA identified (*Lnc-Ang52*). For each genomic locus, shown are scoring profiles from ChIP-seq of H3K4me3 (red), H3K36me3 (blue), and scoring profiles from RNA-seq (green) in both control and Ang II-treated VSMCs for 3 hours. Above each set of tracks is a schematic of the chromosome location. Below the tracks

is the structure of the assembled transcript (black). Each data track shown is on the same scale for both control and Ang II.

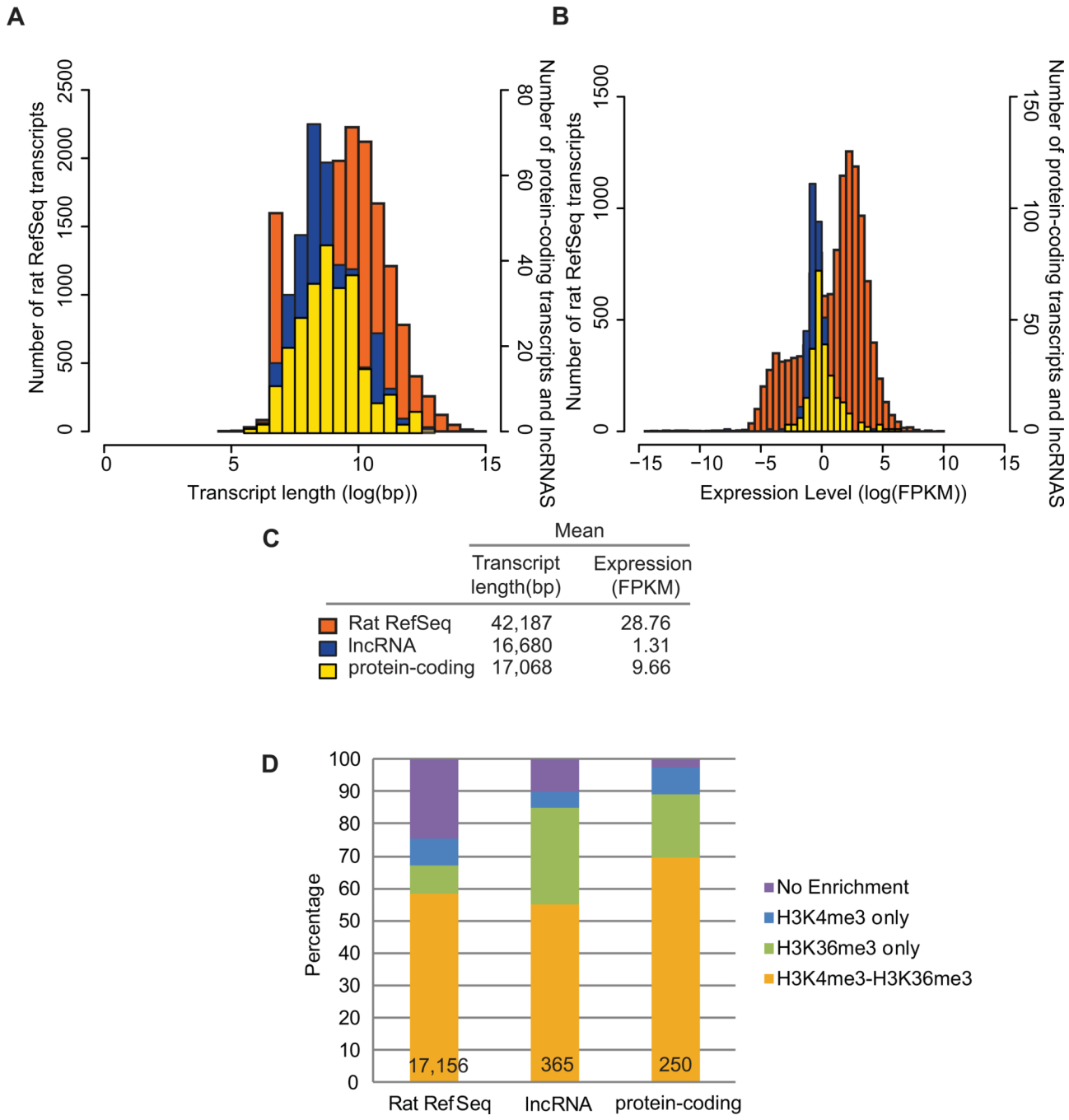

Figure 2. Characteristics of novel protein-coding transcripts and lncRNAs

A) Histogram of transcript length determined by distance from transcriptional start site and transcription end site for Rat RefSeq transcripts (orange), lncRNAs (blue), and protein-coding transcripts (yellow). Left axis is the number of rat RefSeq transcripts and right axis is the number of protein-coding transcripts and lncRNAs for each length. **B**) Histogram of expression level (FPKM) for Rat RefSeq transcripts, lncRNAs, and protein-coding transcripts. Left and right axes as in panel A). **C**) Mean length and expression are shown for each category of transcripts. **D**) Cumulative bar plots of Rat RefSeq transcripts, novel protein-coding transcripts, and novel lncRNAs that are enriched for H3K4me3 only,

H3K36me3 only, and both H3K4me3 and H3K36me3. At the base of each bar is the total number of transcripts in each category.

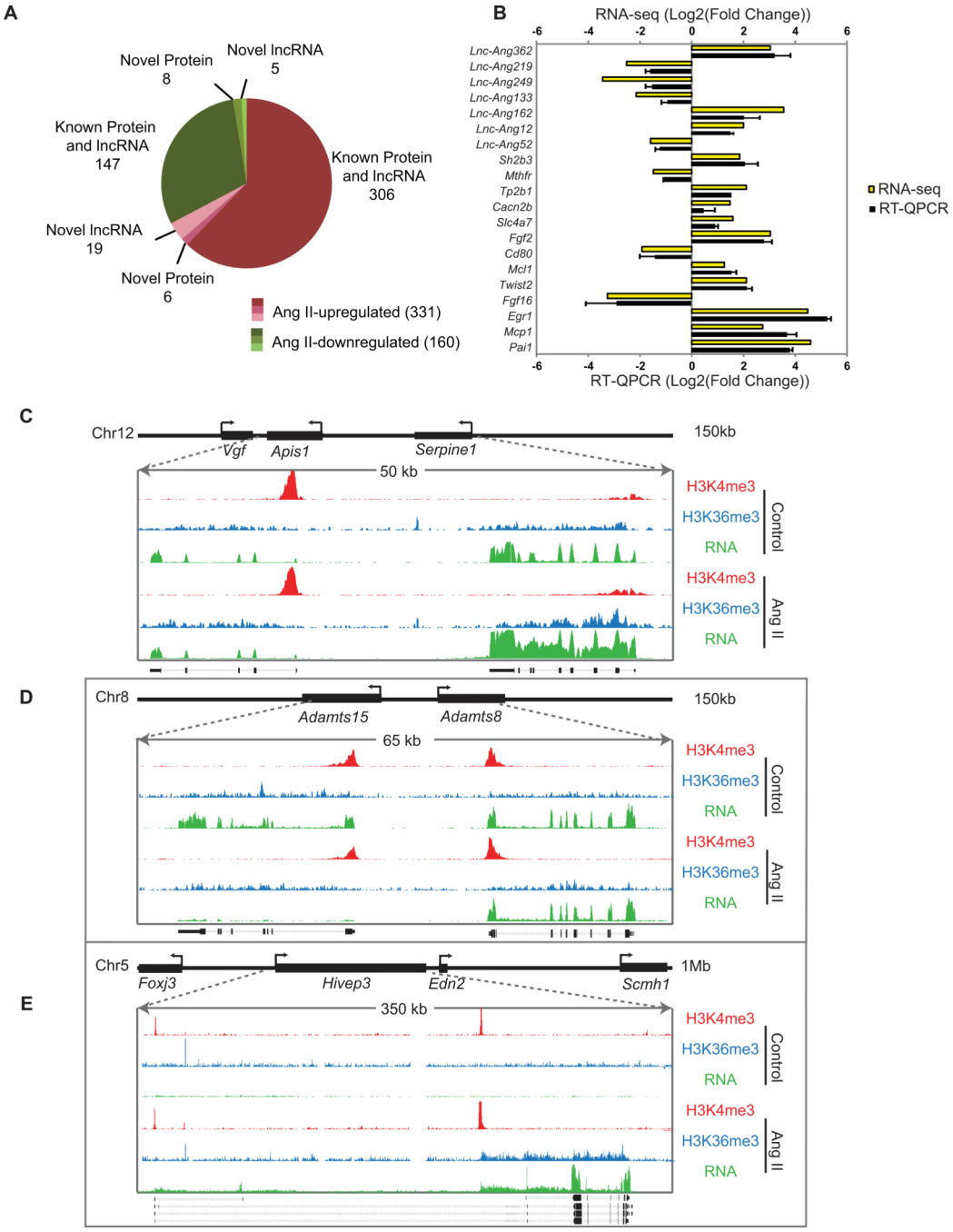

Figure 3. Identification of Ang II-regulated transcripts

A) Pie chart with number of differentially expressed genes from each category of transcripts: Known (Rat RefSeq, with human and mouse XenoRefSeq transcripts), novel protein-coding transcripts, and lncRNAs. Ang II-upregulated transcripts are in red hues, with Ang II-downregulated transcripts in green hues. Numbers indicate total transcripts in each group. **B**) Validation of RNA-seq data with RT-QPCR ($N \geq 3$). Shown are twenty transcripts that were tested. Pearson's correlatioN= 0.907. (**C–E**) Genome browser image of H3K4me3 (red) and H3K36me3 (blue) ChIP-seq and RNA-seq scoring profiles (green) in both control and Ang II-treated VSMCs for **C**) *Apis1-Serpine1(Pai1)* locus, **D**) *Adamts15-Adamts8* locus, and for

E) *Hivep3* locus. Above each set of tracks is a schematic of the chromosome location. Below each track are the structures of the RefSeq transcripts (black). Each data track shown is on the same scale for both control and Ang II.

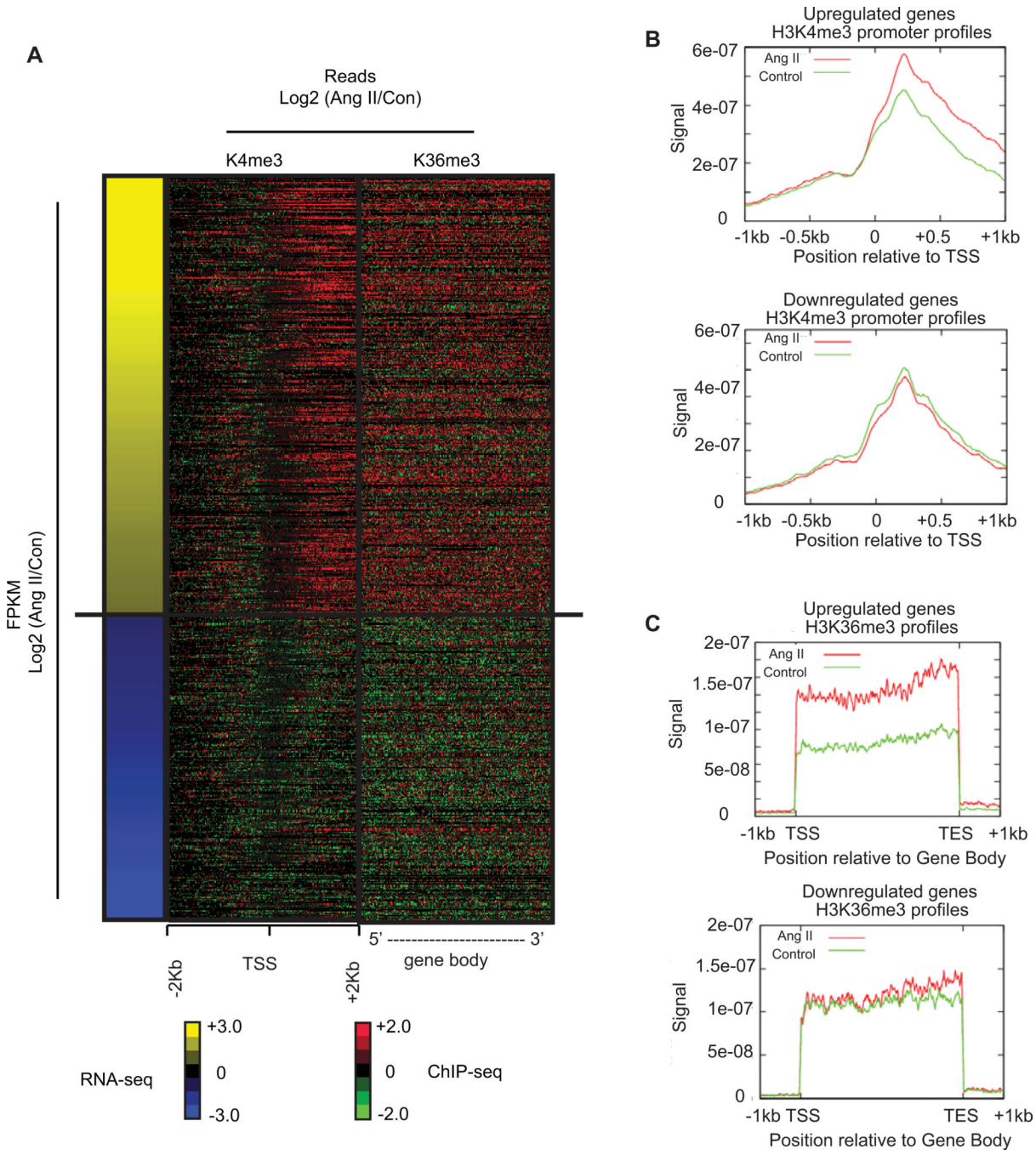

Figure 4. Ang II treatment increased enrichment of H3K4me3 and H3K36me3 at genomic regions of Ang II-upregulated trancripts

A) For each Ang II-regulated gene, the relative enrichment of H3K4me3 surrounding the TSS and H3K36me3 across the gene body was assessed (see Methods for details). Normalized enrichment of H3K4me3 surrounding the TSS for **B**) Ang II-upregulated genes (top) and Ang II-downregulated (bottom) genes in control (green) and Ang II-treated (red) cells. Averaged enrichment of H3K36me3 across the gene body for **C**) Ang II-upregulated genes (top) and Ang IIdownregulated (bottom) genes in control (green) and Ang II-treated (red) cells.

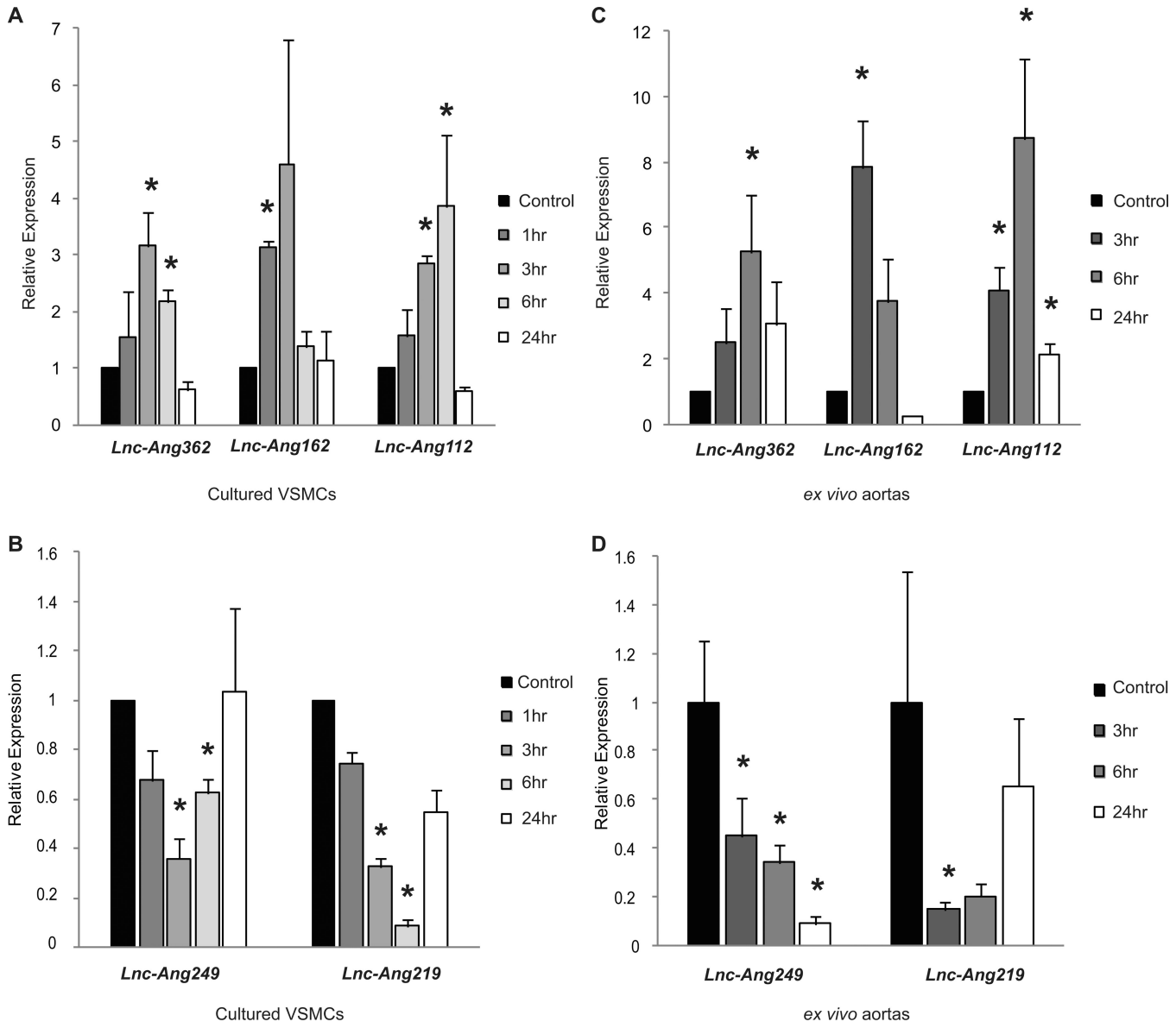

Figure 5. Ang II regulates expression of lncRNAs in VSMCs in vitro and *ex vivo*

VSMCs were treated with Ang II for the indicated times and expression of **A**) upregulated lncRNAs (*Lnc-Ang362*, *Lnc-Ang162*, and *Lnc-Ang112*) and **B**) downregulated lncRNAs (*Lnc-Ang249*, and *Lnc-Ang219*, was assessed by RT-QPCR. Expression of these lncRNAs was also assessed in VSMCs of *ex vivo* Ang II-treated aortas. Expression of **C**) *Lnc-Ang362*, *Lnc-Ang162*, and *Lnc-Ang112*, **D**) *Lnc-Ang249* and *Lnc-Ang219* were analyzed by RT-QPCR. Relative values are shown normalized to *Cypa* transcript levels. Error bars represent SEM calculated from the means of at least 3 biological replicates (N≥3) from independent experiments. (*= $p < 0.05$ using student's t-test comparing the means of each timepoint to the mean of control)

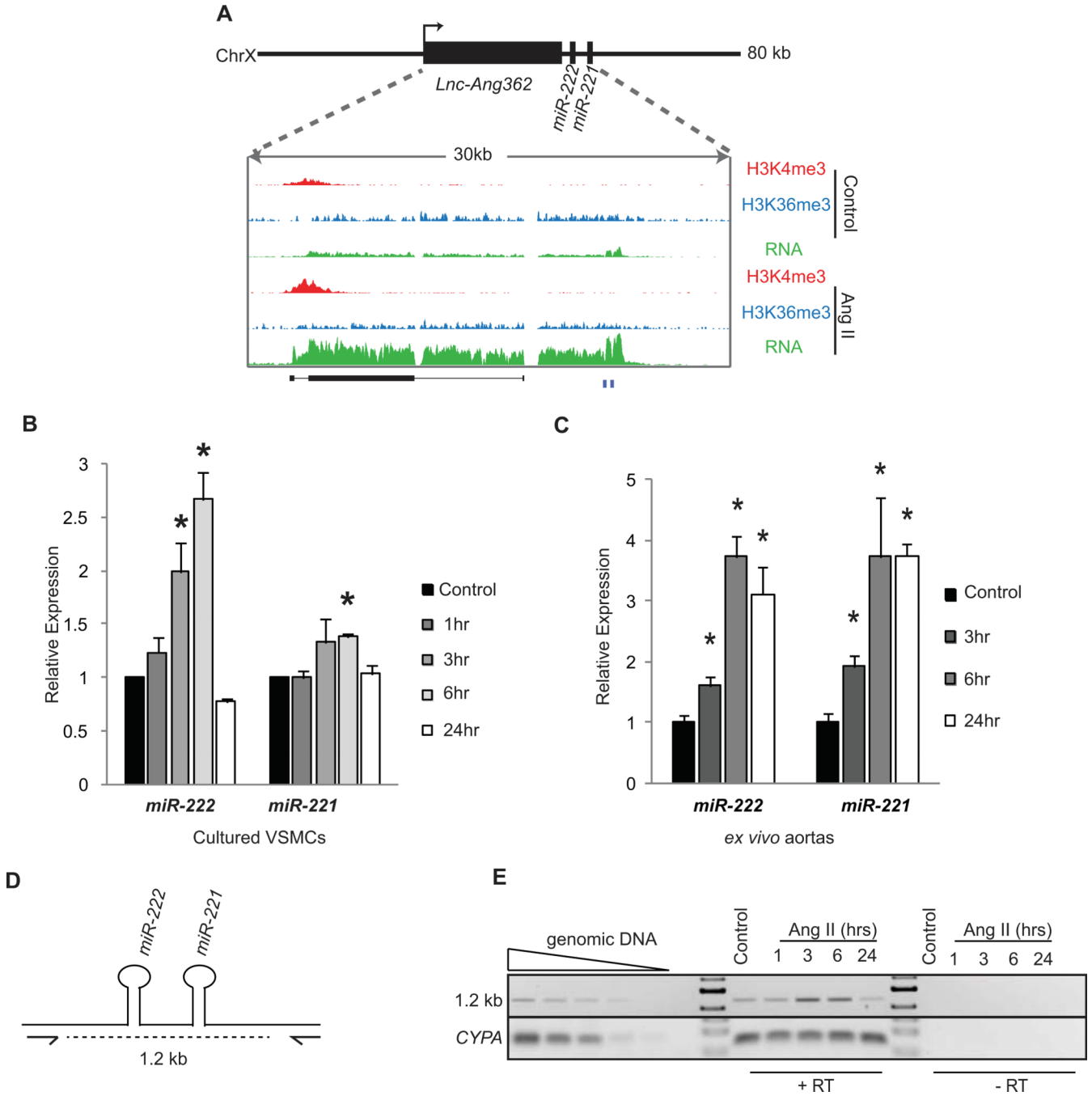

Figure 6. miR-222 and miR-221 which are upregulated by Ang II are transcribed from a large transcript

A) Genome browser images of the region of Chr. X that harbors *lnc-Ang362* and *miR-221/222* with H3K4me3 (red) and H3K36me3(blue). ChIP-seq scoring profiles and RNA-seq scoring profiles (green) from control and Ang II-treated cells aligned to the genomic region with the Cufflinks assembled transcript schematic (black) and annotated miRNAs (blue). Above the tracks is a schematic of the chromosome location. Each data track shown is on the same scale for both control and Ang II.

(B–C) VSMCs were treated with Ang II for times indicated and RT-QPCR was performed to assess the abundance of mature miR-222 and miR-221 **B**) in vitro and **C**) *ex vivo*.

Relative values are normalized to abundance of U6 RNAs. Error bars represent SEM calculated from the means of at least 3 biological replicates (N≥ 3) from independent experiments (* = $p< 0.05$ with student's t-test comparing mean of each timepoint to mean of control) D) Illustration of primers (arrows) used to amplify region surrounding *miR-222* and *miR-221* in PCR to produce 1.2 kb product. E) Region surrounding miR-222 and miR-221 were amplified from cDNA (with (+RT) or without (−RT) reverse transcriptase) generated from VSMCs treated with Ang II for the indicated times.

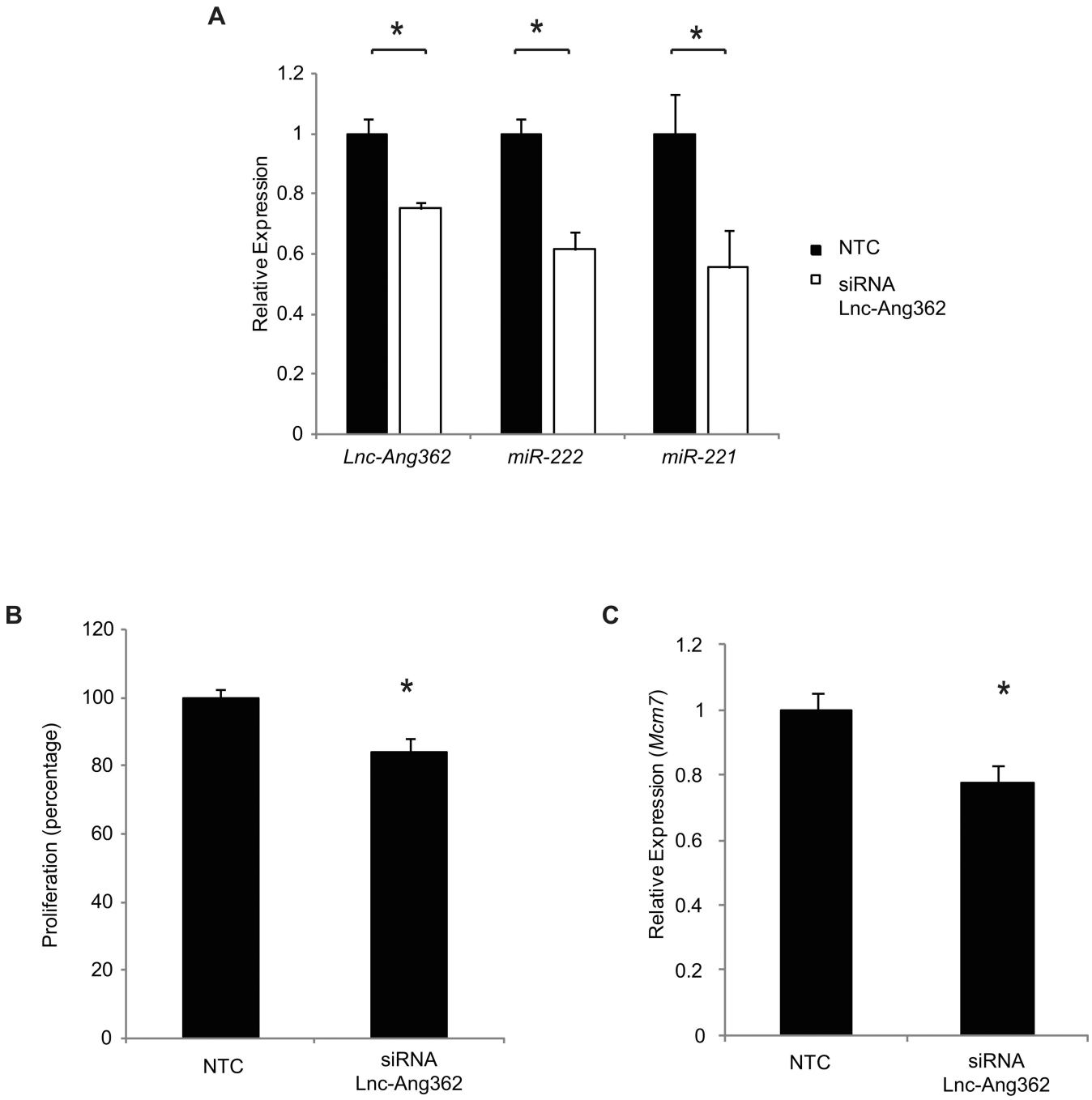

Figure 7. Knockdown of *Lnc-Ang362* decreases expression of *miR-221* and *miR-222* and reduces cell proliferation

VSMCs were transfected with siRNAs targeting *Lnc-Ang362* or non-specific control siRNA(NTC) and treated with Ang II for 3 hours. Expression of **A**) *Lnc-Ang362* (normalized to *18S* RNA) mature miR-221 and miR-222 (normalized to *U6* RNA) were analyzed with RT-QPCR. Error bars represent SEM calculated from the means of three biological replicates (*N*= 3) from independent experiments **B**) Proliferation of VSMC transfected with NTC or siRNA targeting *Lnc-Ang362* was determined using methyl thiazole tetrazolium assay. Error bars represent SEM calculated from the means of six biological replicates (*N*= 6). **C**) Expression of *Mcm7* (normalized to *18S* RNA) was

analyzed as in panel A). Error bars represent SEM calculated from the means of the three biological replicates (N= 3) from independent experiments. (* = p< 0.05 with student's t-test comparing the mean of the NTC-transfected to the mean of siRNA *Lnc-Ang362*-transfected cells)

Table 1

Ang II-regulated lncRNAs and their neighboring Ang II-regulated genes

LncRNA Coordinates	ID	Control FPKM	Ang II FPKM	Fold Change (Log2) (adjusted p-value)	Nearby Gene (accession ID)	Coordinates of Nearby Gene	Control FPKM	Ang II FPKM	Fold Change (Log2) (adjusted p-value)
chr1:134662306–134670128	*Lnc-Ang26*	0.1±0.1	2.7±1.3	4.82 (1.5E-06)	*Acan* (NM_022190)	chr1:134787340–134848992	22.9±6.1	84.2±33.0	2.07 (0.00105)
chr14:87451545–87453951	*Lnc-Ang383*	0.1±0.1	2.2±1.6	5.21 (0.00066)	*Ramp3* (NM_020100)	chr14:87405900–87434789	2.8±2.9	14.5±4.1	2.40 (0.00172)
chr2:211696297–211839561	*Lnc-Ang58*	0.8±0.6	3.5±1.7	2.20 (0.02768)	*S1pr1* (NM_017301)	chr2:211851303–211855511	9.2±3.7	45.5±12.0	2.38 (7.1E-6)
chr3:125702412–125765671	*Lnc-Ang219*	1.5±0.5	0.2±0.2	−2.62 (0.00252)	*Jag1* (NM_019147)	chr3:125181062–125216481	99.6±29.0	28.7±7.2	−1.78 (0.00884)
chr6:92749933–92751244	*Lnc-Ang202*	0.02±0.04	0.9±0.8	5.36 (0.02655)	*Frmd6* (NM_028127)	chr6:92793365–93027451	51.8±23.6	216±106.7	2.06 (0.00187)
chr5:24157867–24177726	*Lnc-Ang249*	0.8±0.5	1.7±0.3	−1.54(0.04877)	*Gdf6* (NM_013526)	chr5:23739174–23756140	161.5±44.8	33.7±8.7	−2.09 (0.00028)
chrX:14911144–14928968	*Lnc-Ang362*	3.1±1.3	25.6±11.4	3.03 (3.14E-09)	*miR-221* (NR_032052)	chrX:14935616–14935720	*	*	*
chrX:14911144–14928968	*Lnc-Ang362*	3.1±1.3	25.6±11.37	3.03 (3.14E-09)	*miR-222* (NR_031935)	chrX:14935055–14935158	*	*	*

LncRNAs and neighboring genes that are within 500kb are listed.

* *miR-221* and *miR-222* have been assessed by RT-QPCR (Fig. 6).

Shown are FPKM for each transcript in control and Ang II-treated VSMCs (± 95% confidence intervals) and the fold change (Log_2) with the *p* adjusted for FDR with Benjamini-Hochberg.