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Long non-coding RNA-SRA promotes neointimal hyperplasia and vascular smooth muscle cells proliferation *via* MEK-ERK-CREB pathway

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ABSTRACT

Long noncoding RNA-steroid receptor RNA activator (LncRNA-SRA) is transcribed from a class of noncoding genes, and plays a critical role in regulating cell proliferation. However, the effect of lncRNA-SRA remains unclear in vascular proliferative diseases. In the present study, we overexpressed lncRNA-SRA *in vitro*, then investigated the biological consequences. A vascular damage mice model was constructed by performing femoral artery wire injury. LncRNA-SRA was overexpressed in the injured arteries, and significantly promoted the expression of ki67, thereby caused an overall increase in neointima formation. LncRNA-SRA overexpression led to the proliferation and migration of vascular smooth muscle cells (VSMCs). By stimulating the phosphorylation of MEK, ERK and CREB (cyclic nucleotide responsive element binding protein), lncRNA-SRA promoted VSMC proliferation. Meanwhile, these effects were blocked by the MEK inhibitor U0126. Therefore, lncRNA-SRA promoted VSMC proliferation by activating the MEK-ERK-CREB pathway. LncRNA-SRA could be a promising therapeutic target in vascular diseases characterized by neointimal hyperplasia.

1. Introduction

Vascular smooth muscle cells (VSMCs) proliferate at a very low rate within blood vessels, exhibit a very low synthetic activity, and express unique functions as contractile proteins, ion channels, and signaling molecules [1]. In a cellular phenotype switching process, VSMCs switch between a contractile (also termed differentiated) state and a synthetic/dedifferentiated state [2]. For example, in response to vascular injury, the proliferation, migration, and synthetic capacity of VSMCs dramatically increase. VSMCs also have plasticity and thus play a critical role in vascular repair [3].

The discovery of long non-coding RNAs (lncRNAs) and their role in epigenetic regulation are the key breakthroughs for the study of gene expression regulation. LncRNAs are widely defined as RNA molecules with length of > 200 nt and participate in a variety of pathological processes [4].

> 20 years ago, the first study of lncRNA in VSMCs reported the H19 cloning. H19 is mainly expressed in SMCs of developing arteries [5]. CDKN2B-AS1 (aka *ANRIL*) is another well-known lncRNA expressed in VSMCs. It is a hotspot that linked to cardiovascular diseases [6]. More recently, by using next generation sequencing, several lncRNAs have been identified in VSMCs, including MIR221HG [7], SENCR [8], the snoRNA-containing GAS5 [9], ATG9B, MIR221HG, HIF1A-AS1, LincRNA-p21, and HAS2-AS1. These lncRNAs play important role in maintenance of vascular homeostasis [10].

In 1999, the steroid receptor RNA activator (SRA) has been firstly identified, and lncRNA-SRA is able to increase the activity of steroid receptors [11]. Since then, increasing studies investigate the effect of

Abbreviations: LncRNA-SRA, Long noncoding RNA-steroid receptor RNA activator; VSMCs, vascular smooth muscle cells; CREB, cyclic nucleotide responsive element binding protein; ERKs, extracellular signal regulated kinases; MAPK, mitogen-activated protein kinase; PPI, protein–protein interaction; DEGs, differentially expressed genes; CDK4, cyclin-dependent kinases 4; MMP, matrix metalloproteinase

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lncRNA-SRA in gene transcription regulation. According to these studies, lncRNA-SRA can regulate a variety of important cellular functions, such as proliferation, migration, and apoptosis; lncRNA-SRA is also implicated into the development of cardiovascular diseases [12]. Moreover, lncRNA-SRA acts as a novel transcriptional coactivator. Mediated by the steroid receptors, lncRNA-SRA acts as an RNA transcript to regulate eukaryotic gene expression, thus plays critical roles in eukaryotic development, metabolism, reproduction, and diseases [13].

Cyclic nucleotide responsive element binding protein (CREB) is a transcription factor that integrates with diverse extracellular signals [14]. Multiple intracellular signaling kinases can induce the phosphorylation of CREB, including extracellular signal regulated kinases (ERKs). The phosphorylated CREB can also promote gene transcription, cell proliferation and survival [15]. Moreover, the ERK signaling cascade is a central mitogen-activated protein kinase (MAPK) pathway that plays a role in the regulation of various cellular processes, such as proliferation, differentiation, survival and, apoptosis under some conditions [16].

However, it is not yet clear which signal molecules are involved in VSMC proliferation. Thus, the aim of the present study was to evaluate the role, if any, of lncRNA-SRA in VSMC proliferation *in vitro* and in response to neointimal hyperplasia in wire injured-femoral arteries *in vivo*.

2. Materials and methods

2.1. Animal and mouse femoral artery injury model

All *in vivo* experiments were performed on adult male C57BL/6 mice purchased from Beijing Weitong Lihua Experimental Animal Ltd.Co.. All mice were randomly allocated to following groups: model group, lncRNA-SRA overexpression group, negative group (NEG). Each group contained ten mice and kept at 25 °C on a 12-h light-dark cycle. Mice were injected with lncRNA-SRA overexpressed adenovirus or empty vector at a dose of 9×10^{11} PFU/mL per mice once. After 14 days, the mice were anesthetized with isoflurane inhalation (2%). The dilation of the femoral artery was performed by inserting a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN, USA) for ~10 mm toward the iliac artery, as previously described [17]. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 8023, revised 1978) and approved by the Animal Ethics Committee of the Hunan University of Chinese Medicine.

2.2. Haematoxylin-eosin (H&E) staining

The histopathology examination of femoral artery was performed H &E staining. Femoral artery was put in 10% formaldehyde solution, dehydrated in ethanol gradient, embedded in paraffin and cut down into slices of 4 μm. After deparaffinage, the samples were stained with haematoxylin and eosin. Then, the slices were mounted and observed under a light microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Immunohistochemical staining analysis

The arteries were excised at the time-points indicated in the results section. The arteries dedicated to morphometric analyses and immunohistochemistry were fixed in 4% paraformaldehyde and embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands). Sections were deparaffinised with xylene and rehydrated in a graded series of ethanol, and endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 min, followed by incubation with an antibody directed against PCNA overnight at 4 °C [18]. Tissue sections were blocked in 10% goat serum, incubated with primary antibody in 1% BSA/PBS for 1 h, followed by incubation with secondary antibody (1:1000). The staining section was visualized using DAB (Vector Labs) as the chromogenic substrate, which produces a reddish-brown stain. The sections were counterstained with haematoxylin.

2.4. Cell lines and cultures

VSMCs were cultured in DMEM media (Dulbecco's modified Eagle's medium, Gibco, America) containing 10% (*v*/v) fetal bovine serum (FBS, Gibco BRL, America), 100 U/mL penicillin and 100 mg/mL streptomycin. All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

2.5. Cell transfection and lentivirus transduction

To overexpress the lncRNA-SRA, the full-length sequence of lncRNA SRA was subcloned into the lentiviral vector LV5 (GenePharma, Shanghai, China) and an empty vector as a control. The harvested viruses were employed to infect VSMCs for overexpression of lncRNA-SRA. The infection efficiency was confirmed by quantitativ real-time polymerase chain reaction (qPCR).

2.6. qPCR

Total RNA was extracted with the RNeasy kit (Qiagen GmbH, Hilden, Germany), and cDNA was obtained from 500 ng RNA with the Omniscript RT kit (Qiagen). qPCR was carried out in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green kit (Roche Diagnostics GmbH) and the following specific primer sets: lncRNA-SRA forward, S-AGCCCACAAGTTTCCCAGTC; reverse, AS-GGCTTGAAAGCTCTTGCACC.

Relative mRNA levels were quantifed using the comparative Ct method. A melting curve analysis was performed for each amplicon to verify the specificity of each amplifcation step. The normalized fold expression levels of the lncRNA-SRA in different cancerous tissues were calculated in comparison to the lncRNA-SRA expression levels in the VSMC using the $2^{-\Delta\Delta CT}$ calculation method according to the manufacturer's protocol (Perkin-Elmer) [19].

2.7. EdU staining

Cell proliferation assay was performed using Click-iT EdU Imaging Kit (Thermo Scientific) according to the manufacturer's protocol. VSMCs were incubated with 10 mmol/L EdU for 2 h at 37 °C. VSMCs were permeabilized with 0.5% Triton X-100 for 20 min after fixing with 4% formaldehyde for 15 min at room temperature. After wash with PBS for three times, VSMCs were incubated with 1Click-iT reaction cocktails overnight at 4 °C. VSMC nuclei stained by 40,6-diamidino-2- phenylindole (DAPI) were used to cell count and visualized using a laser scanning confocal microscope (Leica, Heidelberg, Germany). For quantification of VSMC proliferative rate, six randomly selected views from each sample image were used to calculate the relative EdU-positive ratio [20].

2.8. MTS assay

Cell proliferation was assessed at 0, 24, 48, 72 and 96 h upon treatments by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium inner salt (MTS) cell proliferation colorimetric assay kit (Sigma, USA), according to the manufacturer's instructions.

2.9. Cell migration assay

VSMCs were seeded into 12-well plates for the wound-healing assays. Plastic tips were used to make wounds across the cell monolayer. Wounded cells were treated with or without over-expressed lncRNA-

SRA for 12 and 24 h. Phase-contrast photographs were taken with an inverted phase-contrast microscope (Olympus, Japan).

2.10. Flow cytometry detection of cell cycle

Cell cycle was analyzed with a commercial kit (Beyotime, China) according to the manufacturer's instructions. Briefly, the cells were collected and fixed with pre-cooling 70% alcohol for 2 h at 4 °C. After discarding the supernatant, the precipitate was washed with PBS and recentrifuged. We used 500 μL of staining buffer to resuspend cells. 25 μL propidium iodide staining solution were added followed by 100 μL RNase A to the resuspended cells and mixed them in well, followed by incubation for 30 min at 37 °C away from light. Then, the samples were detected immediately with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.11. Western blot analysis

RIPA lysate (Beyotime, China) was used to extract total proteins, 100 μg of which were segregated with SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. 5% skim milk was used for membrane incubation for 1 h. Then, the membranes experienced incubation with primary antibodies including anti-PCNA, anti-CyclinD1 at 4 °C overnight. The membranes were incubated with anti-rabbit IgG H&L (HRP) secondary antibody at room temperature for 1.5 h. Finally, the membranes were subjected to color reaction by ECL Plus from Life Technology.

2.12. Constructing the protein–protein interaction (PPI) network of differentially expressed genes (DEGs) and selecting the hub genes

Genes involved in the same PPI network often work together to perform one biological function [21]. To further investigate the underlying molecular mechanisms of lncRNA-SRA, PPI networks for the DEGs were constructed using the STRING database (http://www.string-db.org/). We selected the interactions whose integrated scores were > 0.4 (the default threshold in the STRING database) to construct the PPI network. Finally, the obtained PPI networks were visualized using Cytoscape 3.6.1 (http://cytoscape.org/).

2.13. Statistical analysis

Data are expressed as the mean ± SD from at least three independent experiments. Statistical significance was assessed by the two-tailed unpaired Student's *t*-test and a *P*-value of < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 4.0 (La Jolla, CA, USA). The experimental data were visualized by prism 5 software.

3. Results

3.1. Overexpression of lncRNA-SRA accelerates neointimal hyperplasia in vivo

Intimal hyperplasia is the direct factor that leads to the formation of VSMC proliferation. In this study, a well-established femoral artery injury mice model was constructed. Histologic assays were applied to observe the state of intimal hyperplasia. Compared to the arteries negative control group (NEG), the injured carotid in model group showed obviously narrowed vascular cavity, and with thicker intima and media (Fig. 1A). These histomorphological changes in the carotid arteries indicated that the model of vascular remodeling induced by wire injury was established successfully. To investigate whether lncRNA-SRA plays a role in vessel injury-induced neointima formation *in vivo*, lncRNA-SRA-overexpressed mice model was constructed by tail vein injection of lncRNA-SRA adenovirus. The upregulated mRNA expression of lncRNA-

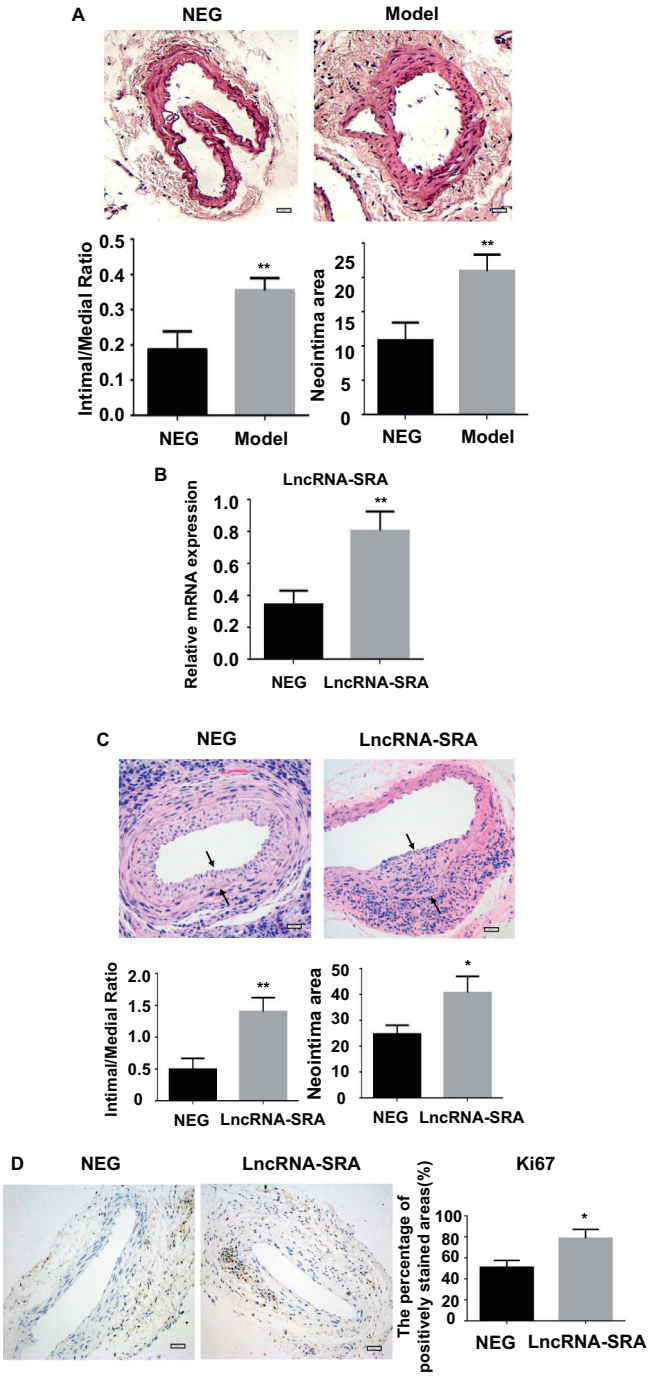

Fig. 1. Neointima formation is facilitated by overexpression of lncRNA-SRA in the injured arteries. (A) Microscopic images after H&E staining and typical region in the cross-section of femoral arteries ($n = 10$). The intima-to-media ratio and neointima area of injured femoral arteries were analyzed. $^{**}P < 0.01$ (B) The mRNA expression of lncRNA-SRA in injured femoral arteries was detected by qPCR (n = 10). (C) Morphometric analysis of intima-to-media ratio and neointima area of injured femoral arteries from mice injected with empty vector or lncRNA-SRA overexpressed adenovirus. The increased ratio of intimal to medial area represented neointima formation. $^{*}P < 0.05$,$^{**}P < 0.01$ (D) Immunohistochemical analysis revealed the expression of Ki67 in wire-injured femoral arteries. The positive area from immunostaining was determined using IMAGEPRO PLUS Software. Images of representative sections from each group were present. $^{*}P < 0.05$. Statistical significance was assessed by the Student's *t*-test.

SRA in injured vessels was confirmed by qPCR (Fig. 1B). In this model, wire-induced vessel injury resulted in rampant VSMC migration and proliferation. Accordingly, H&E staining revealed attenuated intimal thickening in the neointima area in lncRNA-SRA overexpressed adenovirus treated arteries. Morphometric analyses corroborated these histological observations where lncRNA-SRA treatment significantly promoted neointima area and intima/media ratio. Pathological vascular remodeling following injury is attributed to increased VSMC proliferation. (Fig. 1C).

Ki67 protein is widely used as a proliferation indicator in the clinic and basic research. Previous study found that Ki67 accumulated during S, G2, and M phases [22]. Consistently, further analysis by immunohistochemistry assay showed that lncRNA-SRA was significantly upregulated the level of Ki67, suggesting that VSMC proliferation was promoted by lncRNA-SRA overexpression (Fig. 1D). All of these findings indicated that lncRNA-SRA over-expression promoted neointimal hyperplasia in wire-injured femoral arteries *in vivo*.

3.2. LncRNA-SRA promotes VSMC proliferation in vitro

To confirm the biological effects of lncRNA-SRA on VSMCs proliferation, *in vitro* analyses were performed. As shown in Fig. 2A, the baseline level of lncRNA-SRA was low in VSMCs. Then, the adenovirus with lncRNA-SRA overexpression vector or empty vector was infected into the VSMCs. The lncRNA-SRA adenovirus-infected VSMC became fully-activated in culture (Fig. 2A). Immunofluorescent staining with EdU confirmed that lncRNA-SRA-overexpressed VSMCs contained more EdU-positive cells, compared to the control VSMCs (Fig. 2B). MTS assay showed that cell proliferation of lncRNA-SRA-transfected VSMCs was significantly faster compared to the control VSMCs (Fig. 2C).

We further performed flow cytometry to detect cellular DNA content changes during cell cycle. In lncRNA-SRA-overexpressed VSMCs, the proportions of G1-phase cells decreased, while the percentage of cells in S phase increased significantly (Fig. 2D). In control group (empty vector tranfection), 39.51%, 7.63%, and 52.86% of the VSMCs were in G1, S, and G2 phases, respectively (Table 1). In lncRNA-SRA overexpression group, the proportion of cells in G1 phase was 37.49%, whereas the proportion of cells in S phase was 16.55% (Table 1). Moreover, we found that in lncRNA-SRA-overexpressed cells, well-characterized VSMC proliferation molecules, such as proliferating cell nuclear antigen (PCNA) and CyclinD1, were upregulated (Fig. 2E and F). Altogether, these findings showed that lncRNA-SRA exerted a positive role in stimulating VSMC proliferation.

3.3. LncRNA-SRA accelerates VSMC migration in vitro

In order to investigate the role of lncRNA-SRA in VSMC migration, wound-healing assay and matrix metalloproteinase-2(MMP-2)/MMP-9 mRNA level quantifications were performed. As shown in Fig. 3A, the scratch wounds in VSMCs with lncRNA-SRA overexpression healed significantly faster compared with the control cells (Fig. 3A). Consistent with the increased migration phenotype, the mRNA levels of MMP-2 and MMP-9 also increased in parallel in lncRNA-SRA–overexpressed VSMCs (Fig. 3B). Thus, these results suggest that lncRNA-SRA promotes VSMCs migration *in vitro*.

3.4. CREB is the potential downstream of lncRNA-SRA

The Search Tool for the Retrieval of Interacting Genes/Protein (STRING) database is used to retrieve the predicted interactions for the identified genes and allows visualizing complex networks [23]. Version 10.5 of STRING, which contains information on approximately 9.6 million proteins from > 2000 organisms, is a biological database and web resource of known and predicted protein–protein interaction (PPI) [15]. The constructed PPI network was shown in Fig. 4A. We set the required interaction score at > 0.4 as the threshold. Subsequently, the protein interaction was read into Cytoscape 3.6.1, which allowed the network to be integrated into any type of attribute data and implement topology analysis (Fig. 4B). Of these hub genes, cAMP-response element binding protein (CREB) had the highest score (0.995). qPCR analysis incorporation confirmed that the expression of CREB increased in lncRNA-SRA-overexpressed VSMCs (Fig. 4C). Moreover, the protein level of SRA1 was also upregulated in VSMCs after introducing lnRNA-SRA (Fig. 4D).

3.5. MEK-ERK-CREB signaling pathway is involved in lncRNA-SRA-induced VSMCs proliferation

Accumulating evidence has indicated that MEK-ERK signaling pathway is associated with various protein kinase cascades, contributing to cell proliferation [24,25]. As shown in Fig. 5, lncRNA-SRA promoted MEK-ERK signaling by increasing phosphor-MEK expression (Fig. 5A) as well as phosphor-ERK levels (Fig. 5B), resulting in increased phosphorylated-CREB (p-CREB) expression in VSMCs (Fig. 5C). Furthermore, we blocked MEK/ERK pathway in lncRNA-SRA-overexpressed VSMCs by exposing the lncRNA-SRA-overexpressed VSMCs to U0126, a potent MEK-ERK inhibitor. The results showed that U0126 dramatically inhibited the ERK pathway (Fig. 5D), simultaneously reduced CREB phosphorylation (Fig. 5E), and ultimately decreased the protein expressions of PCNA and Cyclin D1 (Fig. 5F and G). Taken together, our findings indicated that MEK-ERK-CREB signaling pathway was involved in lncRNA-SRA-mediated VSMCs proliferation.

4. Discussion

The proliferation and migration of SMCs from the tunica media to the intima ultimately results in neointima formation, which is the key process in the atherosclerosis, the post-coronary angioplasty/stent implantation restenosis, and the vein graft diseases. Many aspects of vascular proliferative diseases remain poorly understood, including the activation of the cells of the neointima and media layer after acute vascular injury, the underlying signaling mechanisms and the proliferation effect of VSMCs and neointima formation [26]. A detrimental consequence of the high degree of plasticity exhibited by adult VSMCs is that it can lead to an adverse phenotypic switch and acquisition of characteristics that can contribute to development or progression of vascular diseases in human, including atherosclerosis, restenosis, cancer, and hypertension [3]. De-differentiated SMCs are highly proliferative and migrate toward the luminal side of the vessel, thus leading to a stenotic process, which result in the progression of neointimal lesion. Moreover, the upregulation of pro-proliferation genes in neointimal SMCs leads to increased cell viability and further promotes neointima formation [17]. Therefore, the identification of the pathophysiological and molecular mechanisms to counter balance VSMC proliferation would facilitate the development of new and selective therapy strategies for neointima formation and the subsequent vascular diseases.

In the present study, to explore whether lncRNA-SRA participate in the progression of vascular diseases, we firstly overexpressed the lncRNA-SRA in wire-injured model and assessed its effects on cell proliferation and invasion *in vivo*. It has been reported that overexpression of lncRNA-SRA showed strong promoting activities on cellular proliferation and differentiation in epithelial cell [27]. In accordance with previous studies, following vascular injury, VSMCs from the arterial tunica media are activated and migrate to the intima, where they massively proliferate, and eventually lead to neointimal thickening and restenosis. Therefore, our data indicate that the combined facilitating effects of lncRNA-SRA on VSMC migration and proliferation contribute to the increased neointima hyperplasia after injury at later stage. As indicated by staining of the proliferation marker Ki-67, suggesting that overexpression of lncRNA-SRA promoted neointima formation *in vivo*.

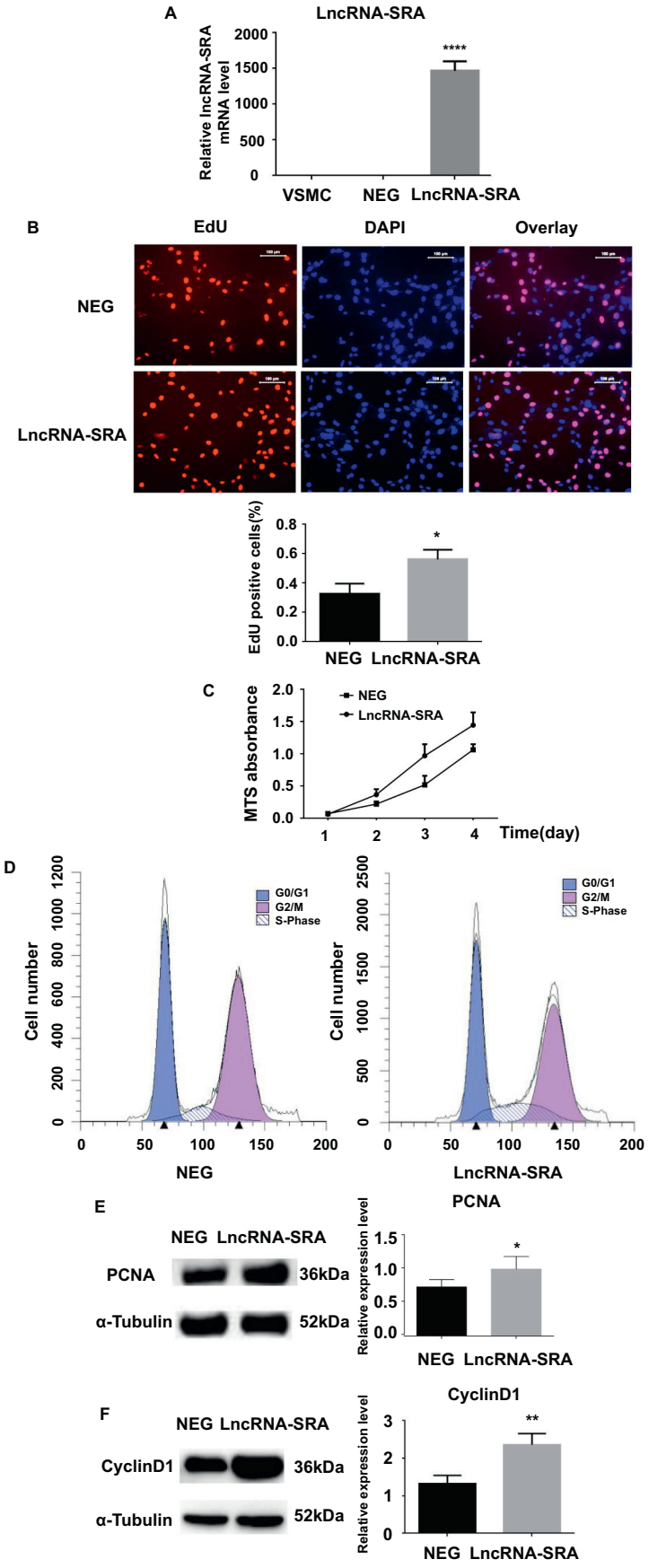

Fig. 2. LncRNA-SRA accelerates VSMC proliferation. (A) qPCR results showed the efficiency of lncRNA-SRA expression in stable lncRNA-SRA-overexpressing VSMCs. ****$P < 0.0001$ compared to negative control group (NEG). (B) Cellular proliferation was estimated from immunofluorescence staining with EdU and the percentage of EdU-positive nuclei was quantified. *$P < 0.05$ compared to NEG. (C) The viability of the cells was measured using an MTS assay at different time points. (D)The phases of the cell cycle were measured by flow cytometry analysis. (E and F) Western blot was used to identify the protein expressions of PCNA and CyclinD1 in VSMCs expressing lncRNA-SRA. *$P < 0.05$, ***$P < 0.001$ compared to NEG.

Table 1
The percentage of cells in each cell cycle.

	G1(% ± SD)	S(% ± SD)	G2(% ± SD)	G1/S ratio
NEG	39.51 ± 2.74	7.63 ± 7.66	52.86 ± 0.07	5.17
LncRNA-SRA	37.49 ± 4.29	16.55 ± 5.86	45.96 ± 6.39	2.27

Consistent with these findings, we also found that overexpression of lncRNA-SRA activated cell proliferation and migration in VSMCs. Additionally, the protein expressions of PCNA and CyclinD1 were significantly upregulated in VSMCs with lentivirus-mediated lncRNA-SRA overexpression. PCNA is well known as a cell cycle marker. Replication of the genome is an essential step in cell cycle progression and proliferation. Failure in this process will lead to abnormal cell proliferation, cell cycle arrest, and genomic instability. At the heart of DNA replication, PCNA has an essential role in orchestrating normal DNA synthesis by providing a platform to which DNA polymerases and other factors bind *via* the PIP box [28]. Moreover, Cyclin D1 plays an important role in cell cycle progression through the association with cyclin-dependent kinases 4 (CDK4) and CDK6, which phosphorylate and inactivate the retinoblastoma protein pRb, leading to the expression of a subset of proliferation-associated target genes [29]. In addition, MMPs are a family of zinc-dependent endopeptidases that are involved in the degradation of various proteins in the extracellular matrix. Increases in specific MMPs could play a role in arterial remodeling, as well as influence cell function such as VSMCs cell migration, proliferation, Ca^{2+} signaling, and contraction. Herein, while lncRNA-SRA promoted VSMCs migration and proliferation, the mRNA levels of MMP-2 and MMP-9 were also upregulated.

For the enriched lncRNA-SRA protein interaction, our results showed that various identified DEGs matched the lncRNA-SRA signaling pathway. Among them, CREB shows the high level in differentiated VSMCs but the expression of CREB is decreased during phenotypic modulation. Several studies have identified the mitogenic properties of CREB in VSMCs [30]. Enhancing cAMP-signaling and VSMCs proliferation are associated with CREB activation. Consistent with this, CREB inhibition with siRNA or dominant-negative mutants is sufficient to block VSMCs proliferation, whereas overexpression of constitutively-active mutants of CREB induces cell-cycle progression. Our study provided direct evidence that lncRNA-SRA induced upregulation of p-CREB expression *via* MEK-ERK signaling pathway in VSMCs. Firstly, lncRNA-SRA activated MEK-ERK signaling by activating MEK and ERK phosphorylation, resulting in cell proliferation and migration. In contrast, the MEK inhibitor U0126 completely inhibited upregulation of p-CREB, PCNA, and Cyclin D1 induced by lncRNA-SRA in VSMCs. Moreover, the protein level of p-ERK1/2 was significantly decreased in overexpressed lncRNA-SRA cells. While U0126 reversed the effects of lncRNA-SRA on expressions of p-ERK1/2, p-CREB, and representative proliferation-related proteins such as PCNA and Cyclin D1, suggest that the MEK-ERK signaling pathway was suppressed definitely. Once MEK1/2 was phosphorylated and activated, ERK1/2 subsequently enters the nucleus to phosphorylate transcription factors that mediate expression of genes which attenuate cell proliferation [31].

5. Conclusions

Taken together, our data highlight a currently underestimated impact of lncRNA-SRA on neointima formation, proliferation and migration, as well as link this mechanism to MEK-ERK signal pathway and activation of CREB. The present results suggest that appropriate reduction of MEK-ERK-CREB signaling pathway induced by lncRNA-SRA could alleviate VSMCs proliferation. Thus, the further exploring the role of lncRNA-SRA may be an effective and promising strategy for prevention and treatment of vascular proliferative diseases.

Author contributions

Zhang analyzed the data and wrote the manuscript. Liu and Wang performed the experiments. Hu and Liao revised the manuscript. Qin was responsible for the concept and design of the manuscript. All authors read and approved the final version of the manuscript.

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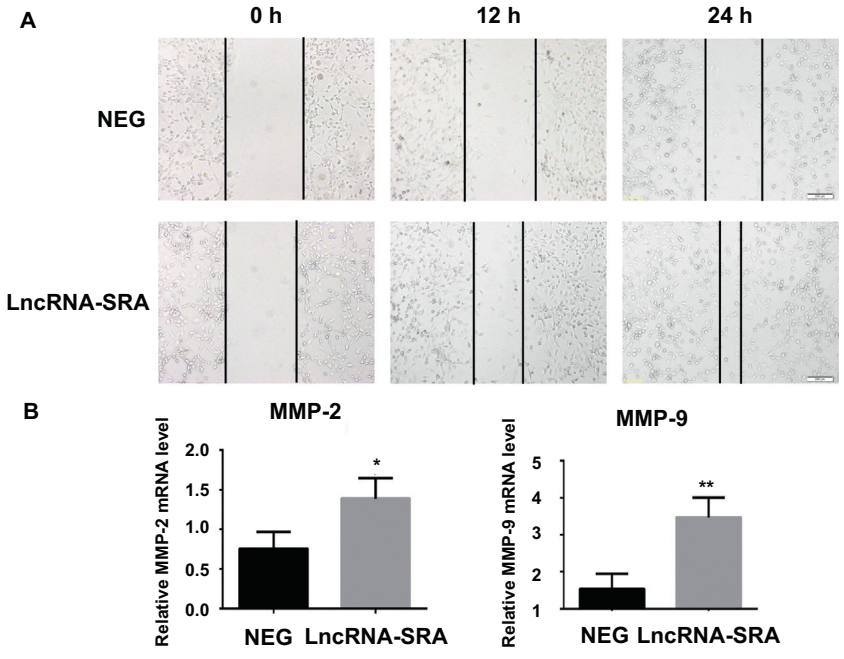

Fig. 3. LncRNA-SRA promotes VSMC migration. (A) A wound-healing assay was performed to investigate the migration ability of VSMCs induce by lncRNA-SRA overexpression. (B) qPCR analysis showed relative mRNA levels of MMP-2 and MMP-9 in VSMCs with lncRNA-SRA overexpression. Results are shown as mean ± s.d. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to NEG.

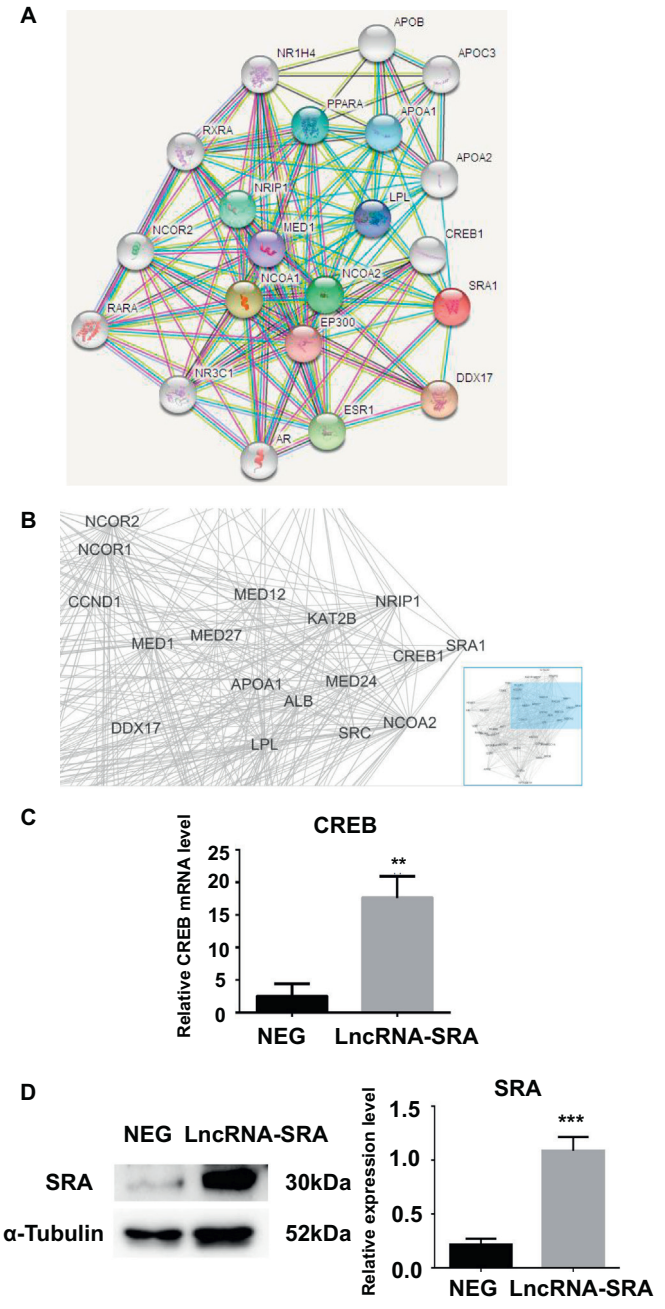

Fig. 4. CREB is the potential downstream of lncRNA-SRA. (A)PPI network for DEGs. The magnitude of the circle of each node indicated the degree to which each gene was connected. Larger circles indicated a greater number of connections between the protein coded by the gene and other proteins. (B) The obtained PPI networks were visualized using Cytoscape software. (C) qPCR analysis was performed to detected the expression of CREB in VSMCs. (D) The expression of SRA in lncRNA-SRA overexpressed VSMCs was measured by western blot analysis $^{**}P < 0.01$,$^{***}P < 0.001$ compared to NEG.

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Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

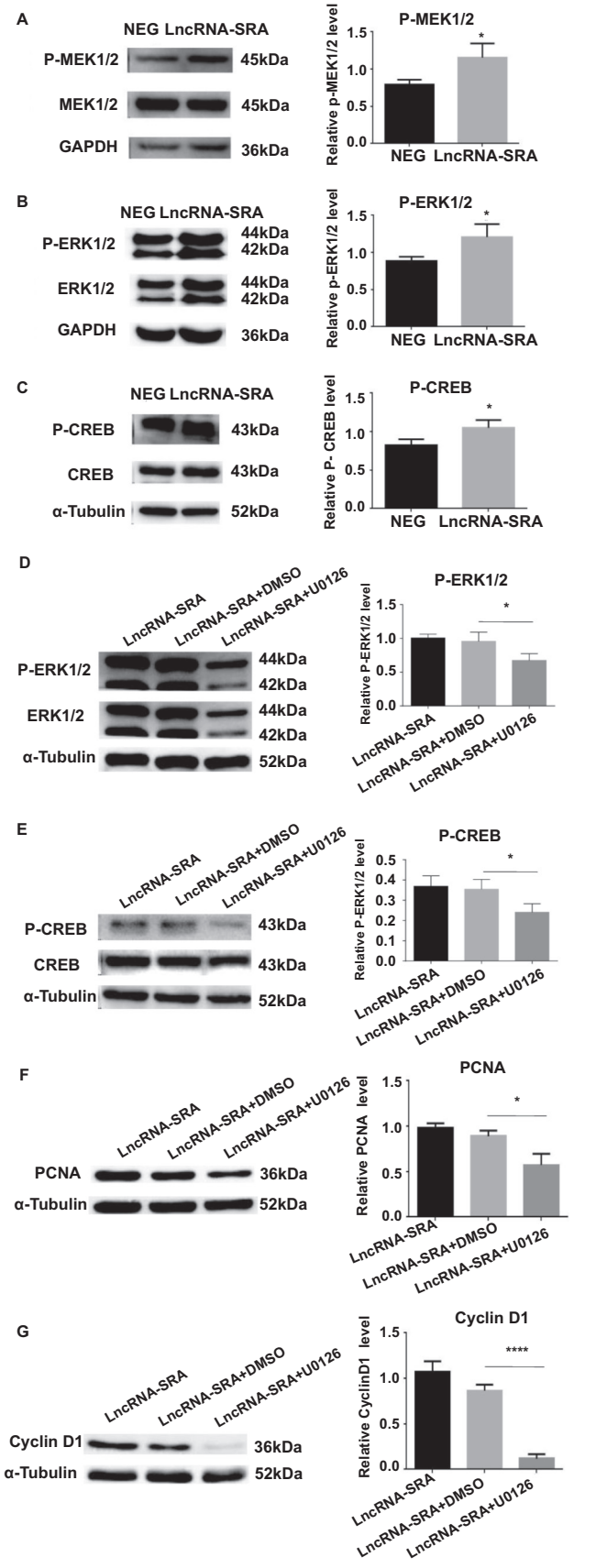

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Fig. 5. LncRNA-SRA induces VSMCs proliferation *via* MEK-ERK-CREB pathway. The protein expressions (A) P-MEK1/2, MEK1/2, (B) P-ERK1/2, ERK1/2, and (C) P-CREB, CREB were analyzed by western blot analysis. LncRNA-SRA overexpressed VSMCs treated with 40 μM U0126 or DMSO for 24 h. The expressions of relative protein were detected by western blot analysis. (D) P-ERK1/2, ERK1/2 (E) P-CREB, CREB and (F) PCNA, (G) Cyclin D1, $^{*}P < 0.05$ compared to NEG, $^{*}P < 0.05$, $^{****}P < 0.0001$ compared to lncRNA-SRA-DMSO.

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