ORIGINAL ARTICLE

Geminin Interference Facilitates Vascular Smooth Muscle Cell Proliferation by Upregulation of CDK-1

Yuanyuan Zhang · Zhouqin Jiang · Linyu Li · Yeping Zhou · Zhiyuan Song · Maoqin Shu

Abstract

Purpose Geminin has been correlated with vascular smooth muscle cell (VSMC) proliferation, but its mechanism is unclear. We selectively silenced the geminin gene of rat VSMCs by using RNAi technology and examined how geminin regulated VSMC proliferation.

Methods By using RNA interference in A10 cells and flow cytometry, ^{3}H-thymidine and 5-ethynyl-2'-deoxyuridine (EdU) measurements were used to detect VSMC proliferation. We performed a Western blot, polymerase chain reaction, and immunohistochemistry to detect the expression and location of geminin and cyclin-dependent kinase-1 (CDK1) in VSMCs.

Results Silencing geminin significantly increased ^{3}H-thymidine and EdU incorporation in VSMCs. We observed a significant increase in ^{3}H-thymidine incorporation 24 h after a serum challenge in the geminin-RNAi-lentiviral vector group (4401.38 ± 438.39 cpm/mg), versus the non-targeting geminin-lentiviral vector (2836.88 ± 476.18 cpm/mg) and control groups (3069.50 ± 508.18 cpm/mg; $P < 0.05$). In the geminin-RNAi-lentiviral vector group, the EdU-positive cell rate was significantly increased (0.75 ± 0.03; $P < 0.05$), versus the non-targeting geminin-lentiviral vector (0.41 ± 0.0) or control group (0.40 ± 0.03). Geminin promoted VSMC proliferation, accelerating G0/G1-S cell-cycle progression (G0/G1 cells, 10 % decrease; S-phase cells, approximate 6 % increase) 12 h after serum withdrawal. Both CDK1 protein and mRNA expression were significantly increased in the positive group versus the controls. The immunofluorescence and co-immunoprecipitation results revealed a close interaction existed between CDK1 and the geminin gene in VSMC proliferation.

Conclusions Geminin gene inhibition could augment VSMC proliferation by increasing CDK1 expression; thus, geminin may be a potential target for treating vascular diseases, specifically VSMCs.

Keywords Geminin · Vascular smooth muscle cells (VSMCs) · RNA interference (RNAi) · Proliferation · Cyclin-dependent kinase-1 (CDK1)

Introduction

Vascular smooth muscle cell (VSMC) proliferation plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, vein graft occlusion, in-stent restenosis, and hypertension [1, 2]. VSMCs represent a class of plastic muscle cells that normally have 2 phenotypes: dedifferentiated phenotype (synthetic phenotype) and differentiated phenotype (contractile phenotype). Given certain conditions, both phenotypes can mutually switch with certain conditions [3]. In the arterial wall, VSMCs are normally quiescent (contractile phenotype), seldom proliferate (<0.05 %), and mainly stay in G0/G1 phase. Following vascular injury, VSMCs migrate to the intimal layer of the arterial wall, where they exit their quiescent state and re-enter the cell cycle (synthetic phenotype) [4]. Therefore, understanding the molecular mechanisms of VSMC proliferation may lead to the development of new therapeutic strategies to treat vascular diseases.

Genome stability is maintained by high-fidelity replication of the genome and faithful separation of replicated DNA into daughter cells. DNA replication is a tightly controlled process. One critical step is the licensing reaction, which assembles pre-replication complexes (pre-RC) through the stepwise

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recruitment of origin recognition complexes, Cdc6, cyclin-dependent kinase-1 (CDK1), and minichromosome maintenance proteins (MCMs) to the replication origins [5]. Once the MCMs are loaded, cyclin-dependent kinases (CDKs), together with a second kinase, Dbf4/Cdc7, promote pre-RC activation and initiate DNA replication. After DNA replication is initiated, replication licensing is inhibited by CDKs and CDK1 degradation, as well as the binding of the inhibitory protein, geminin, to CDK1. CDK1 is expressed during G1 phase, phosphorylated at the onset of S phase, and mostly degraded through Skp1-Cullin-F-box protein/Skp2 and CUL4-DDB1-CDT2 ubiquitin E3 ligases [6, 7]. During the S and G2 phases, non-proteolytic CDK1 is inactivated by binding to geminin, thereby preventing MCMs from reloading onto chromatin [8–11]. Then, geminin is recruited onto chromatin via CDK1 and forms a complex that is unable to recruit MCMs to the origins [11, 12]. Geminin inactivation, as well as the partial degradation of the metaphase-anaphase transition mark, re-initiates the licensing period [12, 13]. This process ensures replication origins fire only once per cell cycle. Loss of geminin results in genomic over-replication [14, 15].

Geminin expression correlates strongly with the proliferative cell state in a wide variety of cancers, including B-cell lymphomas, invasive breast cancer, renal cell carcinoma, and colon and rectal tumors [16–19], as well as in neural cell-fate determination during embryonic development [20]. A few prior studies have shown that similar correlations exist in VSMCs. However, the involvement of geminin in VSMC DNA replication has not yet been demonstrated. We hypothesized that geminin was necessary for precise DNA replication in VSMCs. Given the strong correlation of geminin expression in proliferative cell states in various cancers, as well as in embryonic development, we considered the important role geminin might have in VSMC proliferation with respect to other diseases, in particular, vascular diseases. Therefore, we thought geminin might be a potential new clinical target for the treatment of vascular diseases. To test this hypothesis, we selectively silenced the geminin gene of rat VSMCs by using RNAi technology [21–23] and examined how geminin regulated the proliferation of rat VSMCs.

Materials and Methods

VSMC Culture

A10 cells (thoracic aortic smooth muscle cell line of rats) were bought from the laboratory of Dr Pedro A. Jose of Georgetown University (USA). They were cultured in 60-mm petri dishes containing Dulbecco's modified essential medium (DMEM) (Gibco, USA) that was supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA) and a 1 %

penicillin/streptomycin/epidermal growth factor, along with 5 % CO_2 and 95 % humidity for 2 weeks, in order to allow for A10 cell migration from the vessel onto the dish. At 80 % confluence, the cells were then cultured in a serum-free medium. The specific markers for the synthetic and contractile phenotypes were osteopontin and alpha-smooth muscle actin (α-actin), respectively [24]. The morphology (multilayer sheets, *hills and valleys*) and protein markers were examined at 0 h, 24 h, and 48 h.

siRNA-Mediated Silencing of Geminin in A10 Cells

Three 19-nucleotide siRNA sequences that were specifically targeting rat geminin were designed online (http://www.genscript.com/) and synthesized by Shanghai Genesail Biotech (China).

The mRNA sequences that were utilized are listed below:

A -Geminin siRNA:

upstream 5'-GGUCCUGAAGCCAAUGAAA-3',
downstream 5'-UUUCAUUGGUUUAGGAUU-3'

B -Geminin siRNA:

upstream 5'-GUAUUGGAAAGAAGUGGCA-3'
downstream 5'-UGCCACUUCUUUCCAAUAC-3'

C -Geminin siRNA:

upstream 5'-GUAAUCGAGAGGCUGAGUA-3'
downstream 5'-UACUCAGCCUCUCGAUUAC-3'

Irrelevant siRNA sequences provided by Shanghai Genesail Biotech (China) are listed below:

upstream 5'-CGGCUUCGCGGGCGACGGA-3'
downstream 5'-GAGGAGCUGGAAGCAGCCG-3'

The siRNAs were dissolved in a suspension buffer with a 20-μM concentration and stored in aliquots at −20 °C before use. Freshly isolated A10 cells were seeded onto 6-well plates in DMEM that was supplemented with 10 % FBS and 2 mM of L-glutamine. Upon 50 % confluence, the cells were transfected with siRNA by using an RNAi transfection kit (Invitrogen, USA). The medium was replaced, and the cells were transfected with siRNA by using a cationic liposome 2000 transfection reagent according to the manufacturer's protocol (Invitrogen, USA). Immediately before complexing with Lipofectamine 2000, the siRNA was incubated for 5 min at room temperature. After transfection, the serum-free, antibiotic-free medium was replaced by a medium that contained DMEM plus 10 % FBS, as well as 1 %

streptomycin/penicillin. The irrelevant siRNA served as an siRNA control. In addition, the cells that were treated with the Lipofectamine 2000 transfection reagent, as opposed to siRNA, were used as a normal control.
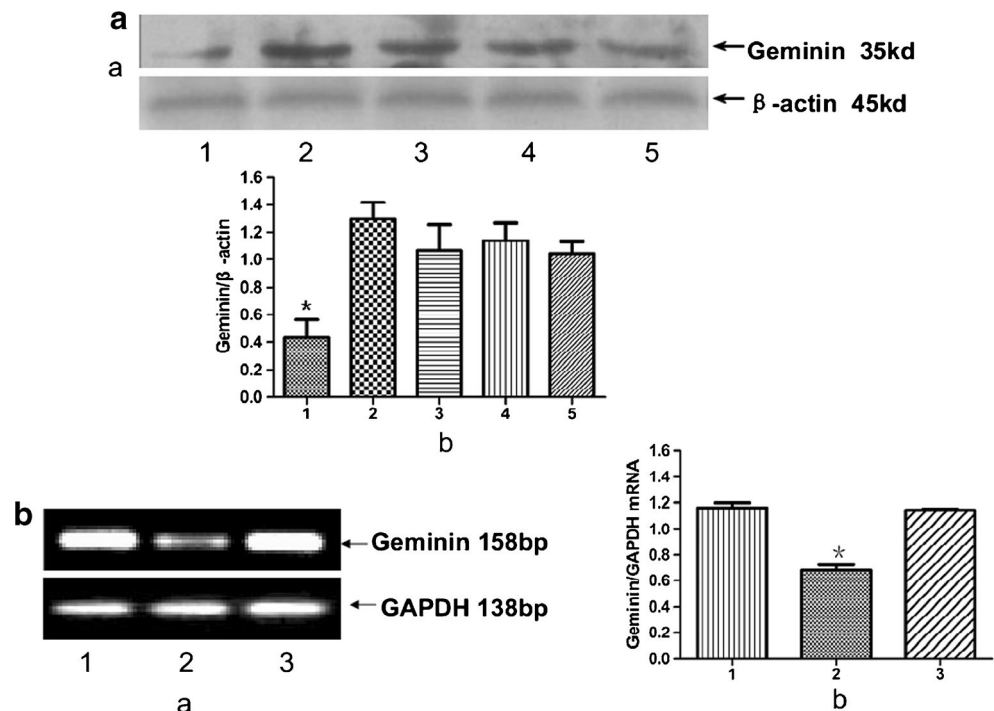
Results

Efficient Silencing of Geminin in A10 Cells

To identify siRNAs that could be efficiently silenced by the expression of geminin in A10 cells, we transiently transfected A10 cells with several siRNAs that targeted geminin. Based on Western blots (Fig. 1A(a)) and a densitometry analysis of these blots (Fig. 1A(b)), the downregulation of geminin protein expression was significantly detected 72 h after transfection with A-geminin siRNA (Lane 1; geminin/β-actin ratio, 0.4; $P < 0.05$), but not with B-geminin siRNA (Lane 2; geminin/β-actin ratio, 1.3) or C-geminin siRNA (Lane 3; geminin/β-actin ratio, 1.1), as compared with that of the negative group (Lane 4; geminin/β-actin ratio, 1.2) and control group (Lane 5; geminin/β-actin ratio, 1.1).

To further validate the effect of A-geminin siRNA, geminin mRNA levels were examined by RT-PCR (Fig. 1B). A-geminin siRNA could efficiently and consistently downregulate geminin mRNA levels in the A10 cells ($P < 0.05$). With geminin and GADPH, we determined geminin/GADPH mRNA ratios of approximately 1.2, 0.7, and 1.2 for irrelevant siRNA, A-geminin siRNA, and non-transfected A10 cells, respectively.

Based on this screening, we used an A-geminin siRNA sequence to construct geminin shRNA lentiviruses. We transfected A10 cells with these viruses (RNAi group) and non-targeting control viruses (NC group). More than 90 % of the A10 cells was transfected by 2–3 days (Fig. 2A). As seen with a laser-scanning confocal microscope, A10 cells in the RNAi group significantly downregulated the expression of geminin, as compared with those of the NC group and the non-transfected cells (control group) (Fig. 2B).
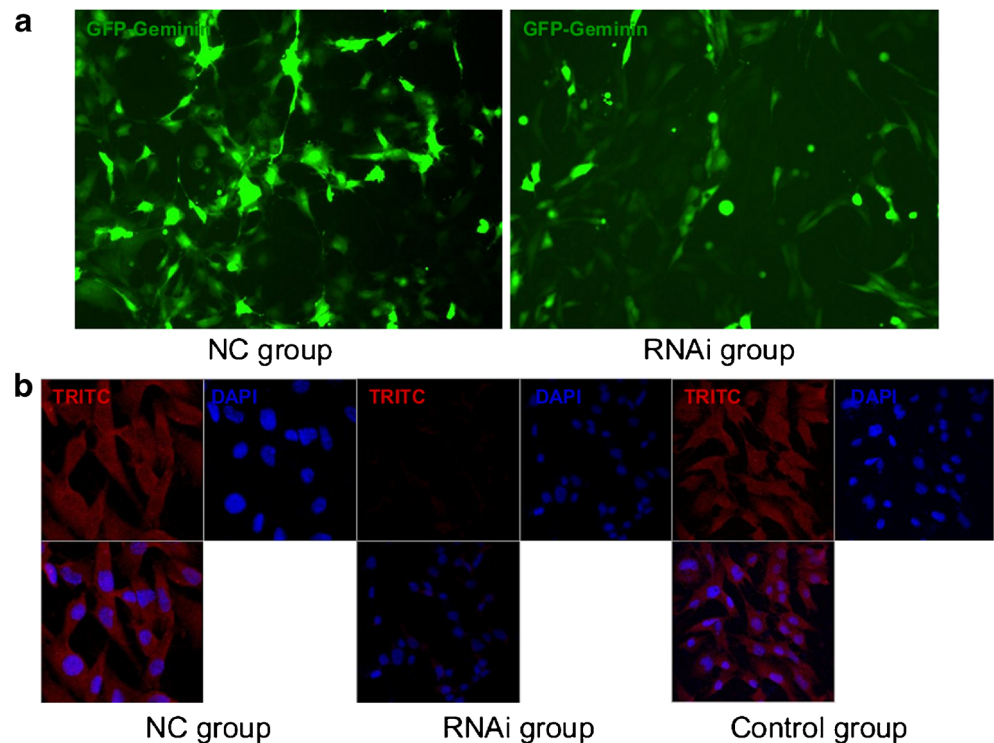
Geminin Gene Silencing Promoted A10 Proliferation

To determine the role of geminin expression in DNA synthesis, A10 cells that were grown in a serum-free culture for 24 h were stimulated in a medium containing 10 % FBS, and ^{3}H-thymidine was added to measure DNA synthesis. Compared with the NC group (2836.88±476.18 cpm/mg protein) and control group (3069.50±508.18 cpm/mg protein), a significant increase in ^{3}H-thymidine incorporation was observed 24 h after a serum challenge in the RNAi group (4401.38±438.39 cpm/mg protein; $P < 0.05$) (Fig. 3A).

To determine the effect of geminin silencing on A10 cell proliferation, we used an EdU incorporation assay. As shown in Fig. 3B, the rate of the EdU-positive cells in the RNAi group increased significantly (0.75±0.03; $P < 0.05$), as compared with the NC group (0.40±0.03) or control group (0.41±0.01).

These results indicate that the knockdown of geminin promoted the proliferation of A10 cells.

Fig. 1 Effects of siRNAs on geminin in A10 cells. **A(a)** A10 cells were transfected with A-geminin siRNA, B-geminin siRNA, C-geminin siRNA (lanes 1–3), an irrelevant siRNA (lane 4), and non-transfected (lane 5). Western blotting was performed to examine geminin protein levels 72 h after transfection. **A(b)** A densitometry analysis of Western blots in A(a); $n=4$; *$P < 0.05$ versus lanes 4 and 5; analysis of variance (ANOVA), Duncan's test. **B(a)** Geminin mRNA of A10 cells that were transfected with A-geminin siRNA (lane 2) and irrelevant siRNA (lane 1), as well as non-transfected A10 cells (lane 3). **B(b)** A densitometry analysis of the results of B(a); $n=4$; *$P < 0.05$ versus lanes 1 and 3; analysis of variance (ANOVA), Duncan's test. The results are expressed as mean±SD

Fig. 2 Lentiviral shRNA-mediated silencing of geminin protein expression in transfected A10 cells. **A** A10 cells were transfected with non-interfering control shRNA lentiviruses (NC group) and geminin-shRNA lentiviruses (RNAi group). Green fluorescent protein (GFP)–geminin-expressing living cells were imaged with a fluorescence microscope. **B** An immunofluorescence examination of geminin expression in A10 cells after a lentiviral infection


Geminin Gene Silencing Induced A10 Cell Proliferation in a Time-dependent Manner and Promoted G0/G1-S Cell-Cycle Progression

Cell proliferation was further analyzed by using flow cytometry. We found that geminin gene silencing was associated with a 10 % decrease in G0/G1 cells and an approximate 6 % increase in S-phase cells 12 h after serum withdrawal (Fig. 4A). The PI was calculated according to the following formula: PI=(S+[G2/M])/([G0/G1]+S+[G2/M]). The A10 cells were cultured in a serum-free medium for 24 h before being stimulated by 10 % FBS. The PI was determined at the

Fig. 3 Effects of geminin gene silencing on the proliferation of A10 cells. **A** The proliferation of VSMCs was determined by ^{3}H-thymidine incorporation. Results are expressed as cpm/mg protein; $n=6$; $*P<0.05$ versus NC group and control group; analysis of variance (ANOVA), Duncan's test. **B** Representative images of EdU staining. (**a**) Edu staining was revealed with a red fluorescence signal; (**b**) bar diagrams showing the number of EdU-positive cells; $n=6$; $*P<0.05$; analysis of variance (ANOVA), Duncan's test
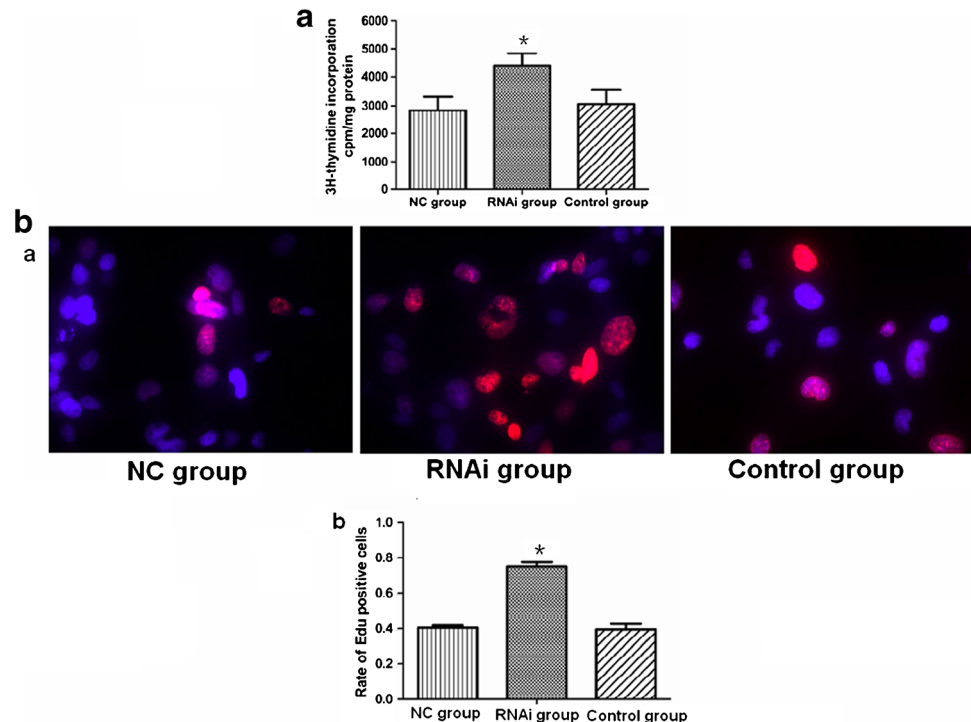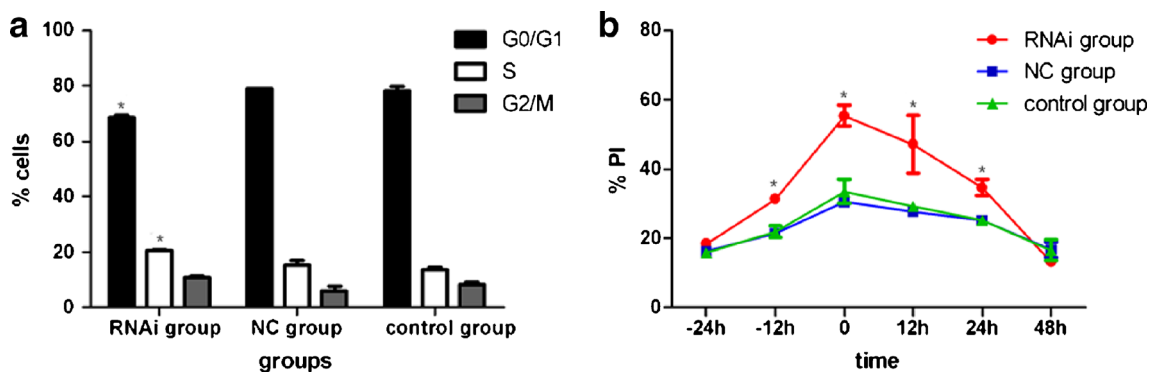
Fig. 4 Flow cytometry of the cell cycle distribution of the treated A10 cells. **A** Percentage of cells in each cell cycle; **B** The PI of A10 cells. The cells were cultured serum-free for 24 h and then stimulated by 10 % FBS. PI was determined at the following time points: −24 h, −12 h, 0 (prior to FBS stimulation), as well as 12 h, 24 h, and 48 h (post-FBS stimulation); $n=3$; *$P<0.05$ versus NC group or control group; analysis of variance (ANOVA), Duncan's test

following time points: −24 h, −12 h, and 0 h before FBS stimulation and 12 h, 24 h, and 48 h after FBS stimulation. The PI increased in the RNAi group and reached its peak at 0 h, at approximately 55.43 %±3.07 % PI, in a time-dependent manner (Figs. 4B and 5). Forty-eight hours post-FBS stimulation, the PI increase returned to control levels at approximately 13.16 %±1.17 % PI (Figs. 4B and 5). Meanwhile, as seen in Fig. 5, in the RNAi group, more cells were in G2/M (26.34 % at 0 h) and S phases (24.74 % at 0 h) than those cells in the NC (10.50 and 20.61 %, respectively, at 0 h) and control (11.65 and 20.08 %, respectively at 0 h)

groups. These data collectively suggest that geminin gene silencing promoted the proliferation of A10 cells by facilitating G0/G1-S cell-cycle progression.

Geminin May Interact with CDK1 to Regulate A10 Cell Proliferation

Considering the close relationship between geminin and CDK1, we examined whether geminin silencing of A10 cells influenced CDK1 expression. Indeed, geminin silencing increased the protein (Fig. 6A) and mRNA (Fig. 6B) levels of
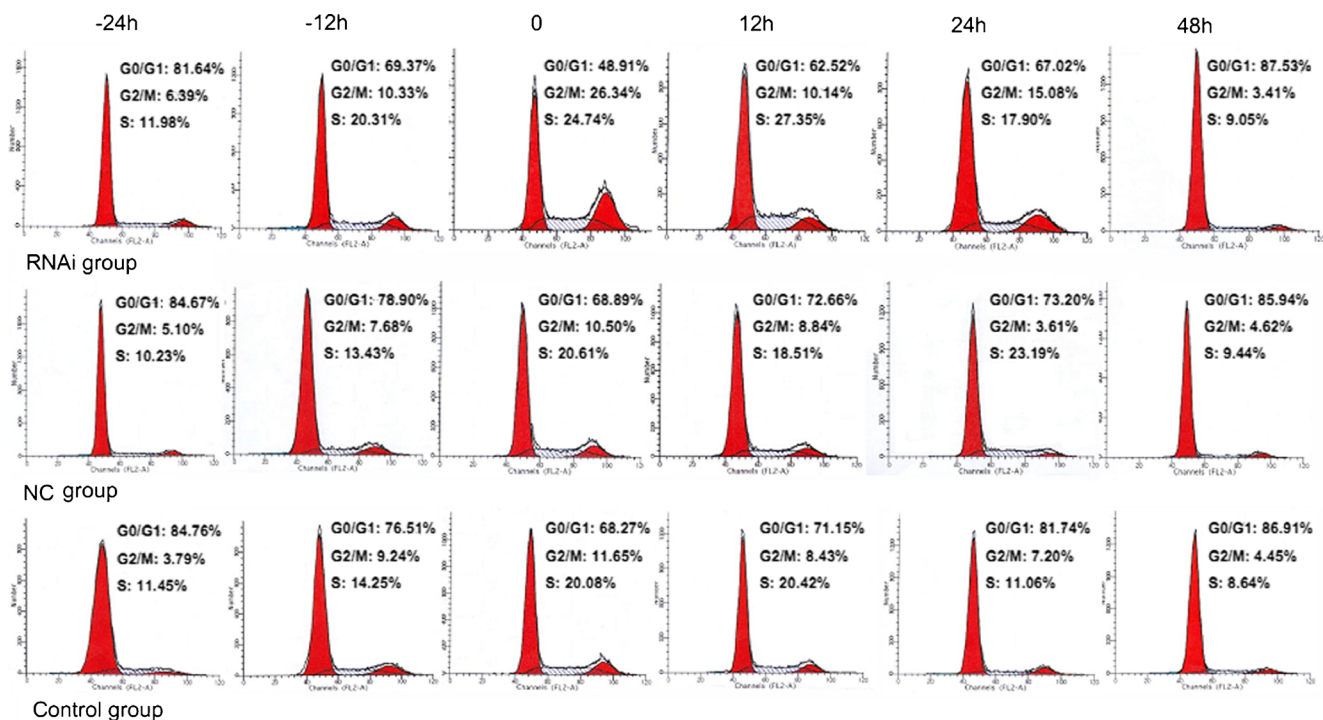

Fig. 5 Geminin gene silencing regulated the cell cycle at different time points. RNAi group, NC group, and control group were cultured in both serum-free medium and medium containing 10 % FBS. The cells were harvested at −24 h, −12 h, 0 (prior to FBS stimulation), 12 h, 24 h, and 48 h (post-FBS stimulation). The cells were labeled with propidium iodide. Triplicate samples of 1×10^4 cells were analyzed by using a 488-nm excitation wavelength and a 610-nm emission wavelength. Representative histograms of DNA contents are shown for the RNAi group, NC group, and control group
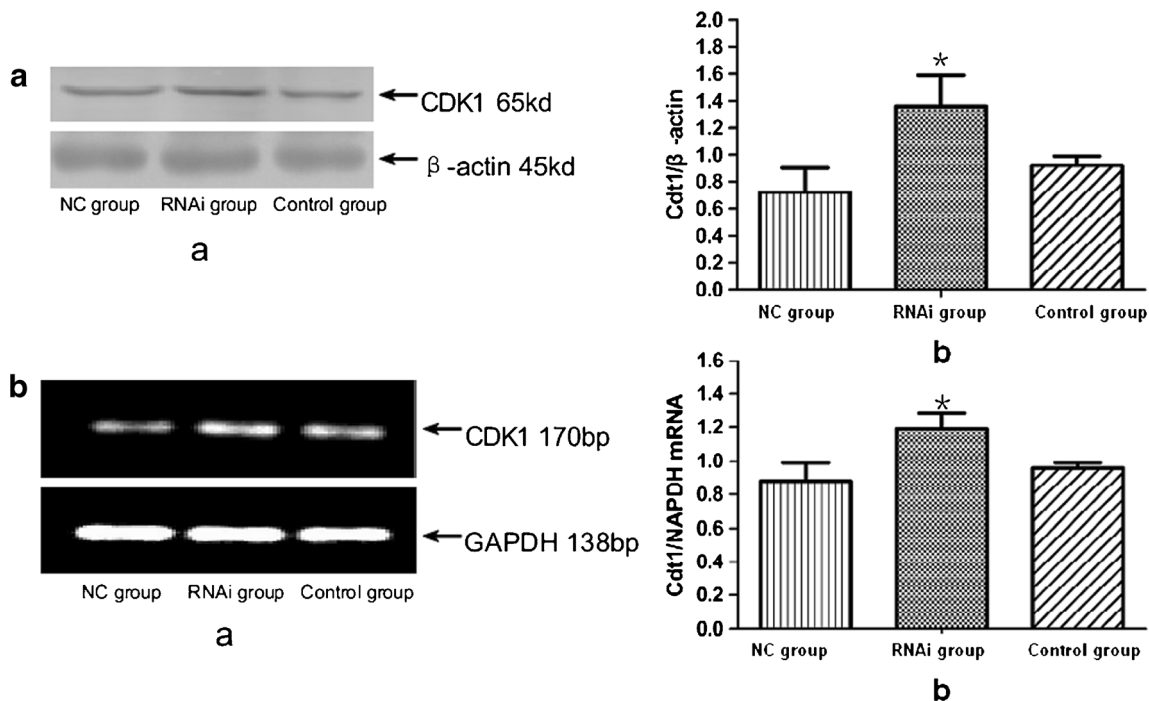
Fig. 6 CDK1 protein and mRNA levels in transfected A10 cells **A** Western blots (**a**) and densitometry (**b**) of CDK1 in A10 cells 72 h post-transfection; $n=4$; *$P<0.05$ versus NC group and control group; analysis of variance (ANOVA), Duncan's test. CDK1 mRNA levels determined by RT-PCT (**a**) and densitometry (**b**) in A10 cells 72 h post-transfection; $n=4$; *$P<0.05$ versus lanes 4 and 5; analysis of variance (ANOVA), Duncan's test. The results are expressed as mean±SD

CDK1 expression, suggesting that geminin knockdown promoted CDK1 expression. The CDK1/β-actin ratios were 0.7, 1.4, and 0.9 in the NC, RNAi, and control groups, respectively (Fig. 6A), while the CDK/NADPH mRNA ratios were 0.9, 1.2, and 1.0 in the NC, RNAi, and control groups, respectively.

We found that geminin and CDK1 co-localized in A10 cells (Fig. 7A). Co-IP experiments showed that geminin could interact with CDK1 in A10 cells with human immunoglobulin heavy locus (IGH; 55 kDa), geminin (35 kDa), and human immunoglobulin light locus (IGL; 25 kDa) (Fig. 7B). Given that geminin binds to CDK1 to prevent CDK1 degradation,

Fig. 7 CDK1 interacted with geminin A10 cells. **A** Co-localization of tetramethyl rhodamine isothiocyanate-Geminin (red) and fluorescein isothiocyanate-CDK1 (green). **B** Co-IP of CDK1 and geminin (triplicate samples). Geminin was immunoprecipitated with CDK1 antibody and analyzed by Western blotting by using an anti-geminin antibody. IgG was used as a negative control
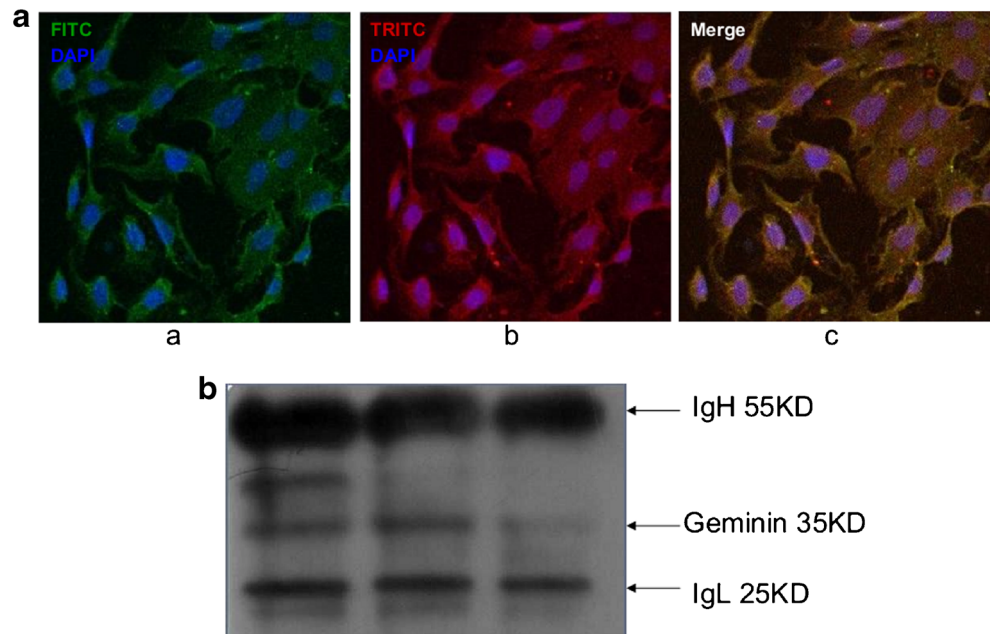
our results suggest that geminin gene silencing might stimulate CDK1 expression, promoting VSMC proliferation in rats.

Discussion

The proliferation of VSMCs plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, vein graft occlusion, in-stent restenosis, and hypertension [1, 2]. The expression of geminin also correlates strongly with the proliferative cell state of various cancers [16–19] and is also involved in neural cell-fate determination during embryonic development [20]. A few prior studies have demonstrated similar correlations with VSMCs, but the involvement of geminin in the DNA replication of VSMCs has not yet been shown. Given the pertinent role of geminin in many diseases, we considered how geminin might be a potential, new clinical target for the treatment of vascular diseases. Therefore, in this study, we selectively silenced the geminin gene in the VSMCs of rats by using RNAi technology [21–23] and examined how geminin regulated VSMC proliferation in rats.

Given that CDK1 is expressed during G1 phase and phosphorylated at the onset of S phase [6, 7] and that during the S and G2 phases, non-proteolytic CDK1 is inactivated by binding to geminin, which prevents the reloading of MCMs onto chromatin [8–11], we investigated whether geminin binds to CDK1 to prevent CDK1 degradation. Moreover, we determined that geminin does bind to and prevents the degradation of CDK1. Therefore, our results suggest that geminin gene silencing might stimulate CDK1 expression and promote VSMC proliferation in rats.

The major finding of this study was that geminin gene silencing promoted A10 cell proliferation by facilitating G0/G1-S cell cycle progression in both serum-free and serum-containing mediums. Furthermore, silencing geminin significantly increased the incorporation of ^{3}H-thymidine and EdU in VSMCs. We observed a significantly increased ^{3}H-thymidine incorporation 24 h after a serum challenge in the geminin-RNAi-lentiviral vector group (4401.38±438.39 cpm/mg), as compared with the non-targeting geminin-lentiviral vector (2836.88±476.18 cpm/mg) and control groups (3069.50±508.18 cpm/mg; $P<0.05$). The CDK1/β-actin ratios were 0.7, 1.4, and 0.9 in the NC, RNAi, and control groups, respectively, while the CDK1/NADPH mRNA ratios were 0.9, 1.2, and 1.0 in the NC, RNAi, and control groups, respectively. Moreover, geminin silencing increased the protein and mRNA levels of CDK1 expression, suggesting that geminin knockdown promoted CDK1 expression. We provided evidence that supports the involvement of CDK1 in this geminin silencing-associated effect.

As for the geminin-RNAi-lentiviral vector group, the EdU-positive cell rate was significantly increased (0.75±0.03;

$P<0.05$), as compared with the non-targeting geminin-lentiviral vector (0.41±0.0) or control group (0.40±0.03), indicating that the knockdown of geminin promoted A10 cell proliferation. Furthermore, both CDK1 protein and mRNA expression were significantly increased in the positive group versus the controls. Moreover, the immunofluorescence and co-immunoprecipitation results revealed the existence of a close interaction between CDK1 and the geminin gene in the proliferation of VSMCs.

Interestingly, the facilitation of G0/G1-S cell-cycle progression was time-dependent (24–48 h). We believe this result was not caused by our research method because we used shRNA lentiviruses, instead of liposomes. A lentivirus could integrate into the host genome to achieve the long-term expression of an integrated gene [25]. Furthermore, geminin promoted the proliferation of VSMCs, accelerating G0/G1-S cell-cycle progression (G0/G1 cells, 10 % decrease; S-phase cells, approximate 6 % increase) 12 h after serum withdrawal. Forty-eight hours after FBS stimulation, the increased PI returned to control levels at approximately 13.16 %±1.17 % PI. In the RNAi group, more cells were in G2/M (26.34 % at 0 h) and S phases (24.74 % at 0 h) than those cells in the NC (10.50 and 20.61 %, respectively, at 0 h) and control (11.65 and 20.08 %, respectively at 0 h) groups. These data suggest that geminin gene silencing promoted A10 cell proliferation by facilitating G0/G1-S cell-cycle progression.

The short-term and reversible effects of VSMC proliferation is of great significance. For example, in 2002, the advent of drug-eluting stents loaded with sirolimus and paclitaxel revolutionized interventional cardiology. Sirolimus, developed as an antibiotic with potent immunosuppressive properties, blocks cell-cycle progression from G1 to S phase and also blocks cell migration, resulting in the cessation of cell-cycle progression [1]. Paclitaxel, developed as an antineoplastic drug, inhibits the disassembly of microtubules, blocking mitotic progression, and thus causes G2/M-phase arrest [2, 3]. Halting cell-cycle progression is thought to be the main mode of action in the reduction of neointimal thickening; the antiproliferative properties of drug-eluting stents impair endothelialization, delay endothelialization, or both, hence leading to late stent thrombosis following stenting. Similarly, if excessive proliferation is inhibited, many different problems could develop.

In Drosophila, Xenopus, and mammals, geminin binds CDK1, preventing the loading of the MCMs onto chromatin and thereby suppressing the inappropriate re-assembly of pre-RC during S, G2, and M phases [8, 9, 26]. As an inhibitor of CDK1, geminin provided a safeguard mechanism to ensure that the activity of CDK1 was restrained only in G1 phase. If geminin was downregulated, we observed a significant increase in CDK1 expression. A high-level CDK1 expression alone was sufficient to induce re-replication [27]. In our study, geminin gene silencing was consistently associated with a

10 % decrease in G0/G1 cells and an approximate 6 % increase in S-phase cells.

In summary, geminin gene silencing promoted G0/G1-S cell-cycle progression and facilitated VSMC proliferation by increasing the expression of CDK1. Moreover, given that geminin gene silencing promoted G0/G1-S cell-cycle progression and facilitated VSMC proliferation in this study, geminin might serve as a potential new target for the treatment of vascular diseases by affecting VSMC proliferation.

Conflict of Interest The authors declare that they have no conflict of interest.

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