High Glucose-Induced Vascular Smooth Muscle Cell Proliferation and Migration are Regulated by the miR-34a-Notch1 Pathway

Liping Zhang

Zhejiang University School of Medicine First Affiliated Hospital

Rongrong Pan

Wenzhou Medical University Affiliated Cixi Hospital: Cixi People's Hospital

Qing Zhang

Zhejiang University School of Medicine First Affiliated Hospital

Linling Gu

Zhejiang University School of Medicine First Affiliated Hospital

Gongde Shi

Zhejiang University School of Medicine First Affiliated Hospital

Yutao Wu (✉ banshuirentaotao@126.com)

Zhejiang University School of Medicine First Affiliated Hospital

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High glucose-induced vascular smooth muscle cell proliferation and migration are regulated by the miR-34a-Notch1 pathway

Liping Zhang^{1, *}, Rongrong Pan^{2, *}, Qing Zhang^{1, *}, Linling Gu¹, Gongde Shi¹, Yutao Wu¹

¹ Department of Cardiology, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

² Department of Cardiology, CiXi Affiliated Hospital of WenZhou Medical College, NingBo Zhejiang 315300, China

Corresponding to:

Yutao Wu, Department of Cardiology, the First Affiliated Hospital, School of Medicine, Zhejiang University, 79th Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China E-mail: wuyutao@zju.edu.cn;

* Contributed equally

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Running title

ZHANG et al: HIGH GLUCOSE-INDUCED VSMC PROLIFERATION AND MIGRATION ARE REGULATED BY MIR-34A-NOTCH1 PATHWAY

Abstract

High glucose(HG)-induced excessive proliferation and migration of the media vascular smooth muscle cell(VSMC) are the main pathological characteristics in diabetes related vascular injuries. Previous studies have shown that microRNA-34a (miR-34a) is involved in cancer metastasis, proliferation and invasion and plays an essential role in cardiovascular disease. However, little is known about the regulating role miR-34a in HG-induced proliferation and migration of VSMC. Here we demonstrated that miR-34a was downregulated at different timepoints under HG stimulation. Then, HG induced proliferation and migration was found to be impaired

by miR-34a overexpression using transwell, CCK8 and RT-qPCR assays. Furthermore, the HG-induced depression of “contractile” VSMC-specific markers were reversed by the overexpression of miR-34a. Moreover, we confirmed that miR-34a regulated HG-induced VSMC proliferation and migration through its target gene, Notch1, which has been shown to be associated with cell proliferation and migration in previous studies. Taken together, we propose that the miR34a-Notch1 axis plays an important role in regulating HG-induced VSMC proliferation and migration.

Introduction

With an exponentially increasing prevalence, diabetes has become a major global health threat. As the main components of the vascular wall, VSMCs are closely related to the occurrence and progression of diabetic atherosclerosis, which is characterized by vascular remodeling, including the phenotypic switching of VSMC from contractile to synthetic phenotype. Vascular remodeling also plays an essential role in other vascular diseases, such as hypertension, vascular injury and arteriosclerosis(1, 2). During vascular remodeling, VSMCs express reduced specific genes, including smooth muscle-actin(SMA), smooth muscle 22 α (SM22), smooth muscle myosin heavy chain (SMMHC) and h1-calponin, but gain greater capacity in cell proliferation and migration, and produce abundant cytokines and extracellular matrix.

microRNAs(miRNAs) are short, endogenous non-coding RNAs, which regulate gene expression post-transcriptionally(3). miRNAs have been reported to be involved in development(4), differentiation(5), proliferation(6), migration(7), apoptosis(8), metabolism(9), immune response(10) and angiogenesis(11). Recently, increasing studies have revealed that miRNAs play an essential role in cardiovascular disease through regulating VSMC phenotypic transition. For instance, Albinsson et al.(12) reported on the essential role of miRNAs in VSMC in deleting the rate-limiting enzyme in miRNA synthesis. miR-143/145 cluster was reported to be a molecular key in the phenotype switching of VSMC(12). Moreover, miR-21 can regulate VSMC function via targeting tropomyosin1 in arteriosclerosis obliterans of the lower

extremities(13). More recently, a study in our department demonstrated that miR-22 was related to VSMC phenotypic modulation and neointima formation by targeting MECP2 and EVI-1(14). These findings suggested the significant roles of miRNAs in vascular disease and provided novel insights into studying the phenotypic transition of VSMC.

miR-34a is a well-studied miRNA in cancer metastasis, proliferation and invasion. miR-34a acts as a suppressor of neuroblastoma tumorigenesis by directly targeting E2F3 mRNA, which is a potent transcriptional inducer of cell-cycle progression and leads to the induction of a caspase-dependent apoptotic pathway(15). Different studies have demonstrated that p53 can bind directly to the precursor gene of miR-34a and activate its transcription during p53-induced apoptosis and G1-arrest(16, 17). Zenz et al.(19) suggested miR-34a was part of the resistance network in chronic lymphocytic leukemia. Recently, our group reported that miR-34a played an important role both in VSMC differentiation from stem cells and in vessel injury-induced neointima formation(18). In another study, down-regulation of miR-34a was associated with the alleviation of mesangial proliferation and glomerular hypertrophy by targeting growth arrest-specific1(GAS1)(19). Notch signaling pathways are important targets of miR-34a. High levels of miR-34a suppress the self-renewal of colon cancer stem cells through the down-regulation of Notch expression(20). These findings have revealed a vital role of miR-34a in regulating cell fate. However, the role of miR-34a in VSMC proliferation and migration induced by high glucose(HG) levels, and the related signaling pathways, remain elusive. In this study, we demonstrated for the first time that miR-34a inhibits HG-induced VSMC proliferation and migration through targeting Notch1.

Materials and Methods

Cell isolation and culture

Primary mouse VSMCs were isolated from C57BL/6 mice (purchased from the Shanghai Institutes for Biological Sciences, Shanghai, China) aorta, and cultured in DMEM (Genom, Hangzhou, China) containing 5.5 mmol/L glucose and

supplemented with 10% fetal bovine serum (FBS: cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as described previously(18). Anesthesia was induced and maintained using 100% O₂ / 4% isoflurane. All mice were euthanized by deep anesthesia with 100% O₂ / 5% isoflurane, followed by dislocated execution. All animal experiments were conducted according to the ARRIVE guidelines. All the animal procedures were approved by the National Institutes of Health Guide for the Care and Use of Laboratory animals(NIH Publications No. 8023, revised 1978). DMEM containing 25 mmol/L glucose was used as the HG medium in this study, and DMEM containing 5.5 mmol/L glucose was defined as normal glucose medium(NG). Cells between passages 5 and 10 were sampled for assays. In all assays, cells were serum-starved for 12-24 hours without FBS and then subjected to the NG or HG with 10% FBS.

VSMC proliferation and migration assays

To verify the effect of HG on VSMC proliferation and migration, the primary VSMCs were cultured in NG or HG medium for 4, 8 and 12 hours and subjected to further studies. Four groups including NG+mimic normal control (NC) , NG+miR-34a mimic , HG+mimic NC and HG+miR-34a mimic were set to detect the role of miR-34a in HG-induced VSMC proliferation and migration. Cells were transfected with mimic NC or miR-34a mimic respectively and then cultured in NG or HG medium for 24 hours. VSMC proliferation was evaluated using a Cell Counting Kit-8 (cat. no. C0037; Beyotime, China) according to the manufacturer's instructions. VSMC migration was evaluated using transwell migration assays(18). In short, the VSMC suspensions were prepared with serum-free medium at the density of 5×10^5 cells/ml and the membranes of the Costar Transwell-24 well plates (8.0 μ m pore size, cat.no.3422, lifesciences, USA) were coated with Matrigel (cat. no. 356234 ; BD Biosciences, San Jose, CA, USA). 100 μ l of cell suspension was added to the upper chamber and 600 μ l of medium containing 20% FBS was added to the lower chamber. The VSMCs were incubated for 24 hours at 37°C. The cells were stained with 0.1%

crystal violet for 20 min and the upper unigrated cells were wiped off with a cotton swab before the stained cells were counted under microscope (400x).

miRNAs and siRNA transfection

Either siRNAs (siRNA-Notch1 and siRNA NC, Ribobio, China, 50 nM) or miRNAs (50 nM, Baiao, China, miR-34a mimic: forward sequences: 5' -UGGCAGUGUCUUAGCUGGUUGU-3', reverse sequences: 5'-ACAACCAGCUAAGACACUGCCA-3'; miR-34a inhibitor mimic (miR-34a Int mimic) : 5'-ACAACCAGCUAAGACACUGCCA-3') were transfected into VSMCs using Lipofectamine 3000 (cat. no. L3000-015; Thermo Fisher Scientific, Inc.) using the methodology described previously (5, 21, 22). Briefly, cells were grown in 6-well plates and the growth media replaced with 1.0 ml empty medium without antibiotics or FBS and maintain for 30 min after reaching 60-70% confluency. The cells were transfected with siRNAs (mixture of A and B; A: 50 µl DMEM /well+ 1 µl siRNA/well; B: 50 µl DMEM /well+3.75 µl Lipofectamine 3000/well) or miRNAs (mixture of A and B; A: 50 µl DMEM /well+ 1 µl miRNA/well; B: 50 µl DMEM /well+3.75 µl Lipofectamine 3000/well) for 24 hours. The cells were then cultured in NG or HG with 10% FBS for an additional 24 or 48 hours.

Real time quantitative PCR (RT-qPCR)

Total mRNA isolation and real-time quantitative PCR (RT-qPCR) were performed as we described previously(23-25). Total mRNAs containing miRNAs were isolated from cells using Trizol (cat. no. 12183555; Invitrogen; Thermo Fisher Scientific, Inc. USA) according to the manufacturer's instructions. Total RNAs and miRNAs specific cDNA synthesis was performed using a PrimeScript RT Master Mix (Perfect Real Time) Kit (cat. no. RR047A; Takara Biomedical Technology Co., Ltd., Beijing, China) and miDETECT A Track™ miRNA qRT-PCR Starter Kit (cat. no. C10712-1; Ribobio, Guangzhou, China). Real-time PCR for mRNA was performed using Takara premix Ex Taq II (cat. no. DRR820A; Takara Biomedical Technology Co., Ltd, Beijing, China) and was run on ABI Prism 7500 system (Applied Biosystems, USA) in a total volume of 10 µl containing 5 ul Takara premix Ex Taq II

and 5 ng cDNA template. Real - time PCR for miRNA was performed with miDETECT A Track™ miRNA qRT-PCR Starter Kit (cat. no. C10712-1; Ribobio) and was run on an ABI Prism 7500 system (Applied Biosystems, USA) in a total volume of 20 µl containing 10ul SYBR Green Mix and 10ng cDNA template. The PCR thermal cycling parameters for mRNA were 2 min at 50 C, 30 sec at 95 C, 40 cycles of 95 C for 5 sec and 60 C for 34 sec and for miRNA were 10 min at 95 C and 40 cycles of 95 C for 2 sec, 60 C for 20 sec and 70 C for 10 sec. Expression of mRNA/miRNA was normalized to the expression of mouse GAPDH (mGAPDH)/U6 and quantified using the $2^{-\Delta\Delta C_q}$ method. Primers used in the experiments are listed in the Table I.

Western blot analysis

Protein extraction and immunoblotting were performed similar to our previous reports(24, 26). Briefly, total proteins were extracted from VSMCs using RIPA lysis buffer (cat. no. P0013B; Beyotime, China) and quantified using a BCA Protein Assay Kit (cat. no. P0012S; Beyotime, China). An equal volume of protein mixed with 5X SDS loading buffer (cat. no. P0015; Beyotime, China) was boiled for 10 min at 100 C. 10-30 ug of the protein were separated on 10% SDS-PAGE gel (cat. no. P0012A; Beyotime, China) and transferred from the gel to the membrane. The membrane was blocked overnight in 5% skim milk followed by incubation with primary antibodies overnight at 4°C. The primary antibodies against Notch1 (cat. no. A-8:sc-376403; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; monoclonal antibody from mouse), PCAN (cat. no. 15014-1-AP; D3-3, No.666 Gaoxin Avenue Wuhan East Lake Hi-tech Development Zone Wuhan, Hubei, P.R.C) and GAPDH (cat. no.14C10; Cell Signaling Technology, Inc.) were diluted 1:1000, 1:500 and 1:1000, respectively. The membrane was washed thrice with 1X PBST, for 10 min per wash, and incubated with the secondary antibody from Santa Cruz Biotech for 1 hour at room temperature. The membrane was washed again 3 times with 1X PBST. The ECL substrate was added and the target proteins observed with the Compact X4 in the dark room.

Statistical analysis

All data were analyzed using a two-tailed student's t-test or one-way ANOVA followed by Tukey's HSD multiple comparison post-hoc test.

Results

miR-34a expression is down-regulated during HG-induced VSMC proliferation and migration

VSMCs cultured in HG media showed a greater growth density than those grown in NG medium after 12 and 24 hours (Figure 1A). Transwell migration assays revealed that HG stimulation promoted VSMC migration when compared against NG (Figure 1B and C). Furthermore, transcript profiles of miR-34a were downregulated under HG stimulation (Figure 1D), suggesting a role of miR-34a in HG-induced VSMC proliferation and migration.

HG-induced VSMC proliferation and migration may be impaired by miR-34a

We performed a series of experiments in order to reveal the further role of miR-34a in HG-induced VSMC proliferation and migration. As transwell assay showed, HG-induced migration may be inhibited by miR-34a overexpression (Figure 2A). CCK8 assays also demonstrated that miR-34a overexpression could abolish the proliferation induced by HG stimulation (Figure 2B). The transcript levels of osteopontin (OPN) and PCAN were determined by RT-qPCR. OPN has been reported to be synthesized by smooth muscle cells in primary and restenotic human coronary atherosclerotic plaques(27) and be associated with cell proliferation(28, 29) and migration(30, 31). PCNA is also expressed in VSMC which has more capacity of proliferation and migration and has been thought to be responsible for the proliferation and migration in many kinds of cells(32, 33). As expected, HG induced the upregulation of both OPN and PCAN could be abated by miR-34a mimic while there was no significance at OPN and PCAN mRNA level between mimic NC group and miR-34a mimic group under NG condition (Figure 2C and D). These results support the hypothesis that miR-34a plays an essential role in regulating VSMC proliferation and migration under HG condition.

HG-induced downregulation of VSMC specific markers is modulated by miR-34a

During the phenotypic switching of VSMC, the increasing capacities for proliferation and migration result in the downregulation of VSMC specific genes, such as SMA, SM22 and SMMHC. Thus, expression of these marker genes was determined by RT-qPCR. HG stimulation inhibited SM22 mRNA expression, while miR-34a overexpression almost abolished this effect (Figure 3A). In contrast, miR-34a Int mimic enhanced the inhibitory effect of HG stimulation (Figure 3B). Meanwhile, the HG-induced downregulation of SMA and SMMHC was affected by miR-34a Int mimic (Figure 3C and D). These data indicated that HG-induced downregulation of VSMC specific markers was modulated by miR-34a.

miR-34a regulates HG-induced VSMC proliferation and migration through targeting Notch1

According to TargetScan (<http://www.targetscan.org>, Figure 4A) and our previous study(18), we examined whether or not miR-34a modulates Notch1 during HG-induced VSMC proliferation and migration. Consistent with our previous study, Notch1 gene and protein levels were significantly downregulated by the overexpression of miR-34a (Figure 4B and C). Conversely, Notch1 levels were significantly upregulated by the inhibition of miR-34a (Figure 4F). Meanwhile, the transfection of siRNA- Notch1 into VSMCs significantly downregulated Notch1 mRNA levels (Figure 4E) and protein levels (Figure 4D). To study the interactions between Notch1 and miR-34a in HG-induced VSMC proliferation and migration further, miR-34a Int mimic and siRNA- Notch1 were co-transfected into VSMCs cultured in HG. Cell counting assays (Figure 4G) and western blotting (Figure 4H) showed that miR-34a Int mimic induced VSMC proliferation and PCAN mRNA overexpression were suppressed by inhibition of Notch1 in these cells. Since we have demonstrated that miR-34a inhibited PCAN expression, VSMC proliferation and Notch1 levels, whereas miR-34a Int mimic reduced these effects, the reduced proliferation in these co-transfected VSMCs was likely due to the downregulated Notch1 levels by its siRNA. Therefore, our results implied that miR-34a modulated HG-induced VSMC proliferation and migration through its target gene Notch1.

Discussion

Excessive proliferation and migration of VSMCs in the tunica media is one of the main pathological characteristics in diabetes-induced vascular injuries, which leads to the formation of new intima, vascular stenosis and eventually causes disability and death in large numbers of patients. Despite great efforts in research into HG-induced VSMC proliferation and migration in the past decades, a comprehensive molecular mechanism in this pathological process remains elusive. In the present study, we report for the first time that HG-induced VSMC proliferation and migration are regulated by miR-34a. The overexpression of miR-34a impaired VSMC proliferation and migration, and alleviated the HG-induced inhibition of gene expression of VSMC specific markers. Moreover, Notch1 was confirmed to be the target gene of miR-34a in regulating HG-induced VSMC proliferation and migration.

The phenotypic transition of VSMC plays an important role in the physiological and pathological process of vascular complications in type 2 diabetes. However, the precise mechanisms have remained largely unclear. Consistent with several other studies(34, 35), HG promoted VSMC proliferation and migration in the present study. As expected, HG induced the expression of OPN and PCAN, which are related to VSMC proliferation and migration(28). Moreover, HG inhibited the expression of SMA, SM22 and SMMHC, which are key markers of VSMC function and are often downregulated in several vascular diseases (36, 37). These changes in gene expression may contribute to the impaired normal function and enhanced proliferation and migration in VSMC under HG stimulation. It was also reported that HG can cause oxidative stress and impair apoptosis in VSMC (38-40) and antioxidants can inhibit HG-induced VSMC proliferation and migration(35). However, the apoptosis level of VSMCs induced by HG was not determined in this study.

Decreased miR-34a expression was also observed under HG stimulation. miR-34a is a member of the highly conserved miR-34 family, which plays a major role in regulating cell-cycle progression, apoptosis, and DNA repair in cancer(41-43), neurophysiology and neuropathology studies(44). Recently, miR-34a has been reported to play an essential role in cardiovascular physiological processes and diseases. Shi et al.(39) has reported that miR-34a can promote apoptosis in

cardiomyocytes and alleviate the injury caused by hypoxia. The research on miR-34a mediated p53 pathways has also deepened our understanding of miR-34a in determining cell fate. p53 can directly bind to the miR-34a precursor gene and modulate its transcription(16, 17, 45). In this study, overexpression of miR-34a largely eliminated the suppressing effects of HG stimulation on SM22 mRNA expression level while inhibition of miR-34a could enhance the suppressing effects of HG stimulation on SM22 mRNA expression. Meanwhile, inhibition of miR-34a could strengthen inhibitory effect of HG stimulation on SMA mRNA expression though it reduced the inhibitory effect of HG stimulation on SMMHC mRNA expression. Moreover, overexpression of miR-34a largely eliminated the promoting effects of HG stimulation on VSMC proliferation and migration ability as transwell and CCK8 assay showed and related genes (OPN and PCAN). Interestingly, miR-34a had no effect on VSMC proliferation and migration under NG condition in our study, suggesting that miR-34a could regulate VSMC proliferation and migration under HG condition rather than under NG condition. This prompts that miR-34a may be an effective and safe therapeutic target for patients suffering diabetes-related vascular diseases. Additionally, the absence of data on VSMC marker genes mRNA expression in the NG+miR-34a mimic group and protein level of OPN and PCAN is a limitation of our study.

A better understanding of the miRNA targets can help to reveal their complex mechanisms. Notch signaling pathways which were activated in HBZY-1 cells(46)and podocytes(47) exposed to high glucose play prominent roles in VSMC proliferation, migration and apoptosis(48). The targeting of Notch1 by miR-34a has been well studied in glioma cells(49), colon cancer stem cells(20) and breast cancer cells(50). miR-34a can downregulate Notch1 protein levels by binding to mRNA 3'-UTRs(49). Yan Tang et al. has reported that miR-34a inhibits pancreatic cancer progression through regulating epithelial-mesenchymal transition (EMT) by targeting Snail1 and Notch1(51), while they do not check the relationship between Snail1 and Notch1 under the EMT process. In the previous study from our team(18), Notch1

repression has been reported to be required for miR-34a mediated VSMC proliferation and migration induced by PDGF or serum. Thus, we guess Notch1 may also act as the functional target of miR-34a under HG stimulation. For the first time, in the present study, we reported that miR-34a overexpression reduced Notch1 mRNA and protein levels in VSMC under HG stimulation while an opposite trend was observed under miR-34a inhibition. Since the data of variation of Notch1 in HG was not completed, it was hard to exclude the effect of HG on the expression level of Notch1. From the existing experimental data and previous studies(46, 47), it is speculated that the effect of miR-34a Int mimic and HG on the expression level of Notch1 should be consistent from the trend. Moreover, In the 'rescue' experiments, the transfection of siRNA- Notch1 into VSMC weaken the miR-34a Int mimic induced PCAN mRNA overexpression and VSMC proliferation. These results indicated that siRNA inhibition of Notch1 resulted in reduced VSMC proliferation. Combining this with the fact that overexpression of miR-34a downregulated Notch1 expression, the targeting of Notch1 by miR-34a in HG-induced VSMC proliferation can be proven. In our present study, we have firstly demonstrated VSMC proliferation and migration under HG condition may be regulated by the miR-34a-Notch1 pathway, which would provide novel insights into diabetes mellitus(DM)-induced vascular complications. However, it is also well acknowledged that miR-34a targets multiple signaling pathways in addition to the Notch1 pathway(20). Hence, the complex mechanism in miR-34a modulated VSMC proliferation and migration requires further study. It is also a limitation that only primary VSMC was used in our study rather than two or more cell lines.

In conclusion, our results provide novel insights into DM-induced vascular complications. We have shown that miR-34a expression is reduced in VSMCs under HG stimulation accompanying increased cell proliferation and migration. Overexpression of miR-34a inhibited VSMC proliferation and migration induced by HG stimulation and alleviated the inhibition on the expression of VSMC specific markers under HG condition. Moreover, of miR-34a targets and downregulates

expression of Notch1. Taken together, we conclude that miR-34a regulated HG-induced VSMC proliferation and migration through the miR-34a-Notch1 pathway. Thus, there is potential to develop novel therapeutic strategies in treating DM-induced vascular complications through modulating miR-34a activities by targeting Notch1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

YW designed the research; LZ, RP, QZ, LG, GS performed the research; YW analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests and all authors should confirm its accuracy

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Figure legends:

Figure 1. Proliferation and migration of vascular smooth muscle cells (VSMCs), and miR-34a expression under high glucose stimulation. (A and B) VSMCs cultured in HG medium showed a greater growth density compared with NG group under microscopy. (C and D) Transwell migration analyses of VSMCs cultured in HG or NG medium for 24 hours. (E) miR-34a expression of VSMCs cultured in HG medium or NG medium (0 hour) determined at different timepoints using RT-qPCR. Data is representative or means $\pm$ S.E.M. of at least three independent samples. * $P < 0.05$, ** $P < 0.01$.

Figure 2. High glucose-induced vascular smooth muscle cell(VSMC) proliferation and migration are impaired by miR-34a mimic. (A-B) miR-34a mimic reduced HG induced VSMC migration while the migrated cells between NG+mimic NC group and NG+miR-34a mimic group seemed no significance. (C) VSMC transfected with mimic NC or miR-34a mimic respectively were cultured in NG or HG medium for 24 hours and then subjected to CCK8 analyses. (D and E) VSMC transfected with mimic NC or miR-34a mimic respectively were cultured in NG or HG medium for 12 hours. Then, cells were harvested to examine the OPN and PCAN

mRNA expression levels by RT-qPCR. Data is representative or means $\pm$ S.E.M. of at least three independent samples. * $P < 0.05$, ** $P < 0.01$.

Figure 3. High glucose-induced downregulation of VSMC specific markers was modulated by miR-34a mimic or miR-34a Int mimic. (A-D) Expression of VSMC specific markers SM22, SMA and SMMHC were detected. Data is representative or means $\pm$ S.E.M. of at least three independent samples. * $P < 0.05$, ** $P < 0.01$.

Figure 4. miR-34a regulated high glucose-induced VSMC proliferation and migration through its target gene Notch1. (A) The potential wild type binding sites of miR-34a within Notch1 3' -UTR predicted by TargetScan. (B - D) VSMCs were transfected with miR-34a mimic or miR-34a Int mimic and their NC for 48 hours and then harvested for western blot and RT-qPCR. (E and F) Transfection of siRNA-Notch1 into VSMCs significantly downregulated Notch1 mRNA and protein level. (G) miR-34a Int mimic and siRNA- Notch1 were co-transfected into VSMCs cultured in HG. Cell counting assays showed miR-34a Int induced VSMC proliferation was eliminated by inhibition of Notch1 in these cells. Data is representative or means $\pm$ S.E.M. of at least three independent samples. * $P < 0.05$, ** $P < 0.01$.

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