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Article

MicroRNA expression profile and functional analysis reveal their roles in contact inhibition and its disruption switch of rat vascular smooth muscle cells

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Abstract

Contact inhibition and its disruption of vascular smooth muscle cells (VSMCs) are important cellular events in vascular diseases. But the underlying molecular mechanisms are unclear. In this study we investigated the roles of microRNAs (miRNAs) in the contact inhibition and its disruption of VSMCs and the molecular mechanisms involved. Rat VSMCs were seeded at 30% or 90% confluence. MiRNA expression profiles in contact-inhibited confluent VSMCs (90% confluence) and non-contact-inhibited low-density VSMCs (30% confluence) were determined. We found that multiple miRNAs were differentially expressed between the two groups. Among them, miR-145 was significantly increased in contact-inhibited VSMCs. Serum could disrupt the contact inhibition as shown by the elicited proliferation of confluent VSMCs. The contact inhibition disruption accompanied with a down-regulation of miR-145. Serum-induced contact inhibition disruption of VSMCs was blocked by overexpression of miR-145. Moreover, downregulation of miR-145 was sufficient to disrupt the contact inhibition of VSMCs. The downregulation of miR-145 in serum-induced contact inhibition disruption was related to the activation PI3-kinase/Akt pathway, which was blocked by the PI3-kinase inhibitor LY294002. KLF5, a target gene of miR-145, was identified to be involved in miR-145-mediated effect on VSMC contact inhibition disruption, as it could be inhibited by knockdown of KLF5. In summary, our results show that multiple miRNAs are differentially expressed in contact-inhibited VSMCs and in non-contact-inhibited VSMCs. Among them, miR-145 is a critical gene in contact inhibition and its disruption of VSMCs. PI3-kinase/Akt/miR-145/KLF5 is a critical signaling pathway in serum-induced contact inhibition disruption. Targeting of miRNAs related to the contact inhibition of VSMCs may represent a novel therapeutic approach for vascular diseases.

Keywords: smooth muscle cells; contact inhibition; microRNAs; miR-145; PI3-kinase/Akt; LY294002; KLF5; vascular diseases

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Introduction

Contact inhibition, the arrest of growth induced by confluence, is characteristic of vascular smooth muscle cells (VSMCs). It is well known that contact inhibition of VSMCs is important in keeping vascular homeostasis and preventing excessive vascular neointimal growth, a key pathological change in many proliferative vascular diseases such as atherosclerosis, restenosis after angioplasty, and diabetic vascular complications[1, 2]. On the other hand, disruption of the VSMC contact inhibition is required for angiogenesis and collateral circulation formation in ischemic cardiovascular disease, stroke and cancer development[3, 4]. Although the contact inhibition of VSMCs is very important in vascular disease, the molecular mechanisms involved in VSMC contact inhibition and its disruption under disease conditions are still unclear.

MicroRNAs (miRNAs), with tremendous biological functions, are a class of endogenous, small, noncoding RNAs. It is believed that miRNAs may directly regulate at least 30% of genes and indirectly regulate the other 70% genes in a cell; so it would not be surprising that miRNAs are involved in the regulation of almost all major cellular functions, such as cell differentiation, proliferation, migration, and apoptosis. Accordingly, miRNAs could be pivotal regulators in normal physiology, as well as diseases states including vascular disease[5]. Indeed, the studies from us[6-10] and other groups[11-14]

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have demonstrated that miRNAs play critical roles in vascular integrity, vascular inflammation and vascular disease, which are related to their strong regulatory functions on vascular cells including VSMCs. For example, miR-145 is a master controller for VSMC differentiation via its target gene Kruppel-Like Factor 5 (KLF5)[7]. MiR-221/222 are able to regulate VSMC proliferation and migration via their target genes, p27(Kip1), p57(kip2) and c-kit[8, 10]. In addition, miR-21 is a strong anti-apoptosis gene in VSMCs via its target gene, programmed cell death 4 (PDCD4)[9]. However, to date, the roles of miRNAs in contact inhibition and its disruption switch of VSMCs have not been studied.

In the current study, we found that multiple miRNAs were differentially expressed in VSMCs with high density, compared with those in low density. Among them, the expression of smooth muscle cell specific/enriched miR-145 is significantly increased in confluent, contact-inhibited VSMCs. In contrast, miR-145 levels were strongly downregulated in sub-confluent VSMCs and in serum-induced, contact inhibition disrupted VSMCs. We further identified that miR-145 plays an important role in controlling the switch of contact inhibition and contact inhibition disruption of VSMCs via its target gene.

Materials and methods

Cell culture

VSMCs were obtained from the aortas of male Sprague-Dawley rats (5 weeks old) by using an enzymatic dissociation method as described previously[6-10]. VSMCs were cultured with DMEM containing 10% fetal bovine serum (Thermo-Fisher Scientific, Grand Island, NY, USA). For all experiments, rat VSMCs from passages 3 to 6 were used.

Contact inhibition and contact inhibition disruption models of VSMCs

Contact inhibition was induced in confluent (100% confluence) VSMCs cultured with 0.2% FBS. Contact inhibition disruption was induced as described in a research article, in which contact inhibition of VSMCs was disrupted by 5.0% FBS in confluent VSMCs[15].

miRNA expression signature array via microarray in cultured VSMCs

miRNAs were isolated from cultured VSMCs. miRNA expression profiling was done by miRNA microarray analysis using a chip containing probes covering all the miRNAs in the latest version of the miRBase database (LC Science, Houston, TX, USA) as described previously[16, 17]. The miRNA expression was demonstrated by the mean of the six biological replicates. Proprietary "spike-in" controls were used at each step of the process.

RNA isolation and qRT-PCR

RNA levels were determined by qRT-PCR[6-10]. Briefly, RNA from VSMCs was isolated with TRIzol (Invitrogen; Carlsbad, CA, USA). qRT-PCR for miRNA was performed on cDNA generated from 100 ng of total RNA using TaqMan MiRNA Reverse Transcription and TaqMan MiRNA assays (Applied Biosystems, Foster City, CA, USA). Amplification and detection of specific products were performed with a Roche Light-cycler 480 Detection System. As an internal control, U6 was used for miRNA template normalization. Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. Relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the U6 Ct value, which provided the ΔCt value. Relative expression between treatments was then calculated using the following equation: relative gene expression=$2-(\Delta C_{t\ sample}-\Delta C_{t\ control})$.

Northern blot analysis of miR-145

Ten micrograms of total RNA from VSMCs were loaded onto a precast 15% denaturing polyacrylamide gel (Bio-Rad) as described in our previous study [7]. The RNA was then electrophoretically transferred to Bright-Star blotting membranes. The probe sequences of miR-145 and U6 are 5'-AAGGGATTCCTGGGAAAACTGGAC-3' and 5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3'. Probes were end-labeled with [alpha-^{32}P]ATP by T4 polynucleotide kinase. Prehybridization and 3 hybridization were carried out in Ultrahyb Oligo solution (Applied Biosystems, Foster City, CA, USA) containing 106 cpm/mL probes overnight at 37 °C. The most stringent wash was with 2×SSC and 1% SDS at 37 °C. For reuse, blots were stripped by boiling and re-probed. U6 was used as a loading control to normalize expression levels.

Oligo transfection, knockdown or overexpression of miR-145 and KLF5 knockdown, in cultured VSMCs

Oligo transfection was performed according to the established protocol[6-10]. Briefly, cells were transfected using a transfection reagent (Qiagen, Chatsworth, CA, USA) for 4 h. Transfection complexes were prepared according to the manufacturer's instructions. For miR-145 knockdown, the miR-145 inhibitor, 2'OMe-miR-145 (Integrated DNA Technologies, Coralville, IA, USA), was added to the complexes at final oligonucleotide concentration of 30 nmol/L. For miR-145 overexpression, pre-miR-145 (Integrated DNA Technologies, Coralville, IA, USA) was added to the complexes at final oligonucleotide concentration of 30 nmol/L. KLF5 knockdown was performed using its siRNA (si-KLF5, 50 nmol/L, Invitrogen, Carlsbad, CA, USA).

Western blot analysis and phosphoinositide 3-kinase (PI3-kinase) inhibition

Proteins were isolated from cultured VSMCs, and the protein levels were determined by Western blot analysis. Briefly, equal amounts of protein were subjected to SDS-PAGE. Standard Western blot analysis was conducted using antibodies for Akt (1:1000 dilution; Cell Signaling, Danvers, MA, USA), phosphor-Akt (p-Akt, ser473, 1:1000 dilution; Cell Signaling) and KLF5 antibodies (1:1000 dilution; Abcam, Cambridge, MA, USA). GAPDH antibody (1:5000 dilution; Cell Signaling,

Danvers, MA, USA) was used as a loading control. In cultured VSMCs, PI3-kinase/Akt pathway was inhibited by its inhibitor, LY294002 (20 μmol/L, Sigma-Aldrich, St Louis, MO, USA).

VSMC proliferation

VSMC proliferation was measured by two methods, cell counting and flow cytometry. For cell counting, the cells were detached by trypsinization and re-suspended in PBS. The cells were then counted under a microscope. For flow cytometry, Click-iT EdU Flow Cytometry Assay Kits (invitrogen, Carlsbad, CA, USA) were used. In brief, VSMCs were cultured with DMEM and 10 μmol/L EdU was added. After 6 h, cells were suspended at 1×10^7 cells/mL in 1% BSA in PBS. Cell suspension 100 μL and 100 μL Click-iT fixative were added to flow tubes, incubated for 15 min at room temperature, and protected from light. Cells were washed once with 3 mL 1% BSA in PBS. 1×saponin-based permeabilization 100 μL and wash buffer was added, incubated for the 20 min at 4 °C temperature, and protected from light. Each tube was washed. Click-iT reaction cocktail 0.5 mL was added, mixed well, incubated for 30 min at room temperature, and protected from light. Cells were washed once. 1×saponin-based permeabilization 0.5 mL and wash reagent were added. For the detection of EdU, 633/635 nm excitation with red emission filter (i.e., 660/20 nm or similar) was used.

Statistics

All data are presented as mean±standard error. For relative gene expression, the mean value of the vehicle control group is defined as 100% or 1. Two-tailed unpaired Student *t* tests and ANOVAs were used for statistical evaluation of the data. SPSS 17.0 was used for data analysis. A $P<0.05$ was considered significant.

Results

The effects of cell density on miRNA expression in VSMCs

To test the potential relationship between contact inhibition and miRNA expression, rat VSMCs were seeded at 30% or 90% confluence. After synchronization with serum-free medium for 24 h, the RNAs in the cells were isolated for miRNA microarray. Intriguingly, compared with those in low-density (30%) cells, 88 miRNAs were differentially expressed with $P<0.05$; 44 miRNAs were upregulated, and 44 miRNAs were downregulated. Thirty-five miRNAs that were highly expressed in VSMCs and over 30% difference in their expression signals between low density and high density cells were shown in supplementary Figure 1. Among the 35 differentially expressed miRNAs, we selected 8 miRNAs for verification by qRT-PCR. As shown in Figure 1A, miR-145, miR-22, miR-29a, miR-199a were indeed upregulated, whereas the levels of let-7b, miR-10a, miR-290 and miR-374 were downregulated in high density VSMCs.

The effect of cell density on miR-145 expression in VSMCs

Among the differentially expressed miRNAs between low and high density VSMCs, miR-145 has been reported by us and others to have strong biological functions in VSMCs. Its expression change in high-density VSMCs was thus further verified by Northern blot analysis as shown by Figure 1B and 1C. To further confirm the relationship between VSMC density and the expression of miR-145, the cells were cultured to reach 20%, 40%, 70% or 100% confluence and synchronized for 24 h with serum free medium. Then, the expression of miR-145 in VSMCs was determined by qRT-PCR. As shown in Figure 1D, the expression of miR-145 in VSMCs was increased in a cell density dependent manner. The maximal level of miR-145 was found in VSMCs when they reached a 100% conflu-

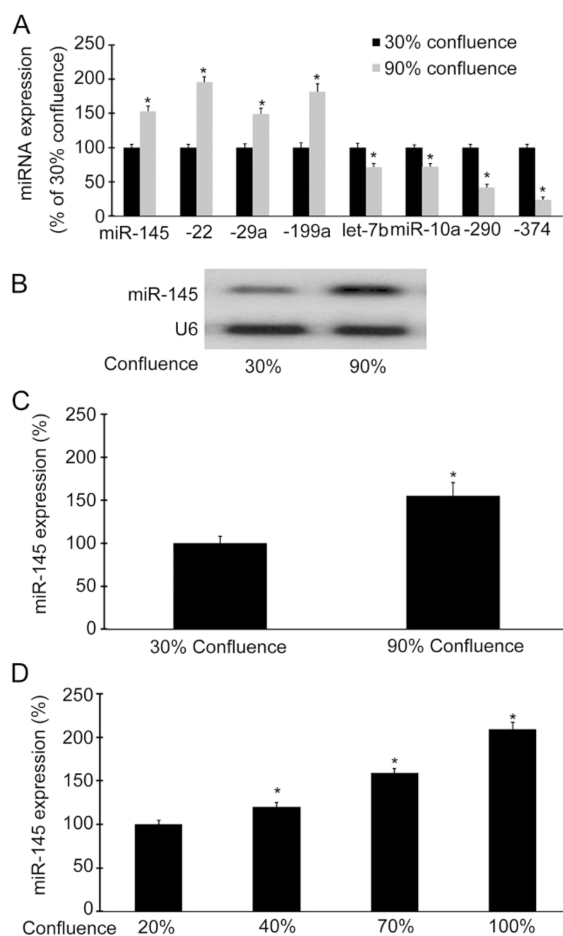

Figure 1. The effects of cell density on miRNA expression in VSMCs. Rat VSMCs were seeded at 30% or 90% confluence. (A) Verification of expression differences of 8 selected miRNAs from miRNA microarray by qRT-PCR between low density and high-density cells, which included miR-145, miR-22, miR-29a, miR-199a, let-7b, miR-10a, miR-290 and miR-374. Note: $n=8$. $^*P<0.05$ compared with that in low-density cells. (B) Representative Northern blots of miR-145 in low density (30% confluence) (low) and high density (90% confluence) (high) VSMCs. (C) Expression levels of miR-145 in low density (30% confluence) (low) and high density (90% confluence) (high) VSMCs determined by northern blot. Note: $n=6$. $^*P<0.01$ compared with low density cells. (D) Expression of miR-145 in VSMCs at 20%, 40%, 70% or 100% confluence determined by qRT-PCR. Note: $n=8$. $^*P<0.05$ compared with that in low density cells (20% confluence).

ence.

Confluent VSMCs have a strong contact inhibition, which could be disrupted by high concentrations of serum

It is well established that VSMCs have a strong contact inhibition. Indeed, we also found that confluent VSMCs stopped their proliferation when cultured with serum free (0% FBS) DMEM or low concentrations of serum (≤ 0.2% FBS). One recent report demonstrated that the contact inhibition of VSMCs could be disrupted by high concentrations of serum [15]. To verify this phenomenon, confluent VSMCs (100% confluence) were synchronized for 24 h in serum-free DMEM. Then, the cells were treated with 0.0%, 0.2%, 0.5%, 2.0% or 5.0% of FBS. Cells were counted before (0 h) and 24 h after treatment with different concentrations of FBS. As expected, contact inhibition was found in 0.0% FBS and 0.2% FBS cultured confluent VSMCs, as shown by the consistent cell number of VSMCs at 0 h and 24 h after culture with 0.0% FBS and 0.2% FBS (Figure 2). However, the contact inhibition of VSMCs was disrupted by high concentrations of FBS (0.5%–5.0%) in a concentration-dependent manner (Figure 2). We thus defined 0.2% FBS-cultured confluent VSMCs as VSMCs with intact contact inhibition. In contrast, 5.0% FBS-cultured confluent VSMCs were defined as VSMCs without contact inhibition or the contact inhibition-disrupted VSMCs.

Contact inhibition disruption of VSMCs accompanies with a significant downregulation of miR-145

To test the potential involvement of miR-145 in contact inhibition and its disruption of VSMCs, we first determined the expression levels of miR-145 in VSMCs with intact contact inhibition (0.2% FBS-cultured confluent VSMCs), and in contact inhibition disrupted VSMCs (5.0% FBS-cultured VSMCs), and the expression changes of miR-145 from contact inhibition intact VSMCs to contact inhibition disrupted VSMCs. As shown in Figure 3A, the expression of miR-145 in the contact inhibition disrupted VSMCs was significantly lower than that in contact inhibition intact VSMCs. Moreover, there was a remarkable drop in miR-145 expression in VSMCs from intact contact inhibition status to contact inhibition disrupted status (Figure 3B).

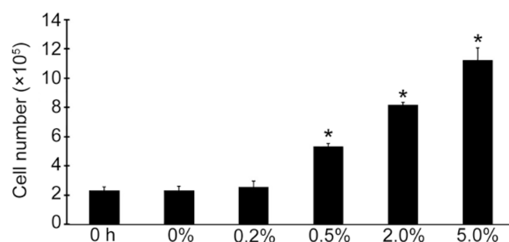

Figure 2. Contact inhibition of confluent VSMCs could be disrupted by serum. Confluent VSMCs (100% confluence) were treated with 0.0%, 0.2%, 0.5%, 2.0% or 5.0% of FBS, and cells were counted before (0 h) or 24 h after treatment with different concentrations of FBS. Contact inhibition was found in serum-free (0.0% FBS) and low concentration of serum (0.2% FBS) cultured confluent VSMCs, as shown by the consistent cell number in 0 h and 24 h after culture with 0.0% FBS and 0.2% FBS. However, the contact inhibition of VSMCs was disrupted by high concentrations of FBS (0.5%–5.0%) in a concentration-dependent manner. Note: n=8. $^{*}P$<0.05 compared with the cell number before treatment (0 h).

Contact inhibition disruption of VSMCs induced by serum is blocked by overexpression of miR-145

To test the role of miR-145 in contact inhibition and its disruption of VSMCs, miR-145 in confluent VSMCs was overexpressed by pre-miR-145 as shown in Figure 3C. Interestingly, serum (5.0% FBS)-induced contact inhibition disruption of VSMCs was blocked by miR-145 overexpresion as shown by the decreased proliferation of VSMCs determined by cell counting (Figure 3D) and flow cytometry (Figure 3F, n=6, P<0.05 compared with that in oligo control group). Representative flow cytometry images are shown in Figure 3E.

Downregulation of miR-145 in confluent VSMCs is sufficient to disrupt the contact inhibition of VSMCs

To test if downregulation of miR-145 in confluent VSMCs could disrupt the contact inhibition, miR-145 expression was downregulated by its inhibitor, 2'OMe-miR-145 (30 nmol/L) in 0.2% FBS-cultured, contact inhibited confluent cells (Figure 4A). Interesting, downregulation of miR-145 in confluent VSMCs is sufficient to disrupt the contact inhibition of VSMCs as shown by the increased cell proliferation determined by cell counting (Figure 4B) and flow cytometry (Figure 4D). Representative flow cytometry images are shown in Figure 4C.

PI3-kinase/Akt pathway is involved in serum-induced downregulation of miR-145 and the resulted contact inhibition disruption in confluent VSMCs

Our recent study has revealed that PI3-Kinase/Akt is a critical signaling pathway related to the downregulation of miR-145 in PDGF-stimulated proliferative VSMCs and in arteries with proliferative arterial diseases such as balloon-injured arteries and in arteries with atherosclerosis[18]. To test whether PI3-kinase/Akt pathway is related to serum-induced downregulation of miR-145 and the resulted contact inhibition disruption, we first determined the activity of Akt (p-Akt) in confluent VSMCs treated with 0.2% FBS or 5.0% FBS. The result demonstrated that in 5.0% FBS-treated, contact inhibition disrupted VSMCs, the expression of p-Akt was increased (Figure 5B). Representative Western blots are shown in Figure 5A. In addition, inhibition of PI3-kinase/Akt pathway by LY294002 (20 μmol/L) could effectively block the miR-145 downregulation (Figure 5C).

KLF5, a target gene of miR-145, is involved in miR-145 downregulation and serum-induced contact inhibition disruption in confluent VSMCs

Our previous study has identified that KLF5 is a target gene of miR-145 that is involved in miR-145-mediated effect on VSMC differentiation[7]. However, whether KLF5 is involved in miR-145 downregulation- and serum-induced contact inhibition disruption in confluent VSMCs is currently unknown. To test

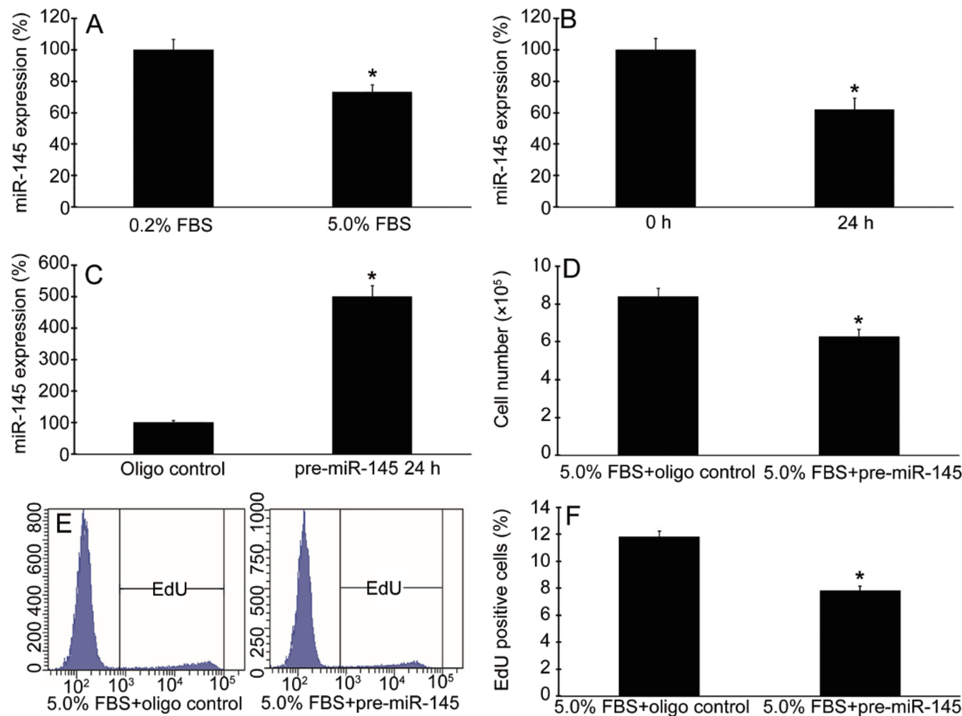

Figure 3. Contact inhibition disruption of VSMCs by serum accompanies with a significant downregulation of miR-145 and the disruption of VSMCs is blocked by overexpression of miR-145. (A) Confluent VSMCs were synchronized for 24 h at serum-free medium. Then, the cells were cultured with 0.2% FBS (which were cells with intact contact inhibition) or 5.0% FBS (which were cells with disrupted contact inhibition) for 24 h. The expression of miR-145 was determined by qRT-PCR. Note: n=6. $^{*}P$<0.05 compared with the expression in 0.2% FBS group. (B) Confluent VSMCs were treated with 0.2% FBS for 24 h to reach the contact inhibition. Some of these cells were isolated to determine the level of miR-145 (before the disruption of contact inhibition). The remaining cells were then treated with 5.0% FBS for another 24 h to disrupt the contact inhibition. After that, the expression of miR-145 in these cells with the disrupted contact inhibition was determined by qRT-PCR. Note: n=6, $^{*}P$<0.05 compared with the expression in cells before the disruption of the contact inhibition. (C) miR-145 expression in confluent VSMCs was upregulated by pre-miR-145 (30 nmol/L, 24 h). Note: n=6. $^{*}P$<0.05 compared with that in oligo control group. Serum (5.0% FBS)-induced contact inhibition disruption of VSMCs was blocked by miR-145 overexpression via pre-miR-145 (30 nmol/L) as shown by decreased proliferation of VSMCs induced by 5.0% FBS for 24 h determined by cell counting (D) and flow cytometry (F). Note: n=6. $^{*}P$<0.05 compared with that in oligo control group. (E) Representative flow cytometry images.

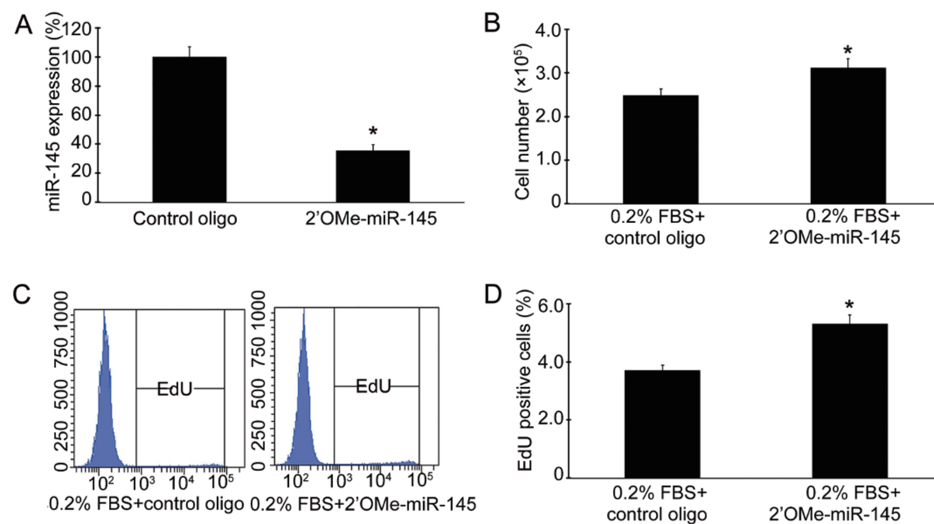

Figure 4. Downregulation of miR-145 in confluent VSMCs is sufficient to disrupt the contact inhibition of VSMCs. (A) miR-145 expression in confluent VSMCs cultured with 0.2% FBS was downregulated by 2'OMe-miR-145 (30 nmol/L, 24 h). Note: n=6. $*P$<0.05 compared with that in oligo control group. Downregulation of miR-145 itself in confluent VSMCs cultured with 0.2% FBS is sufficient to disrupt the contact inhibition as shown by the increased cell proliferation determined by cell counting (B) and flow cytometry (D). Note: n=6. $^{*}P$<0.05 compared with that in oligo control group. (C) Representative flow cytometry images.

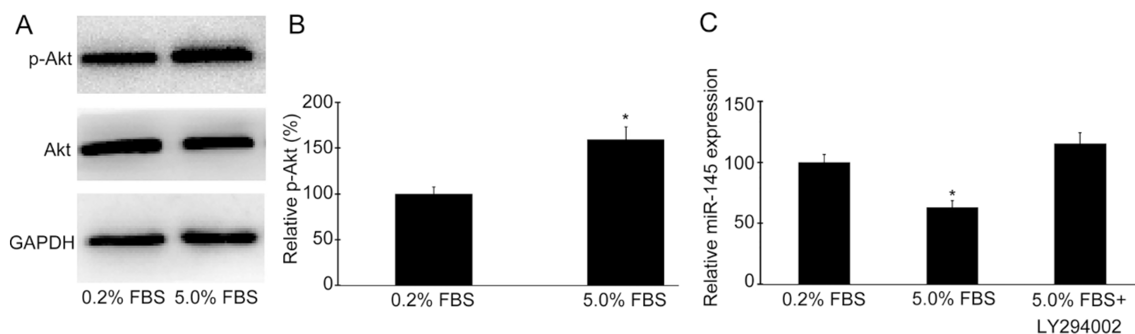

Figure 5. PI3-kinase/Akt pathway is involved in serum-induced downregulation of miR-145 and the resulted contact inhibition disruption in confluent VSMCs. (A) Representative Western blots of Akt and p-Akt. in confluent VSMCs treated with 0.2% FBS (contact inhibited cells) or 5.0% FBS (contact-inhibition disrupted cells). (B) The expression levels of p-Akt in confluent VSMCs treated with 0.2% FBS (contact-inhibited cells) or 5.0% FBS (contact inhibition-disrupted cells). Note: *n*=6. [*]*P*<0.05 compared with that in 0.2% FBS group. (C) inhibition of PI3-kinase/Akt pathway by LY294002 (20 μmol/L) could effectively block the miR-145 downregulation induced by 5.0% serum. Note: *n*=6. [*]*P*<0.05 compared with that in vehicle control.

it, we first determined the expression of KLF5 in confluent VSMCs treated with 0.2% FBS or 5.0% FBS. The result demonstrated that in 5.0% FBS-treated, contact inhibition disrupted VSMCs the expression of KLF5 was significant increased (Figure 6B). Representative Western blots of KLF5 were shown in Figure 6A. To further determine the potential involvement of KLF5 in miR-145 downregulation-induced contact inhibition disruption in confluent VSMCs, the upregulated KLF5 was knocked down by its siRNA (KLF5-siRNA, 50 nmol/L) as shown in Figure 6C and 6D. Interestingly, serum-induced contact inhibition disruption in confluent VSMCs was inhibited by knockdown of miR-145 target gene, KLF5 as shown by the decreased VSMC proliferation compared with that in siRNA control-treated group (Figure 6E).

Discussion

It has long been appreciated that contact inhibition is a process of arresting cell growth when cells encounter each other[10]. As a result, normal cells stop proliferating when they form a monolayer in a culture dish *in vitro* or stop growing when they have reached a certain density in an organ *in vivo*. It is well known that the contact inhibition is a critical endogenous cellular mechanism in keeping normal tissue structure and normal tissue size, as well as in preventing over growth of cells such as cancer. However, the contact inhibition could be disrupted under some pathological conditions such as cancer[19, 20].

In a cardiovascular system, contact inhibition of normal cardiovascular cells is critical to keep normal size of heart and vessels. The disrupted contact inhibition could result in the development of some cardiovascular diseases. For example, loss of the contact inhibition of VSMCs in arterial walls could lead to the over proliferation of VSMCs and their products of extracellular matrix. Finally, it may induce vascular neointimal formation and arterial stenosis, which are basic pathological changes in atherosclerosis, coronary artery disease, stroke, diabetic vascular complications, and peripheral artery disease[21,22]. However, disruption of contact inhibition in cardiovascular cells is not always a bad cellular event. For example, contact inhibition disruption of VSMCs and vascular endothelial cells and its resulted proliferation of these cells are critical for repair of injury and wound, myocardial infarction and stroke, and are important for collateral circulation formation in ischemic cardiovascular disease. Although contact inhibition and its disruption are very important for normal physiology and disease development such as cancer and vascular disease, how proliferation is inhibited or is not inhibited at high cellular densities remains poorly characterized. Recent studies have revealed that hippo pathway, 4E-BP1, RNA polymerase I transcription, reactive oxygen species (ROS), p27 (Kip1), and tyrosine phosphorylation of adherens junction components might be involved in contact inhibition in different cell types under different conditions[23-28]. To date, few studies have been performed to determine the molecular mechanisms involved in the contact inhibition and its disruption of VSMCs. One recent report has demonstrated that high concentrations of serum could overcome the contact inhibition in confluent human pulmonary artery smooth muscle cells[15]. They further identified that serum-induced VSMC contact inhibition disruption was related to PI3-kinase activation.

miRNAs have strong biological functions in VSMC biology, vascular physiology and vascular diseases as described in our review article[29]. However, the potential roles of miRNAs in contact inhibition and its disruption of VSMCs and their molecular mechanisms involved are currently completely unknown. To test whether miRNAs are involved in VSMC contact inhibition, we first determined the expression profiles of miRNAs in low density, non-contact inhibited VSMCs and in high density, contact inhibited VSMCs. We identified that multiple miRNAs were differentially expressed in VSMCs between two different conditions. The results suggest that miRNAs might be important modulators for the contact inhibition of VSMCs.

miR-145 is a VSMC specific/enriched miRNA. The studies from us and other groups have demonstrated that miR-145 plays important roles in phenotypic modulation, proliferation and migration of VSMCs and the development of proliferative

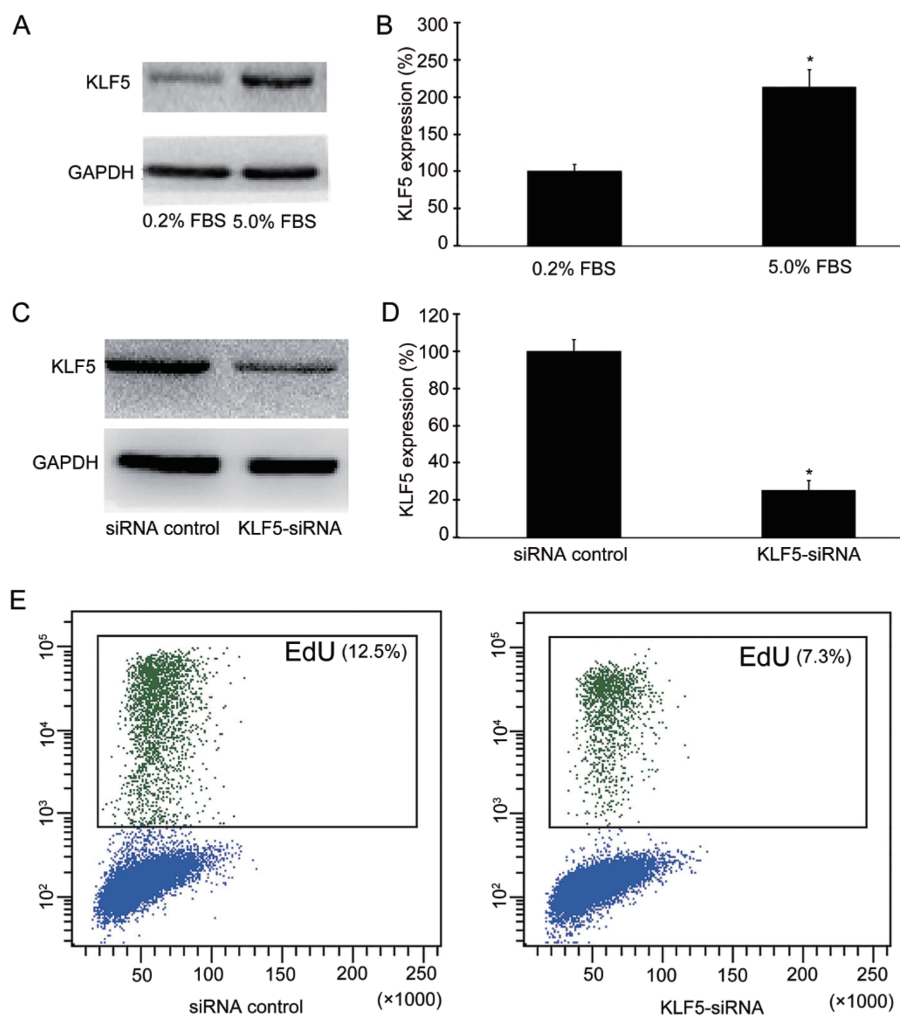

Figure 6. KLF5, a target gene of miR-145, is involved in miR-145 downregulation and serum-induced contact inhibition disruption in confluent VSMCs. (A) Representative Western blots of KLF5 in confluent VSMCs treated with 0.2% FBS or 5.0% FBS. (B) The expression levels of KLF5 in confluent VSMCs treated with 0.2% FBS or 5.0% FBS. Note: n=6. $^*P$<0.05 compared with that in 0.2% FBS group. (C) Representative western blots of KLF5 confluent VSMCs with 5.0% FBS and treated with KLF5 siRNA (KLF5-siRNA, 50 nmol/L) or its control siRNA. (D) Downregulation of KLF5 by its siRNA in confluent VSMCs treated with 5.0% FBS. Note: n=6. $^*P$<0.05 compared with that in siRNA control group. (E) Serum-induced contact inhibition disruption in confluent VSMCs was inhibited by knockdown of miR-145 target gene KLF5, as shown by the decreased VSMC proliferation compared with that in siRNA control-treated group.

vascular diseases such as atherosclerosis and restenosis after angioplasty[7, 12, 13]. Interestingly, miR-145 was among the most upregulated miRNAs in contact-inhibited confluent VSMCs as shown by miRNA microarray analysis. To date, the biological roles of miR-145 in contact inhibition and its disruption of VSMCs have not been reported. To test them, we determined the expression of miR-145 in VSMCs at 20%, 40%, 70% or 100% confluence. Clearly, the expression of miR-145 in VSMCs was increased in a cell density dependent manner. The maximal level of miR-145 was found in VSMCs when they reached a 100% confluence. The result suggested that miR-145 might be associated with the contact inhibition of VSMCs.

To provide a direct evidence that miR-145 could be critical for the contact inhibition and its disruption of VSMCs, we applied two cell models: contact-inhibited VSMCs (0.2% FBS-cultured confluent VSMCs), and contact inhibition-disrupted VSMCs (5.0% FBS-cultured VSMCs). We found that the expression of miR-145 in the contact inhibition-disrupted VSMCs was significantly lower than that in contact inhibition intact VSMCs. Moreover, there was a remarkable drop in miR-145 expression in VSMCs from intact contact inhibition status to contact inhibition disrupted status. Finally, downregulation of miR-145 itself in confluent VSMCs is sufficient to disrupt the contact inhibition of VSMCs. Our study has clearly demonstrated that miR-145 indeed plays important roles in contact inhibition and its disruption of VSMCs. It should be noted that although we were focused on miR-145, the roles of other miRNAs that were differentially expressed in contact inhibited confluent VSMCs and non-contact inhibited low density VSMCs in our miRNA microarray analysis in VSMC contact

inhibition should be determined in future studies.

Our recent study has revealed that PI3-Kinase/Akt is a critical signaling pathway related to the downregulation of miR-145 in PDGF-stimulated proliferative VSMCs and in arteries with proliferative arterial diseases such as balloon-injured arteries and in arteries with atherosclerosis[18]. Our current study revealed that PI3-kinase/Akt pathway was an upstream signaling in serum-induced downregulation of miR-145 and the resulted contact inhibition disruption. It is well established that a miRNA achieves its biological functions via its target genes. Our recent study has reported that KLF5 is a direct target gene of miR-145 that is related to its biological effect on VSMC differentiation. To date, the effects of KLF5 on the contact inhibition and its disruption of VSMCs have not been studied. In current study, we have identified that KLF5 is involved in miR-145 downregulation- and serum-induced contact inhibition disruption in confluent VSMCs. The roles of other target gens of miR-145 in the contact inhibition of VSMCs should be determined in future studies.

In summary, we identified for the first time that multiple miRNAs are differentially expressed in contact inhibited confluent VSMCs and in non-contact inhibited low density VSMCs. Among them, the VSMC specific/enriched miR-145 is a critical gene in contact inhibition of VSMCs. Downregulation of miR-145 in confluent VSMCs is sufficient to disrupt the contact inhibition of VSMCs. PI3-Kinase/Akt/miR-145/KLF5 is a critical signaling pathway in serum-induced contact inhibition disruption of VSMCs. Targeting of miRNAs that are related to the contact inhibition VSMCs may represent a novel therapeutic approach for cardiovascular diseases.

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Author contribution

Ye-ying SUN and Shan-shan QIN were responsible for the most of experiments; Yun-hui CHENG, Chao-yun WANG, Xiao-jun LIU, Ying LIU, Jia-xin ZHAN, Shuai SHAO, Bi-hui LUO, Dong-feng LU, and Jian YANG were responsible for the animal experiments, data collection and analysis; Xiu-li ZHANG, Wendy ZHANG, Wei-hua BIAN, and Chun-hua WANG were responsible for data analysis; Wendy ZHANG was responsible for English edit; and Chun-xiang ZHANG was responsible for experiment design and the manuscript writing.

Supplementary information

Supplementary information is available at the website of Acta Pharmacologica Sinica.

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