Nonadherent culture method promotes MSC-mediated vascularization in myocardial infarction via miR-519d/VEGFA pathway

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Abstract

Background: Mesenchymal stem cells (MSCs) can provide therapeutic benefits for myocardial infarction (MI) recovery; however, the molecular mechanism by which MSCs improve the heart function is unclear.

Methods: Microarray analysis was performed to examine the expression profiling of human MSCs (hMSCs) grown as adherent cultures (AC-hMSCs) or nonadherent cultures on ultra-low-adherent plates (nonAC-hMSCs). Real-time quantitative polymerase chain reaction (RT-qPCR), western blotting, and enzyme-linked immunosorbent assays (ELISA) were used to assess VEGFA expression and secretion in the AC-hMSCs and nonAC-hMSCs. The paracrine effect of VEGFA-overexpressing AC-MSCs (AC-VEGFA-hMSCs) or VEGFA-knockdown nonAC-hMSCs (nonAC-shVEGFA-hMSCs) on the angiogenic ability of human umbilical vein endothelial cells (HUVECs) was evaluated using tube formation assay. AC-VEGFA-hMSCs or nonAC-shVEGFA-hMSCs were transplanted into myocardial infarction rats to investigate the therapeutic effect of AC-VEGFA-hMSCs or nonAC-shVEGFA-hMSCs. Luciferase reporter assay was used to confirm the association of VEGFA with miR-519d.

Results: Microarray analysis revealed that VEGFA is downregulated in AC-hMSCs compared to nonAC-hMSCs. Functional assays revealed that high levels of VEGFA produced from AC-VEGFA-hMSCs increased the tube formation capacity of HUVECs in vitro, improved angiogenesis and cardiac performance, and reduced infarct size in a rat MI model. Low levels of VEGFA secretion from nonAC-shVEGFA-hMSCs had the opposite effects. Mechanistically, we found that miR-519d directly targets VEGFA. High levels of VEGFA secreted from VEGFA-overexpressing nonAC-hMSCs abolished the repressive effect of miR-519d on HUVEC angiogenesis.

Conclusion: Our findings indicate that nonadherent culture-induced secretion of VEGFA plays an important role in MSCs via the miR-519d/VEGFA pathway and may provide a novel therapeutic strategy for MI treatment.

Keywords: Mesenchymal stem cells, Myocardial infarction, VEGFA, Angiogenesis, miR-519d, Nonadherent culture
**Introduction**

Myocardial infarction (MI) is a major cause of mortality and disability in the world [1]. Pharmacologic intervention and conventional revascularization techniques, such as coronary artery bypass grafting, balloon angioplasty, and stenting, can restore blood flow and maintain myocardial viability and function; however, these treatment methods cannot salvage the dying myocardium or repair cardiac function [2]. In recent years, stem cell therapy has become a promising approach to treat MI, as it has the potential to restore damaged myocardium [3, 4]. Mesenchymal stem cells (MSCs) exist in the bone marrow, umbilical cord blood, adipose, and many other tissues [5] and have multilineage differentiation potential, self-renewal capacity, and immunomodulatory properties [6], while posing a low immunorejection risk [7]. Thus, MSCs have been commonly used in experimental research and clinical trials for treating MI. Often for this application, a large amount of MSCs must be isolated from the same tissues and expanded in plastic adherent culturing containers [7, 8]. It was recently reported that in vitro expansion in conventional monolayer cultures can alter the phenotype of MSCs, which may cause cell trapping within the lung and a low rate of delivery to target organs [9–12]. Qian et al. [13] showed that primary MSCs lacked CD44, whereas culture-expanded MSCs acquired CD44 expression. Compared to primary MSCs, cultured MSCs with high levels of CD44 displayed decreased targeting to the bone marrow [13]. Our previous research revealed that stem cell antigen 1 (Sca-1) is expressed at higher levels in adherent cultured mouse MSCs (AC-mMSCs) compared to mMSCs in nonadherent cultures maintained in ultra-low-adherent plates (nonAC-mMSCs) [14]. Sca-1+ mMSCs play a crucial role in improving cardiac function in MI [14, 15]. Compared with nonadherent cultured human MSCs (nonAC-hMSCs), adherent cultured hMSCs (AC-hMSCs) with high protein expression of caspase-3, caspase-7, and caspase-9 had a marked decrease in cell apoptosis [16]. Therefore, it is important to investigate the changes in MSC phenotype that occur during in vitro cultivation.

MSC therapy with angiogenic factors holds a great promise for ischemic disease treatment due to neovascularization [17, 18]. Vascular endothelial growth factor A (VEGFA) is a major driver of angiogenesis and vasculogenesis [19]. VEGFA could regulate angiogenesis and myogenensis in cardiac repair [20, 21]. A growing body of research demonstrated that VEGFA promotes MSC viability in the infarcted hearts via decreasing cellular stress and enhancing cell survival factors [22, 23]. Therefore, it is anticipated that VEGFA-gene-modified MSCs may provide a potentially valuable approach for MI treatment due to increase survival and angiogenic capacity.

In this study, we identified that VEGFA expression is significantly higher in nonAC-hMSCs than in AC-hMSCs, as detected by microarray analysis. The increased levels of VEGFA secreted from VEGFA-overexpressing AC-hMSCs (AC-VEGFA-hMSCs) facilitated tube formation of human umbilical vein endothelial cells (HUVECs), while the decreased levels of VEGFA secreted by nonAC-hMSCs with VEGFA knockdown (nonAC-shVEGFA-hMSCs) led to the opposite effect. In a rat MI model, the increased levels of VEGFA released from AC-VEGFA-hMSCs promoted angiogenesis, decreased infarct size, and improved myocardial function, while the decreased levels of VEGFA secreted by nonAC-shVEGFA-hMSCs reduced angiogenesis, increased infarct size, and impaired cardiac remodeling. Further studies on the mechanism underlying these changes indicated that VEGFA is a direct target of miR-519d. High levels of VEGFA produced by VEGFA-overexpressing nonAC-hMSCs reversed the inhibitory effect of miR-519d on the tube formation capability of HUVECs.

**Materials and methods**

**Isolation and expansion of hMSCs and in vitro nonadherent culture conditions**

hMSCs were isolated from the bone marrow of adult donors (ages 18–25) who underwent orthopedic surgery as described previously [16]. hMSCs were seeded in adherent culture plates (AC-hMSCs) or ultra-low-adherent tissue culture plates (nonAC-hMSCs; Corning®, Corning, NY) and grown in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY) for 24 h or 72 h. All cells were incubated in a humidified incubator with 5% CO₂ at 37 °C. This study was approved by the Ethics Committee of Affiliated Hospital of Guilin Medical University (Guilin, China), and informed consent was obtained from all participants.

**Flow cytometry**

Cells were harvested, washed, and resuspended. The antibodies included anti-CD11b (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD14 (BD Biosciences), anti-CD34 (BD Biosciences), anti-CD45 (BD Biosciences), anti-CD73 (BD Biosciences), anti-CD90 (BD Biosciences), and anti-CD105 (BD Biosciences) were added to the cells at 4 °C for 1 h. Then, cells were analyzed on a BD FACSCalibur (BD Biosciences) and data was analyzed by BD FACSComp software (BD Biosciences). An isotype control immunoglobulin (BD Biosciences) was used as control.

**mRNA microarray analysis**

AC-hMSCs and nonAC-hMSCs grown for 24 h and 72 h were sent to the Shanghai Bohao Biotechnological Co.,
further experiments. The secreted levels of VEGFA in
subjected to two freeze-thaw cycles, and centrifuged for
Rat heart tissues were homogenized in 20 mL of PBS,
MSC groups and centrifuged for further experiments.
Enzyme-linked immunosorbent assays (ELISA)

Measurement of LV infarct size
The heart tissues were fixed in 10% formalin, embedded
in paraffin, and cut into 5 μm slices. Sections were then
the CM and rat heart tissues were examined using a
VEGFA ELISA Kit (R&D System, Los Angeles, CA) ac-
cording to the manufacturer’s protocol, and the absorb-
ance at 450 nm was measured using a microplate reader.
Each sample was assayed three times.

Tube formation assay
HUVECs were obtained from the Jennio Biotech
(Guangzhou, China). HUVECs (2 × 10^4 cells) were sus-
pended in CM obtained from different hMSC groups,
added to the 96-well plates coated with Matrigel (BD
Biosciences, Franklin Lakes, NJ) at a density of 2 × 10^4
cells/well, and incubated at 37 °C for 12 h. Images of
tubular structures were captured and analyzed using
Image-Pro Plus 6.0 software.

Real-time quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted from cultured cells using Tri-
zol reagent (Invitrogen). A microRNA Reverse Trans-
scription Kit (Promega, Madison, WI, USA) or a
PrimeScript RT-PCR kit (Takara, Dalian, China) was
used to synthesize cDNA. RT-qPCR was carried out
using SYBR Premix Ex Taq II (TaKaRa). Relative quanti-
fication of gene expression was determined using the 2^-△△CT
method [24]. The following primers were used:
VEGFA primers: forward 5′-GCAGAATCATCAGC
AAGTGGTG-3′, reverse 5′-CTCTTGATGATGGC
CAGTAGCT-3′; β-actin primers: forward 5′-CTTGC
CCTGGCCTCCTGT-3′, reverse 5′-ACTAGTCA
AGTCCGCTCTAGA-3′; miR-519d RT primers: 5′-
GTGCAGAATCATCACGTGC
GTC
AGGCAGGTATTTCGCAC
TGGATACGACCACCTCT-3′, PCR primers: forward 5′-
GCCAAAGTGGCCTCCCTGT-3′, reverse 5′-GTCG
AGGCGAGGT-3′; U6 primers: forward 5′-GTCGC
TCCCGAGGATTTGTCA
CGCT-3′, reverse 5′-AACGCTTCACGAATT
TTGCCGT-3′.

Enzyme-linked immunosorbent assays (ELISA)
Conditioned medium (CM) was obtained from different
MSC groups and centrifuged for further experiments. Rat heart tissues were homogenized in 20 mL of PBS,
subjected to two freeze-thaw cycles, and centrifuged for
further experiments. The secreted levels of VEGFA in

Plasmid construction, oligonucleotide, and transfection
The full-length VEGFA cDNA was PCR-amplified and
inserted into a pcDNA3.1 plasmid. miR-519d mimics,
miR-519d inhibitors, and the appropriate controls were
purchased from Ribobio (Guangzhou, China). Transient
transfection was performed using Lipofectamine 2000
ware (Agilent Technologies, Santa Clara, CA). The log 2-
fold change was determined by calculating gene expres-
sion at 450 nm was measured using a microplate reader.
Each sample was assayed three times.

Tube formation assay
HUVECs were obtained from the Jennio Biotech
(Guangzhou, China). HUVECs (2 × 10^4 cells) were sus-
pended in CM obtained from different hMSC groups,
added to the 96-well plates coated with Matrigel (BD
Biosciences, Franklin Lakes, NJ) at a density of 2 × 10^4
cells/well, and incubated at 37 °C for 12 h. Images of
tubular structures were captured and analyzed using
Image-Pro Plus 6.0 software.

Rat MI model and hMSC transplantation
Male Sprague-Dawley rats weighing 250–350 g were
subjected to MI using left anterior descending coronary artery (LADCA) ligation. After anesthetized with 10% chloral hydrate (250 mg/kg weight), the rats were intu-
bated with polyethylene-16 tube and connected to the
rodent ventilator (Harvard Apparatus, Holliston, MA).
The chest was opened between the third and fourth ribs,
and the pericardium was split to expose the left ventricle (LV), aorta, and left atrium. The LADCA was ligated
using a 7-0 polypropylene suture 2 mm below the edge
of the left atrium. Forty-eight rats were randomly di-
vided into six treatment groups: rats that received AC-
VEGFA-hMSCs (n = 8), nonAC-shVEGFA-hMSCs (n = 8),
AC-hMSCs (n = 8), nonAC-hMSCs (n = 8), saline (MI
group, n = 8), or saline (a sham operation, n = 8) in the
infarct border region immediately after LAD ligation.
The chest and skin were then closed. All experiments
were approved by the Ethics Committee of the Affiliated
Hospital of Guilin Medical University.

Cardiac function assessment
Four weeks after LADCA ligation, an echocardiography
was performed using a Vevo 2100 system (Visualsonics,
Toronto, ON, Canada) with a 21-MHz transducer. Rats
were anesthetized with 10% chloral hydrate (250 mg/kg
weight) for the exam. LV end-diastolic and end-systolic di-
ensions (LVEDD, LVESD) from the parasternal short-
axis view at the papillary muscle were measured using M-
mode tracing [25]. LV ejection fraction (LVEF) was calcu-
lated using the following formula: LVEF (%) = (LVEDD^3 -
LVESD^3)/LVEDD^3 × 100. LV fractional shortening (LVFS)
was calculated as LVFS (%) = (LVEDD - LVESD)/
LVEDD × 100. Measurements were done in triplicate.

Measurement of LV infarct size
The heart tissues were fixed in 10% formalin, embedded
in paraffin, and cut into 5 μm slices. Sections were then
stained with Masson’s trichrome according to the manufacturer’s instructions and examined using an optical microscope (Olympus, Tokyo, Japan), and images were acquired using a Retiga CCD camera. Image-Pro Plus 6.0 software was used to measure the infarct area and total LV area of each image. Infarct size was expressed as a percentage of the total LV area.

**Immunohistochemical staining**
Sections were dewaxed with xylene and rehydrated with graded ethanol. Subsequently, sections were pretreated with 0.01 M citrate buffer (pH 6.0) in a microwave oven at 95 °C for 20 min and incubated with 3% H2O2 for 15 min at room temperature. The sections were blocked with 10% sheep serum for 30 min and then incubated with anti-Von Willebrand Factor (vWF, Abcam, Cambridge, MA) primary antibody overnight at 4 °C. After washes with PBS, sections were incubated with secondary antibody for 30 min at room temperature and stained with diamidobenzidine. After counterstaining with hematoxylin, sections were analyzed using an optical microscope (Olympus) and photographed using a digital camera.

**Luciferase reporter assay**
The VEGFA 3′ UTR, which contains a putative miR-519d binding site, was PCR-amplified and inserted into the psiCHECK-2 luciferase reporter plasmid (VEGFA-3′ UTR-WT; Promega). Site-directed mutagenesis (Stratagene, La Jolla, CA) was used to construct the mutant VEGFA 3′ UTR (Mut), which was then cloned into the psiCHECK-2 luciferase reporter plasmid (VEGFA-3′ UTR-Mut). VEGFA-3′ UTR-WT or VEGFA-3′ UTR-Mut vectors were transfected into HEK293T cells. Luciferase activity was analyzed 48 h after transfection using a dual-luciferase reporter assay kit (Promega). Data are represented as the Renilla/firefly luciferase ratio.

**Western blotting**
Tissues and cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitor. Protein extracts were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% non-fat milk and incubated with anti-VEGFA primary antibody (Abcam) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody at room temperature. Protein signals were visualized using an ECL detection kit (Millipore).

**Statistical analysis**
The data analysis was performed using SPSS 20.0 software. The differences between two groups were evaluated using student’s t tests. Values are expressed as the mean ± standard deviation. P values < 0.05 were considered statistically significant.

**Results**
**Increased VEGFA expression and production are observed in nonAC-hMSCs**
We first used flow cytometry to identify MSCs and found that hMSCs were positive for CD73, CD90, and CD105, whereas negative for CD11b, CD14, CD34, and CD45 (Additional file 1: Figure S1). Next, microarray analysis was performed in order to profile mRNAs in AC-hMSCs and nonAC-hMSCs at two different time points (Fig. 1a; Additional file 2: Table S1; Additional file 3: Table S2). Two thousand four hundred ninety-six mRNAs were downregulated (1663 genes at 24 h and 1820 genes at 72 h), and 2400 mRNAs were upregulated (1489 genes at 24 h and 1766 genes at 72 h) in nonAC-hMSCs when compared to those in AC-hMSCs (Fig. 1b). The mRNA expression profiles from nonAC-hMSCs at two different time points showed some overlap, with 987 downregulated mRNAs and 855 upregulated mRNAs shared between both samples (Fig. 1b). The 50 most downregulated and 50 most upregulated mRNAs in nonAC-hMSCs at both time points are shown as a heat map in Fig. 1a and are listed in Table 1. The expression of VEGFA in the nonAC-hMSCs was significantly higher than that in the AC-hMSCs at the two time points (Table 1). This increase in VEGFA mRNA in the nonAC-hMSCs was also verified by RT-qPCR. Western blot results showed that the VEGFA protein level was higher in nonAC-hMSCs than in AC-hMSCs (Fig. 1c, d; Additional file 4: Figure S2A). Moreover, the secretion of VEGFA, as determined by ELISA, was significantly higher in nonAC-hMSCs than in AC-hMSCs (Fig. 1e). Taken together, these results indicate that the nonadherent culture method elevates the expression of VEGFA in hMSCs and facilitates VEGFA secretion.

**Decreased levels of VEGFA produced by VEGFA knockdown nonAC-hMSCs inhibit the angiogenesis of HUVECs**
A lentivirus-based system was used to assess whether the expression of VEGFA in hMSCs plays an important role in HUVEC angiogenesis. VEGFA was stably overexpressed in AC-hMSCs (AC-VEGFA-hMSCs) and depleted in nonAC-hMSCs (nonAC-shVEGFA-hMSCs) (Fig. 2a, b; Additional file 4: Figure S2B). ELISA analysis revealed that forced expression of VEGFA induced VEGFA production, whereas knockdown of VEGFA led to the converse (Fig. 2c). We performed a tube formation assay to detect angiogenesis of HUVECs and found that HUVECs cultured in CM from AC-VEGFA-hMSCs and nonAC-hMSCs formed well-developed networks of capillary-like tubes. In contrast, HUVECs cultured in
CM from nonAC-shVEGFA-hMSCs and AC-hMSCs displayed very few capillary-like structures (Fig. 2d). Collectively, these data suggest that silencing and therefore limiting secretion of VEGFA in nonAC-hMSCs hindered tube formation of HUVECs, whereas excess VEGFA secretion in AC-hMSCs promoted HUVEC tube formation.

**VEGFA production by VEGFA-overexpressing AC-hMSCs increases angiogenesis and reduces infarction size after MI**

To explore the functional significance of VEGFA in MSC-mediated angiogenesis in a rat MI model, we transplanted AC-VEGFA-hMSCs, nonAC-shVEGFA-hMSCs, or their corresponding control cells into the ischemic LV wall border zone and analyzed VEGFA expression and secretion after cell transplantation. Compared to the corresponding control groups, AC-VEGFA-hMSC-transplanted group and nonAC-hMSC-transplanted group had significantly higher levels of VEGFA expression and secretion, whereas nonAC-shVEGFA-hMSC-transplanted group and AC-hMSC-transplanted group exhibited much lower levels of VEGFA expression and secretion (Fig. 3a–c; Additional file 4: Figure S2C). Furthermore, we evaluated angiogenesis in the infarcted heart after hMSC transplantation using immunohistochemistry and found that the number of vWF-positive vessels significantly increased in AC-VEGFA-hMSC- and nonAC-hMSC-treated groups, while they were markedly decreased in nonAC-shVEGFA-hMSC- and AC-hMSC-treated groups (Fig. 3d). Additionally, we used Masson’s trichrome staining to detect the extent of fibrosis after hMSC transplantation. The results showed that the infarct size was significantly smaller in the AC-VEGFA-hMSC-injected hearts and nonAC-hMSC-injected hearts than in the control AC-hMSC-injected hearts, whereas it was significantly larger in the nonAC-shVEGFA-hMSC-injected hearts and AC-hMSC-injected hearts than in the control nonAC-hMSC-injected hearts (Fig. 3e). Therefore, our data demonstrate that implantation of AC-VEGFA-hMSCs promotes angiogenesis and decreases infarction size by stimulating VEGFA production in the rat MI model.

**VEGFA secretion by VEGFA-overexpressing AC-hMSCs improves cardiac functions in a rat MI model**

Echocardiography was performed 4 weeks after hMSC implantation in a rat MI model to assess the therapeutic effects of MSCs on cardiac function (Fig. 4a). LVEDD and LVESD were significantly lower in the AC-VEGFA-hMSC and nonAC-hMSC groups and significantly higher in the AC-hMSC- and nonAC-shVEGFA-hMSC groups compared to the corresponding control groups.
Table 1  Differential expression of genes in AC-hMSCs and nonAC-hMSCs at two different time points

| Gene name | 24 h | 72 h |
|-----------|------|------|
|           | Fold change | p value | Regulation | Fold change | p value | Regulation |
| C2CD4B    | 111.8401912 | 0.000145634 | Up | 1340.34 | 0.000682199 | Up |
| SSTR2     | 48.09021308 | 0.000555557 | Up | 307.6615 | 0.000223379 | Up |
| SLC16A6   | 58.22005835 | 0.000188778 | Up | 244.5447 | 0.000546686 | Up |
| FAM65C    | 19.69493511 | 2.3287E-08  | Up | 213.9348 | 3.43255E-05 | Up |
| WDR86     | 29.35821269 | 2.0396E-05  | Up | 190.5289 | 6.26129E-05 | Up |
| GJB2      | 33.73311298 | 2.37009E-05 | Up | 170.4077 | 0.000560036 | Up |
| CXCR4     | 36.63486255 | 0.000159395 | Up | 156.6291 | 0.000381246 | Up |
| MMP13     | 18.1969967 | 9.3413E-05  | Up | 117.9299 | 2.56801E-05 | Up |
| BMP2      | 14.64162866 | 0.000193438 | Up | 85.52492 | 0.000625896 | Up |
| C2CD4A    | 13.20455766 | 0.000159395 | Up | 78.42749 | 0.002467774 | Up |
| TNFSF10   | 29.04491724 | 0.001267072 | Up | 57.67481 | 0.00386508 | Up |
| PRSS35    | 11.75846586 | 0.000587899 | Up | 56.39598 | 0.00064307 | Up |
| MEGF10    | 21.16086411 | 0.002528035 | Up | 55.27018 | 5.18695E-05 | Up |
| DUSP4     | 13.21396405 | 9.3413E-05  | Up | 54.87385 | 0.001212616 | Up |
| TSPAN11   | 27.83430153 | 0.000297067 | Up | 53.68215 | 9.89532E-07 | Up |
| DRAxin    | 13.45152949 | 0.000251701 | Up | 51.35024 | 0.00127201 | Up |
| PDK4      | 96.98929796 | 5.63716E-05 | Up | 46.77971 | 2.99919E-05 | Up |
| ANKH      | 10.78789239 | 0.004008663 | Up | 46.26112 | 2.57026E-05 | Up |
| RG516     | 39.28019753 | 0.000545809 | Up | 45.46778 | 7.36326E-07 | Up |
| RANBP3L   | 9.229649839 | 0.000587899 | Up | 38.70846 | 7.23795E-05 | Up |
| ITGA2     | 21.59037248 | 9.3413E-05  | Up | 38.41117 | 0.000872801 | Up |
| ADGRE2    | 9.283517067 | 0.008575709 | Up | 34.87146 | 6.97014E-06 | Up |
| MMP11     | 16.87380801 | 0.00285751 | Up | 34.62489 | 0.001476287 | Up |
| KLK1      | 11.95489346 | 0.000405053 | Up | 34.56032 | 0.002009842 | Up |
| BEGAIN    | 33.51924802 | 6.25373E-07 | Up | 30.53104 | 0.000704421 | Up |
| PPL       | 9.869982413 | 0.009031407 | Up | 25.65778 | 0.000467585 | Up |
| ABCG1     | 30.41288703 | 0.00025295 | Up | 24.69536 | 4.16571E-07 | Up |
| CITED1    | 18.69977736 | 0.00160336 | Up | 24.55424 | 0.000276694 | Up |
| SYTL5     | 36.85560246 | 0.000548851 | Up | 23.16011 | 0.001025661 | Up |
| RGS17     | 12.57031107 | 0.00015551 | Up | 22.45184 | 0.000241119 | Up |
| KIAA1211L | 16.30985794 | 1.57458E-05 | Up | 22.31267 | 3.31962E-05 | Up |
| GLDN      | 38.48396376 | 0.000844764 | Up | 21.32752 | 0.000790957 | Up |
| EXP15     | 15.81689248 | 0.001356464 | Up | 20.98551 | 0.001995397 | Up |
| PTGDR     | 14.69735545 | 0.002868926 | Up | 18.19271 | 0.000615557 | Up |
| CCR1      | 19.99007688 | 7.95976E-05 | Up | 16.40048 | 0.002030008 | Up |
| RASSF10   | 32.93415505 | 0.00178622 | Up | 16.05662 | 0.000741795 | Up |
| FAM188A1  | 11.84572522 | 0.005621553 | Up | 15.56768 | 1.4842E-06 | Up |
| COLEC12   | 23.05965011 | 0.000724925 | Up | 15.25867 | 0.00408674 | Up |
| KLRC1     | 10.05486412 | 0.002881202 | Up | 15.20619 | 0.000154447 | Up |
| VEGFA     | 9.752636526 | 4.13371E-06 | Up | 14.73218 | 0.00346357 | Up |
| XKRX      | 16.61076959 | 0.00050139 | Up | 14.38702 | 0.000172706 | Up |
| RASGRP3   | 2.106425558 | 0.028207257 | Up | 13.9441 | 7.03918E-05 | Up |
| Gene name | 24 h | 72 h |
|-----------|------|------|
| ENTPD3    | 9.912879304 0.001624712 Up | 13.76377 0.000782473 Up |
| YPEL4     | 53.6189278 3.544E-05 Up | 13.63856 0.002333755 Up |
| SLC7A8    | 11.49043722 0.011671519 Up | 13.22151 0.002134691 Up |
| CH25H     | 30.44303155 0.001762942 Up | 12.42755 0.004566846 Up |
| CYFIP2    | 2.70902602 0.007297695 Up | 10.76685 8.22518E-05 Up |
| C3AR1     | 8.089947094 0.000110391 Up | 9.559041 0.000110391 Up |
| CPA4      | 0.060443451 0.004894715 Down | 0.005735 3.23423E-05 Down |
| CNN1      | 0.071987351 0.002896126 Down | 0.005827 0.001199281 Down |
| MEST      | 0.041677656 0.002021602 Down | 0.006979 1.73885E-05 Down |
| KRT34     | 0.011913749 0.000539452 Down | 0.015092 0.000257229 Down |
| DKK1      | 0.004639044 0.001686929 Down | 0.012196 0.0006374 Down |
| LMCD1     | 0.029802085 2.33205E-05 Down | 0.012196 0.0006374 Down |
| KRTAP2-3  | 0.011067704 0.000539452 Down | 0.015092 0.000257229 Down |
| FAM46B    | 0.033933237 4.34704E-06 Down | 0.0174 0.00090771 Down |
| MYBL1     | 0.029145858 1.44931E-05 Down | 0.017623 0.001442927 Down |
| NEIL3     | 0.017315518 0.000108138 Down | 0.01963 0.00027453 Down |
| KRTAP1-5  | 0.029877632 0.001686929 Down | 0.019696 0.00129879 Down |
| SPC25     | 0.044301408 0.001309748 Down | 0.027418 0.001322887 Down |
| THBS1     | 0.035292076 0.000388028 Down | 0.028926 0.002471628 Down |
| PTX3      | 0.010465082 0.001271904 Down | 0.028936 3.7597E-05 Down |
| TSPAN18   | 0.053314954 0.000181383 Down | 0.029322 0.000796185 Down |
| KRTAP2-2  | 0.048512804 1.88464E-05 Down | 0.029763 0.00057951 Down |
| MGAM      | 0.034235468 0.00061814 Down | 0.030484 0.00037356 Down |
| ANLN      | 0.05142361 0.001527223 Down | 0.031571 0.001433314 Down |
| KIRREL3   | 0.057630141 0.002922558 Down | 0.03209 0.000359608 Down |
| E2F8      | 0.018025232 1.75885E-05 Down | 0.032301 0.000149089 Down |
| CEP55     | 0.0606109 0.000871389 Down | 0.033875 0.000278821 Down |
| CTGF      | 0.048584673 0.000533036 Down | 0.034772 0.000177803 Down |
| SCA1      | 0.04626685 1.04503E-05 Down | 0.034872 0.000282226 Down |
| KRTAP1-1  | 0.029512423 2.68546E-06 Down | 0.036294 0.001195564 Down |
| KRTAP1-4  | 0.034637793 8.67165E-05 Down | 0.032924 0.000393189 Down |
| SHCBP1    | 0.074874368 0.007927709 Down | 0.037302 4.80244E-05 Down |
| CDC45     | 0.048785792 0.001757321 Down | 0.038191 0.0002567892 Down |
| MKI67     | 0.061895471 0.001531656 Down | 0.039185 0.000627038 Down |
| ERC6L     | 0.031855904 1.35451E-06 Down | 0.040946 0.001070963 Down |
| TNFRSF11B | 0.035805553 0.004205919 Down | 0.041701 0.000375158 Down |
| ZNF367    | 0.048382669 0.003338013 Down | 0.042294 8.8958E-05 Down |
| DMD       | 0.038750757 1.1526E-05 Down | 0.042472 0.001853828 Down |
| CDCA8     | 0.038925122 0.00236484 Down | 0.043372 0.000204384 Down |
| PADI1     | 0.041441366 0.000851724 Down | 0.044515 7.47813E-06 Down |
| HIST2H3C  | 0.045817546 0.00054878 Down | 0.045124 0.000144333 Down |
| TTK       | 0.061307423 7.72508E-05 Down | 0.045391 0.000936671 Down |
Table 1  Differential expression of genes in AC-hMSCs and nonAC-hMSCs at two different time points (Continued)

| Gene name | 24 h Fold change | 24 h p value | Regulation | 72 h Fold change | 72 h p value | Regulation |
|-----------|-----------------|-------------|------------|-----------------|-------------|------------|
| GABBR2    | 0.045546263     | 0.001708625 | Down       | 0.045807        | 9.62305E-05 | Down       |
| FAM111B   | 0.027357567     | 4.93311E-06 | Down       | 0.046582        | 0.000558007 | Down       |
| CCIN      | 0.051329319     | 0.003246805 | Down       | 0.048819        | 5.93123E-05 | Down       |
| KIF15     | 0.066141532     | 0.000117669 | Down       | 0.048866        | 0.001136135 | Down       |
| NCKAP5    | 0.066816867     | 0.000303257 | Down       | 0.049423        | 0.001324705 | Down       |
| NTF3      | 0.042448723     | 0.001215131 | Down       | 0.050437        | 0.005324032 | Down       |
| UBE2C     | 0.065284894     | 4.29692E-06 | Down       | 0.052099        | 0.002768879 | Down       |
| KRT33B    | 0.064358986     | 0.00039797  | Down       | 0.052188        | 0.000124335 | Down       |
| MCM10     | 0.046540673     | 0.000257847 | Down       | 0.054035        | 0.003217037 | Down       |
| LMOD1     | 0.054276425     | 1.14094E-06 | Down       | 0.055313        | 0.000663673 | Down       |
| LTF       | 0.0658482765    | 1.6193E-05  | Down       | 0.057596        | 0.000575741 | Down       |
| HIST1H3G  | 0.035683104     | 0.000992525 | Down       | 0.058962        | 0.000146762 | Down       |
| CCDC8SA   | 0.066799698     | 9.312090059 | Down       | 0.059079        | 0.009067666 | Down       |
| NCAPG     | 0.073153137     | 6.164797229 | Down       | 0.060212        | 0.037036192 | Down       |

Fig. 2 Decreased levels of VEGFA produced by VEGFA knockdown nonAC-hMSCs inhibits the angiogenesis of HUVECs. AC-hMSCs were infected with VEGFA or negative control (NC) lentiviruses and nonAC-hMSCs were infected with shVEGFA or the control lentiviruses. a RT-qPCR and b western blotting were performed to detect mRNA and protein levels of VEGFA, respectively. c ELISA analysis was performed to determine the levels of secreted VEGF. d Capillary-like structure formation of HUVECs cultured in CM from hMSCs with different genotypes was evaluated by tube formation assay. Data are represented as mean ± SD (n = 3 per group). *P < 0.05 compared to AC-hMSC-Ctrl or nonAC-hMSC-NC group. ▲ P < 0.05 compared to AC-hMSC-Ctrl group.
Furthermore, the AC-VEGFA-hMSC and nonAC-hMSC groups exhibited significantly increased LVEF and LVFS, whereas the nonAC-shVEGFA-hMSC and AC-hMSC groups displayed significantly decreased LVEF and LVFS (Fig. 4d, e). Collectively, these findings demonstrate that implantation of VEGFA-overexpressing AC-hMSCs could decrease myocardial remodeling.

VEGFA is a direct target of miR-519d

To elucidate the molecular mechanism of VEGFA in the regulation of angiogenesis, we first employed the online bioinformatics (TargetScan and microRNA.org) to predict the potential microRNA (miRNA) binding sites in VEGFA. miR-519d was found to be a putative miRNA for the regulation of VEGFA (Fig. 5a). We then probed for miR-519d expression in AC-hMSCs and nonAC-hMSCs at two different time points using RT-qPCR and found that miR-519d expression was significantly lower in nonAC-hMSCs compared to AC-hMSCs (Fig. 5b). As shown in Fig. 1c, d, the expression of VEGFA was significantly higher in nonAC-hMSCs than in AC-hMSCs. This negative correlation between VEGFA and miR-519d expression indicates that miR-519d may be involved in the regulation of VEGFA.

To validate a direct interaction between VEGFA and miR-519d, a luciferase reporter plasmid containing wild-type or mutant VEGFA 3′ UTR was constructed and transfected into HEK293T cells, along with miR-519d or control mimics. Results showed that miR-519d led to a significant reduction in the luciferase activity produced by the construct containing the VEGFA putative miR-519d binding site, whereas miR-519d had no significant effect on the construct containing the mutant VEGFA 3′ UTR (Fig. 5c). Furthermore, RT-qPCR, western blot, and ELISA showed that overexpression of miR-519d in nonAC-hMSCs significantly decreased the expression and secretion of VEGFA, and knockdown of miR-519d in AC-hMSCs enhanced VEGFA expression and secretion (Fig. 5d–f; Additional file 4: Figure S2D). Taken together, these results indicate that miR-519d exerts suppressive effects on VEGFA expression by binding the VEGFA 3′ UTR.

The secretion of VEGFA in VEGFA-overexpressing nonAC-hMSCs attenuates miR-519d-mediated inhibition of tube formation of HUVECs

We performed a rescue experiment to determine whether miR-519d regulates cell angiogenesis via
targeting VEGFA. nonAC-hMSCs were co-transfected with miR-519d mimics and VEGFA plasmids for 24 h. RT-qPCR and western blot analyses indicated that ectopic expression of VEGFA attenuated miR-519d-mediated suppression of VEGFA expression (Fig. 6a, b; Additional file 4: Figure S2E) and secretion (Fig. 6c). Furthermore, while miR-519d-overexpressing decreased tube formation of HUVECs, the addition of VEGFA derived from VEGFA-overexpressing nonAC-hMSCs reversed this effect (Fig. 6d). Collectively, these data suggest that miR-519d exerts its biological effects by directly targeting VEGFA.

Discussion
A growing body of evidence suggests that the beneficial therapeutic effects of MSCs are the result of paracrine mechanisms [26, 27]. Various growth factors, cytokines, and angiogenic factors released from MSCs play critical roles in improving the function of infarcted hearts [28, 29]. VEGFA belongs to the VEGF family and is a well-known pro-angiogenic factor. VEGFA has been reported to stimulate endothelial cell proliferation, migration, and tube formation, thereby promoting angiogenesis [30]. Recent studies indicated that VEGFA released from different types of stem cells, including MSCs, have the ability to treat various human diseases, such as myocardial ischemia, by regulation of angiogenesis [31–33]. Markel et al. [31] reported that VEGFA knockdown in MSCs impaired stem cell-mediated myocardial function. Cho et al. [32] reported that VEGFA-secreting human umbilical cord blood-derived MSCs decreased infarct size and improved cardiac function via enhancing angiogenesis in infarct myocardium. Kim et al. [23] also reported that the transplantation of hypoxia inducible VEGFA-expressing MSCs promoted ischemia-responsive VEGFA production, resulting in a significant decrease in apoptosis and a significant increase in micro-vessel formation after MI. In our study, VEGFA was upregulated in nonAC-hMSCs (Fig. 1). The high levels of VEGFA secretion by VEGFA-overexpressing AC-hMSCs improved cardiac functions in a rat MI model. a Cardiac function was determined by echocardiography 28 days after MI induction and hMSC transplantation. b Quantification of LVEDD, c LVESD, d LVEF, and e LVFS. Data are represented as mean ± SD (n = 8 per group). ∗P < 0.05 compared to AC-hMSC-Ctrl or nonAC-hMSC-NC group. ▲ P < 0.05 compared to AC-hMSC-Ctrl group. ▲ P < 0.05 compared to AC-hMSC-Ctrl group.
transpolarization [34, 35]. In skin cancer, VEGFA promotes macrophage recruitment to the tumor and thereby facilitates tumor-associated macrophage development [34]. In chronic kidney disease, ELP-VEGF therapy distinctly shifted renal macrophage phenotype from proinflammatory M1 to VEGF expressing M2, restoring VEGF signaling and sustaining improvement of renal function and microvascular integrity [35]. However, whether VEGFA improves cardiac function by affecting cardiomyocytes or regulating macrophage recruitment and macrophage phenotype needs further investigation.

A number of studies have indicated that VEGFA can be directly regulated by miRNAs [36, 37]. miR-299-3p inhibited cell proliferation and motility and induced apoptosis in renal cell carcinoma by directly targeting VEGFA [36]. miR-199a-5p impaired both the survival and angiogenic capacity of MSCs by directly regulating VEGFA expression [37]. In our study, miR-519d directly affected VEGFA expression (Fig. 5). miR-519d is one member of the chromosome 19 miRNA cluster, which is the largest human miRNA cluster containing 46 pre-miRNAs [38]. There is growing evidence showing that miR-519d is involved in a range of biological processes, including cell proliferation, apoptosis, differentiation, migration, and angiogenesis [39–42]. miR-519d overexpression facilitated the proliferation, migration, invasion, and adhesion of melanoma cells in vitro and accelerated lung metastatic capability in vivo [39]. Expression of miR-519d promoted hepatocellular carcinoma cell proliferation and invasion and inhibited apoptosis [40]. On the other hand, overexpression of miR-519d impaired colorectal cancer cell viability, migration, and invasion and induced G0/G1 phase arrest and apoptosis by downregulating TROAP expression [41]. Additionally, miR-519d expression attenuated endothelial cell growth, migration, and tube formation [42]. In this study, increased VEGFA production in nonAC-hMSCs reversed the suppressive effect of miR-519d on HUVEC tube formation (Fig. 6).
Conclusions
In summary, we determined that the nonadherent culture of MSCs increased the secretion of VEGFA, directly opposing the effects of miR-519d (Additional file 5: Figure S3). VEGFA promoted the tube formation capabilities of HUVECs in vitro, decreased myocardial remodeling and infarct size, and increased angiogenesis in a rat MI model. These findings provide a novel experimental protocol to optimize MSC-based therapies for the treatment of MI.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13287-020-01780-x.

Additional file 1: Figure S1. Characterization of isolated hMSCs. Flow cytometry showing the percentage of CD73, CD90, CD105, CD11b, CD14, CD34 and CD45 hMSCs.

Additional file 2: Table S1. Differential expression of genes in AC-hMSCs and nonAC-hMSCs at 24 h.

Additional file 3: Table S2. Differential expression of genes in AC-hMSCs and nonAC-hMSCs at 72 h.

Additional file 4: Figure S2. Relative VEGFA protein expression in the different groups. Data are represented as mean ± SD (n = 3 per group). *P < 0.05 compared to AC-hMSCs, AC-hMSCs-Ctrl, nonAC-hMSCs-NC, miR-Ctrl or anti-miR-Ctrl group. #P < 0.05 compared to miR-519d group.

Additional file 5: Figure S3. The schematic diagram shows how VEGFA expression is regulated by miR-519d-3p after changes of adhesion.

Abbreviations
MSCs: Mesenchymal stem cells; MI: Myocardial infarction; hMSCs: Human MSCs; AC-hMSCs: hMSCs grown as adherent culture; nonAC-hMSCs: hMSCs grown as nonadherent cultures on ultra-low-adherent plates; AC-VEGFA-hMSCs: VEGFA-overexpressing AC-hMSCs; nonAC-shVEGFA-hMSCs: VEGFA-knockdown nonAC-hMSCs; RT-qPCR: Real-time quantitative polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; HUVECs: Human umbilical vein endothelial cells; Sca-1: Stem cell antigen 1; LADCA: Left anterior descending coronary artery; LV: Left ventricle; LVEDD: LV end-diastolic dimensions; LVESD: LV end-systolic dimensions

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Authors’ contributions
BD collected the samples, performed the experiments, and drafted the manuscript. YL analyzed the data and prepared figure WD designed the research and revised the manuscript. HJ participated in the experiments. WH and YW participated in the study design. The authors read and approved the final version of manuscript.

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Availability of data and materials
All data generated and/or analyzed during the current study are included in this manuscript.
Ethics approval and consent to participate
All experiments were approved by the Ethics Committee of the Affiliated Hospital of Guillin Medical University, and informed consent was obtained from all participants.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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