LncRNA260 siRNA promotes M2 macrophage polarization by reducing IL28RA alternative splicing

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Research Article

Keywords: lncRNA260, IL28RA, transfection, macrophages, polarization, alternative splicing

Posted Date: March 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1402717/v1

Abstract

Background and: Regulating the macrophages toward M2 polarization has become a therapeutic target for promoting cardiac repair after acute myocardial infarction (AMI). Macrophages can promote the transformation from inflammatory M1 type to anti-inflammatory M2 type by activating PI3K/AKT signaling pathway. In our previous study, we found that down-regulation of lncRNA260 could ameliorate hypoxic cardiomyocytes injury by regulating IL28RA through the activation of PI3K/AKT signaling pathways. It was suggested that lncRNA260 siRNA could promote the macrophages toward M2 polarization by regulating IL28RA. In this study, lncRNA260 siRNA was used to observe the polarization of mouse bone marrow macrophages and investigate its related mechanisms.

Objective and methods: LncRNA 260 specific siRNA were designed and synthesized which were transfected into murine bone marrow-derived macrophages (BMDM)with liposomes. The experiment was divided into three groups: hypoxia group, hypoxia + lncRNA 260 specific siRNA transfection group, Normoxia group. The CD206-FITC/CD107b (Mac-3) or CD206-APC/CD11b-FITC double positive proportions were used to compare the M2 polarization ratio in the hypoxia process by using the immunofluorescence staining method. The p-AKT, Arginase 1, PI3KCG, IL28RAV1, IL28RAV2 proteins expression changes were observed by using the western blot method.

Results: Compared with the Normoxia group, the double positive ratio of CD206-FITC/ Mac-3 and CD206-APC/CD11b-FITC were both significantly decreased in Hypoxia group (P<0.05). Compared with the hypoxia group, the double positive ratio of CD206-FITC/ Mac-3 and CD206/CD11b were both significantly increased in the Hypoxia + lncRNA260 siRNA transfection group (P<0.05). In the Hypoxia group, the ratios of Arg 1/β-Actin, p-AKT/β-Actin, PI3KCG/β-Actin, IL28RAV1/β-Actin were significantly lower than those in the Normoxia group (1.00±0.01) (P<0.05). After transfection with lncRNA260 siRNA, the ratios of Arg1/β-Actin, p-AKT/β-Actin , PI3KCG/β-Actin, IL28RAV1/β-Actin were significantly higher than those in the Hypoxia group (P<0.05) .Compared with the Normoxia group, the IL28RAV2/β-Actin in the Hypoxia group was significantly increased. After transfection with lncRNA260 siRNA, the ratio of IL28RAV2/β-Actin was significantly decreased than that in the Hypoxia group (P<0.05).

Conclusion: LncRNA260 siRNA could promote the M2 polarization of the hypoxia macrophages by reducing the IL28RAV2 alternative splicing variant, which might be related to the activation of the JAK-STAT and PI3K/AKT signaling pathways.

Introduction

Acute myocardial infarction (AMI) is a major disease threatening people's health nowadays. With the progress of intervention and drug therapy, the treatment of AMI has made great progress. Nevertheless, cardiac remodeling is one of the major causes of death in AMI patients. Therefore, cardiac remodeling is an urgent problem to be solved. Cardiac remodeling after myocardial infarction is a process of changes in cardiac geometry and function, which is considered to be a common response to increased ventricular

wall adaptability or reduced viable myocardium. Cardiac remodeling would lead to the impaired cardiac function, even heart failure. In the early stage of AMI, inflammation and immune response occur to limit the ischemic infarct size and maintain cardiac output. However, excessive and long-term inflammation aggravates ventricular remodeling after AMI.

Macrophages are an important component of inflammation and play an important role in later heart remodeling.M1 and M2 macrophages play different roles in the inflammatory stage and myocardial remodeling after infarction. The early stage of inflammation is dominated by M1 type, which secretes pro-inflammatory cytokines and promotes the development of inflammation. In the late stage of inflammation, M2 type is dominant, which promotes inflammation subside, proliferation of fibroblasts and formation of new vessels. According to the previous studies, the expression of monocyte chemotaxis protein-1was gradually up-regulated within 3 days after AMI in mice, and reached its peak on the third day. After AMI, the secretion of inflammatory factors and cytokines gradually increased, attracting a large number of macrophages. The recruited M1 macrophages and other inflammatory cells secrete a large number of cytokines and chemokines, which recruit more inflammatory cells. The macrophages M1 cells decreased after AMI three days, the M2 macrophages increased and participated in cardiac remodeling by secreting anti-inflammatory cytokines, promoting the formation and proliferation of myofibroblasts, new vessels and so on [1]. The imbalance of M1 and M2 macrophages during inflammation will lead to poor ventricular remodeling. Therefore, the therapy targeting M1/M2 macrophage transformation can inhibit early inflammation and promote late cardiac repair, thus effectively blocking cardiac remodeling after AMI.

Interleukin-28 receptor α (IL28RA) and IL10RB constitute the receptors of type III interferon (IFN-λs).Type III interferon is a new type of interferon, which belongs to IL10 family of class II cytokines. IL28RA is widely present in various tissues of human body, and is highly expressed in organ tissues such as heart, bone marrow, pancreas, thyroid, skeletal muscle, prostate and testis [2]. Studies have shown that IFN-λs can activate JAK-STAT and PI3K/AKT signaling pathways to play antiviral, anti-tumor proliferation roles and regulate inflammatory responses [3–8]. IL28RA has has two alternative splicing (AS)variants, namely IL28RAVl and IL28RAV2, both of which can bind to type III interferon, but the latter loses the signal transduction function and can inhibit the activity of IFN-λs, so they have opposite functions[9]. It has been confirmed that the M2-type polarization of macrophages is related to the activation of JAK-STAT and PI3K/AKT signaling pathways, which promote the transformation of macrophages from the inflammatory M1-type to the anti-inflammatory M2-type [10–12]. Different IL28RA AS variants promotes different phenotypic transformation of macrophages. IL28RAVl promotes M2-type polarization of macrophages, while IL28RAV2 inhibits this function.

In our early study, we found elevated expression of lncRNA260 and IL28RA in patients with AMI through high-throughput chip screening, suggesting that IL28RA gene is the target gene regulated by lncRNA260. It was suggested that lncRNA260 might regulate IL28RA gene through trans effect and participate in the signal transmission process in the post AMI process. Our previous studies have shown that down-

regulation of lncRNA260 regulates IL28RA and activates the JAK-STAT and PI3K/AKT signaling pathways, thus improving the injury of hypoxic cardiomyocytes in rats [13], which is thought to be related to the reduction of IL28RAV2. Therefore, we speculate that lncRNA260-specific siRNA could reduce the expression of *IL28RAV2* gene, activate JAK-STAT and PI3K/AKT signaling pathways, promote the polarization of macrophages to M2-type, inhibit inflammatory response, and thus achieve certain biological effects.

In order to investigate whether lncRNA260 siRNA can promote the M2 polarization of in hypoxia murine BMDM by reducing the expression of IL28RAV2, this study was performed. In the current study, lncRNA260 specific siRNA was designed and transfected into macrophages with liposome transfection method for hypoxia intervention to explore the effect and related mechanism of macrophage polarization in hypoxia model.

Materials And Methods

Materials

Collagenase and DMEM cell culture medium (Gibco), Lipofectamine^{TM}2000 (Invitrogen), IL28RA rabbit antibody (Sigma). Arginase 1, FITC rat CD206 antibody(Biolegend), CD107b (Mac-3)rat antibody (Biolegend),β-actin rabbit antibody (Cell Signaling),phospho-AKT (pAKT)(Ser 473) rabbit antibody (Cell Signaling), Arginase 1 goat antibody (Santa Cruz), PI3KCG mouse antibody (Santa Cruz), Donkey F(ab)2 Anti-Rat IgG H&L(Alexa Fluor® 568) preadsorbed (Abcam),Donkey F(ab)2 Anti-Rabbit IgG H&L(Alexa Fluor® 568) preadsorbed (Abcam).

The healthy C57BL/6 mice, 6-8 weeks old, 16-20g weight were from the Jiangsu Province Animal Center. The lncRNA260-specific siRNA was synthesized by Shanghai Gene Pharma Co., Ltd. The nucleotide sequence is listed as follows: sense chain (5’-CCCAGUGAAGGAGACGAAATT-3’), anti-sensechain (5’-UUUCGUCUCCUUCACUGGGTT-3’).

Methods

Mononuclear macrophages from murine bone marrow isolation and culture [14]:

The current research was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (NO. 2021-SRFA-007).BALB/C57 mice were sacrificed by using CO2 inhalation in a rodent euthanasia device. Their hind limbs were cut off, and placed in petri dishes containing sterile PBS and moved to a super-clean platform. The hind limbs were rinsed with PBS twice, and the muscles of the hind limbs were removed successively. The hind limbs were rinsed with PBS again 1-2 times, and then placed in a small amount of PBS. The ends of the thigh and tibia were cut open at the joint, and PBS was absorbed with a 1ml syringe to rinse the bone marrow cavity until it turned white. Bone marrow rinses collected and filtered through a 200-mesh filter. The bone marrow cells were centrifuged at room temperature at 300g for 5 min, andthe supernatant was discarded. After cell counting, an appropriate

amount of Dulbecco Modified Eagle medium (DMEM) was added and the final concentration of cell suspension was adjusted to 1×10^6 cells/mL. The cells were inoculated in 24-well plates, 6-well plates and 3.5cm petri dishes. On the third day, the experiment was randomly divided into groups. The experiment was divided into three groups: hypoxia + lncRNA260 siRNA transfection group, hypoxia group and normal control group.

Transfection of murine BMDM with lncRNA260 siRNA:

After culturing murine BMDM for 3 days, penicillin/streptomycin was removed from the DMEM culture medium and lncRNA260siRNA were transfected into the cells at the 100 nmol/L concentration by lipofectamine transfection method. In each group, triplicate parallel wells were set up. After the lipofectamine 2000 was dissolved in DMEM medium and mixed with lncRNA260 siRNA, the lipofectamine-lncRNA260siRNA mixture was added into the mononuclear macrophages culture wells respectively and gently blended. After transfection for 4 hours at 37℃ in the incubator, the lipofectamine 2000was removed. The culture medium was then changed with new complete DMEM medium penicillin/streptomycin.

Establishing the murine BMDM Hypoxia model:

72 hours after bone marrow macrophages transfection, all groups except controls were treated with hypoxia for 24 hours by using Anaerobic bag (Becton Dickinsonand Inc, USA) (95% N_2 and 5% CO_2) to simulate the Hypoxia process.

Measuring CD107b、Mac-3、CD11b-FITC、CD206-APC、CD206-FITC、proteins expression in cultured murine BMDM by immunofluorescence staining

After the **Hypoxia** process, the 24-well cell plate was removed from the hypoxia bag and rinsed with PBS 2-3 times. 4% paraformaldehyde (PFA) was added to each well and fixed at room temperature (RT) for 15min and rinsed with PBS 3 times. 0.2% triton was added to each well, and the triton was permeated at RT for 5min. After Triton was discarded and the wells were rinsed with PBS for 3 times, 3% normal lamb serum (3% in PBS) was added. After the cells were blocked at RT for 1h, and the serum was discarded. The antibodies CD11b-FITC, Mac-3 and CD206-APC, CD206-FITC were diluted with 3% lamb serum at a ratio of 1:100. The cells were double stained with the CD11b-FITC and CD206-APC antibodies, Mac-3 and CD206-FITC antibodies respectively. 3% lamb serum containing antibodies were added into each well, and incubated overnight at 4℃dark for more than 18h. Then the wells were rinsed with PBS for 3 times. Alexa Fluor® 568-conjugated donkey anti-rat IgG(H+L) antibody was diluted with 3% lamb serum at the ratio of 1:500. The diluted fluorescent secondary antibody was added to each well and incubated for 1 h at RT, away from light. Then the wells were rinsed with PBS for 4 times. The 4’, 6-diamidino-2-phenylindole (DAPI) dyeing solution was added to each well. The anti-fade fluoresence mounting medium was dropped on the slide. Then the slide was removed from the wells and covered back on the glass slide. The macrophages glass slides were observed under the fluorescent microscope and photographed.

Measuring protein expression of cultured murine BMDM by Western Blot

The phenylmethylsulfonyl fluoride (PMSF): RIPA lysis buffer was prepared in a ratio of 1:100 for full protein extraction lysis buffer. The total protein was extracted from cultured mouse bone marrow macrophages by using this RIPA lysis buffer. The total protein was quantified by using Bicinchoninic acid method. The total protein was separated through 10% SDS-PAGE electrophoresis. Then they were transferred to polyvinylidene fluoride (PVDF) membrane. The transferred PVDF membrane was blocked overnight at 4 °C by using the 5% skim milk. The 1:1000 diluted primary antibodies (goat anti-mouse Arginase 1 antibody, rabbit anti-mouse IL28RA antibody, rabbit anti-mouse β-actin antibody, rabbit anti-mouse pAKT antibody, mouse anti-mouse PI3KCG antibody) were incubated with the proteins on the shaker at 4 °C overnight. The horseradish peroxidase (HRP) labeled secondary antibodies (rabbit anti goat IgG antibody, goat anti rabbit IgG antibody, goat anti mouse IgG antibody) were diluted at a ratio of 1:5000 and incubated with the proteins on the shaker for 2 hours at 4°C. The Thermo scientific pierce Super Signal West Femto Chemiluminescent Substrate was used to develop the antibodies incubated PVDF membrane for 1 min. After the PVDF membrane was washed in the double-distilled water, it was exposed and photographed with the Gel Imaging System. The optical density (OD) of the proteins was calculated and compared.

Statistical analysis

SPSS23.0 statistical software was used for statistical analysis, and all measurement data were expressed as mean ± standard deviation ($\overline{x}$ ±s). T test was used for comparison of measurement data between the two groups, and one-way ANOVA was used for comparison between multiple groups. P<0.05 indicated that the difference was statistically significant.

Results

1. Culture of BMDM from adult C57 BL/6 mice

The non-adherent cells of murine BMDM just extracted were found to be round under the microscope. After 48 hours of culture, the adherent cells were observed to be round or irregular under the microscope, with pseudofoot protrusion, and the cell size was basically uniform (Fig. 1).

2. The M2 murine BMDM proportion changes during the hypoxia process by immunofluorescence method

CD107b (MAC-3) and CD11b-FITC mainly expressed in all macrophage populations, and CD206 was a specific surface marker of M2-type macrophages. DAPI staining is the nucleus.

2.1 The CD11b-FITC, CD206-APC proteins localization and relative levels by immunostaining in the murine BMDM hypoxia process

Compared with normal group (0.45 ± 0.04), the double positive ratio of CD206/CD11b was decreased in hypoxia group (0.26 ± 0.01) ($P < 0.05$). Compared with the hypoxia group, the CD206/CD11b double positive ratio (0.80 ± 0.04) was significantly increased in the hypoxia + lncRNA260 siRNA transfection group ($P < 0.05$) (Fig. 2A, 2B) .

2.2 The Mac-3, CD206-FITC proteins localization and relative levels by immunostaining in the murine BMDM hypoxia process

Compared with normal group (0.48 ± 0.04), the double positive ratio of CD206/ Mac-3 was decreased in hypoxia group (0.28 ± 0.01) ($P < 0.05$). Compared with the hypoxia group, the CD206/CD11b double positive ratio (0.81 ± 0.04) was significantly increased in the Hypoxia + lncRNA260 siRNA transfection group ($P < 0.05$) (Fig. 3A, 3B).

3. The expression changes of Arginase 1(Arg 1), p-AKT and PI3KCG proteins in the murine BMDM hypoxia process detected by Western blot

After transfection of lncRNA260 siRNA into murine BMDM for 72h and 24h hypoxia intervention, the expression of Arg 1, p-AKT and PI3KCG proteins was detected by Western blot, and the expression changes of Arg 1, p-AKT and PI3KCG proteins were compared. All of them were statistically analyzed by the grayscale ratio with internal reference β-Actin (Fig. 4A).

Arg 1 protein is one of the characteristic proteins of M2 macrophages. In the Hypoxia group, the ratios of Arg 1/β-Actin, p-AKT/β-Actin, PI3KCG/β-Actin were 0.81 ± 0.05, 0.67 ± 0.01, 0.62 ± 0.01 respectively, which were significantly lower than those in the Normoxia group (1.00 ± 0.01) ($P < 0.05$). After transfection with lncRNA260 siRNA, the ratios of Arg1/β-Actin, p-AKT/β-Actin, PI3KCG/β-Actin were increased to 1.65 ± 0.03, 2.30 ± 0.03, 1.03 ± 0.02 which were significantly higher than those in the Hypoxia group ($P < 0.05$) (Fig. 4B).

4. The expression changes of IL28RAV1 and IL28RAV2 proteins in the murine BMDM hypoxia process detected by Western blot

The protein gray levels of IL28RAV1/β-Actin and IL28RAV2/β-Actin were both based on the Normoxia group. Compared with the Normoxia group, the ratio of IL28RAV1/β-Actin in the Hypoxia group was significantly decreased (0.59 ± 0.01). After transfection with lncRNA260 siRNA, the ratio of IL28RAV1/β-Actin was significantly higher than that in the Hypoxia group (0.90 ± 0.02) ($P < 0.05$). Compared with the Normoxia group, the IL28RAV2/β-Actin in the Hypoxia group was significantly increased (1.31 ± 0.03). After transfection with lncRNA260 siRNA, the ratio of IL28RAV2/β-Actin was significantly decreased than that in the Hypoxia group (0.82 ± 0.02) ($P < 0.05$) (Fig. 5A, 5B).

Discussion

In the current study, we found that the M2 murine BMDM proportion was significantly decreased in the Hypoxia process ($P < 0.05$). After transfection with lncRNA260 siRNA, the M2 murine BMDM proportion

was significantly increased ($P < 0.05$). Meanwhile, the proteins expression of the Arg 1, p-AKT, PI3KCG, IL28RAV1 changed in the same way to the M2 murine BMDM proportion. Conversely, the IL28RAV2 protein expression presented the changes in the opposite direction in the murine BMDM hypoxia process. It was indicated that lncRNA260 siRNA could promote the M2 polarization of the hypoxia murine BMDM by reducing the IL28RAV2 alternative splicing variant, which might be related to the activation of the JAK-STAT and PI3K/AKT signaling pathways.

The degree of inflammation after AMI is positively correlated with the size of myocardial infarction, but the research on blocking inflammation has not obtained ideal results [15–22]. It was indicated that moderate suppression of inflammatory storm after AMI could reduce myocardial injury and improve prognosis, while blocking the inflammatory process was not conducive to myocardial repair, but increased the risk of pathological cardiac remodeling and cardiac rupture. The latest evidence for anti-inflammatory therapy after AMI is available. After PCI treatment of AMI, anti-inflammatory treatment with colchicine could reduce infarct size and improve prognosis [23]. Intravenous application of metoprolol after AMI could reduce the inflammatory response and limit the infarct size by reducing neutrophil tissue infiltration and its interaction with platelets in myocardial infarction area [24].

Peripheral blood mononuclear macrophages are recruited into damaged myocardium after AMI. This process was critical for cardiac repair because they could adopt pro-inflammatory or repair phenotypes to regulate inflammatory and repair responses, respectively [25, 26]. At first, M1 type macrophage was dominant, and reached the peak on 3–4 days after AMI, mainly producing inflammatory cytokines such as TNF-α, IL-1β, IL-6, chemokines such as CCR2, CXCL1, IL-8, and so on, thus aggravating the degree of inflammation. Then entering the fiber proliferation stage, Arginase 1, IL-10, VEGF and TGF-β1were mainly produced, and necrotic cells were cleared through cell burial [27, 28], inflammation was inhibited, neovascularization was promoted, and damaged tissue repair was promoted. The peak value was reached on 6–7 days after AMI. Finally, cardiac remodeling, or scar formation, occurs, and the necrotic areas of the myocardium are replaced by fibrous scar tissue formed by crosslinked fibers. The early stage of inflammatory response after AMI is a critical time for cardiac remodeling. It is very important to find genes or drugs that can promote the Mφ phenotype from pro-inflammatory M1 to anti-inflammatory M2. Macrophages can promote the transformation from inflammatory M1 type to anti-inflammatory M2 type by activating PI3K/AKT signaling pathway [29–30]. In our previous study, we found that down-regulation of lncRNA260 could ameliorate hypoxic cardiomyocytes injury by regulating IL28RA through the activation of PI3K/AKT signaling pathways. It was suggested that lncRNA260 siRNA could promote the macrophages toward M2 polarization by regulating IL28RA.

Studies have shown that IL28RA has a variety of alternative spliceosomes with different functions [9]. IL28RAVl is a normal functioning CRF2-12. IL28RAV2 lacks 29 amino acids in the intracellular region and can bind to type III interferon, but it loses the signal transduction function. IL28RAV2 can inhibit the activity of IFN-λ1, and is a negative regulator of type III interferon, which plays an anti-cell proliferation and promotes inflammatory response. In the current study, it was found that mouse macrophages also

expressed two AS variants, namely, 59KD and 55KD respectively. After 24h hypoxic, the full length of 59KD IL28RAVl was significantly reduced, and the expression of 55KD IL28RAV2 was significantly increased. It is speculated to be related to IL28RA mRNA alernative splicing caused by hypoxia, which inhibits JAK-STAT and PI3K/AKT signaling pathways, promotes polarization of macrophages to M1, enhances inflammatory response, significantly increases apoptosis and necrosis of myocardial cells, and increases the risk of myocardial fibrosis and heart rupture. After lncRNA-260 siRNA intervention, the IL28RAV2 AS was significantly reduced and IL28RAV1 was significantly increased compared with that of the hypoxia group, which promoted the activation of JAK-STAT and PI3K/AKT signaling pathways, caused the M2 polarization of macrophages, and reduced the inflammatory response. IL10RB is a common receptor ligand of IL28RA and IL10RA. During AMI, the expression of IL28RAV2 is up-regulated in macrophages, resulting in a competitive increase in the heterodimer formed by IL10RB and IL28RAV2, while the binding of IL10RA receptor is reduced. In addition, when hypoxia occurs, the expression of IL10RA is down-regulated due to the inhibition of type III interferon signaling pathway, which further reduces the activation of IL10/STAT3/IL4RA/STAT6, decreases M2 macrophages, further reduces the secretion of IL10, and aggravates inflammation.

CatRAPID omics is a server for large-scale calculations of protein-RNA interactions [31]. It was showed that lncRNA260 could bind to helicase-like transcription factor (HLTF), which has helicase and ATPase activity, and could bind to IL28RA promoter by using the catRAPID omics assay. HLTF could cause the chromatin structure around IL28RA gene change to promote the transcription of *IL28RA* gene. Therefore, it is speculated that lncRNA260 can promote the transcription of *IL28RA* gene through trans action by recruiting HLTF to the promoter site of *IL28RA*. These results suggest that lncRNA260 can regulate *IL28RA* gene and participate in the signal transduction process of AMI through JAK-STAT and PI3K/AKT signaling pathways.

The catRAPID omics predicted that lncRNA260 also competitively could bind to the SF3B2, SF3B3 splicing complex with IL28RA. The ESE finder predict that lncRNA260 also has the exon splicing enhancer (ESE) sequence of the SRSF protein recognition site of the splicing enhancer, which competitively binds to the SRSF protein with IL28RA (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) [32, 33]. Thus, it affected the recognition and splicing of the 7th exon of IL28RA mRNA by the splicing body, resulting in partial loss of the 7th exon, and increased the IL28RAV2 AS variant.

LncRNA260 is distributed in both nucleus and cytoplasm. According to the bioinformatics prediction of miRDB (http://mirdb.org/cgi-bin/custom.cgi) and Targetscan databases (http://www.targetscan.org/mamm_31/), both of lncRNA260 and *IL28RAV2* mRNA can bind to miR-3622. LncRNA260 acts as competing endogenous RNAs (ceRNA). Hence, the degradation of *IL28RAV2* gene by miR-3622 is weakened, and the expression of *IL28RAV2* gene is further increased. In contrast to IL28RAV1, the function of this IL28RAV2 variant blocked JAK-STAT, PI3K/AKT signaling pathways, resulting in decreased M2-type polarization of macrophages and enhanced inflammatory response, resulting in myocardial cell damage and ventricular remodeling during AMI.

In a word, lncRNA260 siRNA promotes M2 macrophage polarization by reducing IL28RAV2 AS in the murine BMDM hypoxia process. Considering the current study is an in vitro experiment, this conclusion will be further confirmed in the animal experiments. This study will lay a foundation for future in vivo research.

Declarations

Acknowledgements

This work was funded by the National Natural Science Foundation of China (NSFC 81100073 to Dr Yan-yan Li), Excellent Young and Middle-Aged Teachers Assistance Program of Nanjing Medical University for Dr Yan-yan Li (2013-2015, JX2161015034), Jiangsu Overseas Research Training Program for University Prominent Young Middle-aged Teachers and Presidents, and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). Thank all our colleagues working in the First Affiliated Hospital of Nanjing Medical University.

Author Contributions:

X.Y. researched data. Y.L. and X.Y. wrote manuscript, researched data. Y.L. and X.Y. reviewed/edited manuscript. Y.L., X.Y., and G.G. contributed to discussion, reviewed/edited manuscript. Y.L., X.Y. G.G., and H.G. researched data, contributed discussion.

Disclosures

The authors have no other conflicts of interest to disclose.

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Figures

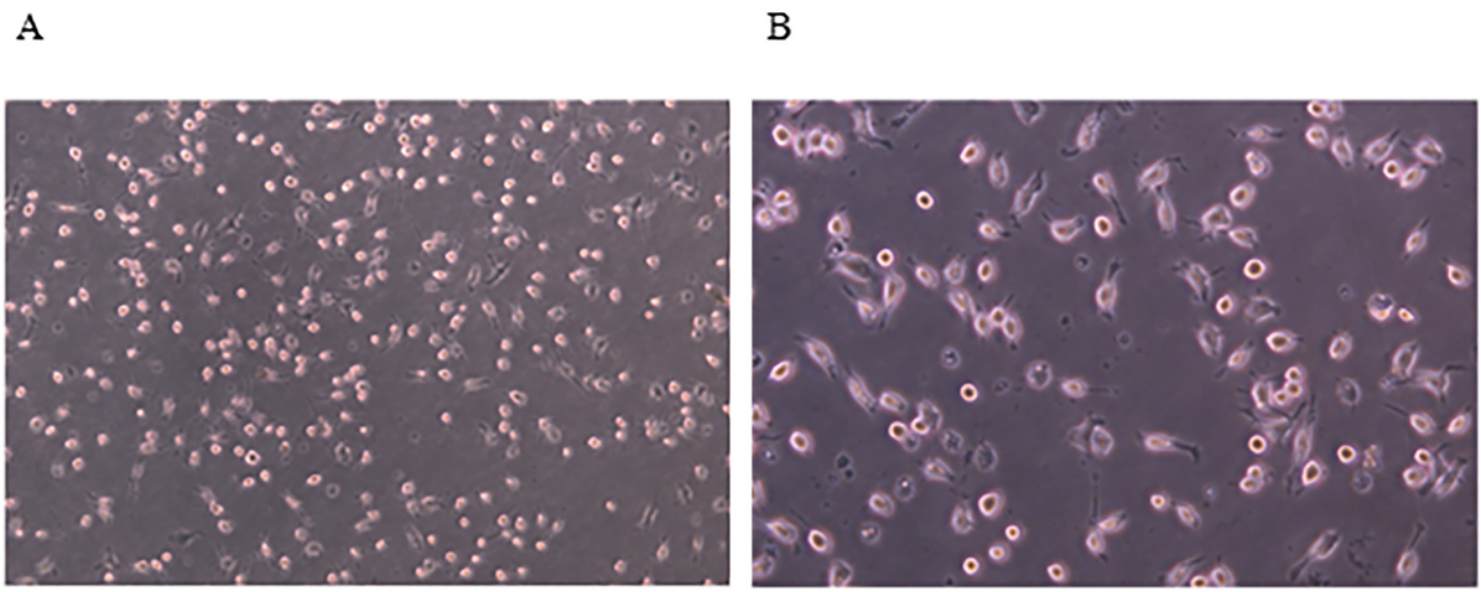

Figure 1

The morphological characteristics of murine BMDM

A. The normal cells shape under 100 x magnification

B. The normal cells shape under 200 x magnification

Figure 2

The CD11b-FITC, CD206-APC proteins localization and relative levels by immunostaining method in the murine BMDM hypoxia process

A. The M2 macrophages changes during the hypoxia process by immunofluorescence method

In the CD11b-FITC and CD206-APC double staining group, positive CD11b-FITC was green fluorescence, positive CD206-APC was red fluorescence, and blue was DAPI staining. The scale in each picture means 100um.

B. The M2 macrophages proportion changes during the hypoxia process

*P<0.05, compared with Hypoxia group; △P<0.05, compared with normal control group.

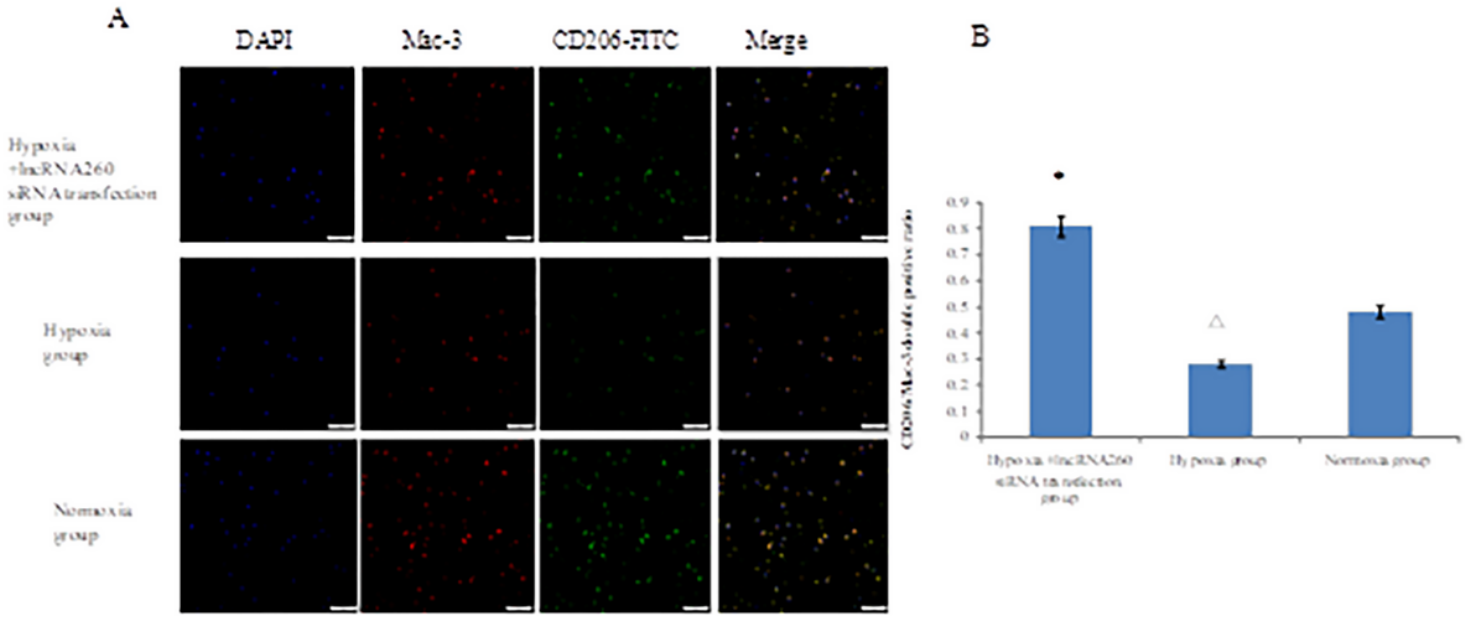

Figure 3

The Mac-3, CD206-FITC proteins localization and relative levels by immunostaining in the murine BMDM hypoxia process

A. The M2 macrophages changes during the hypoxia process by immunofluorescence method

In Mac-3 and CD206-FITC double staining group, positive CD206-FITC was green fluorescence, positive Mac-3 was red fluorescence, and blue was DAPI staining. The scale in each picture means 100um.

B. The M2 macrophages proportion changes during the hypoxia process

*P<0.05, compared with Hypoxia group; △P<0.05, compared with normal control group.

Figure 4

The expression changes of Arg 1, p-AKT and PI3KCG proteins detected by Western blot in the murine BMDM hypoxia process

A. The western blot developing figure for the Arg 1, p-AKT and PI3KCG proteins in the murine BMDM hypoxia process

B. The ratios changes of Arg 1/β-Actin, p-AKT/β-Actin, PI3KCG/β-Actin in the murine BMDM hypoxia process

*P<0.05, compared with Hypoxia group; △P<0.05, compared with normal control group.

Figure 5

The expression changes of IL28RAV1 and IL28RAV2 proteins detected by Western blot in the murine BMDM hypoxia process

A. The western blot developing figure for the IL28RAV1 and IL28RAV2 proteins in the murine BMDM hypoxia process

B. The ratios changes of IL28RAV1/β-Actin, IL28RAV2/β-Actin in the murine BMDM hypoxia process

*P<0.05, compared with Hypoxia group; △P<0.05, compared with normal control group.