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Low-expression of miR-1929-3p mediates murine cytomegalovirus-induced fibrosis in cardiac fibroblasts via targeting endothelin A receptor/NLRP3 inflammasome pathway

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Abstract

It has shown that mouse cytomegalovirus (MCMV) infection induced low-expression of miR-1929-3p is closely related to hypertensive myocardial remodeling in our previous research. In this study, we further explored the molecular mechanism of myocardial remodeling induced by miR-1929-3p after MCMV infection on cellular level. We built MCMV infected mouse cardiac fibroblasts (MMCFs) as main cell model. First, MCMV infection caused low-expression of miR-1929-3p and higher mRNA and protein expression of its target gene endothelin receptor type A (ETAR) in mouse cardiac fibroblasts (MCFs), which showed an internal relation to myocardial fibrosis (MF) on high proliferation, phenotypic transformation (α-SMA) and collagen expression in MMCFs. Then, transfection of miR-1929-3p mimic down-regulated the high expression of ETAR and relieve these adverse effects in MMCFs. Inversely, these effects were exacerbated by miR-1929-3p inhibitor. Second, transfection of endothelin receptor type A over-expressed adenovirus (adETAR) reversed these positive effects of miR-1929-3p mimic about MF improvement. Third, it presented strong inflammatory response in MMCFs with more expression of NOD like receptors pyrin domain containing 3 (NLRP3) and secretion of interleukin-18 (IL-18). However, we found ETAR antagonist BQ123 and selected NLRP3 inflammasome inhibitor MCC950 effectively eliminated the inflammatory response induced by both MCMV infection and miR-1929-3p inhibitor. Moreover, the supernatant of MCFs was shown to be related to cardiomyocyte hypertrophy. Our research revealed that MCMV infection promotes MF by inducing down-regulation of miR-1929-3p and high expression of ETAR which activate NLRP3 inflammasome in MCFs.

1. Introduction

Hypertension is an important risk factor for cardiovascular disease, and the aggravation and deterioration of target gene damage caused by hypertension is one of the important factors leading to death. Hypertension causes up to 7.6 million deaths worldwide every year. Studies have shown that the incidence of hypertension increases synchronously with the aging of the population. Regarding the pathogenic factors of hypertension, it has been reported that there are genetic genes, gender and race, environmental and diet, disease status and so on [1, 2].

It is worth noting that there is a lot of evidence that hypertension is associated with human cytomegalovirus (HCMV) infection. HCMV is a ubiquitous β-herpesvirus as a member of the CMV family. It has the largest human herpesvirus genome (230kb) and encodes a variety of genetic products, many of which play an immunomodulatory role in the host [3, 4]. Several studies have found that HCMV infection can increase arterial blood pressure by inducing the expression of renin and angiotensin Ⅱin human vascular endothelial cells in a dose-dependent manner [5]. HCMV can damage endothelial function by reducing the expression of mRNA through hypermethylation of regulator of G protein signaling 5 (RGS5) gene. HCMV can also exert adverse effects on cardiovascular system through renin-angiotensin-aldosterone system (RAAS) activation, oxidative stress, inflammatory reaction, endothelial dysfunction, epigenetic modification leading to changes in gene expression and so on. In the end, these effects may independently or jointly lead to the occurrence and development of hypertension [6].

Hypertensive left ventricular hypertrophy plays a very important role in the compensation and decompensation of cardiac structure and function. Histologically, myocardial remodeling is characterized by myocardial hypertrophy and myocardial fibrosis (MF) [7]. In recent years, more and more attention has been paid to the role of cardiac fibroblasts (CFs) in MF. There are large numbers of stable fibroblasts in the myocardial stroma of normal adults. New phenotypic characteristics were obtained by activating cardiac fibroblast subsets after heart injury. They transform into smooth muscle-like fibroblasts, which called myofibroblasts, and express α-smooth muscle actin (α-SMA) [8]. Cardiac myofibroblasts break the homeostasis of extracellular matrix (ECM) synthesis and degradation, and excessive accumulation of collagen and lead to fibrosis at last [9]. Previous study indicated transforming growth factor β (TGF-β) induces the translocation of the SMAD2–SMAD3 complex of transcription factors into the nucleus, where it directly promotes the expression of ECM genes such as COL1A1, COL3A1 and tissue inhibitor of metalloproteinase 1 (TIMP1), as well as about 60 other ECM-related genes [10]. Recent studies have shown an emerging role of reactive oxygen species (ROS) and mitochondrial function in CFs proliferation and activation. Specifically, ROS production induced by mitochondrial fission activates the phosphorylation of p38-MAPK to increase proliferation and collagen production in rat CFs [11, 12]. Another study found EGFR and related downstream signaling pathways were partially activated by increased thrombospondin motif type 8 (ADAMTS8) secretion, thereby promoting fibroblast activation in vitro [13].

MicroRNA (miRNA) is a kind of small endogenous non-coding RNA, that regulates the expression of RNA after transcription [14]. In the heart, miRNA regulates post-transcriptional gene expression and has been shown to regulate cardiovascular development, inflammation (miR-155 and miR-221/222), hypertrophy, fibrosis (miR-21, miR-208b and miR-125b), regeneration (miR-302-367and miR-99/100 family), and vascular function [15, 16]. Our previous studies have found that murine cytomegalovirus (MCMV) induced low expression of miR-1929-3p in C57BL/6 mice and targeted regulation of endothelin receptor type A (ETAR), induced hypertensive myocardial remodeling in mice through inflammatory response dominated by activation of NOD like receptors pyrin domain containing 3 (NLRP3) inflammasome. This phenomenon can be effectively alleviated by the intervention of miR-1929-3p overexpression of adeno-associated virus [17]. Based on the above research, this study intends to explore the role of down-regulation of miR-1929-3p induced by MCMV infection in mouse cardiac fibroblasts (MCFs) and mouse cardiomyocytes (MCMs), and its relationship with inflammation in mice at the cellular and molecular level, and to further clarify the role and mechanism of MCMV in the occurrence and development of hypertension and myocardial remodeling, hoping to provide new ideas for clinical prevention and treatment of hypertension.

2. Materials And Methods

2.1. Cells and Cell Culture

MCFs and MCMs of C57BL/6 mice were purchased from Beinachuanglian BioTechnology Corporation Limited (BNCC, http://www.bnbio.com). MCFs and MCMs were adherent cell, and were given RPMI-1640

medium containing 10% FBS and 100 U/mL penicillin-streptomycin solution, which cultured at 37℃ with 5% CO_2 saturation. When the number of cells grew to about 80% of the culture flask area, the subculture was carried out, and the logarithmic growth phase cells with active proliferation were selected for the experiment.

2.2. Virus Infection and CCK8 Cell Proliferation Assay

MCMV Smith strain was donated by Wuhan Institute of Virology, Hubei Province, and stored at -80℃. MCMV infection was detected by agarose gel electrophoresis. CCK8 Cell Proliferation Assay Kit from Biosharp Life Sciences, China. MCFs were inoculated into the 96-well plate according to the instruction. After adhesion, MCFs were treated at the designed time points (0 h, 12 h, 24 h, 48 h, 72 h) according to the experimental requirements (multiplicity of infection (MOI) = 0, 0.01, 0.05, 0.1). The absorbance value at 450 nm of each well was measured, and the cell viability and proliferation rate were calculated to select the optimal MOI for MCMV infection.

2.3. Real-time Quantitative Polymerase Chain Reaction (qRT-PCR)

Primer design was commissioned by Shanghai Biotechnology Company, China. Total RNA was extracted by Trizol method and controlled by agarose gel electrophoresis. QRT-PCR was performed according to the instructions of SYBR@ Green Reagent.

The sequences of primers (Shanghai living creature, China) are as follows:

β-actin-F: 5'-CTGGCCTCACTGTCCACCTT − 3'

β-actin-R: 5'-CGGACTCATCGTACTCCTGCTT − 3'

Ednra-F: 5'-TCACCGTCTTGAACCTCTGTGC-3'

Ednra-R: 5'-GATGGAGACGATTTCAATGGCGG-3'

mmu-miR-1929-3p-F: 5'-ACACTCCAGCTGGGCAGCTCATGGAGACCT-3'

mmu-miR-1929-3p-R: 5'-TGGTGTCGTGGAGTCG-3'

U6-F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'

U6-R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'

collagen Ⅰ-F: 5'-TGGTACATCAGCCCGAAC-3'

collagen Ⅰ-R: 5'-GTCAGCTGGATAGCGACA-3'

collagen Ⅲ-F: 5'-TCCTAACCAAGGCTGCAAGATGGA-3'

collagen Ⅰ-R: 5'-AGGCCAGCTGTACATCAAGGACAT-3'

All gene expression levels were calculated using the 2 − ΔΔCt method.

2.4. MiR-1929-3p Mimics and Inhibitors Transfection

MiR-1929-3p mimics and inhibitors were customized by Thermo Fisher Scientific, US. MCFs with fusion growth of 60–80% were selected to be planked. According to the instructions, miR-1929-3p mimic and inhibitor were transfected into MCFs with concentrations of 20nM and 30nM, respectively. Dilute miRNA was added to dilute Lipofectamine 2000 at a ratio of 1:1 and incubated for 5 minutes. Add miRNA-lipid complex to cells, Incubate cells for 24 hours at 37°C for subsequent research.

2.5. ETAR Adenovirus Overexpression Vector Transfection and Identification

The ETAR adenovirus overexpression vector with green fluorescent protein was customized by Thermo Fisher Scientific, US. The vector can effectively overexpress ETAR in MCFs within 12–24 hours after adding into the culture system. MCFs with good growth condition was selected to plank with lower cell density. Proper ETAR adenovirus overexpression vector diluted by RPMI-1640 medium was added according to the instruction and cultured for 8–12 hours. The transfection efficiency was evaluated by observing the fluorescence intensity of MCFs.

2.6. Drug Intervention

MCC950 Sodium (CP-456773 sodium, Selleck) is an effective, selective NLRP3 inhibitor that has no effect on AIM2, NLRC4 or NLRP1 inflammasomes. BQ-123 sodium salt (B150, Sigma) is an endothelin receptor antagonist. Their working concentrations were 100 nmol and 10 nmol respectively for MCFs.

2.7. Western Blot

Total protein was extracted from cells for SDS-PAGE electrophoresis, then transferred to PVDF membrane via transmembrane. After the non-specific antigen was sealed, the primary antibody (Abcam, UK) was given and incubated for 2–3 hours. The dilution concentrations of protein antibodies was β-actin: 1:1000, α-SMA: 1:1000, ETAR: 1:500, NLRP3Ⅰ1:1000, caspase-1Ⅰ1:1000, IL-18Ⅰ1:1000, IL-1βⅠ1:1000, respectively. The second antibody was goat anti-rabbit or goat anti-rat labeled by HRP and the dilution concentration was 1:10000. Finally, ECL luminescence reagent was used to obtain the protein bands.

2.7. EDU Cell Proliferation Assay

BeyoClick™ EdU Cell Proliferation Kit (C0088S, Beyotime Biotechnology, China) with TMB was used to detect cell proliferation quantitatively. Cardiac fibroblasts were first labeled with EdU, followed by fixation, washing, permeability and staining. The absorbance was measured at 370nm after the sample was colored.

2.8. Immunofluorescence Staining

The actin in MCMs was stained by Actin-tracker Green-488 probe (C2201S, Beyotime Biotechnology, China). Cells were fixed with 3.7% formaldehyde solution prepared by PBS and incubated with Actin-Tracker Green dilution for 60 minutes in darkness. After washing, the cells were observed under the fluorescence microscope.

2.9. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA kits for IL-1β and IL-18 detection were purchased from Wuhan Hualianke Biotechnology Company, China. The cell supernatant was centrifuged briefly for gradient dilution, the sample and standard were then added to the soaked microplate meter respectively. In this experiment, 6 multiple wells were set up and the detection antibody and streptavidin diluted solution were added to the wells for incubation. the OD value was detected by a microplate meter after the color development stopped.

2.10. Statistical Analysis

The SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA) was utilized for statistical analysis. Measurement data were presented as the mean ± standard deviation. The t test was adopted for comparison between two groups. P 0.05 indicated a statistically significant difference.

3. Results

3.1. Identification of MCMV Infection and Determining MOI of MCMV for MCFs infection.

The detection of an immediate early gene (IE) in the host is the evidence of early CMV infection [18]. Both MCFs and MCMs were infected with MCMV in the same condition. Then, IE was detected in MCFs but not MCMs which reflecting the successful establishment of the infection model in MCFs (supplementary figure 1). It was known that MF was accompanied by excessive proliferation of CFs. The MCMV strain used in this study was not attenuated to simulate the natural infection in the body more accurately. Meanwhile, to minimize the influence of the toxic effect of infection itself on MCFs by promoting proliferation, we screened and identified the optimal MOI of MCMV with CCK8 cell proliferation assay. It showed concentration-dependent and time-dependent manner in the effect of MCMV infection on the proliferation activity of MCFs (P 0.05, Figure 1A and1B). With the prolongation of infection time and the increase of MOI, the number of proliferative cells in the culture system increased gradually. However, this growing tendency was broken while the MOI up to 0.05 and 0.1 after infected for 72 hours and proliferation activity of the MCFs decreased visibly. On this basis, we selected the condition with the maximum cell proliferation rate, namely MOI=0.01 and Time=48h, as the optimal MOI and time.

3.2. ETAR was negatively regulated by miR-1929-3p as its target gene in MCFs.

The results of qRT-PCR showed that the expression of miR-1929-3p was significantly down-regulated after MCFs infection for 48 hours. miR-1929-3p-mimic and miR-1929-3p-inhibitor significantly up-regulated and down-regulated the expression of miR-1929-3p after MCFs infection, respectively, suggesting that they might play a regulatory role in the downstream by directly changing the concentration of miRNAs (P<0.05, Figure 1C). However, it was not significantly changed in MCMs with the same MOI (P>0.05, Figure 1D), which further indicated that MCMs was not the host cell of MCMV. Based on this intervention, we used qRT-PCR and western blot to detect the mRNA and protein expression levels of ETAR in MCFs after different treatments, respectively. The results showed that the expression of ETAR was increased after MCMV infection. Based on infection, we gave appropriate exogenous miR-1929-3p-mimic and inhibitor to increase or decrease the expression of miR-1929-3p in MCFs. Then, it showed that low level of miR-1929-3p led to a surge of ETAR expression in MCFs. On the contrary, high level of miR-1929-3p effectively eliminated the increased expression of ETAR caused by MCMV infection (P<0.05, Figure 1E). These results proved that ETAR was negatively regulated by miR-1929-3p as its target gene in MCFs.

3.3. Down-regulated miR-1929-3p Promoted the Proliferation, Phenotypic Transformation and Collagen Expression of MCFs after MCMV infection.

During cardiac repairment, myofibroblasts express contractile proteins such as α-SMA and secrete ECM which consist largely of collagen I and Ⅲ [19]. To figure out the effect of miR-1929-3p on the pro-fibrosis index of MCFs after infection with MCMV, we explored in three aspects. First, the proliferation activity of MCFs was detected by EDU Cell proliferation assay. It showed that the proliferation activity of MCFs was significantly increased after MCMV infection (P<0.05, Figure 2A and 2B). Second and third, the expression of α-SMA protein was evaluated by western blotting to represent the transformation of MCFs into myofibroblasts, and the mRNA expression of type Ⅰ and Ⅲ collagen was quantified by real-time quantitative PCR. The over-expression of collagen gene occurred with the activation of MCFs (P<0.05, Figure 2C, 2E and 2F), suggesting that the pro-fibrosis effect had appeared. After intervention with miR-1929-3p inhibitor, it was found that the above effects on MCFs proliferation, activation and collagen expression were further strengthened. In contrast, miR-1929-3p mimic effectively reversed the pro-fibrotic effect induced by MCMV infection (P<0.05, Figure 2).

3.4. Overexpressed ETAR could eliminate the beneficial effect of miR-1929-3p mimic on alleviating MF infected with MCMV.

We have demonstrated that MCMV infection promoted MF through down-regulated miR-1929-3p in MCFs. Meanwhile, miR-1929-3p targeted ETAR at the post-transcriptional level. To further explore the

effect of ETAR on MF after MCMV infection, we constructed ETAR adenovirus overexpression vector and intervened on MCFs. We transfected ETAR overexpressed adenovirus vector with multiple of infection (MOI) of 10000, 5000 and 100 into MCFs respectively. The immunofluorescence result showed that when transfected for 12h or 24h with MOI of 5000 or 10000, the transfection efficiency was more than 80%. Then, we selected transfection time of 12h and MOI=5000 as the effective transfection concentration (supplementary figure 1). The results revealed that overexpressed ETAR could even eliminate the beneficial effect of miR-1929-3p mimic on alleviating MF, which was specifically manifested as increased proliferation rate of MCFs (P<0.05, Figure 3A and 3B), promoted MCFs activation that with more protein expression of α-SMA (P<0.05, Figure 3C and 3D), and more mRNA expression of collagen Ⅰ and Ⅲ compared with MCMV+miR-1929-3p-mimic group (P<0.05, Figure 3E and 3F).

3.5. Down-regulated miR-1929-3p Induces NLRP3 Inflammasome Activation Via ETAR was mediated by MCMV infection.

Our previous studies showed that hypertension and pathological myocardial and vascular remodeling in C57 mice after intraperitoneal infection of MCMV were accompanied by NLRP3 inflammasome-mediated inflammatory response. In this study, to investigate whether there is NLRP3 inflammasome activation in MCFs infected with MCMV, we chose the selective NLRP3 inhibitor MCC950 for intervention. First, The result of western blot displayed that the proteins expression of mature NLRP3, caspase-1 and IL-18 in MCFs increased after MCMV infection (P<0.05, Figure 4A-D). Subsequent result of ELISA showed that the concentration of mature IL-18 released into the supernatant from MCFs was also increased (P<0.05, Figure 4F), suggesting that NLRP3 inflammasome were activated in MCFs after MCMV infection. Besides, these inflammatory cytokines were further increased in MCFs with intervention of miR-1929-3p inhibitor (P<0.05, Figure 4A-D, F). Moreover, with the selective NLRP3 inhibitor MCC950 intervention, NLRP3, caspase-1 protein expression as well as IL-18 protein production and secretion were significantly decreased compared with the MCMV + miR-1929-3p inhibitor group (P<0.05, Figure 4A-D, F), which indicated that MCMV infection activated NLRP3 inflammasome by down-regulated miR-1929-3p and MCC950 effectively reversed this effect.

Finally, to further explore the role of ETAR, the target gene of miR-1929-3p, in MCMV-induced inflammasome activation, we used ETAR specific blocker BQ123, which specifically blocked ETAR activity without affecting the binding of ET-1 to endothelin receptor type B (ETBR) [1]. The results of western blot suggested that BQ123 effectively reduced the expression and secretion of NLRP3, caspase-1 and IL-18 in the MCMV group (P < 0.05, Figure 4A-D, F). This indicated that ETAR played the pro-inflammatory effect and participated in MCMV-induced myocardial remodeling. Besides, the results of western blot and ELISA demonstrated that the expression and secretion of IL-1β did not change between the control group with other groups (P>0.05, Figure 4E and 4G), and it was only affected by MCC950 in contrast to the MCMV+ miR-1929-3p-inhibitor group (P<0.05, Figure 4E, G). Above-mentioned results suggested that ETAR, as a

target gene, was involved in the activation of NLRP3 inflammasome by low expressed miR-1929-3p after MCMV infection and so that took part in MF.

3.6. MCFs Promote MCMs Hypertrophy Through Paracrine Effect After MCMV Infection.

The preceding experimental results suggest that MCMs was not the host cell of MCMV. It was known that MCFs has strong autocrine and paracrine functions. So, to understand whether MCFs might affect MCFs through paracrine effect after MCMV infection in the experimental system of this study, we compared the possible effects of MCFs culture supernatant before and after MCMV infection on MCMs cell size and myocardial hypertrophy. Immunofluorescence results showed that MCMs became larger after stimulated by infected supernatant, and the myofilaments in the cells increased and became thick and disordered (P 0.005 Figure 5A and 5B). The mRNA expression of BNP and β-MHC in MCMs was also significantly increased ($P < 0.05$, Figure 5C and 5D). The above experimental results fully suggest that MCFs may stimulate MCMs to produce hypertrophic response through paracrine effect.

4. Discussion

We explored the possible mechanism of pathological myocardial remodeling induced by miR-1929-3p in mice after MCMV infection on the cellular and molecular level. Here, MCMV tended to infect MCFs in myocardial tissue, whereafter, up-regulated the expression of ETAR through the endogenous low expression of miR-1929-3p in MCFs. Subsequently, it caused myocardial remodeling by promoting the proliferation, activation and autocrine of MCFs and paracrine of MCFs on MCMs. The specific mechanism was related to the activation of NLRP3 inflammasome as well as the mature and release of IL-18 induced by high expression of ETAR. HCMV is a kind of double-stranded DNA virus in Herpesviridae, which has species-specific infection to the body. HCMV has a high latent infection rate in the population, which is a conditional pathogenic pathogen. It is related to a variety of cardiovascular diseases including essential hypertension, atherosclerosis, and coronary heart disease [20]. Our previous studies have shown that MCMV infection leads to hypertensive ventricular remodeling in mice, which is closely related to the low expression of cardiovascular miR-1929-3p. urthermore, MCFs are the most abundant cell types in the heart and play a key role in regulating normal myocardial function and adverse myocardial remodeling caused by hypertension, myocardial infarction, and heart failure [21]. HCMV can widespread attack human tissues and cells while MCMV specifically infects mice and is not pathogenic to humans. In the laboratory, MCMV strain can be obtained in large quantities by infection with primary mouse embryonic fibroblasts or homologous cell lines [22]. However, there is few research on MCMV-infected myocardial tissue cells in mice. To explore the cellular and molecular mechanism of myocardial remodeling caused by MCMV infection, MCMs and MCFs were selected as the research objects in the experimental design, and it was found that MCMV was more likely to infect the latter. Moreover, the expression of miR-1929-3p in MCMs after the same MOI intervention at the same time did not show a change consistent with the previous overall level, further indicating that MCMs were not the host cells of MCMV. It is well known that

stable cardiac fibroblasts undergo phenotypic transformation during myocardial remodeling, and their proliferation ability is significantly enhanced. The MCMV Smith strain used in this study is a non-toxic pathogen, which can reduce cell viability. In our vivo studies, MCMV infection caused myocardial remodeling in mice with hypertension, which theoretically promoted the proliferation of MCFs. When MCFs were infected with MOI = 0.1 for 72 hours, its proliferation activity was obviously decreased, and the cell morphology was significantly abnormal or even dead. The CCK8 cell proliferation assay showed that the proliferation rate of MCFs was the highest under the conditions of MOI = 0.01 and time = 48 hours, which was chosen as the optimal condition for infection in subsequent experiments in this study.

Myocardial remodeling is the main mechanism of disability and death caused by cardiovascular diseases such as hypertension, heart failure and myocardial infarction. This involves the overall structure and morphology of the heart, as well as changes in CMs and non-CMs cells and subcellular levels, which adversely affect cardiac function. Our previous studies have shown that MCMV infection can induce hypertensive myocardial remodeling in mice. This study suggests that MCFs are the key cells to induce this effect. On the one hand, it was found that MCMV did not successfully infect MCMs, and the expression of miR-1929-3p in cells did not change significantly after intervention. Namely, MCMV may not induce myocardial hypertrophy by directly inducing the expression changes of related regulatory factors in MCMs. On the other hand, although CMs are essential for cardiac pumping, the main cell type of the heart is CFs. The main role of CFs is to secrete collagen to cause ECM deposition, which maintains the integrity of cardiac structure and function. Excessive collagen deposition or pathological fibrosis is an important cause of left ventricular dysfunction and adverse outcomes in patients with hypertension, myocardial infarction, and heart failure. In fact, in our study, MCMV showed a tendency to infect MCFs, and miR-1929-3p expression was significantly down-regulated. CFs are the major regulator of ECM metabolism, maintaining the balance between the synthesis and degradation of ECM components. Specifically, the key aspects of CFs involved in cardiac remodeling-related functions include proliferation, migration, differentiation, ECM conversion and secretion of growth factors and cytokines [23]. In addition, CFs mediate many biological processes through differentiation into myofibroblasts, which often show high sensitivity to pro-inflammatory cytokines (including IL-1, IL-6, TNF-α) and vasoactive peptides (including Ang II, ET-1) that cause adverse myocardial remodeling [24]. We found that MCFs proliferated actively after MCMV infection and showed phenotypic transformation to myofibroblasts. The outcome of these changes was the synthesis of type I and type III collagen. In other studies, it has been found that there is ‘crosstalk’between CFs and CMs. In a rat model of aortic coarctation, hypoxia-induced mitogens induce CFs migration, proliferation and myofibroblast differentiation through CMs-CFs paracrine [23]. We added the supernatant of MCFs, which was cultured for 48 hours after infection, into MCMs medium and measured cell size of MCMs after 24 hours. It is well known that CFs can secrete various types of cytokines. The above phenomena suggested that MCFs may release some cytokines or gene products to induce myocardial hypertrophy through paracrine effect. MCMV infection could cause myocardial remodeling directly and indirectly. Of course, the specific mechanisms of paracrine mediators and occurrence need further exploration, but the leading role of MCFs in myocardial remodeling induced by MCMV infection in mice with hypertension was beyond doubt.

Studies have found that ET-1 levels in myocardium and circulation are elevated in patients with heart failure and experimental models of heart failure. ET-1 mainly increases collagen synthesis by stimulating CFs from different species (including humans), thereby promoting MF. ETAR and ETBR are both involved in this process. There is evidence that ET-1 can reduce the activity of collagenase through ETAR. These two receptors can be expressed by CFs and myofibroblasts in adult and rat, and the expression of ETBR is dominant. Other studies have shown that ET-1 can promote the proliferation of CFs in neonatal rats and adult rats by activating ETAR and induce the expression of myofibroblast phenotypic marker α-SMA in neonatal rats [24, 25]. In previous study, we found that ETAR is one of the target genes of miR-1929-3p related to hypertension by miRNA microarray combined with bioinformatics analysis and MCMV infection in mice. In this study, MCFs infected with MCMV was used as the cell disease model. We found that the expression of miR-1929-3p was down-regulated after MCMV infection, while the mRNA and protein expression of its target gene ETAR was increased. Over-expression of ETAR by adenovirus vector intervention significantly weaken the beneficial effect of miR-1929-3p mimic on reducing MF, which was manifested as the increase of MCFs proliferation activity, α-SMA protein expression, as well as collagen I and III mRNA levels. However, whether ETBR has a beneficial effect on MF after MCMV infection deserves further exploration. Combined with previous in vivo studies, we believe that the down-regulated miR-1929-3p induced by MCMV infection is involved in the occurrence of hypertensive pathological myocardial remodeling through high expression of ETAR, which may be one of the important links of hypertension and target organ damage induced by MCMV infection.

miRNA plays a negative regulatory role by inhibiting mRNA translation or promoting mRNA degradation. Traditionally, fibrosis is considered as an indirect response to myocardial cell death and/or injury-induced hypertrophy. A report on the role of miR-21 in CFs demonstrated its ability to regulate MAPK pathway. MiR-21 regulated cell proliferation in the reconstructed heart and increased the release of CFs growth factor, thereby promoting cardiomyocyte hypertrophy [26]. In another study, miR-21 negatively regulated the expression of phosphatase and tensin homolog (PTEN) deleted on chromosome 10 in CFs, resulting in an up-regulation of AKT signaling pathway and an increase in matrixmetallo proteinase-2 (MMP-2) expression [27]. According to the results of previous studies, we proposed that MCMV caused the decrease of miR-1929-3p expression in mice by infection, which further led to the increase of ETAR expression, leading to essential hypertension and adverse myocardial remodeling [17]. This study further proved that MCMV in myocardium mainly caused the low expression of miR-1929-3p in CFs, and MF was mainly caused by the activation of NLRP3 inflammasome mediated by ETAR. These new findings suggest that miRNAs expressed in CFs may be targets for the treatment of poor myocardial remodeling in the future. However, due to the individual differences of miRNA expression in the circulation among hypertensive patients, it is difficult to be used as a biomarker for diagnosis or screening of hypertension. Therefore, these multifunctional cells provide attractive but challenging therapeutic targets for future cardiovascular disease diagnosis and treatment. So far, many scholars have begun to develop small non-coding RNA-related therapeutic strategies. For example, miRNA has been developed for the treatment of liver fibrosis. In addition, a variety of viral and non-viral vectors have been developed for miRNA mimics [28].

NLRP3 inflammasome can be stimulated by a variety of signals and trigger inflammatory reactions to participate in myocardial remodeling. NLRP3 inflammasome can be stimulated by a variety of signals and trigger inflammatory reactions to participate in myocardial remodeling. In this study, the expressions of NLRP3, caspase-1 and IL-18 in CFs of MCMV infection group were increased, while miR-1929-3p inhibitor aggravated the above effect. This suggests that the mechanism of MCMV infection promoting the occurrence and development of MF may be related to the low expression of miR-1929-3p caused by MCMV infection and the activation of NLRP3 inflammasome induced by high expression of ETAR. Selective NLRP3 inflammasome MCC950 attenuated NLRP3 inflammasome activation and completely reversed the inflammatory effect induced by miR-1929-3p inhibitor, suggesting that the inflammatory response triggered by MCMV infection in MCFs was mainly mediated by down-regulated miR-1929-3p. In addition, we also compared the expression of inflammasome and their downstream effectors after MCMV infection and after the addition of ETAR antagonist BQ123. The results showed that ETAR as a target gene was indeed involved in the activation of MCMV-miR-1929-3p-ETAR-NLRP3 pathway in MCFs. Unfortunately, expression of IL-1β did not change after MCMV infection and after treatment with miR-1929-3p inhibitors, but it was reduced only by the effects of MCC950 drugs. This phenomenon is most likely to be affected by drugs themselves, but not MCMV infection and miR-1929-3p downregulation. We speculated that MCMV infection may selectively trigger the activation of IL-18 in MCFs and promote the survival and DNA replication of MCMV in host cells. At the same time, IL-18 is also the main effector molecule of NLRP3 inflammasome activation and participation in MF. From the perspective of disease treatment, inhibiting the activation of inflammatory cytokines may be more effective than blocking any of these downstream cytokines. However, this research limitation lies in has not explore the participation of other mesenchymal cells in cardiac tissue in myocardial remodeling caused by MCMV, and the specific mechanism of ETAR activating NLRP3 inflammasome in MCFs.

5. Conclusion

In summary, DNA replication was performed in MCFs after MCMV infection. As the initiating factor, it induced the role of MCFs and MCMs in promoting the occurrence and development of myocardial remodeling. This study showed that MCMV infected MCFs promoted MF by inducing the downregulation of miR-1929-3p expression in MCFs and the high expression of its target gene ETAR, and MCFs might cause myocardial hypertrophy through paracrine effect on MCMs. In short, down-regulated miR-1929-3p induced by MCMV infection is involved in myocardial remodeling through ETAR/NLRP3 inflammasome activation. In recent years, some successful experience has been achieved by interfering with miRNA adjuvant therapy for related diseases. Intervention of miR-1929-3p is expected to become a new method for prevention and treatment of adverse myocardial remodeling caused by MCMV infection.

Declarations

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jiaqi Zhao, Yongjia Wang and Yuanyuan Qu contributed equally to this work.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

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Figures

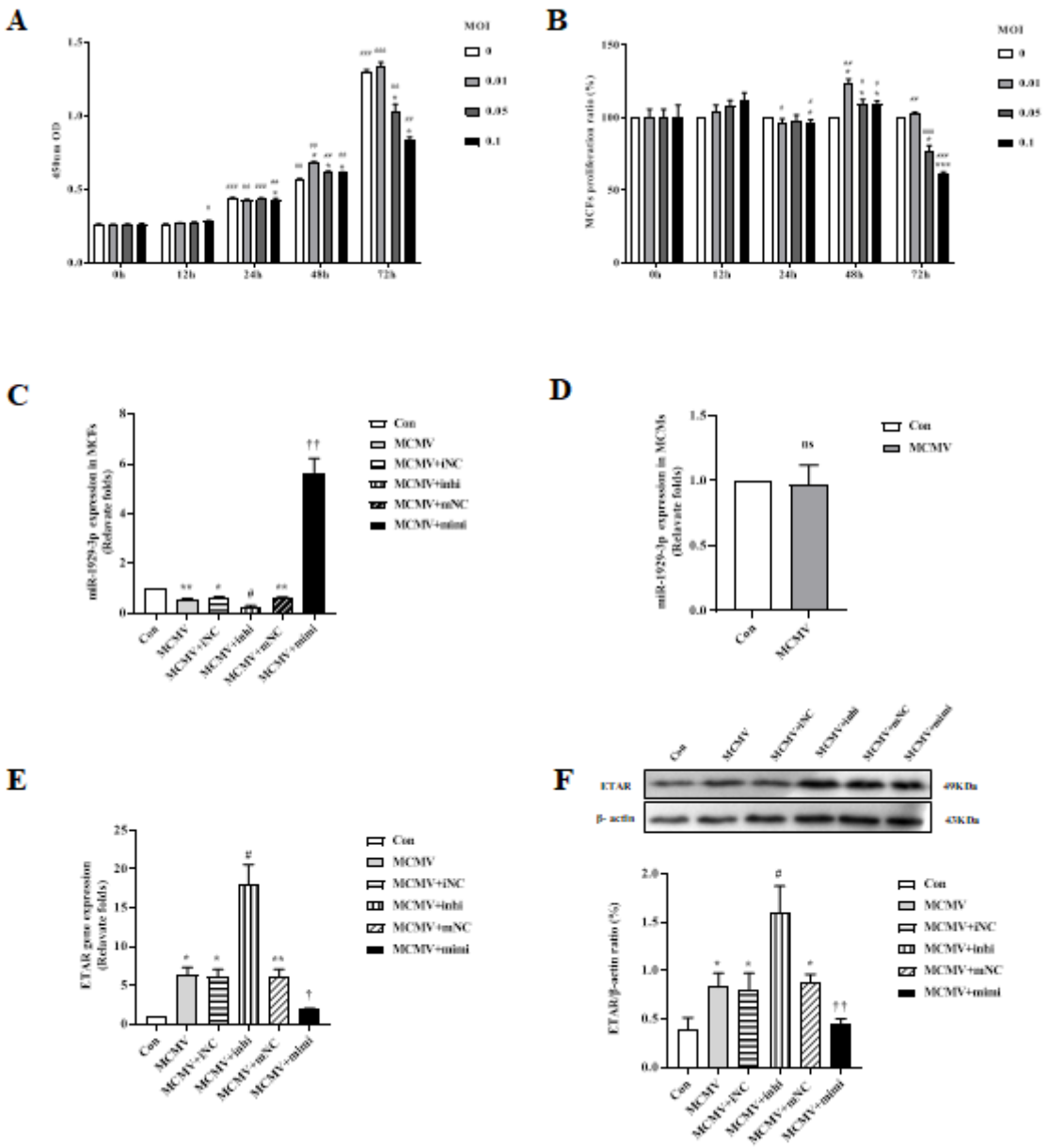

Figure 1

MCMV infection induced down-regulation of miR-1929-3p expression and increased ETAR expression in MCFs. **A-B** CCK8 cell proliferation assay results of MCFs at different MOI and different time infected by MCMV (n=5). $^{*}P<0.05$ and $^{***}P<0.001$ vs MOI=0 at the same point group, $^{\#}P<0.05$ $^{\#\#}P<0.005$ $^{\#\#\#}P<0.001$, vs the previous point with the same MOI group. **C-F** Results from the qRT-PCR analysis of the expression of miR-1929-3p, ETAR mRNA and western blot image and analysis of ETAR in MCFs and MCMs after MCMV infection (n=3). Con: control, MCMV+iNC: MCMV+miR-inhibitor-NC, MCMV+inhi: MCMV+miR-

inhibitor, MCMV+mNC: MCMV+miR-mimic-NC, MCMV+mimi: MCMV+miR-mimic. $^{*}P$<0.05 and $^{**}P$<0.005 vs Con group$^{\#}P$<0.05 vs MCMV+iNC group, $^{\dagger}P$<0.05 and $^{\dagger\dagger}P$<0.005 vs MCMV+mNC group.

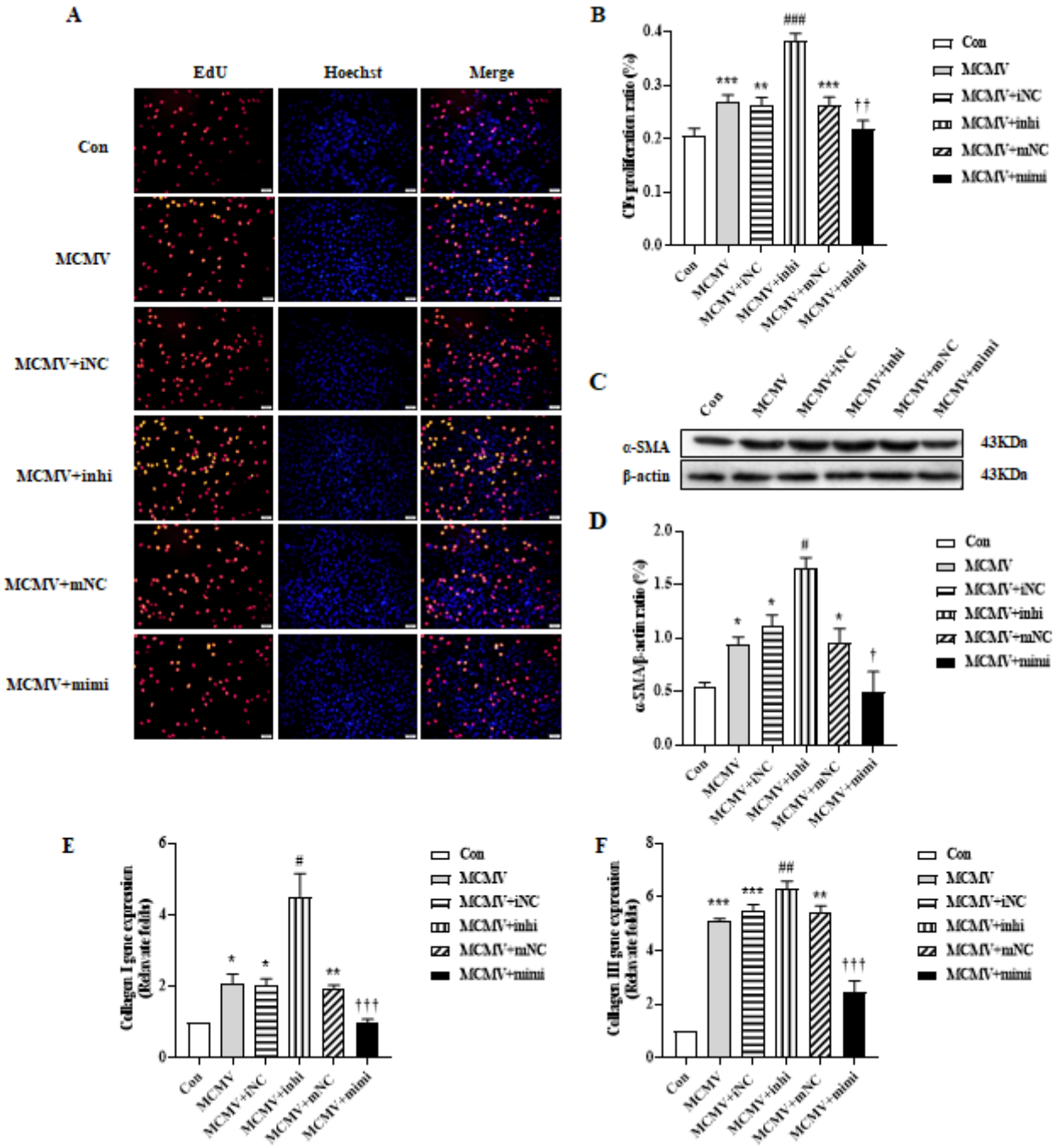

Figure 2

Down-regulated miR-1929-3p promoted MCFs proliferation, phenotypic transformation, and collagen expression. **A-B** Cell proliferation evaluated by EDU assay (n=3). Representative immunofluorescence images of MCFs were stained rad and all the nuclei were stained blue. **C-D** Western blot and the quantitative analysis of α-SMA in MCFs (n=3). **E-F** Results from the qRT-PCR analysis of the expression of

collagen Ⅰ and Ⅲ (n=3). $^{*}P$<0.05, $^{**}P$<0.005, and $^{***}P$<0.001 vs control group, $^{\#}P$<0.05, $^{\#\#}P$<0.005, and $^{\#\#\#}P$<0.001 vs MCMV+iNC group, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.005, and $^{\dagger\dagger\dagger}P$<0.001 vs MCMV+mNC group.

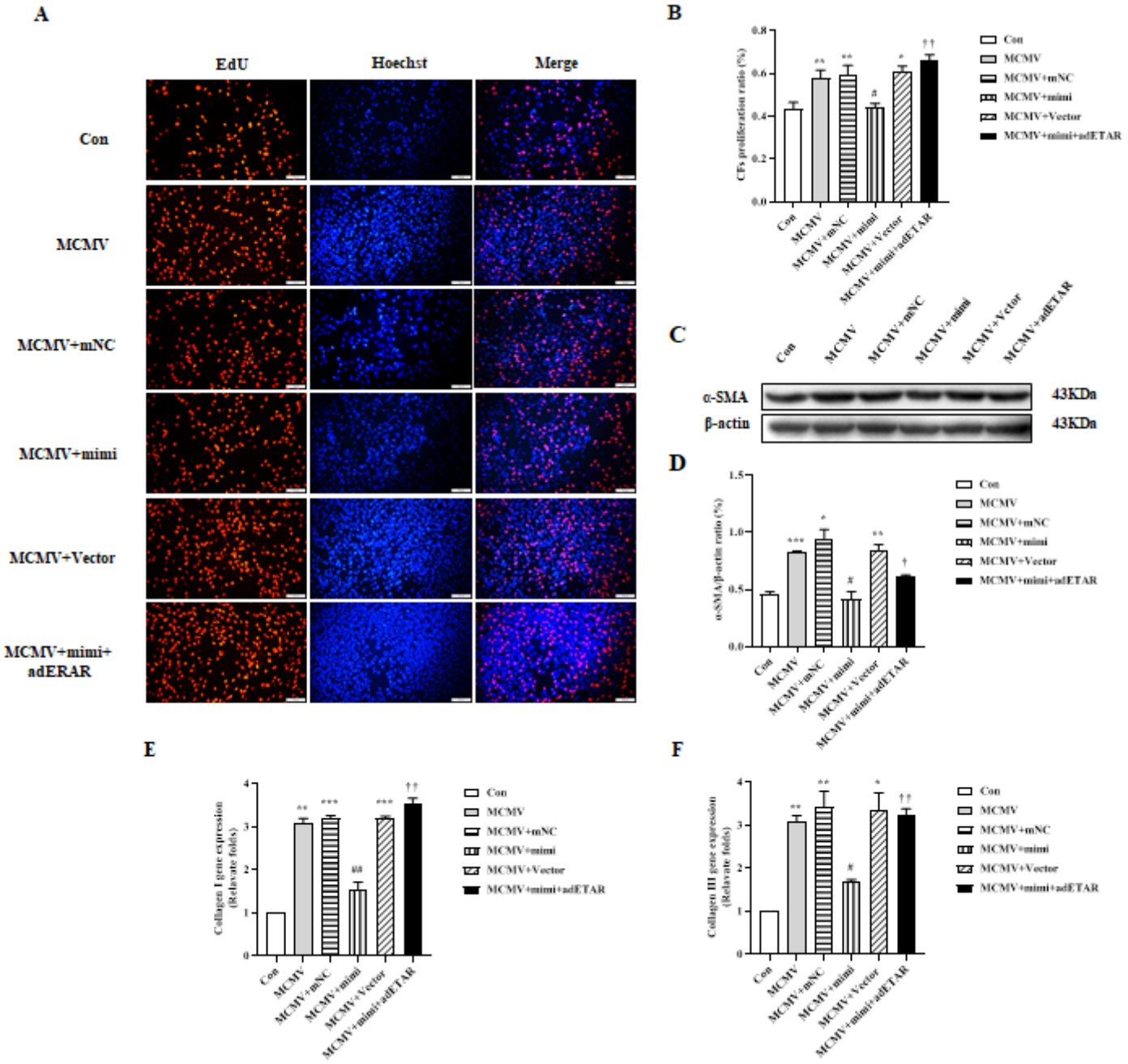

Figure 3

Low expressed miR-1929-3p promote MF through ETAR. **A-B** Cell proliferation evaluated by EDU assay (n=3). Representative immunofluorescence images of MCFs were stained rad and all the nuclei were stained blue. **C-D** Western blot analysis and the quantitative analysis of α-SMA in MCFs (n=3). **E-F** Results from the qRT-PCR analysis of the expression of collagen Ⅰ and Ⅲ (n=3). MCMV+mimi+adETAR: MCMV+miR-mimic+adETAR. $^{*}P$<0.05, $^{**}P$<0.005, and $^{***}P$<0.001 vs control group, $^{\#}P$<0.05, $^{\#\#}P$<0.005, and $^{\#\#\#}P$<0.001 vs MCMV+mNC group, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.005, and $^{\dagger\dagger\dagger}P$<0.001 vs MCMV+mimi group.

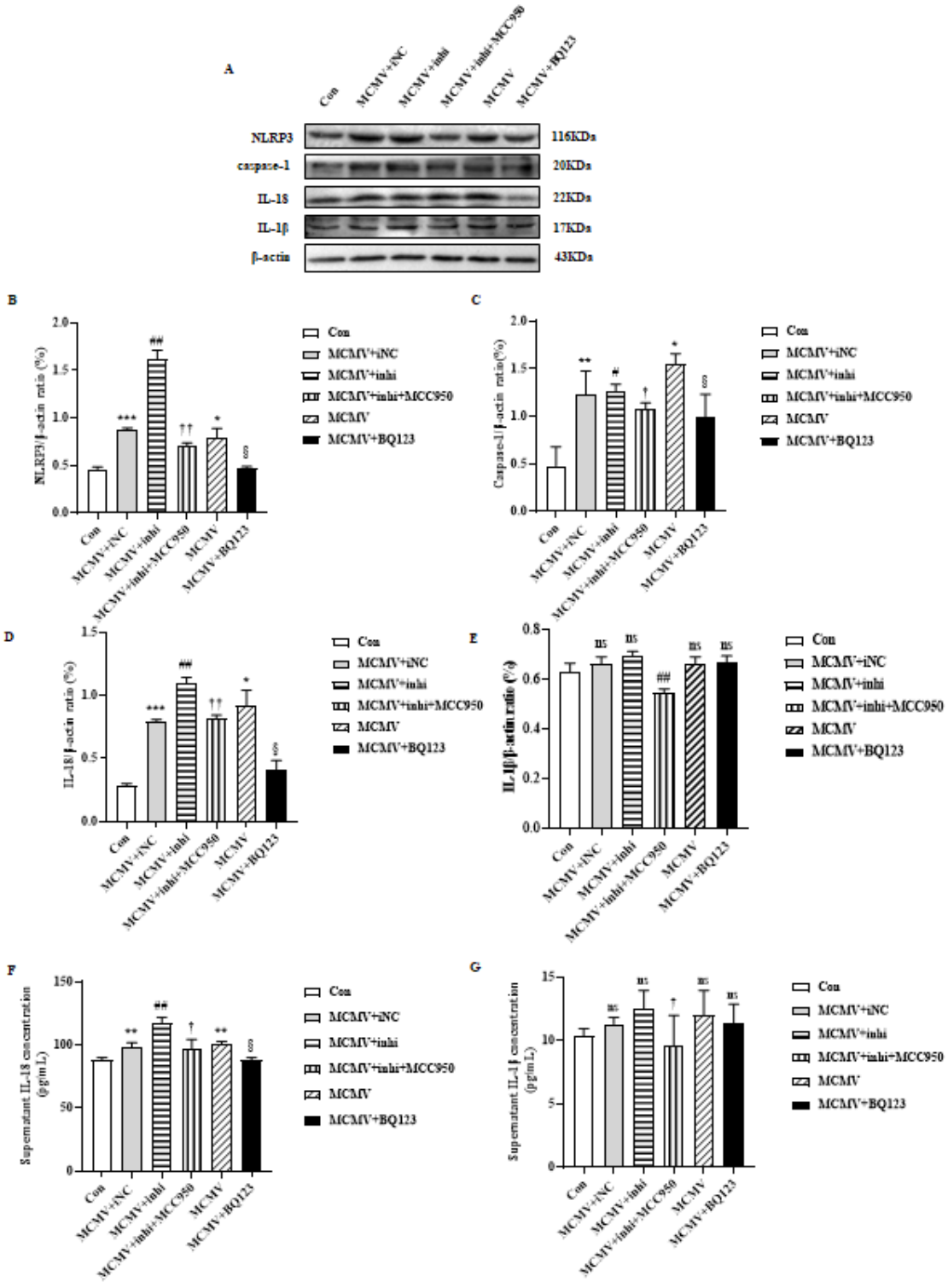

Figure 4

Low expressed miR-1929-3p induces NLRP3 inflammasome activation via ETAR. **A-E** Western blot analysis and quantitative analysis of NLRP3, caspase-1, IL-18 and IL-1β in MCFs (n=3). **F-G** Results from the ELISA analysis of the expression of IL-18 and IL-1β secreted in supernatant of MCFs (n=3). MCMV+inhi+MCC950: MCMV+miR-inhibitor+MCC950. Ns means P□0.05 vs control group, $^{*}P$<0.05,

$^{**}P$<0.005 and $^{***}P$<0.001 vs control group, $^{\#}P$<0.05 and $^{\#\#}P$<0.005 vs MCMV+iNC group, $^{\dagger}P$<0.05 and $^{\dagger\dagger}P$<0.005 vs MCMV+inhi group, $^{\S}P$<0.05, $^{\S\S}P$<0.005 and $^{\S\S\S}P$<0.001 vs MCMV group.

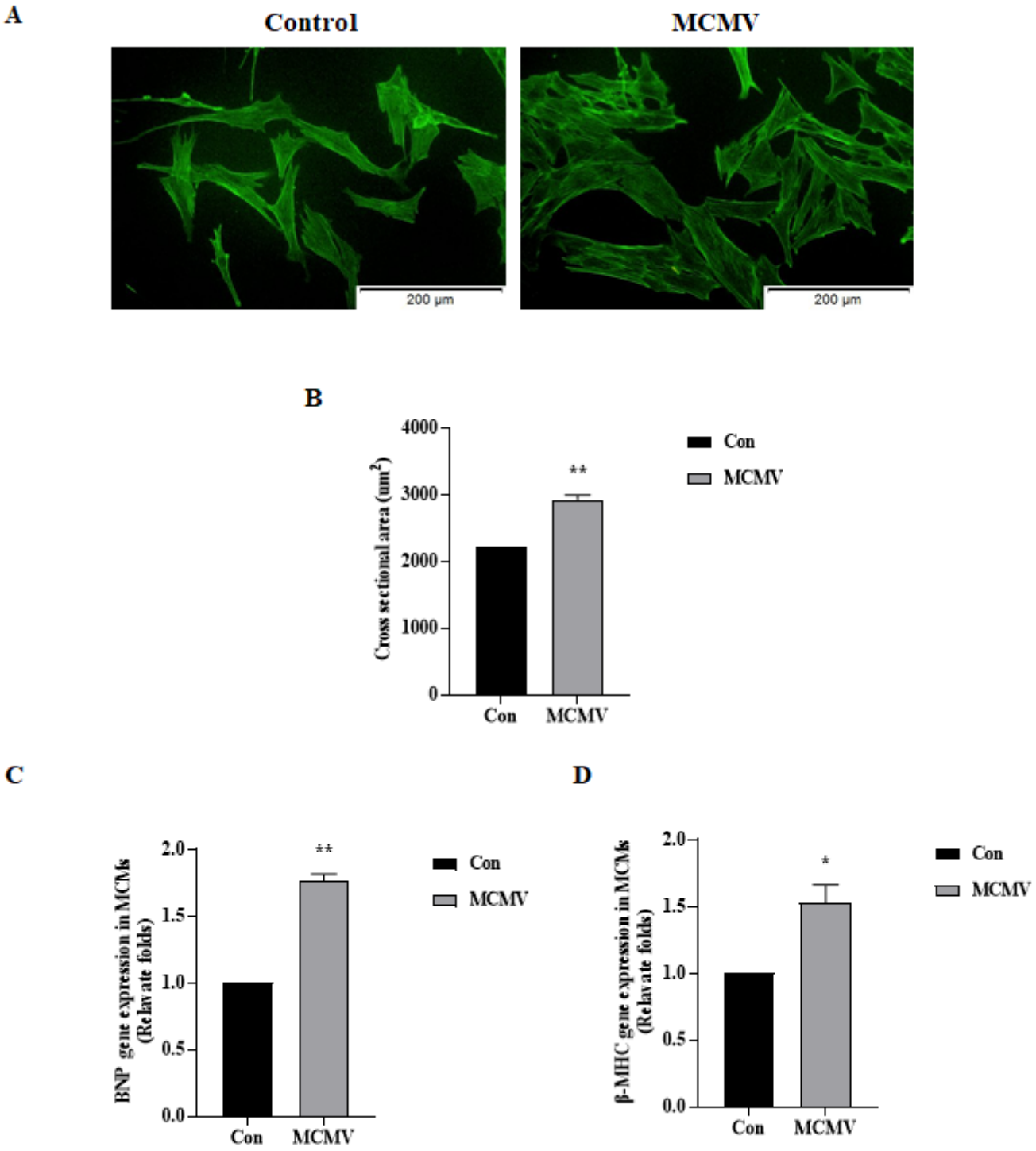

Figure 5

MCFs promote MCMs hypertrophy through paracrine effect after MCMV infection. **A-B**, Representative fluorescence images and analysis of actin green fluorescence staining in MCMs. **C-D**, Results from the

qRT-PCR analysis of the expression of BNP and β-MHC mRNA in MCMs (n=3). * $P<0.05$, ** $P<0.005$ vs control group.

Supplementary Files

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