Wang *et al.*
European Journal of Medical Research (2024) 29:472
https://doi.org/10.1186/s40001-024-02061-6

European Journal of Medical Research

RESEARCH Open Access

MicroRNA-124a promoted the differentiation of bone marrow mesenchymal stem cells into neurons through Notch signal pathway

Daimei Wang[1], Lijun Jing[2], Zhongyan Zhao[1], Shixiong Huang[1], Ling Xie[1], Shijun Hu[1], Hui Liang[1], Yanquan Chen[1] and Eryi Zhao[1*]

Abstract

This study investigated the possible mechanisms of microRNA-124a on the differentiation of bone marrow mesenchymal stem cells (BMSCs) and its underlying mechanism. β-Thiol ethanol induced Notch1 mRNA expression, microRNA-124a inhibitor reduced the effects of β-thiol ethanol on Notch1 mRNA expression in BMSCs. Baicalin induced Hes1 mRNA expression, and microRNA-124a inhibitor reduced the effects of baicalin on Hes1 mRNA expression in BMSCs. Si-Notch1 suppressed Hes1 mRNA expression in BMSCs. Baicalin increased the effects of Notch1 on Hes1 mRNA expression in BMSCs. Si-Notch1 increased cell growth of BMSCs. Baicalin reduced the effects of si-Notch1 on cell growth and the differentiation of BMSCs. We demonstrated that microRNA-124a promoted the differentiation of BMSCs into neurons through Notch/Hes1 signal pathway.

Keywords MicroRNA-124a, BMSCs, Notch, Hes1

Introduction

Ischemic stroke is a serious threat to human health because of its high incidence rate, high disability rate, and high mortality [1]. Therefore, the treatment of ischemic stroke is widely concerned by society [2]. In clinic, the treatment of stroke is mainly drug therapy, while interventional therapy and stem cell therapy are relatively few [3].

Biological characteristics of bone marrow mesenchymal stem cells (BMSCs): bone marrow mesenchymal stem cells (BMSCs) are seed cells commonly used in tissue engineering research [4]. Through in vitro culture, it is found that progenitor cells with multidirectional differentiation ability can be isolated from bone marrow. The bone marrow was isolated, expanded in vitro, and some of the cells adhered to the surface of the culture dish could be highly expanded in vitro [5]. The multidirectional differentiated cell group was named bone marrow mesenchymal stem cells. Until 1999, Pittinger uniformly named bone marrow mesenchymal stem cells (BMSCs) in Science magazine [6]. At present, bone marrow mesenchymal stem cells can be summarized as a subset of cells derived from non-hematopoietic cells, which originate from mesenchymal tissue and mainly exist in the connective tissue and organ interstitium of the whole body, and are the most abundant in bone marrow and have the ability of self-renewal and multidirectional differentiation [7]. They can be expanded in vitro, and can differentiate into chondrocytes, adipocytes, neuroid cells, matrix supporting hematopoietic stem cells and other cells after induction in vitro [8].

*Correspondence:
Eryi Zhao
zhaoguanduozgd@hainmc.edu.cn
[1] Department of Neurology, Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University, No. 19 Xiuhua Road, Xiuying District, Haikou 570311, Hainan, China
[2] Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

MicroRNA (miRNA) is a kind of small endogenous non-coding single-stranded RNA, which can negatively regulate the stability of mRNA and/or inhibit the translation of mRNA by combining with the complementary sequence in the 3′-untranslated region of the target gene, and most miRNAs play a role in ischemic stroke in a tissue-specific manner [9–11]. Its imbalance is closely related to the occurrence and progression of ischemic stroke [12].

Neural stem cells have a wide range of therapeutic potential in stroke, spinal cord injury and other neurological diseases [13]. There are neural stem cells with the ability of self-proliferation and differentiation in adult hippocampal dentate gyrus and subventricular area [14]. After cerebral ischemia, neural stem cells in these regions are activated to participate in the nerve regeneration after cerebral ischemia, thus improving the nerve function [15]. However, due to the influence of the local environment after ischemia, neural stem cells are not enough to fully repair the brain tissue damage, so improving the survival rate of neural stem cells after cerebral ischemia, regulating their proliferation and differentiation has become an urgent problem to be solved [16, 17].

Materials and methods

Isolation and identification of BMSCs

Bone marrow MSCs were harvested from Sprague–Dawley rats, aged 10 weeks and weighing 180–250 g, housed in a controlled environment (24 ± 1 °C, 12 h light/12 h dark cycle) and allowed food and water ad libitum [18, 19]. All procedures were conducted according to the guidelines of the Animal Care Committee. Bone marrow cells were flushed out and collected from the femur and tibia of rats, plated in T25 flasks, and cultured overnight in a 37 °C incubator with 5% CO_2. BMSCs were incubated with alpha minimum essential medium (α-MEM, Gibco, CA, USA) containing 20% fetal bovine serum (FBS), 2 mM Glutamax, and 1% penicillin and streptomycin (PS) in a 5% CO_2 atmosphere at 37 °C. BMSCs were incubated with PE-conjugated anti-CD29 (BD Biosciences, USA), anti-CD34 (BD Biosciences, USA), PE-conjugated anti-CD90 (BD Biosciences, USA), and then analyzed via flow cytometry (BD Biosciences, USA).

Cell culture and transfection

BMSCs were incubated in a 5% CO_2 atmosphere at 37 °C. Si-Notch1 plasmid and microRNA-124a inhibitor plasmid was transfected into BMSCs cell lines, respectively,

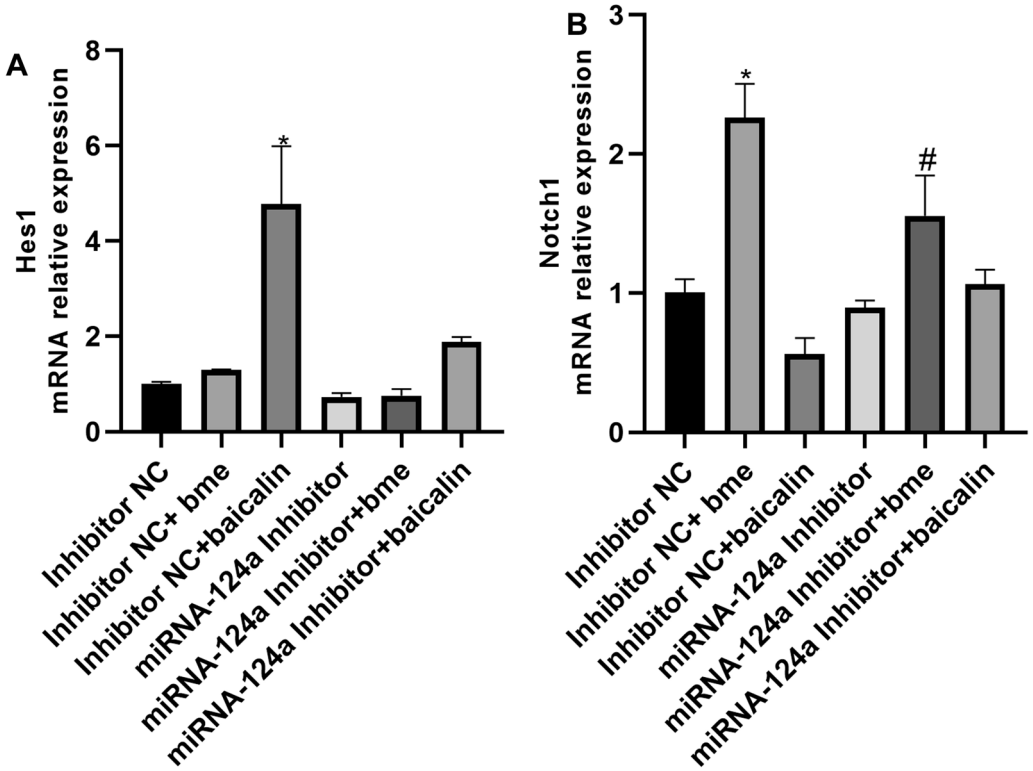

Fig. 1 Baicalin induced Hes1 mRNA expression and β-thiol ethanol induced Notch1 mRNA expression in the differentiation of BMSCs into neurons by microRNA-124a. Hes1 (**A**) and Notch1 (**B**) mRNA expression. *$p < 0.01$ compared with inhibitor NC, #$p < 0.01$ compared with microRNA-124a inhibitor

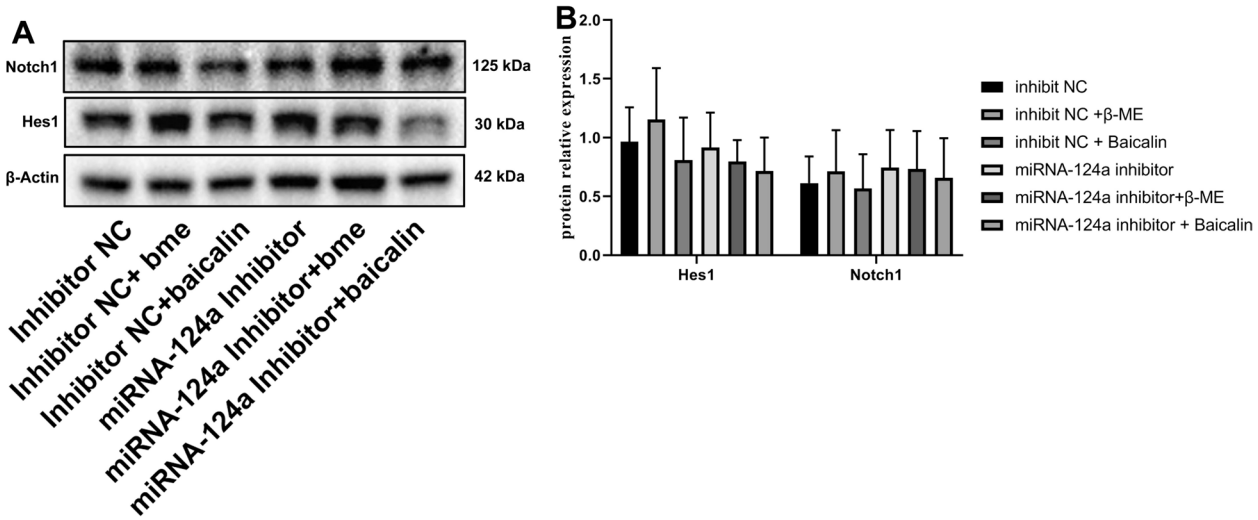

Fig. 2 Hes1 and Notch1 protein expression. Hes1 and Notch1 protein expression (Western blot, **A**) and (statistical analyses, **B**). *$p < 0.01$ compared with inhibitor NC, $^{\#}p < 0.01$ compared with microRNA-124a inhibitor

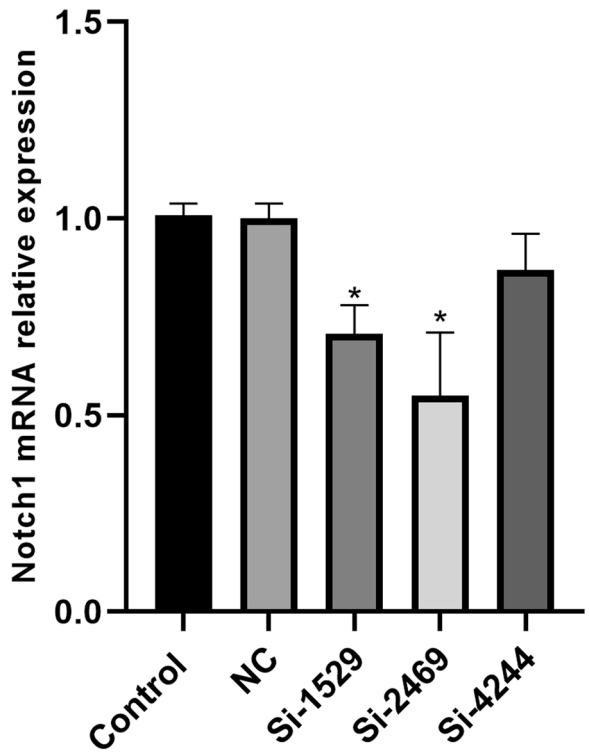

Fig. 3 Notch1 mRNA expression. *$p < 0.01$ compared with NC

by using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Transfection was performed according to the manufacturer's instructions. Specifically, the plasmid was mixed with Lipofectamine 2000 and allowed to rest for 30 min. Subsequently, it was added to the cells, and 24 h after transfection, the cells were cultured using a fresh medium. Transfection was completed 48 h later and cultured for subsequent experiments. The experiment was performed in triplicate. The cells used in this study were within 10 passages.

Quantitative polymerase chain reaction (qPCR)

Total RNAs were isolated with RNA isolator total RNA extraction reagent (Takara, Tokyo, Japan), and cDNA was synthesized using PrimeScipt RT Master Mix(Takara, Tokyo, Japan). qPCR were performed with the ABI Prism 7500 sequence detection system according to the PrimeScriptTM RT detection kit. The reaction mixtures were incubated at 50 °C for 15 min, followed by 95 °C for 5 min; then, 35 PCR cycles were performed with the following temperature profiles: 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min. Relative levels of the sample mRNA expression were calculated and expressed as 2-DDCt. The experiment was performed in triplicate.

Proliferation assay

For Cell Counting Kit-8 (CCK-8), after 48 h of transfection, a total of approximately 5×10^3 cells/well was seeded in 96-well plate. After culturing at indicated time (0, 1, 2, 3 and 4 day), the cellular proliferation was detected using CellTiter-GloR Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to manufacturer's instructions. The experiment was performed in triplicate.

Cellular induction and treatment

For cellular induction and treatment, 5×10^5 cells/well was seeded in 6-well plate. Cells which the cellular

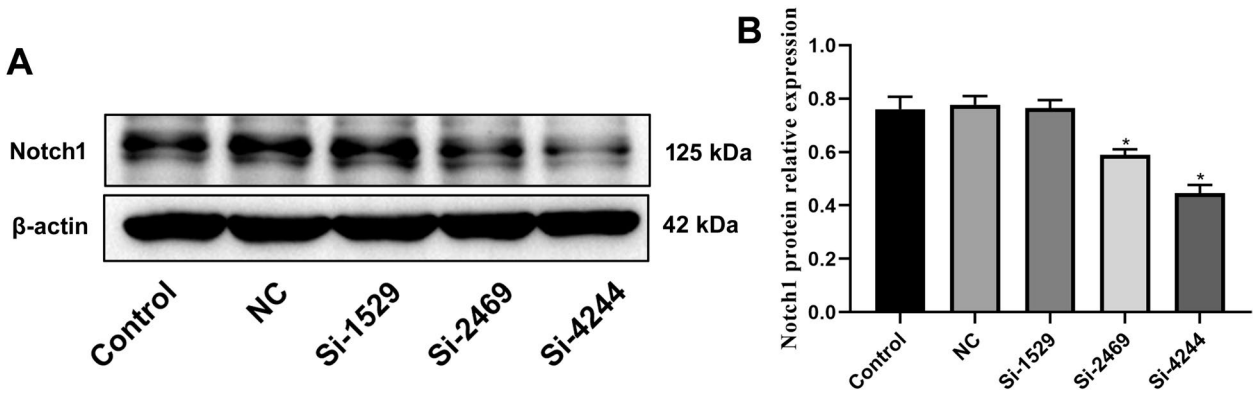

Fig. 4 Notch1 protein expression. Notch1 protein expression (Western blot, **A**) and (statistical analyses, **B**). *$p < 0.01$ compared with NC

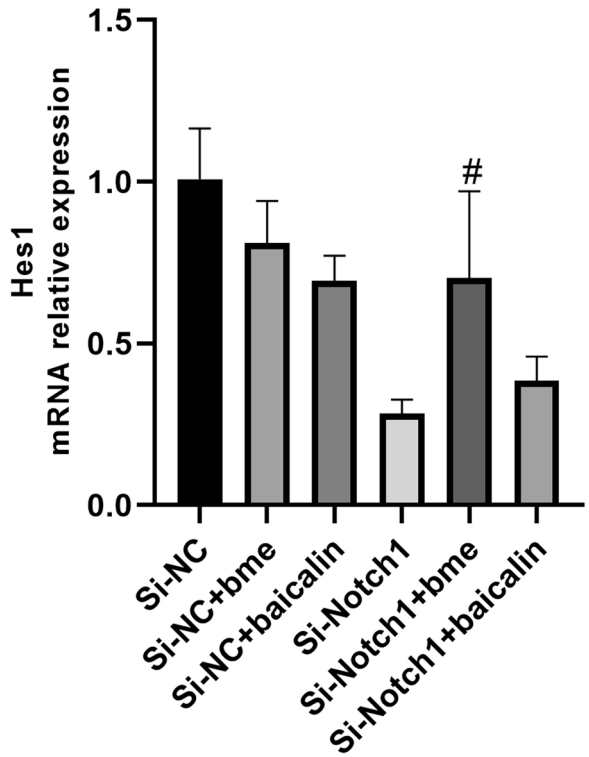

Fig. 5 Notch1 is one target for the baicalin on Hes1 mRNA expression. #$p < 0.01$ compared with si-Notch1 group

density reaches 70% were treated by si-Notch1, miRNA-124a inhibit or miRNA-124a mimc. After 48 h of transfection, a total of approximately, cells were induced by β-thiol ethanol and baicalin for 6 h. The experiment was performed in triplicate.

Immunofluorescence detection

Cells treated after crawling tablets, were fixed with 4% paraformaldehyde for 30 min, permeated with 0.5% Triton X-100 for 20 min, and sealed with 5% BSA at 37 °C for 30 min. They were incubated with NSE (1:200), MAP-2 (1:200) and GFAP (1:200) at 4 °C overnight. After washing, fluorescent secondary antibody was added, the film was sealed with DAPI and observed under fluorescence microscope (CKX53, Olympus).

Western blot

Tissue or cells samples were lysed with ice-cold RIPA buffer with complete protease and phosphatase inhibitors. The protein concentrations were measured using BCA protein assay kit. Total proteins were separated by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies after blocking with 5% BSA in TBS, followed by incubation with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The signals were detected with the ECL system and exposed by the ChemiDoc XRS system with Image Labsoftware (Bio-rad).

Statistical analyses

Graphad Prism 6 was used for the statistical analysis. $p < 0.05$ was considered statistically significant. Comparisons of data between groups were followed using Student's t test or one-way analysis of variance (ANOVA), followed by Tukey's post hoc test.

Results

Baicalin induced Hes1 mRNA expression and β-thiol ethanol induced Notch1 mRNA expression in the differentiation of BMSCs into neurons by microRNA-124a

This study explored that the mechanism of microRNA-124a on the differentiation of BMSCs into neurons. Baicalin induced Hes1 mRNA expression, and microRNA-124a inhibitor reduced the effects of baicalin on

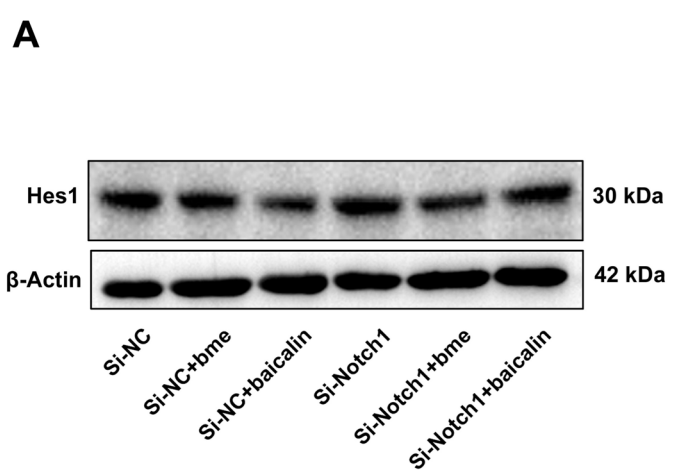

Fig. 6 Notch1 is one target for the baicalin on Hes1 protein expression

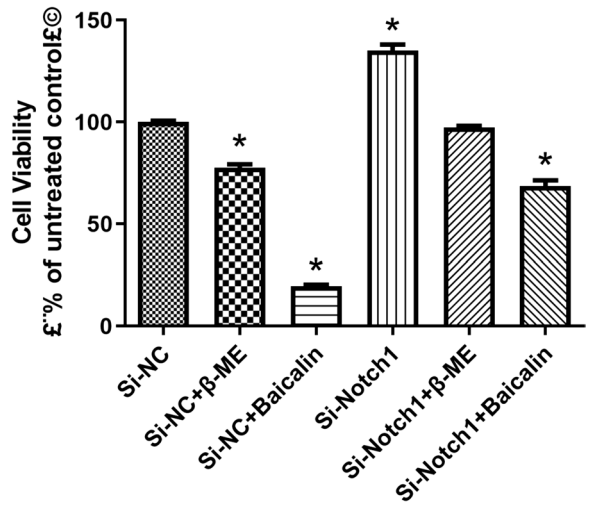

Fig. 7 Notch1 is one target for the baicalin on cell growth of BMSCs into neurons. *$p < 0.01$ compared with si-NC

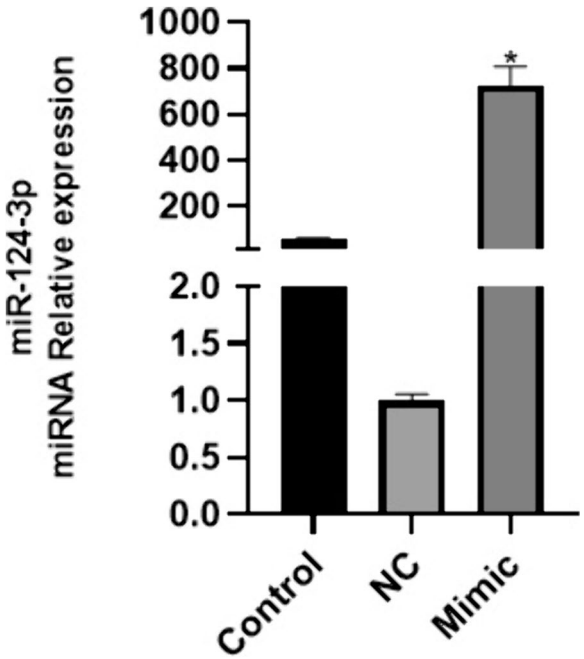

Fig. 8 MicroRNA-124a mimic increased microRNA-124a expression in BMSCs. The qPCR results of microRNA-124a expression. *$p < 0.05$ compared with NC group

Hes1 mRNA expression in BMSCs (Fig. 1A). β-Thiol ethanol induced Notch1 mRNA expression, microRNA-124a inhibitor reduced the effects of β-thiol ethanol on Notch1 mRNA expression in BMSCs (Fig. 1B). However, baicalin or β-thiol ethanol did not affect Hes1 and Notch1 protein expressions in BMSCs (Fig. 2).

Notch1 is one target for the baicalin on the differentiation of BMSCs into neurons

Next, Si-Notch1 plasmid reduced the Notch1 mRNA and protein expression in BMSCs (Figs. 3, 4). Si-Notch1 suppressed Hes1 mRNA expression in BMSCs (Fig. 5). Baicalin increased the effects of Notch1 on Hes1 mRNA expression in BMSCs (Fig. 5). However, baicalin did not affect Hes1 protein expression in BMSCs (Fig. 6). We found that si-Notch1 increased cell growth of BMSCs (Fig. 7). Baicalin reduced the effects of si-Notch1 on cell growth of BMSCs (Fig. 7).

MicroRNA-124a regulated the cell growth and neuronal differentiation of BMSCs

Lastly, the results of qPCR showed that microRNA-124a mimic treatment could increase microRNA-124a expression in BMSCs (Fig. 8). MicroRNA-124a increased cell

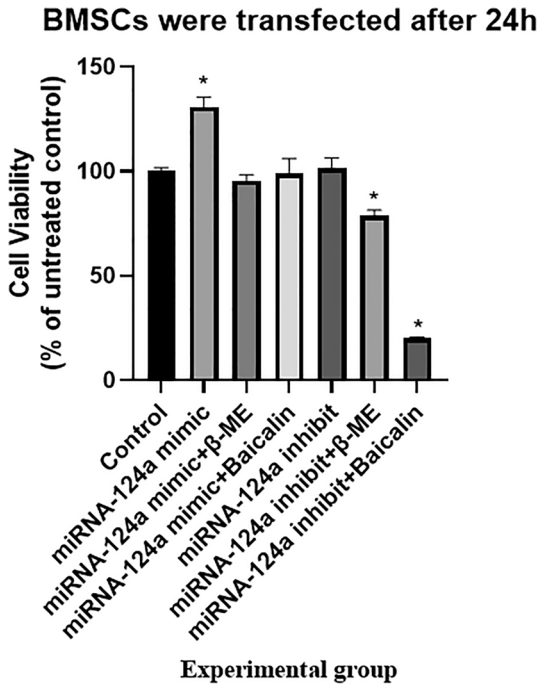

Fig. 9 MicroRNA-124a regulated the cell growth of BMSCs into neurons. *$p < 0.01$ compared with Control group

growth of BMSCs (Fig. 9). Baicalin and β-thiol ethanol reduced the effects of microRNA-124a on cell growth of BMSCs (Fig. 9). MiRNA-124a inhibitor reduced the effects of β-ME and baicalin induced neuronal differentiation of BMSCs, and decreased expression of neuronal markers NSE and MAP-2. Conversely, MiRNA-124a mimic increased the effects of β-ME and baicalin induced neuronal differentiation of BMSCs, and promoted expression of neuronal markers NSE (Fig. 10A) and MAP-2 (Fig. 10B).

Discussion

BMSCs, also known as pluripotent stem cells, are stem cells derived from the mesoderm and have the ability of multidirectional differentiation and self-renewal to osteoblasts, chondrocytes, adipocytes and other multidirectional differentiation, and can differentiate into neural cells and glial cells in vivo and in vitro under specific conditions [20, 21]. Studies have confirmed that compounds, growth factors, gene modification, co-culture and in vivo transplantation can induce bone marrow mesenchymal stem cells to differentiate into neurons, which indicates that the neural differentiation process of BMSCs may involve multiple signal pathways, which require various signal pathway conduction and information integration to promote the reconstruction of cell structure [22–24]. In this study, baicalin induced Hes1 mRNA expression, and microRNA-124a inhibitor reduced the effects of baicalin on Hes1 mRNA expression in BMSCs. β-Thiol ethanol induced Notch1 mRNA expression, microRNA-124a inhibitor reduced the effects of β-thiol ethanol on Notch1 mRNA expression in BMSCs. Both of these show that microRNA-124a regulates Notch signaling pathway during MSCs differentiated into neurons. As in the case with these, Xu et al. showed that microRNA-124a protected against ischemia reperfusion injury through Notch signaling pathway [25]. However, the mechanism of the effects of baicalin on the promoting Hes1 mRNA expression in BMSCs was not clear.

NOTCH signal transduction pathway is one of the most important pathways that determine the fate of cells and plays an important role in cell differentiation, proliferation and apoptosis [26]. At present, it has been reported that cerebrovascular disease are sensitive to ferroptosis inducers, and its occurrence and development are closely related to ferroptosis [27, 28]. Notch1/Hes1 signal transduction plays an important role in cell differentiation, proliferation and apoptosis, and is also an important regulatory signal of ferroptosis. In the study of human small cell lung cancer, it was found that cMyc activated NOTCH signaling pathway can promote the sensitivity of small cell lung cancer cells to ferroptosis, suggesting that NOTCH signaling pathway is a potential regulatory molecular mechanism of ferroptosis [29–31]. In the present study, si-Notch1 increased cell growth of BMSCs. Baicalin reduced the effects of si-Notch1 on cell growth of BMSCs.

MicroRNAs, a short non-coding single-stranded RNA with a length of about 20–24 nucleotides, combine with mRNA through the principle of base complementary pairing to directly target the cleavage of mRNA or inhibit the translation of target genes to regulate the expression of post-transcriptional genes [32–34]. MicroRNAs can regulate the development and function of the nervous system by combining with mRNA [35]. Brain-specific microRNA-124 plays a regulatory role in the development stages of neurite generation [36]. MiR-124 affects the cell fate of glial cells, and induces their differentiation into neurons [37]. We found that microRNA-124a increased cell growth of BMSCs. Baicalin and β-thiol ethanol reduced the effects of microRNA-124a on cell growth of BMSCs. Underexpression of MiRNA-124a reduced the effects of β-ME and baicalin induced neuronal differentiation of BMSCs, and decreased expression of neuronal markers NSE and MAP-2. Conversely, overexpression of MiRNA-124a increased the effects of β-ME and baicalin induced neuronal differentiation of BMSCs, and promoted expression of neuronal markers NSE and MAP-2.

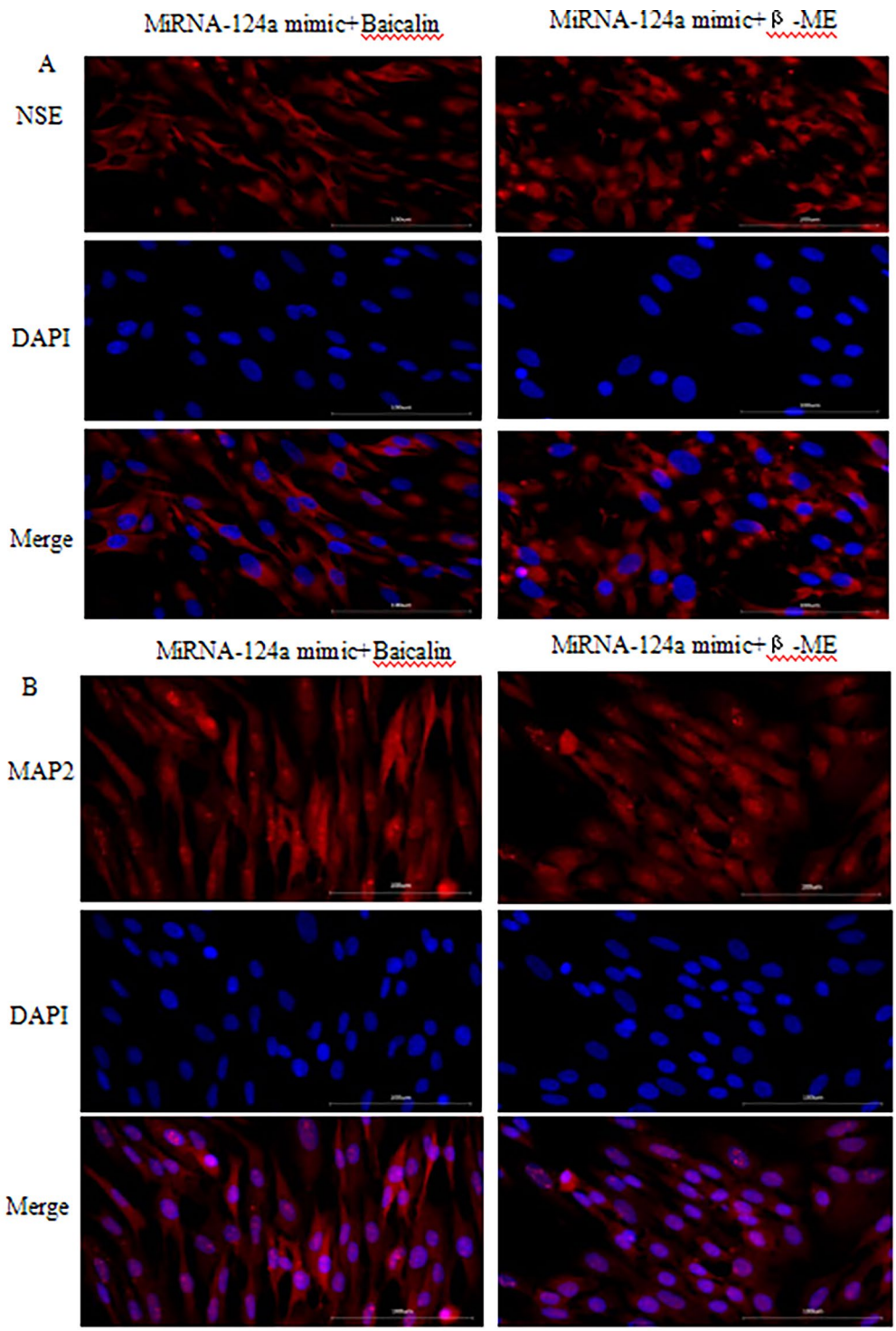

Fig. 10 Overexpression of MiRNA-124a promoted expression of neuronal markers NSE and MAP-2 of BMSCs into neurons

This study has some limitations. First, the molecular mechanism of how baicalin impacts on Hes1 mRNA expression in BMSCs requires in-depth investigation. Second, it needs to be explored more deeply how MiRNA-124a regulates Notch signaling pathway. Third, the experimental conclusion will need to be confirmed in vivo.

On the basis of these findings, we conclude that microRNA-124a promoted the differentiation of BMSCs into neurons through Notch signal pathway. The observations that microRNA-124a plays a broad role in the differentiation of BMSCs, and microRNA-124a might benefit the treatment of the differentiation of BMSCs into neurons. Also, baicalin might induce neuronal differentiation of BMSCs by regulating Notch signal pathway.

Acknowledgements
Not applicable.

Author contributions
DMW designed the experiments. LJJ, ZYZ and SXH performed the experiments. LX and SJH collected and analyzed the data. EYZ, HL and YQC drafted manuscript. All authors read and approved the final manuscript.

Funding
This study is supported by Hainan Province Science and Technology Special Fund (No. ZDYF2021SHFZ112), Hainan Province Clinical Medical Center.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of the Hainan General Hospital. Mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The study is in accordance with ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 23 September 2023 Accepted: 12 September 2024
Published online: 28 September 2024

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