Article

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From Silencing to Differentiation: SOCS3 Knockdown as the Key to MSC Conversion Into Cardiomyocytes

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Background: Stem cell transplantation has emerged as a widely recognized therapeutic approach for myocardial infarction (MI). The primary objective of this study was to investigate the specific role of suppressor of cytokine signaling 3 (SOCS3) in regulating the differentiation potential of mesenchymal stem cells (MSCs) into functional cardiomyocytes. This research lays important groundwork for the development of novel therapeutic strategies aimed at post-injury cardiac regeneration.
Methods: Isolated from human umbilical cord tissue, MSCs were characterized through confirmation of positive expression of surface markers (CD90, CD73, CD44) in flow cytometry. Their multipotency was verified by demonstrating osteogenic and adipogenic differentiation potential, which was confirmed using Alizarin red and oil red O staining approaches, respectively. To elucidate the role of SOCS3, its expression was knocked down using shRNA (shSOCS3). Subsequently, cell morphological changes and expression of key cardiomyocyte-specific genes and proteins were analyzed. Furthermore, STAT3 knockdown (siSTAT3) and GATA binding protein 4 (GATA4) overexpression plasmids were constructed and employed *in vitro* to explore the underlying mechanism. Putative binding interaction between the transcription factor STAT3 and the promoter of *GATA4* was predicted via the JASPAR database and subsequently validated experimentally by means of chromatin immunoprecipitation (ChIP).
Results: ShSOCS3 promoted cardiomyocyte-like morphological alterations in MSCs. Upregulation in the expression of connexin-43, myosin heavy chain (MHC), cardiac troponin T, NK2 homeobox 5 (NKX-2.5), and GATA4 was also detected following the transfection of shSOCS3. These effects attributable to shSOCS3 were partially reversed by siSTAT3. GATA4, which was bound to STAT3, partially counteracted the effect of siSTAT3.
Conclusion: SOCS3 plays an inhibitory role in the cardiomyogenic differentiation of MSCs, which is mechanistically mediated by the STAT3/GATA4 signaling axis, wherein SOCS3 modulates STAT3 activity, which in turn directly influences the expression of the critical cardiac transcription factor GATA4.

Keywords: mesenchymal stem cells; cardiomyocytes; suppressor of cytokine signaling 3; signal transducer and activator of transcription 3; GATA binding protein 4

Introduction

Myocardial infarction (MI) remains one of the leading causes of death and disability worldwide [1,2]. Although the disease progression can be hampered by utilizing existing drugs and therapeutic interventions, both acute and severe myocardial ischemia can irreversibly impair cardiomyocytes of the affected organs, causing adverse remodeling and cardiac function decline, which negatively impact the quality of life of the patients [3,4]. Through fundamental and clinical research, stem cell transplantation has emerged as a widely recognized therapeutic approach for MI. This is because stem cells possess multilineage differentiation potential, self-renewal ability, immune regulation and tissue repair capacity [5,6]. These characteristics make stem cell transplantation a promising therapeutic strategy, in addition to the existing drug treatments and interventional procedures. Among the various types of stem cells, mesenchymal stem cells (MSCs) represent one of the most extensively studied cell populations in the MI context.

Among the advantages of MSCs are a wide range of source availability, ease of isolation and expansion, and low immunogenicity [7]. MSCs can be induced to undergo cardiomyocyte-like differentiation under various *in vivo* and *in vitro* environments, a trait that is clinically beneficial to patients with cardiovascular diseases [8,9]. Nevertheless, a range of fundamental and clinical issues associated with cell transplantation remain to be resolved [10,11]. These problems have contributed to the tremendous growth of stem cell research, with enhancing cell differentiation efficiency using small-molecule compounds emerging as one of the hot topics in recent years [12].

Interestingly, a recent study has shown that enhancement of STAT3 phosphorylation by cardiac-specific dele-

tion of suppressor of cytokine signaling 3 (SOCS3) may prevent post-MI myocardial apoptosis through both the mitochondrial pathway and cytosolic/nuclear signal transduction [13]. SOCS3 is a member of the SOCS family, whose gene is situated in chromosome 17q25.3. Besides, a previous study has shown that MSCs inhibited Th17 cells differentiation via SOCS3 activation mediated by interferon-gamma (IFNγ) [14]. Moreover, SOCS3 plays an anti-inflammatory role in cardiomyocytes [15], and MSC-derived exosomes upregulate SOCS3 levels in SW982 cells [15]. Our analysis of differentially expressed genes in the GSE59867 data package revealed that SOCS3 expression was significantly increased on the first day after MI and significantly downregulated after the 4th–6th day (compared to the 1st day). Therefore, we propose a reasonable speculation that changes in SOCS3 levels may influence the cardiomyocyte-like differentiation of MSCs.

GATA binding protein 4 (GATA4) is a tissue-specific transcription factor abundant in cardiomyocytes [16]. It plays a crucial role in the early development of the heart, especially in the regulation of many genes encoding cardiomyocyte-specific proteins. GATA4 is regarded as one of the important factors for the differentiation of MSCs into cardiomyocytes [17]. GATA4 is expressed in the mesoderm of early cardiac development, representing the earliest signal for the development of cardiac progenitor cells [18,19]. In the mature stage of cardiomyocyte development, GATA4 plays an important role in regulating the transcriptional programs governing cell lineage differentiation [20]. Therefore, we sought to determine whether modulation of SOCS3 expression levels affects the molecular expression levels of GATA4.

Materials and Methods

Ethical Approval and Isolation of MSCs From Human Umbilical Cords

In this study, umbilical cords were collected from 10 healthy pregnant women. Specimens were isolated from each donor and then cultured independently. Umbilical cords were cut into small parts with approximately 3 $\times$ 3 mm in dimension, and subsequently immersed in a 0.25% trypsin solution. After digestion for 20 min, the trimmed tissue specimens were transferred into cell culture flasks (430639, Corning®, Corning, NY, USA) containing Dulbecco's Modified Eagle Medium (DMEM) solution (PM150220A, Procell, Wuhan, China) supplemented with 10% fetal bovine serum (164210, Procell, Wuhan, China) [17]. The flasks were then placed in a CO_2 incubator set at 37 °C (MCO-20AIC, SANYO, Kobe, Japan). During the incubation period, the medium was replaced once every 2–3 days. Following observed cellular attachment to the culture flasks (after approximately 15 days), the umbilical cord tissue specimens were removed and replaced with fresh medium to facilitate cell growth. Upon reaching 80% confluence, MSCs were collected for use in subsequent experiments. The cells were confirmed to be free of mycoplasma contamination and subjected to short tandem repeat (STR) analysis for cell identification.

For all functional experiments described hereafter, cells from passages 3–5 were used. Unless otherwise specified, experiments were randomly performed using MSCs derived from three independent donors (biological replicates, $n = 3$).

Characterization of MSCs

Flow Cytometry

The collected cells were washed and resuspended in fluorescence-activated cell sorting (FACS) buffer. Diluted anti-CD90 antibody (ab23894), anti-CD73 antibody (ab202122) and anti-CD44 antibody (ab264539) were added to the cell suspension (1×10^6 cells), followed by an incubation at 4 °C. The next day, the cells were treated with Alexa Fluor® 488-labeled Donkey Anti-Mouse secondary antibody (ab150105) for 1 h at room temperature. Treated cells were analyzed using a FACScalibur flow cytometer (BD Biosciences, USA) to assess the expression of CD90, CD44 and CD73. All antibodies used in this experiment were obtained from Abcam (Cambridge, UK).

Oil Red O Staining

In this experiment, the cells in 6-well plates were cultured with Procell's adipogenic differentiation medium (PD-019, Procell, Wuhan, China) to induce adipogenic differentiation. The medium was changed every 3 days during the 21-day period of differentiation induction. Afterward, the cells were subjected to oil red O staining using a commercial kit (C0157S, Beyotime, Shanghai, China). The stained samples were observed and photographed under a microscope (LV100ND, Nikon, Tokyo, Japan) set at 200$\times$ magnification. Images were processed through the threshold tool of the ImageJ software (1.47v, National Institutes of Health, Bethesda, MD, USA) to segment out and measure the staining areas as a percentage of the total area.

Alizarin Red Staining

In this experiment, the cells in 6-well plates were cultured with Procell's osteogenic differentiation medium (PD-017; containing Alizarin red S staining solution, Procell, Wuhan, China) to induce osteogenic differentiation. The medium was changed every 3 days. After 21 days, the cells were treated with Alizarin red S solution, and the stained samples were observed under a microscope (LV100ND, Nikon, Tokyo, Japan) set at 200$\times$ magnification. The stained areas following the Alizarin red S staining were quantified as the percentage of the mineralized area over the total area using ImageJ software (1.47v, National Institutes of Health, Bethesda, MD, USA).

Table 1. Sequences of primers used in qRT-PCR assays.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
SOCS3	CACACCGGACCAACCAGC	CTGTCGCGGATCAGAAAGGT
Connexin-43	CTTCATGCTGGTGGTGTCC	ACCACTGGTCGCATGGTAAG
MHC	GTGTCCACCGTGACCCCTGTC	ATTCAGGTTCTTAACTTCAT
Cardiac troponin T	CTGCTGTTCTGAGGGAGAGC	TGAAGGAGGCCAGGCTCTAT
NKX-2.5	AGTGTGCGTCTGCCTTTCC	CACAGCTCTTTCTTTTCGGCTC
GATA4	CTGCCCTCCGTCTTCTGC	CTCGCAGGTCAAGGAGCC
STAT3	CTGCCCCATACCTGAAGACC	TCCTCACATGGGGGAGGTAG
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT

qRT-PCR, Reverse-transcription Quantitative Polymerase Chain Reaction; *SOCS3*, suppressor of cytokine signaling 3; *MHC*, myosin heavy chain; *NKX-2.5*, NK2 homeobox 5; *GATA4*, GATA binding protein 4; *STAT3*, signal transducer and activator of transcription 3; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Cell Transfection

Shanghai GenePharma Co., Ltd (Shanghai, China) provided short hairpin RNA of SOCS3 (shSOCS3; silencing sequence: 5′-CGGAACTTGTTTGCTCTTTGATT-3′; negative control labeled as shNC (5′-TTCTCCGAACGTGCACGTTTC-3′)) and small interfering RNA of STAT3 (siSTAT3; silencing sequence: 5′-AACTGGATAACGTCATTAGCAGA-3′; negative control labeled as siNC (5′-UUCUCCGAACGUGUCACGUTT-3′)), which were employed to silence the specified genes in this study. STAT3 (G102546) and GATA4 overexpression plasmids (G144245), obtained from Youbio (Changsha, China), were used to upregulate the STAT3 or GATA4 expression, with an empty plasmid (pDONR223 vector) utilized as a negative control (labeled as NC). Sequence details for the STAT3, GATA4 overexpression plasmids and the pDONR223 vector are presented in **Supplementary File 1**. Cells were transfected using the Lipofectamine 3000 universal transfection reagent (L3000015, Invitrogen™, Carlsbad, CA, USA).

Reverse-transcription Quantitative Polymerase Chain Reaction (qRT-PCR)

After transfection, the MSCs were re-inoculated in 6-well plates. RNAiso Plus reagent (500 µL per 10^6 cells; Code No. 9108, Takara, Kyoto, Japan) was added to and mixed well with the MSCs. Following treatment with chloroform, isopropanol and other reagents, the precipitated RNA was dissolved in ribonuclease (RNase)-free water. Purity of RNA was determined using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific™, Waltham, MA, USA). The extracted RNA was then immediately reverse-transcribed to cDNA. The cDNA samples were amplified in qRT-PCR, and quantification was performed using the One Step SYBR® Green RT-qPCR kit (QR0100, Sigma-Aldrich, St. Louis, MI, USA) and the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [17]. Sequence details of primers used in the qRT-PCR assays are listed in Table 1. The relative gene expressions were calculated with the $2^{-\Delta\Delta Ct}$ method [21]. For the purpose of group comparisons, mRNA expression was normalized to the corresponding control. All qRT-PCR data are presented as fold change.

STAT3-GATA4 Binding Study

We predicted the *GATA4* binding site for transcription factor STAT3 by using the JASPAR database (https://jaspar.genereg.net/). Chromatin immunoprecipitation (CHIP) assays were conducted to validate the binding site results. The collected MSCs were crosslinked, sonicated and immunoprecipitated according to the instructions provided in the ChIP Assay Kit (P2078, Beyotime, Shanghai, China). After elution and crosslinking, the extracted purified DNA was subjected to qRT-PCR to detect the degree of enrichment. Briefly, MSCs were crosslinked with 1% formaldehyde for 10 min at room temperature and quenched with glycine. Chromatin was extracted and sheared by sonication to an average length of 200–500 bp. For each immunoprecipitation reaction, 10 µg of the sonicated chromatin was incubated overnight at 4 °C with 2 µg of anti-STAT3 antibody (0.526 mg/mL, ab68153, Abcam, Cambridge, UK). Normal immunoglobulin G (IgG, 1.644 mg/mL, ab172730, Abcam, Cambridge, UK) was used as a negative control. Subsequently, protein A/G magnetic beads were added to capture the antibody-chromatin complexes. After extensive washing, the immunoprecipitated DNA was eluted and reverse-crosslinked. The purified DNA was analyzed using qRT-PCR to assess the enrichment of the predicted STAT3 binding sites on the *GATA4* promoter.

Statistical Analysis

GraphPad 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the experimental data. Data comparisons were conducted using one-way analysis of variance (ANOVA) and Bonferroni correction. The results were considered statistically significant at $p < 0.05$. Continuous data are expressed as mean ± standard deviation.

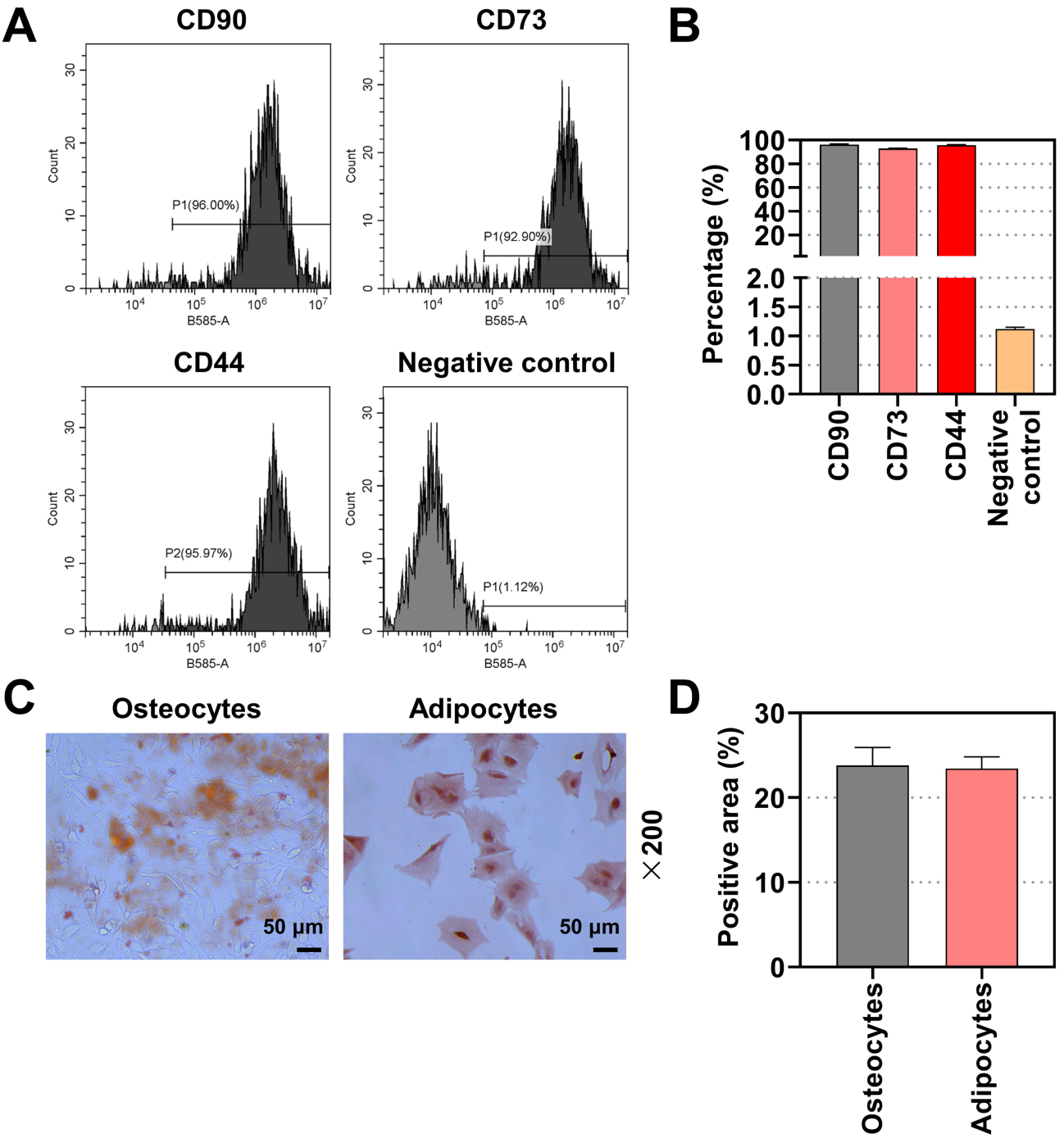

Fig. 1. Characterization of MSCs. (A,B) Expression of MSC marker proteins (CD90, CD73, CD44) in isolated cells was analyzed using flow cytometry. (C,D) Osteogenic and lipogenic differentiation abilities of the isolated cells were tested by means of Alizarin red staining and oil red O staining assays. Magnification: 200×. All experiments were repeated three times to obtain mean values (biological replicates). Abbreviations: MSCs, mesenchymal stem cells.

Results

Confirmed Identity and Multipotency of MSCs for Further Experimental Use

As shown in Fig. 1A,B, the positive expression of CD90 (96.00%), CD73 (92.90%) and CD44 (95.97%) provides evidence that the isolated cells were indeed MSCs. Red calcium deposits (23.80 ± 2.11%) were clearly observed in the Alizarin red S-stained samples (Fig. 1C,D, left), whereas red lipid droplets (23.43 ± 1.38%) were found in the oil red O-stained samples (Fig. 1C,D, right). Additional validation using the staining approaches contributes to further confirmation of the identity of the isolated cells as MSCs.

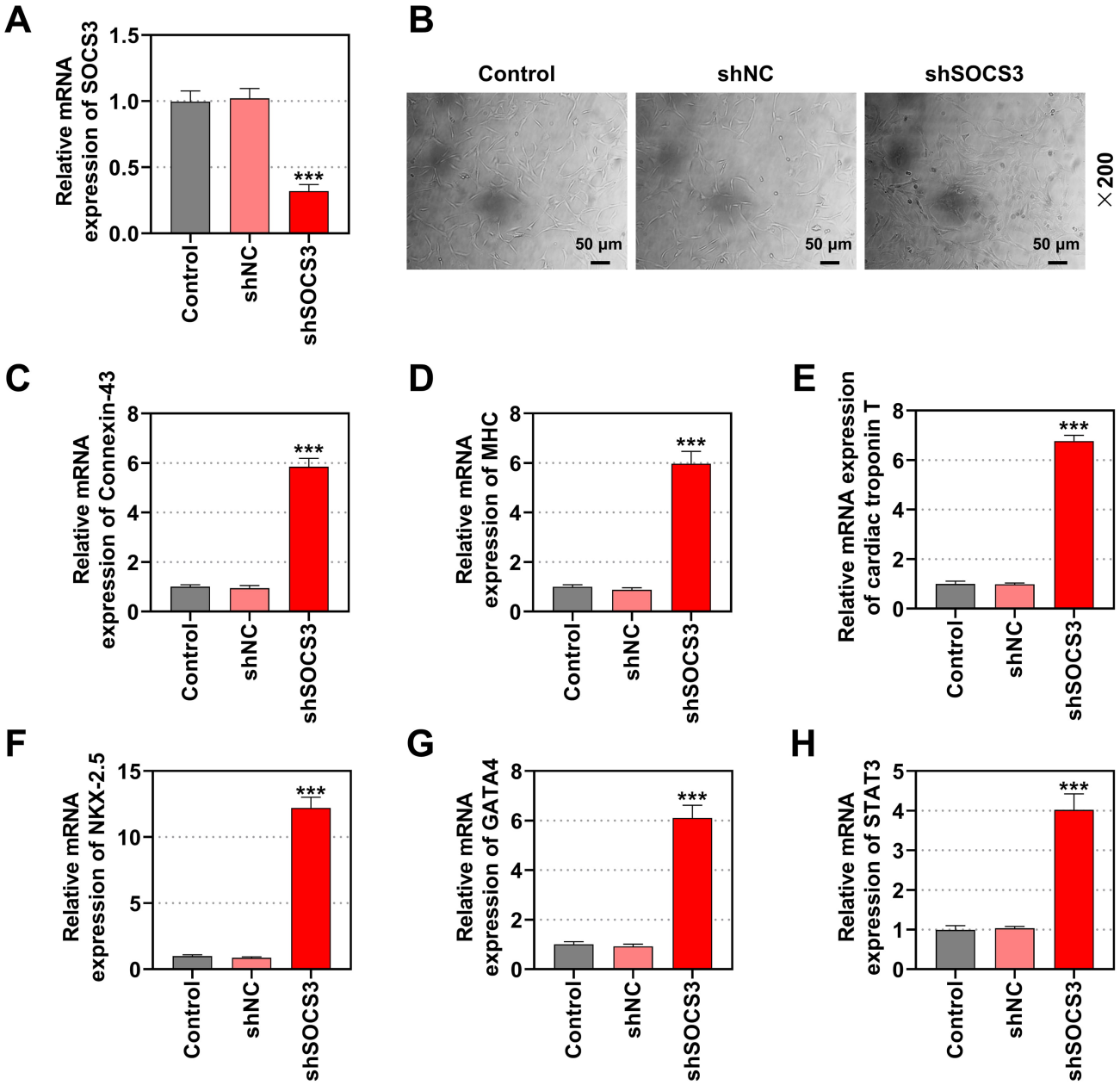

Fig. 2. ShSOCS3 induces cardiomyocyte-like differentiation and upregulates *STAT3* expression in MSCs. (A) The transfection efficiency of shSOCS3 was tested by means of qRT-PCR. (B) Cell morphological changes were observed microscopically 14 days after transfection. Magnification: 200×. (C–G) The effect of shSOCS3 on the expression of genes related to cardiomyocyte differentiation in MSCs was tested using qRT-PCR. (H) The effect of shSOCS3 on *STAT3* expression in MSCs was tested using qRT-PCR. *GAPDH* was used as an internal reference gene in the qRT-PCR experiments. All experiments were repeated three times to obtain mean values (biological replicates). ***$p < 0.001$ vs. shNC. Abbreviations: *GATA4*, GATA binding protein 4; *MHC*, myosin heavy chain; MSCs, mesenchymal stem cells; *NKX-2.5*, NK2 homeobox 5; shNC, negative control of shSOCS3; shSOCS3, short hairpin RNA of SOCS3; *SOCS3*, suppressor of cytokine signaling 3; *STAT3*, signal transducer and activator of transcription 3.

ShSOCS3 Induces Cardiomyocyte-like Differentiation and Upregulates STAT3 Expression in MSCs

In this experiment, MSC samples were divided into three groups based on the treatment designs: MSCs in the Control group received no treatments; MSCs in the shNC group were transfected with the shNC plasmid; and MSCs in the shSOCS3 group were transfected with the shSOCS3 plasmid.

Transfection of shSOCS3 into MSCs significantly downregulated the mRNA levels of *SOCS3* in cells (Fig. 2A, $p < 0.001$). Following a 14-day culture, the MSCs in the shSOCS3 group exhibited typical cardiomyocyte-

like features under the microscope: cytoplasmic protrusions were elongated and exhibited myotube-like structures (Fig. 2B). The expression of cardiomyocyte differentiation-related genes was also altered in these cells. Compared with the shNC, the shSOCS3 significantly promoted the expression of connexin-43, *MHC*, cardiac troponin T, *NKX-2.5* and *GATA4* (Fig. 2C–G, $p < 0.001$). Besides, *STAT3* mRNA expression was also upregulated under the influence of shSOCS3 (Fig. 2H, $p < 0.001$).

SiSTAT3 Partially Reverses the Molecular Regulatory Effect of shSOCS3

This study demonstrated that the siSTAT3 plasmid effectively reduced the mRNA level of *STAT3* in MSCs (Fig. 3A, $p < 0.001$). In this experiment, the MSCs were divided into four groups according to the treatment designs: MSCs in the Control group were not subjected to any treatments; MSCs in the shNC+siNC group were transfected with shNC and siNC plasmids; MSCs in the shSOCS3 group were transfected with shSOCS3 plasmids; and MSCs in the shSOCS3+siSTAT3 group were transfected with siSTAT3 and shSOCS3. Consistent with the previous experiment, shSOCS3 upregulated the expression of cardiomyocyte differentiation-related genes in MSCs, such as connexin-43, *MHC*, cardiac troponin T, *NKX-2.5* and *GATA4* (Fig. 3B–F, $p < 0.001$); however, treatment with siSTAT3 counteracted this particular effect of shSOCS3 (Fig. 3B–F, $p < 0.001$).

STAT3 Binding to GATA4 Counteracts Effects of siSTAT3

The possible binding sites of STAT3 on the *GATA4* promoter were predicted by using the JASPAR database (Fig. 3G). The binding site results were validated by the ChIP assays. The STAT3 overexpression plasmid enhanced the GATA4 expression, and *vice versa*, indicating the STAT3-GATA4 interaction (Fig. 3H, $p < 0.01$). Expression of *GATA4* was significantly upregulated in MSCs after transfection with the GATA4 overexpression plasmid (Fig. 4A, $p < 0.001$). In the rescue experiment, MSCs were divided into four groups based on the treatment designs: MSCs in the shSOCS3 group and shSOCS3+siSTAT3 group were treated in the same way as described in the previous section; MSCs in the shSOCS3+siSTAT3+NC group were transfected with shSOCS3, siSTAT3 and NC plasmids; MSCs in the shSOCS3+siSTAT3+GATA4 group were transfected with shSOCS3, siSTAT3 and GATA4 overexpression plasmids. We found that overexpression of GATA4 counteracted the regulatory effects of siSTAT3 and restored the expression of connexin-43, *MHC*, cardiac troponin T, *NKX-2.5* and *GATA4* (Fig. 4B–F, $p < 0.001$).

Discussion

Previous research has demonstrated that transplantation of genetically modified MSCs into animal models of MI is associated with enhanced transplantation rate, survival rate, and differentiation ability into myocardium and vasculature [22]. In the same vein, future therapeutic optimization efforts should focus on the continued, in-depth exploration of the mechanisms underlying the differentiation of transplanted MSCs, with the aim of addressing the cardiac regeneration conundrum in MI cases. The present study demonstrates that *SOCS3* knockdown induces the cardiomyocyte-like differentiation of human umbilical cord-derived MSCs, which is likely mediated through the activation of the STAT3/GATA4 pathway. Our key findings include: (i) shSOCS3 induces the morphological changes characteristic of cardiomyocytes and upregulates expression of cardiac-specific markers; and (ii) mechanistically, *SOCS3* knockdown activates STAT3, which in turn binds to the *GATA4* promoter.

Clarifying the effect of SOCS3 on cardiomyocyte-like differentiation of MSCs was the primary aim of this study. While the role of SOCS3 as a key negative regulator of inflammatory signaling in cardiomyocytes is well-established [23,24], its specific function in governing the differentiation fate of MSCs remains poorly understood. To explore this knowledge gap, we first analyzed the morphological changes of the cells. A previous study has shown that cardiomyocytes assume a bamboo-like morphology—different from the "fibroblast-like" characteristics of MSCs—and fuse to form myotube-like structures [17]. In addition to morphological examination, molecular analysis involving investigation of cardiomyocyte differentiation-related genes such as connexin-43, *MHC*, cardiac troponin T, *NKX-2.5* and *GATA4* was conducted. The current study also provides direct evidence linking SOCS3 suppression to enhanced cardiomyogenic differentiation.

Connexin-43 is a major gap junction protein during cardiomyocyte differentiation and is directly implicated in cardiomyocyte signaling [25]. During myocardial development, deficiencies in connexin-43 predispose affected individuals to severe cardiac malformations [26,27]. It has been found that MSCs improve cardiac conduction by upregulation of connexin-43 through paracrine signaling [28]. This is due to the fact that MSCs harbor a low-degree expression of connexin-43, which undergoes a gradual upregulation during the differentiation into cardiomyocytes and can reach higher levels after about 1 week of co-culture. MHC and cardiac troponin T are specific markers of cardiac origin, and their upregulation usually indicates early cardiomyocyte development [29]. NKX-2.5, like GATA4, is a transcription factor expressed in the heart during early development [30,31]. However, unlike GATA4, NKX-2.5 is involved in not only early heart formation but

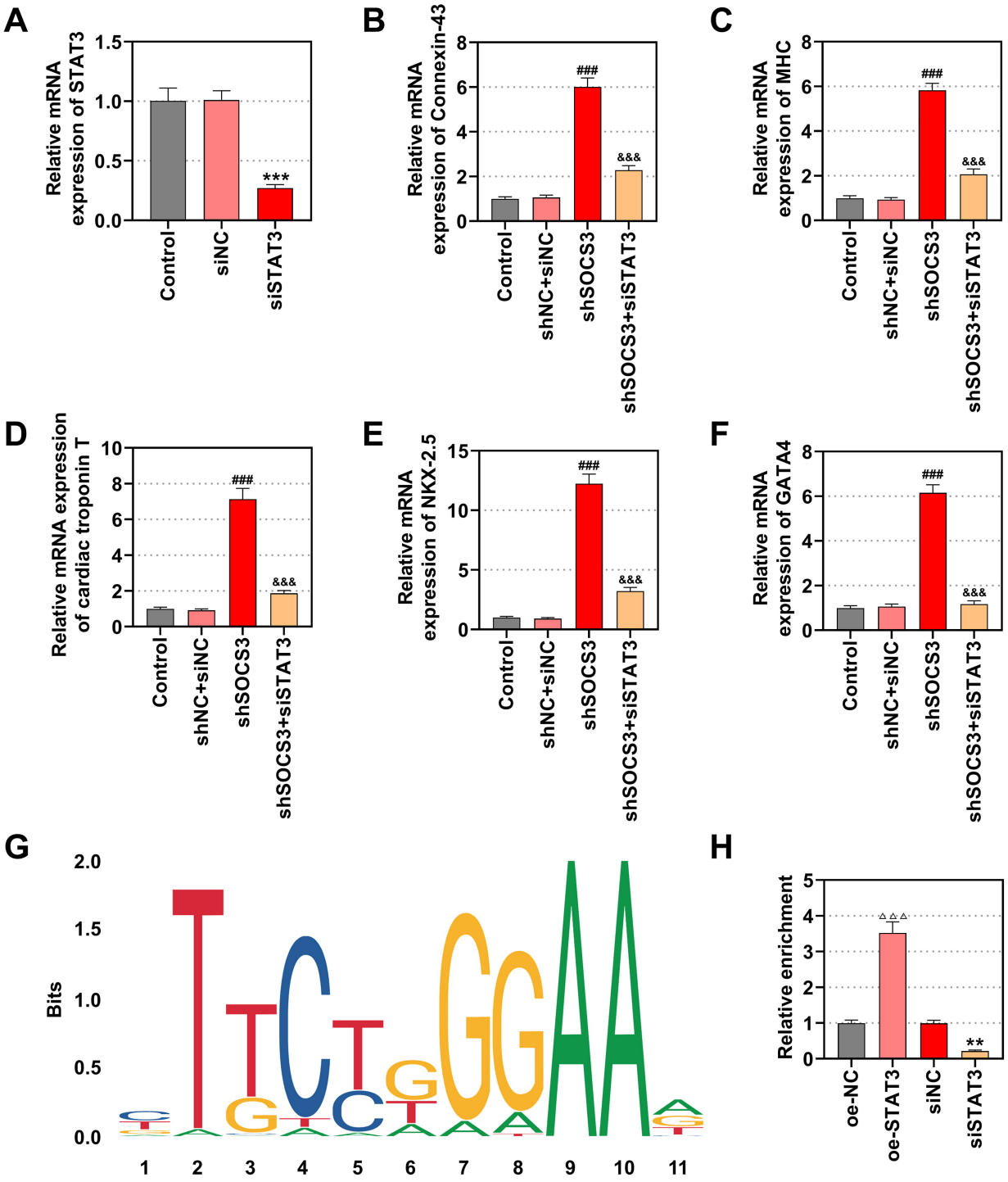

Fig. 3. SiSTAT3 partially reverses the molecular regulatory effect of shSOCS3. (A) The transfection efficiency of siSTAT3 was evaluated using qRT-PCR. (B–F) The effects of siSTAT3 and shSOCS3 on cardiomyocyte differentiation-related gene expression in MSCs were tested using qRT-PCR. (G) STAT3 binding sites on the *GATA4* promoter were predicted using the JASPAR database (https://jaspar.genereg.net/). (H) The binding of STAT3 to GATA4 was verified by means of chromatin immunoprecipitation assay. *GAPDH* was used as an internal reference gene in the qRT-PCR experiments. All experiments were repeated three times to obtain mean values (biological replicates). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. siNC; $^{\#\#\#}p < 0.001$ vs. shNC+siNC; $^{\&\&\&}p < 0.001$ vs. shSOCS3; $^{\triangle\triangle\triangle}p < 0.001$ vs. oe-NC. Abbreviations: *GATA4*, GATA binding protein 4; *MHC*, myosin heavy chain; MSCs, mesenchymal stem cells; *NKX-2.5*, NK2 homeobox 5; oe-NC, negative control of oe-STAT3; oe-STAT3, STAT3 overexpression plasmid; shNC, negative control of shSOCS3; shSOCS3, short hairpin RNA of SOCS3; siNC, negative control of siSTAT3; siSTAT3, small interfering RNA of STAT3; *SOCS3*, suppressor of cytokine signaling 3; *STAT3*, signal transducer and activator of transcription 3.

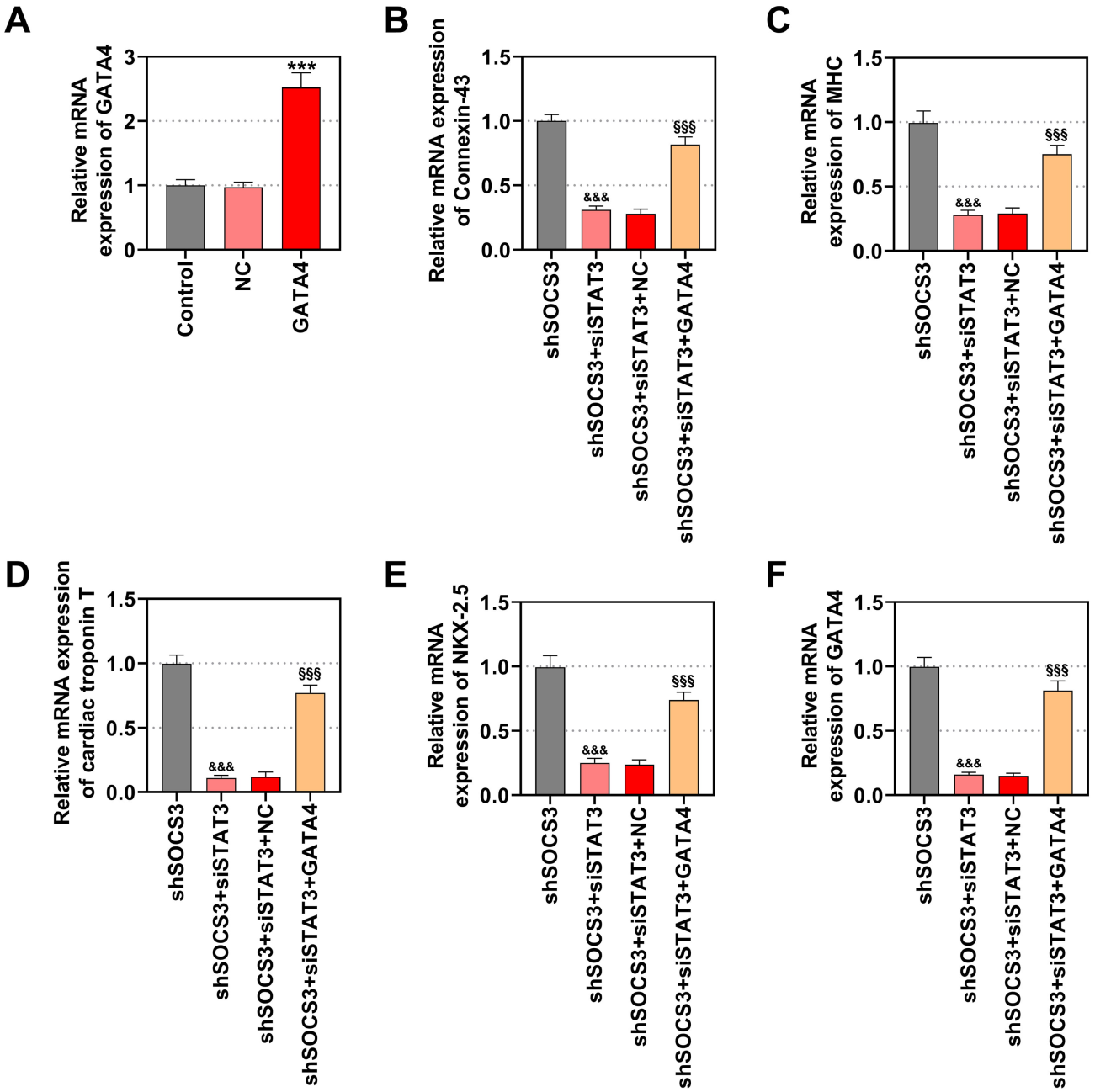

Fig. 4. STAT3 binding to *GATA4* counteracts the effects of siSTAT3. (A) The transfection efficiency of the GATA4 overexpression plasmid was evaluated by means of qRT-PCR. (B–F) Expression of genes related to cardiomyocyte differentiation in MSCs transfected with different combinations of vectors, such as GATA4 overexpression plasmid and shSOCS3, was tested using qRT-PCR. *GAPDH* was used as an internal reference gene in the qRT-PCR experiments. All experiments were repeated three times to obtain mean values (biological replicates). $^{***}p < 0.001$ vs. NC; $^{\&\&\&}p < 0.001$ vs. shSOCS3; $^{\S\S\S}p < 0.001$ vs. shSOCS3+siSTAT3+NC. Abbreviations: *GATA4*, GATA binding protein 4; *MHC*, myosin heavy chain; MSCs, mesenchymal stem cells; NKX-2.5, NK2 homeobox 5; shSOCS3, short hairpin RNA of SOCS3; siSTAT3, small interfering RNA of STAT3; *SOCS3*, suppressor of cytokine signaling 3; *STAT3*, signal transducer and activator of transcription 3.

also in cardiac looping, differentiation, septal development, and maintenance of the cardiac conduction system [30,31]. GATA4 plays an important role in myocardial survival and cardiac hypertrophy. In this study, shSOCS3-induced cardiomyocyte-like morphological changes and upregulation of differentiation-related genes in MSCs both demonstrate that SOCS3 knockdown induces differentiation of MSCs to cardiomyocytes.

The most intriguing finding of our study is the elucidation of the mechanistic link between SOCS3 knock-

down and GATA4 upregulation via STAT3 activation. Despite the well-documented canonical negative regulatory relationship of SOCS3-STAT3 in various cellular contexts [32–34], its specific role in modulating MSC differentiation has not been previously reported. STAT3, as a common acute responsive factor, is involved in a variety of signaling pathways [35]. Its activation has been primarily studied in the contexts of cardioprotection [36,37] and cardiomyocyte regeneration [38], with limited investigation into its role in stem cell differentiation. For example, G-CSF–induced STAT3 activation, which mediates myocardial regeneration, has been utilized clinically to improve cardiac function in patients with angina pectoris or acute MI [39]. Through the current work, we also demonstrated that STAT3 binds to the promoter of GATA4 and thus induces its expression. Altogether, these findings contribute to elucidation of a proposed molecular mechanism: shSOCS3 facilitates MSC differentiation into cardiomyocytes via activation of the STAT3/GATA4 pathway. This newly identified SOCS3/STAT3/GATA4 axis significantly expands our understanding of the molecular mechanisms controlling cardiomyogenic differentiation.

Several limitations of this study should be acknowledged. First, our findings are based primarily on *in vitro* experiments, necessitating validation work using animal models of MI in the future to confirm the therapeutic potential of targeting SOCS3. Second, while we have established the association between STAT3 and GATA4, other downstream effectors that may contribute to the observed phenotypic changes remain unexplored. Third, the temporal dynamics of STAT3 activation and GATA4 expression during the differentiation process warrant further investigations in a bid to optimize potential therapeutic interventions.

Despite these limitations, our study unveils a previously unrecognized role for SOCS3 in regulating MSC differentiation through the STAT3/GATA4 axis. This novel mechanism not only advances our fundamental understanding of cardiac lineage differentiation of MSCs but also presents new directions for enhancing the efficacy of stem cell-based therapies for myocardial regeneration.

Conclusion

Collectively, our findings demonstrate that the newly identified SOCS3/STAT3/GATA4 axis is a crucial regulatory pathway governing cardiac lineage differentiation of MSCs. Specifically, knockdown of SOCS3 presents as a key driver of cardiomyocyte-like differentiation of human umbilical cord-derived MSCs, which is possibly mediated by activation of the STAT3/GATA4 pathway. According to the results, SOCS3 is a potential molecular target for enhancing the efficacy of stem cell-based therapies in myocardial regeneration.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author Contributions

YD and SG designed the research study; HW and MG performed the research; QL and BX collected and analyzed the data. YD has been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity are addressed.

Ethics Approval and Consent to Participate

Consent was obtained from the human subjects and their families prior to cord collection. The study was approved by the Ethics Committee of Shanghai East Hospital (Approval No: 2025YS-159). Procedures were conducted in accordance with the Declaration of Helsinki.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537202.222.

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