

# LncGBP9/miR-34a axis modulates macrophage M1/M2 polarization to affect spinal cord injury repair via STAT1/STAT6 and SOCS3

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## Research

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# Abstract

**Background:** Acute spinal cord injury (SCI) could cause mainly two types of pathological sequelae, the primary mechanical injury, and the secondary injury. The dysregulation of macrophage polarization to a pro-inflammatory (M1) or an anti-inflammatory phenotype (M2) might cause the failure to post-SCI repair.

**Methods:** In the present study, we conducted SCI model in Balb/c mice and observed the deregulation of M1/2 macrophage polarization after SCI; by bioinformative analyses, lncRNA lncGBP9 and SOCS3 have been regarded as potent regulators of the macrophage polarization after SCI. Mouse bone marrow-derived macrophages (BMDMs) were isolated and identified.

**Results:** In M1 macrophages, lncGBP9 silence significantly decreased p-STAT1 and SOCS3 expression and protein levels, as well as the production of IL-6 and IL-12; in M2 macrophages, lncGBP9 overexpression increased SOCS3 expression and protein levels while suppressed p-STAT6 levels and the production of IL-10 and TGF- $\beta$ 1, indicating that lncGBP9 overexpression promotes the M1 polarization of macrophages. In lncGBP9-silenced SCI mice, the M2 polarization was promoted on day 28 after operation, further indicating that lncGBP9 silence revised the predominance of M1 polarization at the late stage of secondary injury after SCI, therefore improving the repair after SCI.

**Conclusions:** In macrophages, lncGBP9 sponges miR-34a to rescue SOCS3 expression, therefore modulating macrophage polarization.

## Background

Spinal cord injury (SCI) is one of the most important global contributors to disability and related death; however, the treatment for SCI and efficiency are still limited [1–3]. Acute SCI could cause mainly two types of pathological sequelae, the primary mechanical injury and the secondary injury [4]. Direct mechanical trauma could cause the primary SCI, followed by the secondary injury through activating several pathophysiological processes, such as inflammation, dysregulation of microvascular perfusion, deregulated generation of free radicals, dysregulation of cell apoptosis, and broken ionic homeostasis [5–9], which would directly lead to the destruction of intact axonal tracts and hinder the structural and functional recoveries after initial SCI.

During both the acute and chronic phases of the secondary injury when the central nervous system (CNS) evokes innate and adaptive immunities [9, 10], inflammatory responses have been regarded as the central issue. Recent studies have shown that the sequential activation of immune cells, including resident and recruited subtypes, may play an important role in the secondary inflammatory/immune responses after CNS injury, including SCI. The macrophage is a critical cell type in the innate immune response within CNS. Macrophages are phenotypically dynamic in both morphology and function, ranging from resting ramified steady-state (M0) to a pro-inflammatory (M1) or an anti-inflammatory phenotype (M2) [11]. Within a few hours after SCI, macrophages first polarized into M1 macrophages in response to IFN- $\gamma$ , LPS, TNF- $\alpha$ , and other stimuli, and reaches a peak on day 1 after SCI. Macrophage surface Toll-like receptors

(TLRs) are activated and then induce the recruitment of downstream protein myeloid differentiation factor 88 (MyD88), the activation of the downstream pathways, including NF- $\kappa$ B, JAK-STAT, JNK, MAPK, and PI3K/Akt [12], and promote the polarized macrophages to release TNF- $\alpha$ , IL- $\beta$ , IL-6 and other inflammatory factors [13] and chemokines (CCL8, CCL9, cyclooxygenase (Cox), which in turn promote the differentiation of more macrophages to M1. Later, the polarized M1 macrophages exhibit stronger phagocytosis and antigen-presenting ability. Moreover, a large number of M1 macrophage-secreted inflammatory cytokines, reactive oxygen species (ROS), reactive nitrogen (RNS), prostaglandin (PGE2) and other active substances cause damage to neurons and glia, leading to neuronal apoptosis [14]. As for M2 macrophages, cell surface receptors bind IL-4 and IL-13 to promote STAT6 phosphorylation, therefore stimulating the macrophage polarization into M2 type [15]. The markers of M2 macrophages include arginase (Arg-1), resistin-like molecules (Fizz-1), IL-10, transforming growth factor- $\beta$  (TGF- $\beta$ ), mannitol receptors (including CD163, CD204, CD206), etc. [16]. M2 macrophages highly express IL-10, IL-4, IL-13, TGF- $\beta$ , and neurotrophic factor (NTF), which can inhibit neuronal apoptosis and the proinflammatory effects of M1 macrophages, promoting nerve tissue repair. There are 3 subtypes of M2 macrophages including M2a/b/c; M2a macrophages appear on day 1 ~ 3 after SCI, highly express CD206 and Arg1 and exert anti-inflammatory and repair functions [17]; M2b appears on day 3 ~ 7 after SCI, express high IL-10 and low Arg1; M2c appears the latest and could inhibit the production of inflammatory cytokines and inflammation [18].

Macrophages are widely malleable in functions, allowing them to convert from one phenotype to another under the broad stimuli in the post-SCI inflammatory microenvironment. M1 macrophages play a detrimental role after SCI, while M2 macrophages play a promotive role in the regenerative growth responses in adult sensory axons. After SCI, M1 macrophages' taking advantage and the decreased number of M2 macrophages may lead to or even aggravate the secondary injury. Previously, it has been reported that the angiotensin-converting enzyme (ACE)-C domain overexpression in macrophages would result in the transformation of macrophages toward M1 phenotype under tumor stimulation, accompanied by enhanced activation of NF- $\kappa$ B and signal transducer and activator of transcription 1 (STAT1) and attenuated activation of STAT3/6 [19]. Yao et al. reported that the M1 polarization could be hindered by a crucial immune inhibitory receptor, namely programmed cell death 1 (PD-1), via suppressing the phosphorylation of STAT1; in the meantime, PD-1 also promoted the M2 polarization via enhancing the phosphorylation of STAT6 [20]. Consistently, the M1 polarization was enhanced by activating STAT1 and NF- $\kappa$ B in PD-1 knockout mice [20]. Therefore, investigating the factors and mechanisms of regulating STAT1/STAT6 signaling may help to understand the mechanism of M1/2 macrophage polarization switch.

During the past decades, non-protein coding RNAs (ncRNAs) have been regarded as key regulators by playing diverse roles in not only fundamental biological but also pathological processes [21, 22]. Among them, microRNA (miRNA) and long noncoding RNAs (lncRNAs) are the most well-known. miRNAs induce either mRNA degradation or block mRNA translation depending on the complete or incomplete complementarity [23], while lncRNAs could serve as competing endogenous RNAs (ceRNAs) to counteract miRNA-mediated inhibition on miRNA downstream transcripts, therefore exerting their biological

functions [24–26]. Like miRNA, there is new evidence that lncRNAs might be a novel type of regulator macrophage immune response [27]. LncRNAs have been reported to be partially responsible for the gene expression dysregulation during macrophage polarization [28]. Huang et al. [29] also identified differentially-expressed lncRNAs in M1 or M2 polarized macrophages. Of these deregulated lncRNAs, lncRNA TCONS\_00019715 has higher expression in M1 macrophages than that in M2 macrophages. When proinflammatory macrophages convert to anti-inflammatory macrophages, TCONS\_00019715 expression decreases. However, it increases when anti-inflammatory macrophages convert to proinflammatory phenotype. Knockdown of TCONS\_00019715 diminishes the expression of proinflammatory macrophage markers and increases the expression of anti-inflammatory markers. TCONS\_00019715 promotes macrophage transition to proinflammatory macrophages by downregulating PAK1, an important regulator of cytoskeletal remodeling and cell motility in mononuclear phagocytic system [29]. Another lncRNA, lncRNA E330013P06, was found to regulate proinflammatory gene expression and foam cell formation in macrophages [30]. Based on these previous findings, we hypothesize that lncRNAs may participate in macrophage polarization by regulating related molecules and STAT1/STAT6 signaling pathways, most possibly in a miRNA-dependent manner.

In the present study, we conducted the SCI model in Balb/c mice and examined the expression of M1/2 macrophage markers on day 1, 3, 7, 14, and 28 after the operation to monitor the changes in the macrophage polarization. Next, by downloading and analyzing online microarray profiles reporting differentially-expressed lncRNAs and genes in M1/2 macrophages, we selected lncRNAs and genes related to macrophage polarization, namely lncGBP9 in mice and SOCS3. Mouse bone marrow-derived macrophages (BMDMs) were isolated, identified, induced to differentiate into M1/2 macrophages, and examined for expression of SOCS3, STAT1 and STAT6 and cytokine production. Next, the effects of lncGBP9 on macrophage polarization, SOCS3 expression, and STAT1/STAT6 signaling were evaluated in vitro and in vivo. Since miR-34a has been reported to promote the M2 macrophage polarization [31] and be predicted to target lncGBP9 and SOCS3, we further investigated whether lncGBP9 could compete with SOCS3 for miR-34a binding, thereby counteracting miR-34a-mediated SOCS3 suppression. The predicted bindings of miR-34a to lncGBP9 and SOCS3 were validated and the dynamic effects of lncGBP9 and miR-34a on SOCS3, STAT1/STAT6 signaling, and macrophage polarization were examined. Finally, we investigated whether STAT6 could bind the miR-34a promoter to activate its transcription. In summary, we provide a novel mechanism by which the lncGBP9/miR-34a axis modulates STAT1/STAT6 to affect macrophage polarization via SOCS3.

## Methods

### Spinal cord injury (SCI) model in Balb/c mice

Balb/c mice (The SLAC experimental animal center, Shanghai, China) received a moderate midthoracic (T9–10) SCI as described previously [32]. Sham mice received a laminectomy without SCI. To identify the SCI model in mice, tissue in lesion epicenter was collected 1, 3, 7, 14, or 28 d after SCI and examined for the M1 macrophage marker CD16/32 and M2 macrophage marker Arg1 by immunofluorescence (IF)

staining, the mRNA expression of M1 macrophage markers iNOS, CD16/32, and IFN- $\gamma$  and M2 macrophage markers Arg1, CD206, and IL-4 by real-time PCR and Immunoblotting.

Locomotor recovery in GBP9-silenced mice was assessed by two persons using the Basso Mouse Scale (BMS) [33] open field test at 1, 3, 7, 14, and 28 d after injury following the methods described previously [33, 34].

### **Isolation and identification of mouse bone marrow-derived macrophages**

Bone marrow-derived macrophages (BMDMs) were isolated from the bilateral femurs and tibias of adult Balb/c mice and then cultured as described previously [35, 36]. 20% supernatant from L929 cells was added to stimulate BMDMs to differentiate into macrophages [37].

### **Induction and identification of M0 macrophages differentiating toward M1/2 macrophages**

The induction of M0 macrophages differentiation into M1 or M2 macrophages was conducted with 100 ng/ml LPS (Sigma-Aldrich) + 20 ng/ml IFN- $\gamma$  (eBioscience) or 20 ng/ml IL-4 (eBioscience), respectively, for 12–24 h following the methods described previously [35-37].

### **Immunoblotting**

Immunoblotting was performed as previous describe [38] using the following primary antibodies: anti-iNOS (ab15323, Abcam, Cambridge, MA, USA), anti-CD16/32 (Catalog # 14-0161-82, Invitrogen, Waltham, MA, USA), anti-IFN $\gamma$  (500-P119, PeproTech, Rocky Hill, NJ, USA), anti-Arg1 (ab212522, Abcam), anti-CD206 (C068C2, BioLegend, San Diego, CA, USA), anti-IL-4 (ab211374, Abcam), anti-STAT1 (ab3987, Abcam), anti-p-STAT1 (ab30645, Abcam), anti-STAT6 (ab32520, Abcam), anti-p-STAT6 (ab28829, Abcam), anti-SOCS3 (ab16030, Abcam), and the HRP-conjugated secondary antibody (1:5000). The plots were visualized by ECL Plus (Thermo).

### **Real-time PCR**

Total RNA was extracted from target cells by using Trizol reagent (Invitrogen) and the expression of miRNA or mRNA was examined following the methods previously described [39] using a Hairpin-it<sup>TM</sup> miRNAs qPCR kit (Genepharma, Shanghai, China) or a SYBR Green PCR Master Mix (Qiagen), respectively. U6 or GAPDH expression was used as an endogenous normalization, respectively. The threshold cycle (Ct) was determined and relative mRNA and miRNA levels were calculated based on the Ct values.

### **ELISA**

Cell culture medium was collected for ELISA assay using human IL-6, IL-12, IL-10, and TGF $\beta$ 1 ELISA kits according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following the methods described previously [40]. The specific binding optical density was assayed immediately at 450 nm with a spectrophotometer (Bio-Rad Laboratories).

## Recombinant adenoviruses

The recombinant adenoviruses expressing IncGBP9 shRNA or IncGBP9-overexpressing fragment or scramble RNA (NC shRNA) were generated using the AdEasy™ Vector System (Invitrogen) following the methods described before [41]. Viruses were diluted in PBS and administered at a dose of  $1 \times 10^7$  pfu/well in 12-well plate.

## Cell transfection

The overexpression or inhibition of miR-34a was conducted by transfection of miR-34a mimics or miR-34a inhibitor (RiboBio, Guangzhou, China). All transfection was performed with the help of Lipo2000 (Invitrogen).

## Luciferase reporter assays

For the validation of the binding of miR-34a to GBP9 or SOCS3 3'-UTR, the wild-type GBP9 or SOCS3 3'-UTR luciferase reporter vector was constructed by cloning the fragment of GBP9 or SOCS3 3'-UTR to the Renilla psiCHECK2 vector (Promega, Madison, WI, USA) and named wt-GBP9/wt-SOCS3 3'-UTR; the mutant-type GBP9 or SOCS3 3'-UTR vector was constructed by mutating the predicted miR-34a binding site in GBP9 or SOCS3 3'-UTR and named mut-GBP9/mut-SOCS3 3'-UTR. These vectors were co-transfected in HEK293 cells with miR-34a mimics/inhibitor; the luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).

## RNA Immunoprecipitation (RIP)

RIP analysis was performed M1 macrophages using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-700, Millipore, Burlington, MA, USA) and a T7 High Yield RNA Synthesis Kit (E2040S, NEB, Ipswich, MA, USA) following the methods described previously [42]. The levels of IgG, GBP9 and miR-34a in the immunoprecipitates were measured by qRT-PCR.

## Chromatin immunoprecipitation (ChIP)

ChIP assays were performed following the methods described previously [43] using antibodies against STAT6 (ab32520, Abcam), a positive control antibody (RNA polymerase II), and a negative control non-immune IgG. The immunoprecipitated DNA was cleaned, released, eluted, and used for downstream applications. The fold-enrichment (FE) was calculated as previously described [43].

## Statistical Analyses

All the data of results from at least three independent experiments in the present study are first processed by SPSS17.0 (IBM, Armonk, NY, USA) and presented as the mean  $\pm$  S.D. The statistical comparison between means was conducted using a Student *t*-test where applicable. Differences among more than two groups were analyzed using one-way ANOVA. \**P* < 0.05; \*\**P* < 0.01.

# Results

## Macrophage polarization during spinal cord injury (SCI)

To investigate the deregulation of macrophage polarization during SCI, we constructed an SCI model in Balb/c mice following the methods described previously [32]. To identify the SCI model, we examined the content and distribution of macrophage marker F4/80, M1 macrophage marker CD16/32, and M2 macrophage marker Arg1 in the lesion epicenter on day 1, 3, 7, 14, 28 after operation. As revealed by IF staining, the fluorescence intensity representing F4/80 (red) and CD16/32 (green on the upper panel) gradually increased in a time-dependent manner (Fig.1A); however, the fluorescence intensity representing Arg1 (green on the lower panel) appeared on day 3, reached a peak intensity on day 7, and then gradually decreased till day 28 (Fig.1A). Consistently, the mRNA expression and the protein levels of M1 macrophage markers CD16/32, iNOS, and IFN- $\gamma$  and M2 macrophage markers Arg1, CD206, and IL-4 emerged similar trend. M1 macrophage markers increased after SCI in a time-dependent manner, while M2 macrophage markers appeared on day 3, reached peak values on day 7, and then decreased gradually (Fig.1B-C). These data indicate that macrophage polarization deregulation exists after SCI and might participate in the dysfunction of SCI repair.

## Selection of lncRNAs and genes related to macrophage polarization

As we have mentioned, the deregulation and dysfunction of lncRNAs in the process of a pro-inflammatory (M1) to an anti-inflammatory phenotype (M2) have been observed [29, 44]. In the present study, we attempted to identify lncRNAs related to the deregulation of macrophage polarization during SCI. The study downloaded and analyzed microarray profiles (GSE117040 and GSE5099) which reported upregulated lncRNAs in M1 macrophages. A total of 4 lncRNAs were reported to be upregulated in M1 macrophages by both profiles; there are homologous genes for lncRNA GBP1P1 (GBP9 in mice) and LINC00869 (Fam91a1 in mice) in mice (Fig. S1A).

Next, to further validate the involvement of these lncRNAs, we isolated BMDMs and identified them by examining macrophage markers F4/80 and CD11b using IF staining (Fig.2A). BMDMs (M0 macrophages) were then induced for differentiating towards M1 or M2 macrophages; the mRNA expression and protein levels of M1 macrophage markers iNOS and CD16/32 and M2 macrophage markers Arg1 and CD206 were examined to identify different subtypes. As shown in Fig.2B-C, iNOS and CD16/32 were significantly upregulated in M1 subtype while downregulated in M2 subtype; Arg1 and CD206 were remarkably upregulated in M2 subtype while downregulated in M1 subtype, indicating the successful induction. As revealed by real-time PCR, the expression of GBP9 and Fam91a1 were both significantly upregulated in M1 macrophages, GBP9 more upregulated (Fig.2D), indicating that GBP9 might be involved in macrophage M1/2 polarization. Reportedly, GBP1P1 is a pseudogene of the guanylate-binding protein of GB (guanylate-binding protein); this family is also involved in macrophage functions, such as IFN- $\gamma$ -mediated macrophage activation and immune defense [45]. More importantly, based on microarray profile or RNA-seq analyses GSE5099 (Fig.S1B), GSE117040 (Fig.S1C), E-GEOD-57494 (Fig.S1D), E-MTAB-2399 (Fig.S1E), and GSE40885 (Fig.S1F), lncRNA GBP1P1 is specifically highly-

expressed in human M1 macrophages and could be rapidly up-regulated after treatment with M1-inducing factors LPS and IFN- $\gamma$ . Thus, GBP1P1 (lncGBP9 in mice) was selected for further experiments.

We then performed STRING analyses on differentially-expressed genes in M1 macrophages reported previously [29, 46] to identify key regulators of the switch from a pro-inflammatory (M1) to an anti-inflammatory phenotype (M2). As revealed by STRING analyses, Tnf, SOCS3, and STAT1 are key factors in macrophage polarization (Fig.2E). Next, the mRNA expression and protein levels of M1-related STAT1 and p-STAT1, M2-related STAT6 and p-STAT6, and SOCS3 were examined in M0, M1, and M2 macrophages. As shown in Fig.2F and G, p-STAT1 and SOCS3 mRNA and protein levels were dramatically upregulated in M1 macrophages while p-STAT6 was upregulated in M2 macrophages. In the meantime, the production of STAT1 downstream cytokines, including IL-6 and IL-12 was increased in M1 macrophages while that of STAT6 downstream IL-10 and TGF- $\beta$ 1 was increased in M2 macrophages (Fig.2H). These data indicate that lncGBP9 and SOCS3 expression are upregulated in M1 macrophages and might be related to M1/2 polarization.

### **Effects of lncGBP9 on macrophage polarization *in vitro***

After selecting lncGBP9 for further experiments, we next evaluated its effects on macrophage polarization *in vitro* and *in vivo*. The silence or overexpression of lncGBP9 was conducted in BMDMs (M0), as confirmed by real-time PCR (Fig.3A and E). lncGBP9-silenced macrophages were stimulated by LPS + IFN- $\gamma$  for M1 polarization. In lncGBP9-silenced M1 macrophages, the mRNA expression and protein levels of p-STAT1 and SOCS3 were significantly reduced by lncGBP9 silence (Fig.3B-C); consistently, the production of IL-6 and IL-12 was also inhibited by lncGBP9 silence in M1 macrophages (Fig.3D). On the contrary, lncGBP9-overexpressing macrophages were stimulated with IL-4 for M2 polarization. In lncGBP9-overexpressing M2 macrophages, the mRNA expression and protein levels of SOCS3 were significantly increased, while those of p-STAT6 were decreased by lncGBP9 overexpression in M2 macrophages (Fig.3F-G); consistently, the production of IL-10 and TGF- $\beta$ 1 was suppressed by lncGBP9 overexpression in M2 macrophages (Fig.3H). These data indicate that lncGBP9 might modulate macrophage M1/2 polarization through affecting STAT1/STAT6 and SOCS3.

To further investigate the speculation, the study then co-transfected M1 macrophages with Adv-lncGBP9 and SOCS3-overexpressing vector (SOCS3 OE) or with Adv-lncGBP9 and si-SOCS3 and examined for macrophage M1/2 polarization. In Adv-sh-lncGBP9 and SOCS3 OE co-transfected M1 macrophages, lncGBP9 silence significantly inhibited SOCS3 mRNA expression (Fig.3I), decreased SOCS3 protein level and STAT1 phosphorylation (Fig.3J), and reduced the concentrations of IL-6 and IL-12 (Fig.3K). SOCS3 overexpression in M1 macrophages exerted opposite effects. The effects of lncGBP9 silence on M1 macrophages were significantly reversed by SOCS3 overexpression. In Adv-lncGBP9 and si-SOCS3 co-transfected M2 macrophages, lncGBP9 overexpression significantly upregulated SOCS3 mRNA expression (Fig.3L), increased SOCS3 protein level, inhibited STAT6 phosphorylation (Fig.3M), and reduced the concentrations of IL-10 and TGF $\beta$  (Fig.3N). SOCS3 silence in M2 macrophages exerted

opposite effects. The effects of lncGBP9 silence on M2 macrophages were significantly reversed by SOCS3 silence.

### **Effects of lncGBP9 on macrophage polarization *in vivo***

Next, the effects of lncGBP9 on macrophage polarization were evaluated *in vivo*. lncGBP9 silence was conducted in the SCI mice model via tail vein injection Ad-sh-lncGBP9; we evaluated the BMS scores on day 1, 3, 7, 14, and 28 after the operation to access the effects of lncGBP9 silence on SCI severity. As shown in Fig.4A, lncGBP9 silence significantly reduced the BMS scores in SCI mice on day 14 and 28 after the operation, indicating lncGBP9 silence in SCI mice promoted the repair after SCI.

At the same time, the content and distribution of M1 macrophage marker CD16/32 and M2 macrophage marker Arg1 determined in lncGBP9-silenced SCI mice by IF staining and Immunoblotting on day 28 after the operation to investigate macrophage polarization. As shown in Fig.4B and C, the protein levels of p-STAT1 and SOCS3 were significantly decreased, while the protein levels of p-STAT6 were increased by lncGBP9 silence in SCI mice on day 28 after the operation. These data indicate that lncGBP9 might affect macrophage polarization via STAT1/6 and SOCS3, therefore modulating the repair after SCI.

### **lncGBP9 modulates SOCS3 through miR-34a in macrophages**

lncRNAs could serve as ceRNAs for miRNAs to counteract miRNA-mediated suppression on miRNA downstream transcripts, therefore exerting their biological functions [24-26]. Essandoh reported a number of miRNAs that might promote M2 polarization [47]; among them, miR-124 and miR-34a were predicted to target SOCS3 and only miR-34a was predicted to target lncGBP9. More importantly, miR-34a could promote M2 macrophage polarization [31]. Thus, we hypothesize that miR-34a might participate in lncGBP9 function on macrophage polarization.

To validate the hypothesis, we examined the expression of miR-34a in M0, M1, and M2 macrophages. As shown in Fig.5A, miR-34a expression was dramatically upregulated in M2 macrophages. In M1 macrophages, miR-34a expression was significantly increased by lncGBP9 silence (Fig.5B). To investigate the cellular effects of miR-34a, we conducted miR-34a overexpression in M1 macrophages by transfection of miR-34a mimics, as confirmed by real-time PCR (Fig.5C). lncGBP9 expression was significantly downregulated by miR-34a overexpression in M1 macrophages (Fig.5D). Consistently, the SOCS3 protein level was also decreased by miR-34a overexpression in M1 macrophages (Fig.5E).

In M2 macrophages, miR-34a expression was significantly downregulated by lncGBP9 overexpression (Fig.5F). Here, we conducted miR-34a inhibition M2 macrophages by transfection of miR-34a inhibitor, as confirmed by real-time PCR (Fig.5G). In M2 macrophages, lncGBP9 expression was significantly upregulated by miR-34a inhibition (Fig.5H). Consistently, the SOCS3 protein level was increased by miR-34a inhibition in M2 macrophages (Fig.5I). These data indicate that lncGBP9 might regulate SOCS3 through miR-34a to participate in M1/2 macrophage polarization.

### **lncGBP9 serves as a ceRNA for miR-34a to counteract miR-34a-mediated SOCS3 suppression**

To validate the predicted targeting of miR-34a to lncGBP9 and SOCS3, we performed luciferase reporter assays by constructing wild- and mutant-type GBP9 and SOCS3 3'-UTR luciferase reporter vectors (wt-GBP9/SOCS3 3'-UTR or mut-GBP9/SOCS3 3'-UTR) as described in M&M section (Fig.6A-B). Next, 293T cells were co-transfected with the above-described vectors and miR-34a mimics/inhibitor and examined for the luciferase activity. As shown in Fig.6A-B, the luciferase activity of wt-GBP9 and wt-SOCS3 3'-UTR vectors could be significantly inhibited by miR-34a overexpression and enhanced by miR-34a inhibition; in responding to the mutation at the putative miR-34a binding sites, the changes in the luciferase activity were abolished. Moreover, in the RNA derived from precipitated AGO2 protein, lncGBP9 and miR-34a levels were significantly higher than those in IgG in M1 macrophages (Fig.6C). We also performed RIP assay in M1 macrophages transfected with control miRNA (NC mimics) or miR-34a mimics and then detected lncGBP9 and miR-34a levels associated with AGO2; the results shown in Fig.6D confirmed the interaction between lncGBP9 and miR-34a. Furthermore, in lncGBP9-overexpressing M1 macrophages, the level of lncGBP9 detected was dramatically higher than that of SOCS3 and  $\beta$ -actin (used as a negative control) (Fig.6E), indicating that lncGBP9 and SOCS3 could bind miR-34a, respectively; lncGBP9 competes with SOCS3 for miR-34a binding.

### **lncGBP9/miR-34a axis modulates macrophage polarization via affecting the balance of STAT1/STAT6**

After confirming the binding of miR-34a to lncGBP9 and SOCS3, next, we evaluated the dynamic effects of lncGBP9 and miR-34a on STAT1/STAT6 and macrophage polarization. M1 macrophages were co-transfected with Ad-sh-lncGBP9 and miR-34a inhibitor and examined for the mRNA expression and protein levels of STAT1, p-STAT1, SOCS3, iNOS, and CD16/32, and the production of IL-6 and IL-12. As shown in Fig.7A, C, and E, lncGBP9 silence significantly reduced, while miR-34a inhibition significantly increased the mRNA expression and protein levels of p-STAT1, SOCS3, iNOS, and CD16/32, as well as the production of IL-6 and IL-12 in M1 macrophages; the effects of lncGBP9 silence could be significantly reversed by miR-34a inhibition.

M2 macrophages were co-transfected with Ad-lncGBP9 and miR-34a mimics and examined for the mRNA expression and protein levels of STAT6, p-STAT6, SOCS3, Arg1, and CD206, and the production of IL-10 and TGF- $\beta$ 1. As shown in Fig.7B, D, and E, lncGBP9 overexpression significantly increased SOCS3 mRNA expression and protein level, decreased p-STAT6, Arg1, and CD206 mRNA and protein levels, and suppressed the production of IL-10 and TGF- $\beta$ 1; miR-34a overexpression exerted opposing effects on these indicators; the effects of lncGBP9 overexpression could be significantly reversed by miR-34a overexpression. These data indicate that the lncGBP9/miR-34a axis modulates M1/2 macrophage polarization through SOCS3 and STAT1/STAT6.

### **STAT6 binds miR-34a promoter to activate its transcription**

As predicted by the online tool, STAT6 might bind the promoter region of miR-34a to activate its transcription. STAT6 overexpression or silence was conducted in M2 macrophages by transfection of STAT6-overexpressing or si-STAT6 vector, as confirmed by Immunoblotting (Fig.8A). In M2 macrophages, the expression of miR-34a was significantly upregulated by STAT6 overexpression while downregulated

by STAT6 silence (Fig.8B). Next, wild- and mutant-type miR-34a luciferase reporter vectors are constructed; mut-miR-34a vector contained a 9-bp mutation in any of the predicted STAT6 binding sites (Fig.8C). STAT6 and wt- or mut-miR-34a were then co-transfected in M2 macrophages; the luciferase activity was determined. As shown in Fig.8D, the promoter activity of wt-miR-34a was dramatically increased by STAT6 overexpression; however, after mutating any of the predicted binding sites, STAT6 overexpression-induced increase in promoter activity was abolished (Fig.8D). Moreover, the ChIP assay showed that the level of STAT6 antibody binding to miR-34a binding element in the miR-34a promoter was much greater than that of IgG in M2 macrophages (Fig.8E), suggesting that STAT6 might bind to the promoter of miR-34a to activate its expression in M2 macrophages.

## Discussion

In the present study, we constructed the SCI model in Balb/c mice and observed the deregulation of M1/2 macrophage polarization after SCI. BMDMs were isolated and identified. LncRNA GBP1P1 (lncGBP9 in mice) has been previously reported to be upregulated and observed overexpressed in M1 macrophages in the present study. SOCS3 and p-STAT1, key factors in macrophage polarization, were also overexpressed in M1 and underexpressed in M2 macrophages while p-STAT6 was underexpressed in M1 and overexpressed in M2 macrophages. Consistently, IL-6 and IL-12 were increased in M1 while IL-10 and TGF- $\beta$ 1 were increased in M2 macrophages. In M1 macrophages, lncGBP9 silence significantly decreased p-STAT1 and SOCS3 expression and protein levels, as well as the production of IL-6 and IL-12; in M2 macrophages, lncGBP9 overexpression increased SOCS3 expression and protein levels while suppressed p-STAT6 levels and the production of IL-10 and TGF- $\beta$ 1, indicating that lncGBP9 overexpression promotes the M1 polarization of macrophages. In lncGBP9-silenced SCI mice, the BMS scores were significantly lower from day 14 after operation and the M2 polarization was promoted on day 28 after operation, further indicating that lncGBP9 silence revised the predominance of M1 polarization at the late stage of secondary injury after SCI, therefore improving the repair after SCI. In macrophages, lncGBP9 competed with SOCS3 for miR-34a binding to counteract miR-34a-mediated suppression on SOCS3, therefore modulating STAT1/STAT6 signaling and the polarization of macrophages. Finally, STAT6 bound the promoter of miR-34a to activate its transcription, therefore forming two different regulatory loops to modulate the polarization of macrophages after SCI (Fig. 8F).

As mentioned earlier, during the occurrence and development of SCI, via enhanced phagocytosis and increased production and release of pro-inflammatory cytokines, M1 macrophages promote innate immunity to remove foreign microorganisms and wound fragments from damaged sites. Differently, M2 macrophages have tissue repair properties, showing a decrease in inflammatory cytokines and in the production of ROS [48, 49]. These media allow M2 macrophages to regulate inflammatory reactions, remove debris and facilitate tissue remodeling and repair. This sequence of M1-M2 macrophages reacts together will lead to success in SCI healing [50]. That is, M1/2 macrophage polarization disorders may cause SCI repair to fail. At the early phase after the injury, the production and release of certain inflammatory cytokines would be induced by macrophages [35, 51]. During the early stage of SCI in the SCI mice model, Kigerl et al. [36] reported that M1 macrophages accounted for the majority. After SCI, M1

and M2 biomarkers all increased rapidly; however, Arg1, one of the M2 biomarkers, was only transient and reverted to the baseline 7 days later post-SCI [36]. On the 14th day after SCI, CD206, another M2 biomarker, was significantly increased in comparison to the normal spinal cord tissue. Differently, M1 marker iNOS transiently increased until day 3 after the injury; CD32, another M1 marker, significantly decreased on day 28 post-SCI. It appears that the expression of iNOS and Arg1 is regulated by each other, suggesting that not all M1 and M2 biomarkers change their expression in a coordinated manner after SCI, possibly because the regulation of macrophage polarization begins at different times and at different phases post-SCI, or the inflammatory microenvironment affects these factors in different manners. In the present study, we observed the mRNA expression and protein levels of M1 markers, including iNOS, CD16/32, and IFN- $\gamma$  increased after SCI from day 3 to day 28 in a time-dependent manner. On the contrary, the expression of one of the M2 markers, Arg1, reached peak values on day 7 and 14 while decreased on day 28 after SCI. Another two M2 markers, CD206 and IL-4, reached peak values on day 7 while decreased since then. These findings indicate that the predominance of M1 macrophages continues to the late phase after SCI. The deregulation of M1/2 macrophage polarization might contribute to the failure of SCI repair.

As we have mentioned, the polarization of macrophages could be regulated by different stimuli and factors, including protein-coding and non-coding RNAs. The deregulation of polarization-related genes might, in turn, contribute to the dysfunction of macrophages. Huang et al. analyzed the differentially-expressed genes in M0, M1, and M2 macrophages and demonstrated that 2528 mRNAs were overexpressed and 4534 mRNAs were underexpressed in the M2 group compared with the M1 group. More importantly, there was a significant steady-state in the expression levels of 275 mRNAs between three groups [29]. Another group identified a total of 1,253 differentially-expressed genes between M1 and M2 macrophages, of which 696 were upregulated and 557 downregulated in M1 macrophages compared with M2 macrophages [46]. Based on these previous findings, we performed Protein-Protein Interaction analysis and revealed that Tnf, SOCS3, and STAT1 were at the core location of macrophage polarization. SOCS proteins are a family of eight intracellular cytokine-inducible proteins [52, 53] obtaining a basal expression in cells. SOCSs could be sharply induced by many stimuli, including cytokines, TLR ligands, immune complexes, and hormones [54]. Although SOCSs are expressed at a very low level in macrophages, they could also be rapidly induced upon activation. SOCS1 and SOCS3 can regulate the polarization of macrophages to M1 and/or M2 subtypes [55, 56]. More importantly, IFN- $\gamma$ /STAT1, IL-4/STAT6, and IL-12/STAT4 signaling pathways in differentiating Th cells may be under feedback regulation by SOCS [57–59]. Yu et al. [60] revealed that in STAT1<sup>-/-</sup> Th2 cells, SOCS1 and SOCS3 protein levels are remarkably reduced; in addition, they also demonstrated that SOCS1 and SOCS3 could lead to the suppression of STAT6 signaling. In the present study, we observed that the phosphorylation of STAT1 dramatically increased in M1 macrophages while STAT6 phosphorylation increased in M2 macrophages. Consistently, SOCS3 expression was upregulated in both M1 and M2 macrophages compared to M0 type but was significantly higher in M1 compared to M2 macrophages. These findings suggest that STAT1/STAT6 signaling and SOCS3 might participate in the deregulation of macrophage polarization. Regarding differentially-expressed lncRNAs, lncRNA GBP1P1 (lncGBP9 in mice) was significantly

upregulated in M1 macrophages according to previous studies (GSE117040 and GSE5099) and our observations, suggesting that lncGBP9 may play a role in M1/M2 macrophage polarization.

As we have mentioned, GBP1P1 is a pseudogene of the guanylate-binding protein of GB (guanylate-binding protein). GBPs account for over 20% of the proteins induced after IFN- $\gamma$  treatment [61, 62]. GBP family is also involved in macrophage functions, such as IFN- $\gamma$ -mediated macrophage activation and immune defense [45]. More importantly, lncRNA GBP1P1 expression was significantly up-regulated in M1 macrophages according to several online data (Fig.S1). In the present study, by conducting lncGBP9 silence in M1 macrophages, we observed a decreased expression of SOCS3 and suppressed phosphorylation of STAT1, as well as reduced production of IL-6 and IL-12. On the contrary, lncGBP9 overexpression in M2 macrophages significantly induced the upregulation of SOCS3 while suppressed the phosphorylation of STAT6 and the production of IL-10 and TGF- $\beta$ 1. Notably, the effects of lncGBP9 silence on M1 macrophages were significantly reversed by SOCS3 overexpression while the effects of lncGBP9 overexpression on M2 macrophages were significantly reversed by SOCS3 silence. Consistently, in SCI mice model, lncGBP9 silence significantly suppressed STAT1 phosphorylation and SOCS3 expression while promoted STAT6 phosphorylation on day 28 after SCI; in the meantime, lncGBP9 silence caused a significant decrease in the BMS scores, indicating that lncGBP9 silence inhibits SOCS3 while promotes STAT6 activation at the late phase of SCI, therefore improving the SCI repair.

It has recently been discovered that lncRNAs act as miRNA "sponges" by sharing common miRNAs responses elements (MRE) and inhibiting the targeting activity of miRNAs on downstream target mRNAs, therefore forming posttranscriptional ceRNA networks to regulate the expression of downstream target mRNAs and participating in biological processes [25]. We have revealed that lncGBP9 plays an essential role in M1/M2 macrophage polarization via SOCS3 and STAT1/STAT6, here, we hypothesize miRNAs might mediate the function of lncGBP9 in macrophage polarization. miR-34a, previously known for its potent tumor-suppressive role, has been regarded as an inflammation regulator. Jiang et al. [31] reported that the expression of miR-34a was downregulated in macrophages after LPS stimulation. MiR-34a overexpression decreased the expression of inflammatory cytokines TNF- $\alpha$  and IL-6 in LPS-treated RAW264.7 cells. Furthermore, LPS-induced NF- $\kappa$ B activation was also significantly suppressed by miR-34a. In the present study, online tools predicted that miR-34a might target both lncGBP9 and SOCS3. Consistent with the previous studies, miR-34a expression was significantly downregulated in M1 macrophages after LPS + IFN- $\gamma$  stimulation while upregulated in M2 macrophages. miR-34a overexpression in M1 macrophages significantly inhibited lncGBP9 expression and reduced SOCS3 protein levels, on the contrary, miR-34a inhibition in M2 macrophages promoted the expression of lncGBP9 and the protein levels of SOCS3. Regarding the molecular mechanism, miR-34a directly targets lncGBP9 and SOCS3 3'-UTR. lncGBP9 competed with SOCS3 for miR-34a binding, therefore abolishing miR-34a-mediated SOCS3 suppression. lncGBP9 silence significantly decreased the levels of SOCS3 and M1 macrophage markers, while lncGBP9 overexpression increased SOCS3 while reduced the levels of M2 macrophage markers. In both macrophage types, the effects of miR-34a were opposite to those of lncGBP9. More importantly, the effects of lncGBP9 could be significantly reversed by miR-34a, indicating

that IncGBP9 exerts its functions in macrophage polarization via serving as a ceRNA for miR-34a to counteracting miR-34a-mediated SOCS3 suppression.

Interestingly, the phosphorylation of STAT6 and miR-34a expression is significantly upregulated in M2 macrophages. Previously, STAT6 has been regarded as the dominant mediator in IL-4-induced transcriptional alterations in macrophages [63]; herein, we speculated that STAT6 might also take the responsibility for the IL-4-induced miR-34a upregulation in macrophages. As predicted by online tools and later confirmed by luciferase reporter and CHIP assays, STAT6 binds the promoter region of miR-34a to activate its transcription.

## Conclusions

In summary, in macrophages, IncGBP9 competed with SOCS3 for miR-34a binding to counteract miR-34a-mediated suppression on SOCS3, therefore modulating STAT1/STAT6 signaling and the polarization of macrophages. STAT6 bound the promoter of miR-34a to activate its transcription, therefore forming two different regulatory loops to modulate the polarization of macrophages after SCI (Fig.8F). We provide a novel strategy for improving the failure in SCI repair.

## Declarations

**Ethics approval and consent to participate:** All procedures performed in studies involving animals were in accordance with the ethical standards of The Third Xiangya Hospital, Central South University and with the 1964 Helsinki declaration. Informed consent to participate in the study has been obtained from participants.

**Consent for publication:** Consent for publication was obtained from the participants.

**Availability of data and materials:** Please contact the authors for data requests.

**Conflict of Interest:** The authors declare that they have no competing interests.

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**Author Contributions:** Zhiyue Li, Qun Zhao made substantial contribution to the conception and design of the work; Tianding Wu, Yong Cao analyzed and interpreted the data; Zhiyue Li, Qiancheng Zhao drafted the manuscript; Jiahui Zhou revised the work critically for important intellectual content; Final approval of the work: all authors.

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## Abbreviations

BMDMs, bone marrow-derived macrophages; ChIP, Chromatin immunoprecipitation; CNS, central nervous system; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; PGE2, prostaglandin; RIP, RNA Immunoprecipitation; RNS, reactive nitrogen; ROS, reactive oxygen species; SCI, spinal cord injury; SOCS3, suppressor of cytokine signaling 3; STAT1, signal transducer and activator of transcription 1; STAT6, signal transducer and activator of transcription 6.

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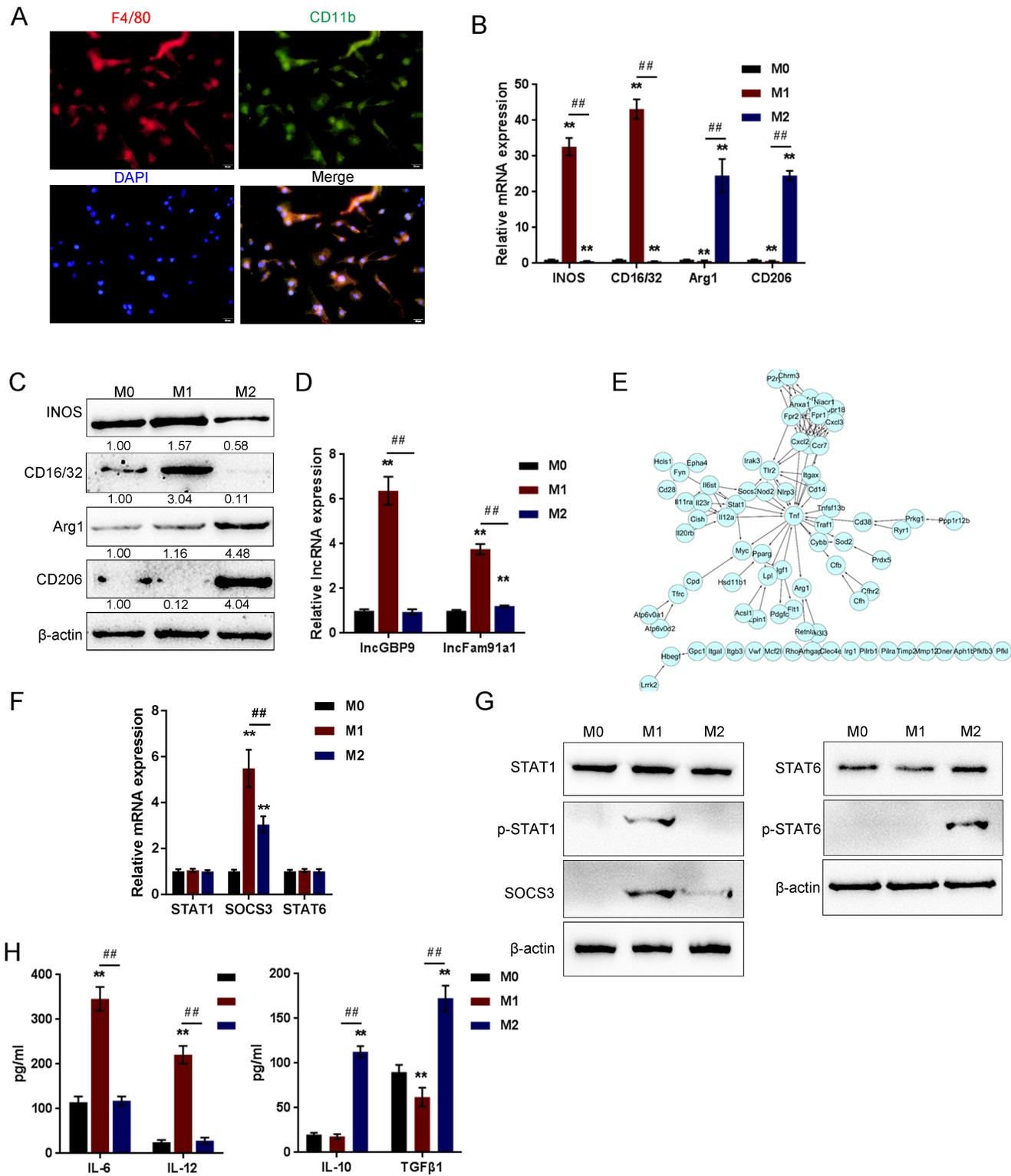
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## Figures



### Figure 1

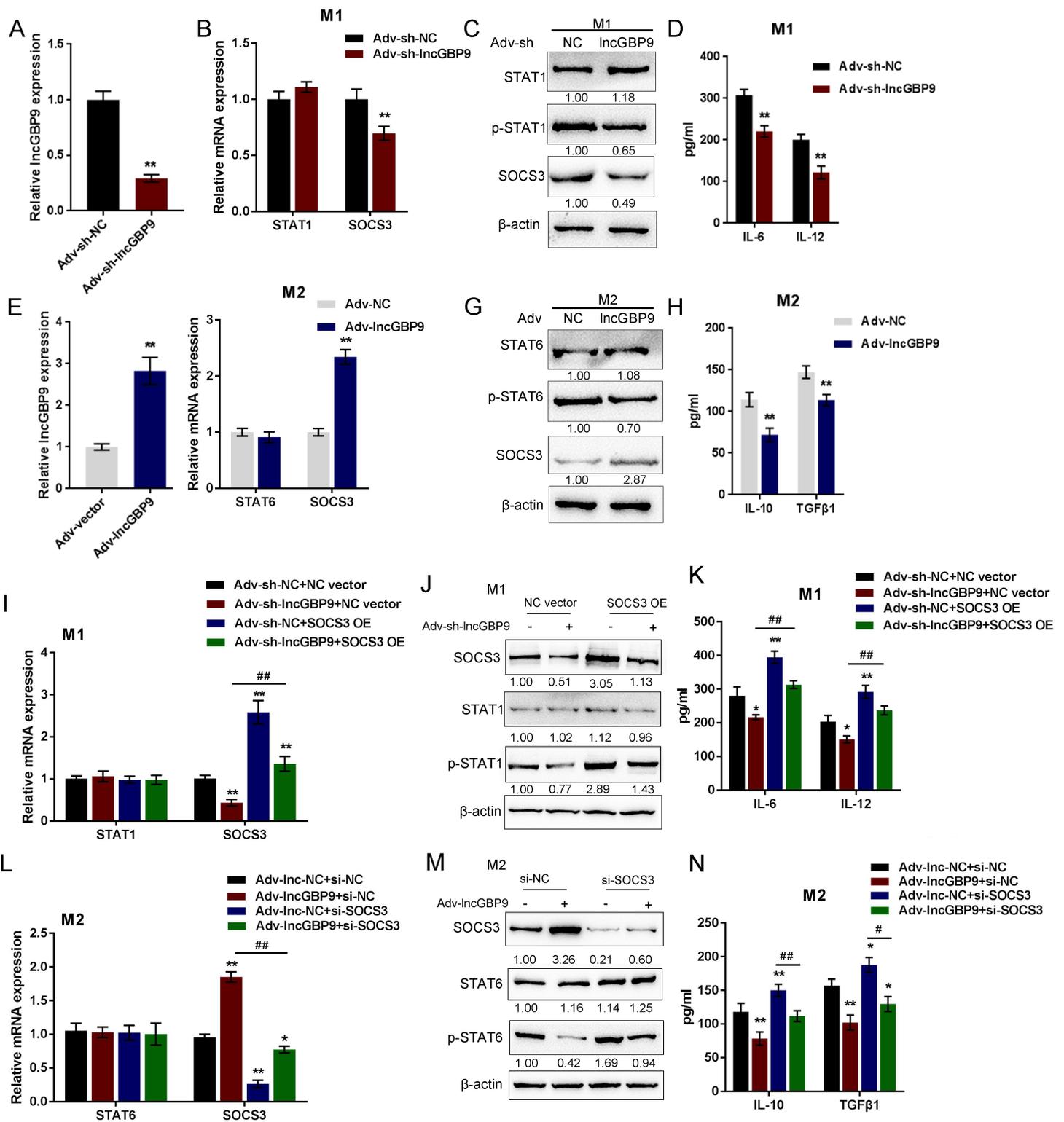
Macrophage polarization during spinal cord injury (SCI) (A) SCI model was constructed in Balb/c mice as described in M&M section. The content and distribution of M1 macrophage marker CD16/32 and M2 macrophage marker Arg1 in the lesion epicenter was examined by Immunofluorescence (IF) staining on day 1, 3, 7, 14, 28 after treatment. (B) The mRNA expression and (C) the protein levels of M1 macrophage markers CD16/32, iNOS, and IFN- $\gamma$  and M2 macrophage markers Arg1, CD206, and IL-4 were examined by real-time PCR and Immunoblotting on day 1, 3, 7, 14, 28 after treatment. \*P<0.05, \*\*P<0.01.



**Figure 2**

Selection of lncRNAs and genes related to macrophage polarization (A) Mouse bone marrow-derived macrophages (BMDMs) were isolated and identified by examining macrophage markers F4/80 and CD11b using IF staining. (B) BMDMs were induced for differentiating towards M1 or M2 macrophages; the mRNA expression of M1 macrophage markers iNOS and CD16/32 and M2 macrophage markers Arg1 and CD206 were examined in M0, M1, and M2 macrophages by real-time PCR; (C) the protein levels of M1

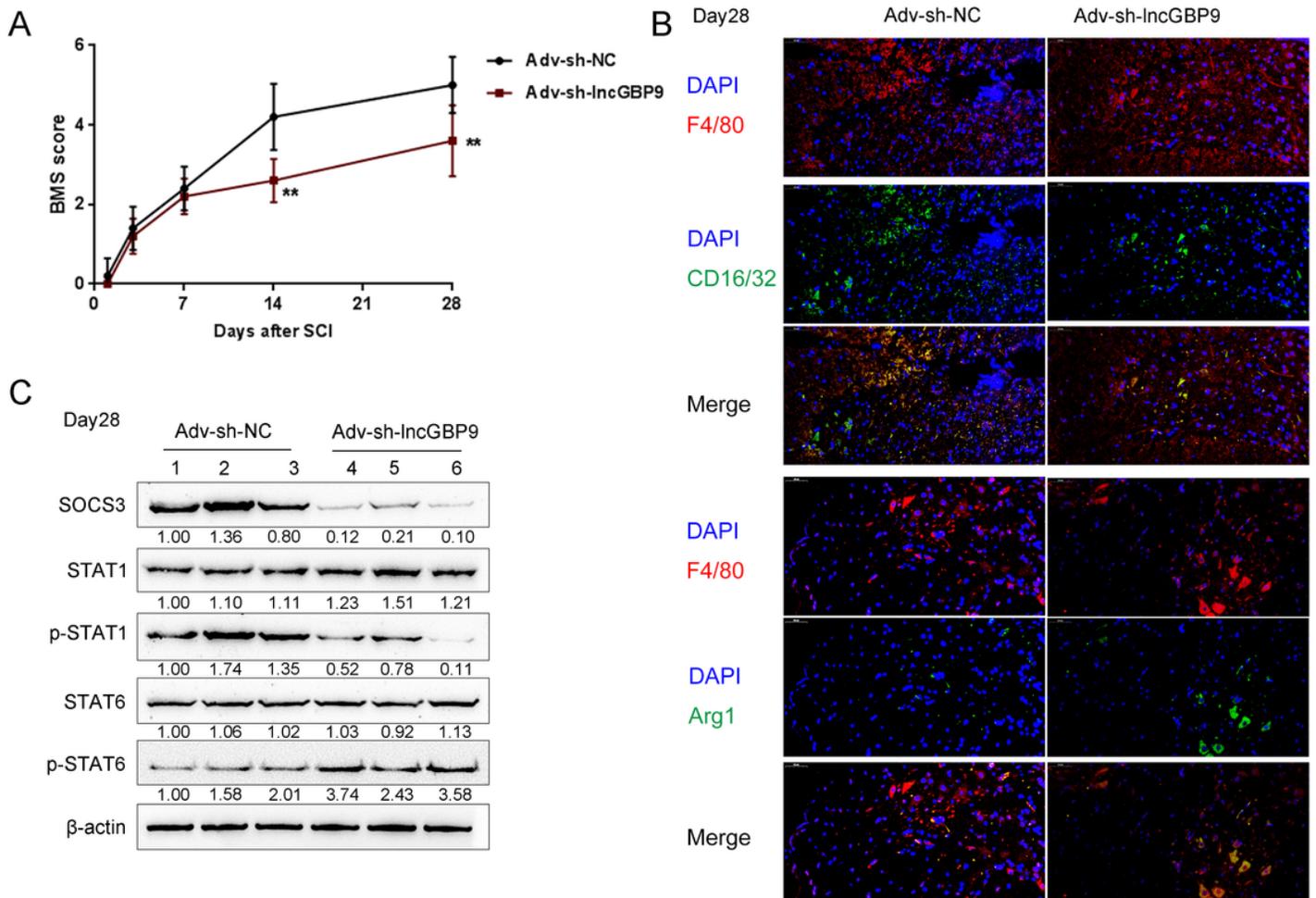
macrophage markers iNOS and CD16/32 and M2 macrophage markers Arg1 and CD206 were examined by Immunoblotting; (D) the expression of GBP9 and Fam91a1 were examined by real-time PCR. (E) STRING analyses on differentially-expressed genes reported previously. Tnf, Socs3, and Stat1 are key factors in macrophage polarization. (F-G) The mRNA expression and protein levels of STAT1, p-STAT1, SOCS3, STAT6, and p-STAT6 in M0, M1, and M2 macrophages determined by real-time PCR and Immunoblotting. (H) The production of cytokines, including IL-6, IL-12, IL-10, and TGF- $\beta$ 1 in M0, M1, and M2 macrophages determined by ELISA. \*P<0.05, \*\*P<0.01, compared to control group; #P<0.05, ##P<0.01, compared to M1 group.



**Figure 3**

Effects of IncGBP9 on macrophage polarization in vitro (A and E) The silence or overexpression of IncGBP9 conducted in BMDMs, as confirmed by real-time PCR. (B-C) The mRNA expression and protein levels of STAT1, p-STAT1, and SOCS3 in response to IncGBP9 silence were determined by real-time PCR and Immunoblotting in M1 macrophages. (D) The production of IL-6 and IL-12 in response to IncGBP9 silence were determined by ELISA in M1 macrophages. (F-G) The mRNA expression and protein levels of

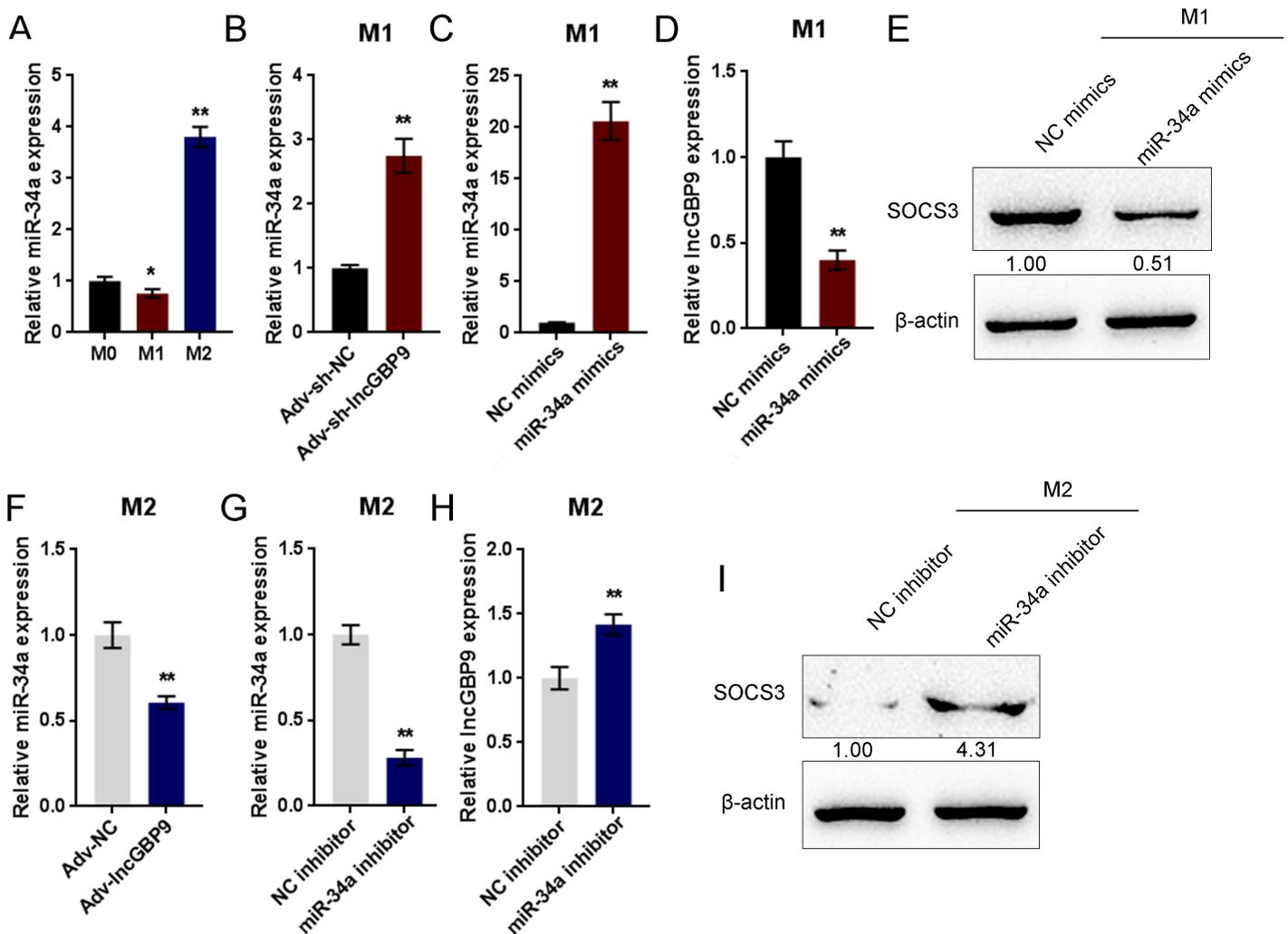
STAT6, p-STAT6, and SOCS3 in response to IncGBP9 overexpression were determined by real-time PCR and Immunoblotting in M2 macrophages. (H) The production of IL-10 and TGF- $\beta$ 1 in response to IncGBP9 overexpression were determined by ELISA in M2 macrophages. Next, M1 macrophages were co-transfected with Adv-IncGBP9 and SOCS3-overexpressing vector (SOCS3 OE) and examined for (I) the mRNA of STAT1 and SOCS3 by real-time PCR; (J) the protein levels of SOCS3, STAT1, and p-STAT1 by Immunoblotting; (K) the concentrations of IL-6 and IL-12 by ELISA. M2 macrophages were co-transfected with Adv-IncGBP9 and si-SOCS3 and examined for (L) the mRNA of STAT6 and SOCS3 by real-time PCR; (M) the protein levels of SOCS3, STAT6, and p-STAT6 by Immunoblotting; (N) the concentrations of IL-10 and TGF $\beta$ 1 by ELISA. \*P<0.05, \*\*P<0.01, compared to the control group; #P<0.05, ##P<0.01, compared to the Adv-IncGBP9 + NC (negative control) vector or Adv-IncGBP9 + si-NC (negative control) group.



**Figure 4**

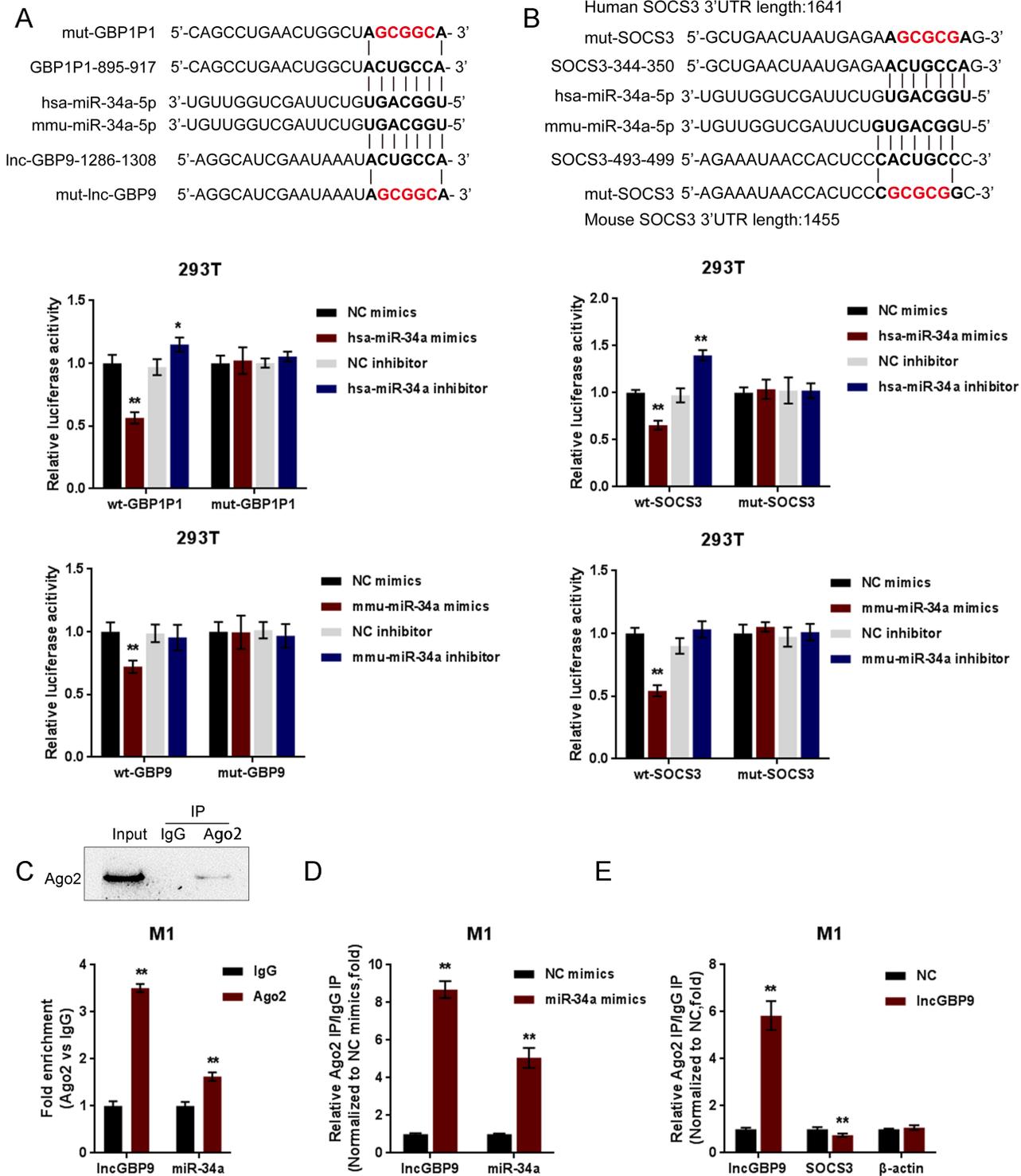
Effects of IncGBP9 on macrophage polarization in vivo LncGBP9 silence was conducted in the SCI mice model via tail vein injection Ad-sh-IncGBP9. (A) The BMS scores were evaluated on day 1, 3, 7, 14, and 28 after operation; (B) The content and distribution of M1 macrophage marker CD16/32 and M2 macrophage marker Arg1 determined by IF staining on day 28 after operation; (C) The protein levels of

STAT1, p-STAT1, STAT6, p-STAT6, and SOCS3 determined by Immunoblotting on day 28 after operation.  
 \*\*P<0.01.



**Figure 5**

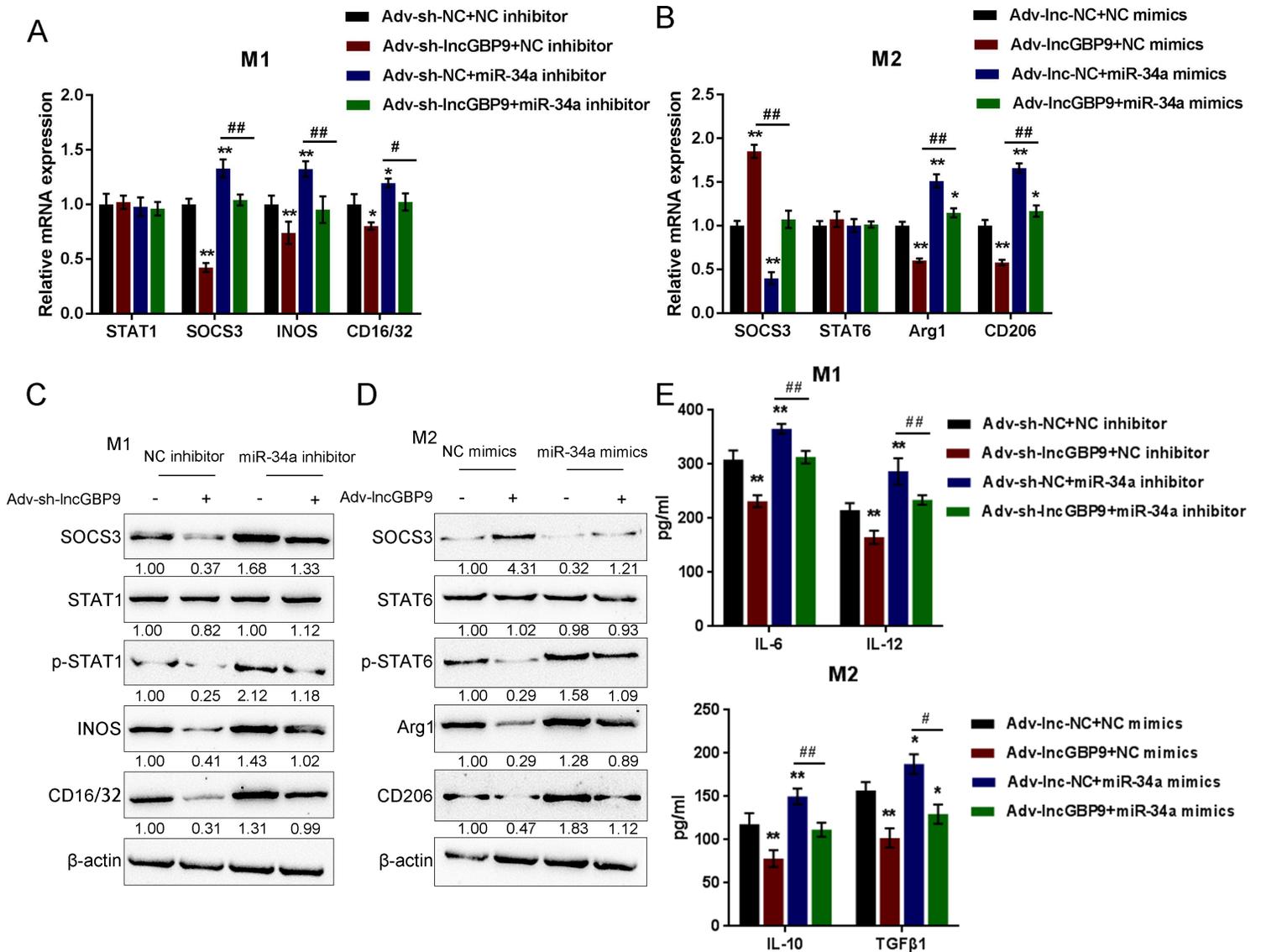
LncGBP9 modulates SOCS3 through miR-34a in macrophages (A) The expression of miR-34a determined in M0, M1, and M2 macrophages by real-time PCR. (B) The expression of miR-34a in response to LncGBP9 silencing determined in M1 macrophages by real-time PCR. (C) miR-34a overexpression conducted in M1 macrophages by transfection of miR-34a mimics, as confirmed by real-time PCR. (D) LncGBP9 expression in response to miR-34a overexpression determined in M1 macrophages by real-time PCR. (E) SOCS3 protein level in response to miR-34a overexpression determined in M1 macrophages by Immunoblotting. (F) miR-34a expression in response to LncGBP9 overexpression determined in M2 macrophages by real-time PCR. (G) miR-34a inhibition conducted in M2 macrophages by transfection of miR-34a inhibitor, as confirmed by real-time PCR. (H) LncGBP9 expression in response to miR-34a inhibition determined in M2 macrophages by real-time PCR. (I) SOCS3 protein level in response to miR-34a inhibition determined in M2 macrophages by Immunoblotting. \*P<0.05, \*\*P<0.01.



**Figure 6**

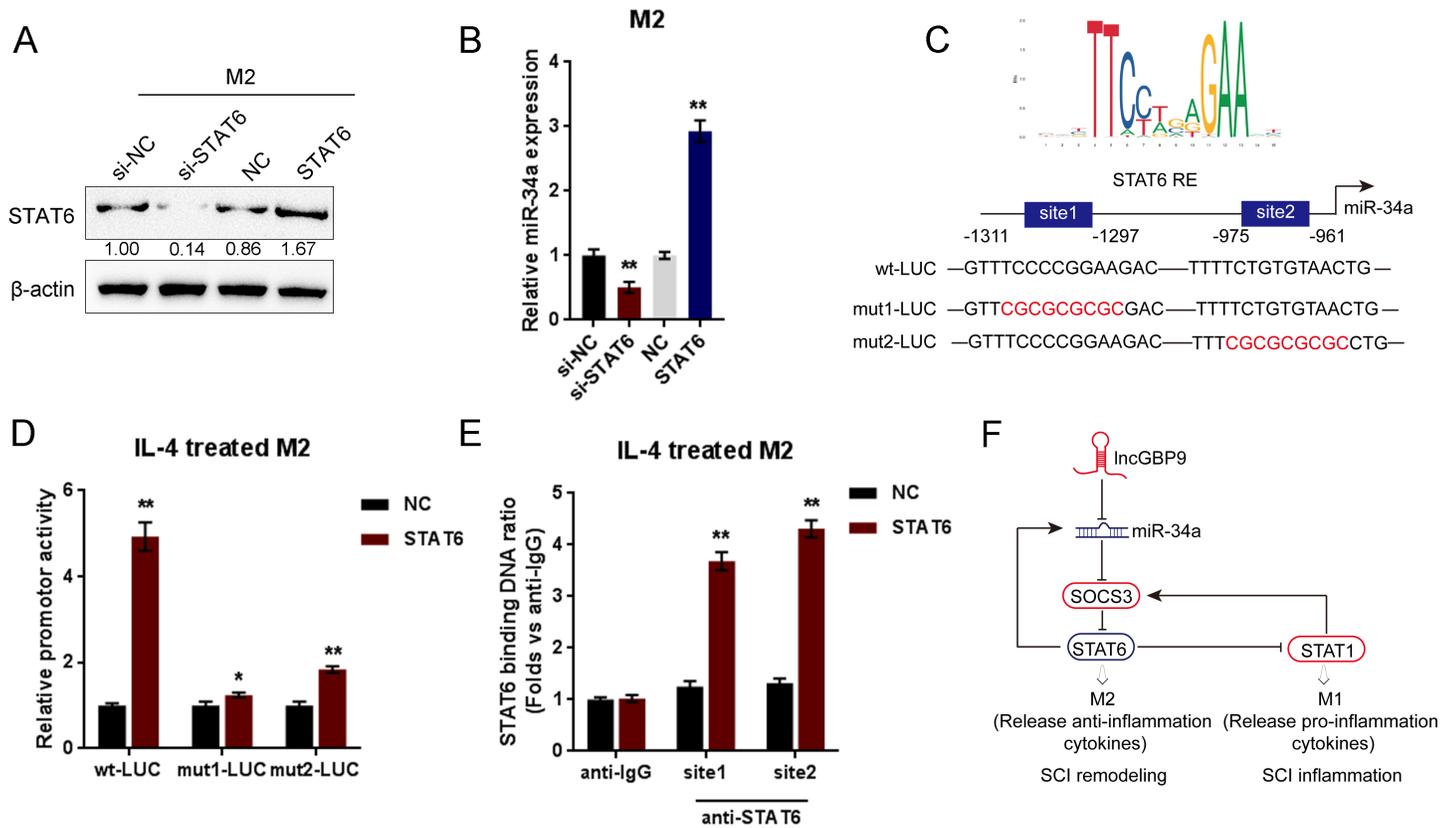
LncGBP9 serves as a ceRNA for miR-34a to counteract miR-34a-mediated SOCS3 suppression (A-B) Schematic diagrams showing the predicted binding site between miR-34a and lncGBP9 and between miR-34a and SOCS3. Wild- and mutant-type GBP9 and SOCS3 3'-UTR luciferase reporter vectors (wt-GBP9/SOCS3 3'-UTR or mut-GBP9/SOCS3 3'-UTR) were constructed and co-transfected in 293T cells with miR-34a mimics/inhibitor; the luciferase activity was determined. (C) Association of miR-34a and

IncGBP9 with AGO2 in M1 macrophages. Detection of AGO2 and IgG using Immunoblotting assays. (D) RIP assay in M1 macrophages transfected with control miRNA (NC mimics) or miR-34a mimics followed by real-time PCR to detect GBP9 and miR-34a associated with AGO2. (E) RIP assay in M1 macrophages transfected with control vector (NC) or IncGBP9-overexpressing vector followed by real-time PCR to detect GBP9, SOCS3, and  $\beta$ -actin associated with AGO2. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 7**

LncGBP9/miR-34a axis modulates macrophage polarization via affecting the balance of STAT1/STAT6 (A, C, and E) M1 macrophages were co-transfected with Ad-sh-IncGBP9 and miR-34a inhibitor and examined for the mRNA expression and protein levels of STAT1, p-STAT1, SOCS3, iNOS, and CD16/32, and the production of IL-6 and IL-12. (B, D, and E) M2 macrophages were co-transfected with Ad-IncGBP9 and miR-34a mimics and examined for the mRNA expression and protein levels of STAT6, p-STAT6, SOCS3, Arg1, and CD206, and the production of IL-10 and TGF $\beta$ 1. \* $P < 0.05$ , \*\* $P < 0.01$ , compared to control group; # $P < 0.05$ , ## $P < 0.01$ , compared to Ad-sh-NC + miR-34a inhibitor or Ad-Inc-NC + miR-34a mimics group.



**Figure 8**

STAT6 binds miR-34a promoter to activate its transcription (A) STAT6 overexpression or silence conducted in M2 macrophages by transfection of STAT6-overexpressing or si-STAT6 vector, as confirmed by Immunoblotting. (B) The expression of miR-34a in STAT6-overexpressing or STAT6-silenced M2 macrophages determined by real-time PCR. (C) A schematic diagram showing the predicted binding sites between STAT6 and miR-34a promoter. Wild- and mutant-type miR-34a luciferase reporter vectors are constructed. (D) STAT6 and wt- or mut-miR-34a were co-transfected in M2 macrophages; the luciferase activity was determined. (E) The real-time ChIP assay showed that the level of STAT6 antibody binding to miR-34a promoter was much greater than that of IgG. (F) In macrophages, IncGBP9 competed with SOCS3 for miR-34a binding to counteract miR-34a-mediated suppression on SOCS3, therefore modulating STAT1/STAT6 signaling and the polarization of macrophages. STAT6 bound the promoter of miR-34a to activate its transcription, therefore forming two different regulatory loops to modulate the polarization of macrophages after SCI.

## Supplementary Files

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- [Fig.S1.tif](#)