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ORIGINAL ARTICLE

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UNC-5 netrin receptor B regulates adipogenesis of human adipose–derived stem cells through JNK pathway

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Abstract

Background: There is a balance between adipogenic differentiation and osteogenic differentiation of human adipose–derived stem cells (hASCs). It is essential to explore the mechanism of hASCs lineage commitment. In our previous study, UNC-5 netrin receptor B (UNC5B) was identified as a positive regulator for osteogenesis.

Objective: To further explore the potential roles and mechanisms of UNC5B during adipogenic differentiation and to provide a new method to regulate adipogenesis and osteogenesis of hASCs.

Methods: Lentivirus containing *UNC5B* shRNA was used for *UNC5B* knockdown. Plasmids overexpressing *UNC5B* gene were used for *UNC5B* upregulation. To investigate the role of UNC5B in adipogenesis in vitro and in vivo, Oil Red O staining, RT-qPCR and transplantation into nude mice were performed. Western blotting analyses were performed to explore the mechanisms of UNC5B in adipogenic differentiation.

Results: UNC5B expression in hASCs was significantly increased during adipogenic differentiation. Knockdown of *UNC5B* enhanced adipogenic differentiation in vitro. Both H&E staining and Oil Red O staining showed more adipose tissue–like constructs in *UNC5B*-knockdown cells in vivo. Upregulation of UNC5B significantly impaired adipogenic differentiation in vitro. Downregulation of *UNC5B* could increase phosphorylation of JNK in hASCs. JNK inhibitors reduced adipogenic differentiation of hASCs.

Conclusion: Our findings showed that UNC5B inhibited adipogenesis of hASCs through JNK signalling. As a whole, UNC5B regulates both adipogenesis and osteogenesis of hASCs.

KEYWORDS

adipogenic differentiation, human adipose–derived stem cells, JNK pathway, netrin receptor

1 | INTRODUCTION

Adipose tissue defects can be found in tumour resection, trauma or genetic diseases. Autologous fat grafting has been widely studied for soft tissue augmentation, which is inefficient and unpredictable. Adipose tissue engineering may be a promising solution to regenerate adipose tissue. Human adipose–derived stem cells (hASCs) are attractive for adipose tissue engineering, with the potential to

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differentiate into mature adipocytes.[1-6] Moreover, there is a balance between adipogenic differentiation and osteogenic differentiation of hASCs.[7,8] It is essential for adipose tissue engineering to explore the mechanism of hASCs lineage commitment, which would be a great help for rational clinical use.

UNC-5 netrin receptor B (*UNC5B*) gene is located in 10q22.1, encoding a transmembrane receptor of netrin-1 and mediating its repulsive effect. In our previous study, we found that UNC5B promoted osteogenesis of hASCs through bone morphogenetic protein signalling.[9] However, the role of UNC5B in adipogenesis is still unexplored. Mitogen-activated protein kinase (MAPK) family is important to regulate the differentiation of stem cells, including ERK, p38 and JNK. It was reported that activated JNK signalling could enhance adipogenic differentiation of MSCs, and IGFBP2 could enhance adipogenesis of WJCMSCs through JNK and Akt signalling pathways.[10]

In the present study, we found that adipogenic differentiation was enhanced in *UNC5B*-knockdown hASCs by activating JNK signalling pathway. Through these findings, we can gain insight into the regulation of adipogenesis and osteogenesis, which is essential in adipose and bone tissue engineering.

2 | MATERIALS AND METHODS

2.1 | Cell culture

hASCs were cultured at 37℃ in proliferation medium (PM), consisting of DMEM, 10% FBS, 100 U/mL penicillin G and 100 mg/mL streptomycin. For adipogenic differentiation, cells were cultured in adipogenic medium (AM), consisting of PM, 50 nmol/L insulin, 100 nmol/L dexamethasone, 200 mmol/L indomethacin and 0.5 mmol/L 3-isobutyl-1-methylxanthine.

2.2 | Cell transfections

Knockdown of *UNC5B* was achieved as previously mentioned.[9,11] *UNC5B* stable knockdown cells were constructed with lentiviruses containing short hairpin (sh)RNAs [7]:

UNC5Bsh#1: CTGTCGGACACTGCCAACTAT;

UNC5Bsh#2: GGAGCCGAAACCGCTAATGTT.

The plasmid that encoding *UNC5B* was purchased from Shanghai Sangon Biotech (Shanghai, China). The plasmid was packaged with Lipofectamine 2000 (Invitrogen), and cells were transfected with either a control or a *UNC5B*-overexpressing vector according to the manufacturer's instructions.

2.3 | Oil Red O staining

After adipogenic induction for 2 weeks, 60% Oil Red O in isopropanol was used for staining. Dissolved with 100% isopropyl alcohol, Oil Red O quantification was performed spectrophotometrically at 520 nm.

2.4 | Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA using Reverse Transcription System (Takara, Kusatsu, Japan). Real-time quantitative PCR assays were performed on the ABI PRISM 7500 Sequence Detection System with SYBR Green Master Mix (Life Technologies).[9] As the internal control, GAPDH expression was detected to normalise gene expression levels. The primer sequences are as follows:

GAPDH:(F)CGGACCAATACGACCAAATCCG,(R)AGCCACATCGCTCAGACACC;

UNC5B:(F)TTACTGGTGCCAGTGCGTGG,(R)TCTTGCGCAGGTAGGCGATG;

C/EBPα:(F)CGCAAGAGCCGAGATAAAGC;(R)CACGGCTCAGCTGTTCCA;

adiponectin:(F)CTTGCAAGAACCGGCTCAGATCCTCCC;(R)GAGCTGTTCTACTGCTATTAGCTCTGC;

LPL:(F)CGGATTAACATTGGAGAAGCTATCCG;(R)AGCTGGTCCACATCTCCAAGTC;

CD36:CGATTAACATAAGTAAAGTTGCCATAATCG;(R)CGCAGTGACTTTCCCAATAGGAC.

2.5 | Western blotting

Western blotting analysis was performed as mentioned earlier.[12] Antibodies against UNC5B, JNK, phospho-JNK, ERK 1/2, phospho-ERK 1/2, PPARγ and GAPDH were purchased from Cell Signaling Technology. The protein bands were quantified with ImageJ analysis software for Windows.

2.6 | Adipose tissue formation in vivo

After adipogenic induction for 1 week, hASCs were seeded on Collagen Sponge for 2 hours and implanted subcutaneously into BALB/c nude mice, with the approval of Peking University Animal Care and Use Committee. The complex was harvested at 8 weeks after implantation.

The harvested implants were fixed in 4% paraformaldehyde. For H&E staining, implants were embedded with paraffin. For Oil Red O staining, implants were embedded in a Tissue-Tek OCT freezing medium (Sakura Finetek Inc, Torrance, CA, USA). 60% Oil Red O in isopropanol was used for staining.

2.7 | Statistical analysis

We performed statistical analysis with GraphPad software and compared two groups by independent two-tailed Student's t test. All experiments were repeated three times, and all data were shown as the mean ± standard deviation (SD).

3 | RESULTS

3.1 | *UNC5B* expression during adipogenesis of hASCs

After 2 weeks of adipogenic induction, Western blotting analysis revealed that endogenous expression of both the UNC5B protein and the adipogenic marker PPARγ were significantly increased ($P < .01$) (Figure 1A,B).

3.2 | Knockdown of *UNC5B* significantly enhances adipogenesis in vitro

To understand the role of UNC5B in adipogenesis, two shRNA sequences were used for *UNC5B* knockdown. Fluorescent staining, real-time quantitative PCR and Western blotting were performed to confirm the knockdown efficiency (Figure S1A-B, Figure 2A-B). Increased lipid deposits were found in *UNC5B*-knockdown cells ($P < .001$) through Oil Red O staining and quantification (Figure 2C,D). Moreover, knockdown of *UNC5B* enhanced the mRNA expression of *CD36*, *LPL*, *adiponectin* and *C/EBPα*, which were known as adipogenic marker genes ($P < .01$) (Figure 2E-H).

3.3 | Upregulation of *UNC5B* inhibits adipogenesis in vitro

To further confirm the important role of UNC5B in adipogenic differentiation, plasmids overexpressing *UNC5B* gene were used for *UNC5B* upregulation. The expression of *UNC5B* was examined by real-time RT-PCR (Figure 3A). In *UNC5B*-overexpressed cells, neutral fat stained with Oil Red O was observed less than control cells ($P < .001$) (Figure 3B,C). In addition, the gene expression of adipogenic markers was significantly suppressed when UNC5B was upregulation, including *CD36*, *LPL*, *adiponectin* and *C/EBPα* ($P < .01$) (Figure 3D-G).

3.4 | *UNC5B* knockdown enhances adipogenesis in vivo

Seeded on collagen sponge, hASCs were implanted subcutaneously into nude mice. H&E and Oil Red O staining showed more adipose tissue–like constructs in *UNC5B*-knockdown hASCs (Figure 4A,B).

3.5 | UNC5B negatively regulates JNK signalling in adipogenesis

In the previous study, no significant changes were found in the expression of p-Akt and p-p38 after *UNC5B* knockdown.[9] Nevertheless, it has been reported that activated JNK signalling could promote adipogenic differentiation of MSCs.[5] So we decided to explore whether UNC5B regulated adipogenesis through JNK signalling. Western blotting indicated that downregulation of *UNC5B* could increase phosphorylation of JNK and ERK 1/2 in hASCs, while no marked change was found in total protein level of JNK and ERK 1/2 (Figure 5A). To further investigate the effect of UNC5B on JNK signalling pathway during adipogenic differentiation, the specific inhibitor of JNK signalling (SP600125, 40 uM) was used to treat *UNC5B*-knockdown hASCs. As expected, SP600125 significantly reduced the promotion of adipogenesis induced by *UNC5B* knockdown (Figure 5B-G).

4 | DISCUSSION

As we know, bone is a dynamic tissue undergoing continuous resorbing and forming by osteoblasts and osteoclasts.[13] Besides, the relationship between bone and fat formation in the bone marrow is considered inverse. Imbalance between the formation of osteoblasts and adipocytes may cause bone diseases such as osteoporosis.[14,15] Mesenchymal stem cells (MSCs) have attracted much attention, which can give rise to both osteoblasts and adipocytes.[16] Li concluded that pathological environmental factors could increase adipose tissue formation from MSCs, including inflammation and osteoblastic inhibitors.[17] Moreover, MSCs are considered as a new target for the treatment of obesity, as MSC dysfunction may induce the progression of obesity.[18]

However, further research is needed to explore the mechanism of MSC lineage commitment.

UNC5B, a transmembrane receptor of netrin-1, mediates the repulsive effect of netrin-1. It was reported to act as a coreceptor for RGMa, activate RhoA and mediate collapse of the neuronal growth cone.[19] Besides, *UNC5B* expression was increased

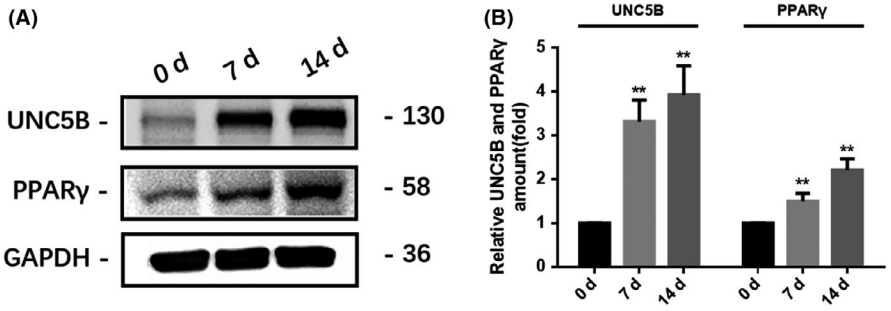

FIGURE 1 Endogenous expression of UNC5B during adipogenic differentiation of hASCs. (A-B) Western blotting (A) and quantification (B) of UNC5B and PPARγ expression in hASCs after 0, 7 and 14 d of adipogenic induction. All data were shown as the mean ± SD, n = 3. $^{**}P < .01$ versus the control group

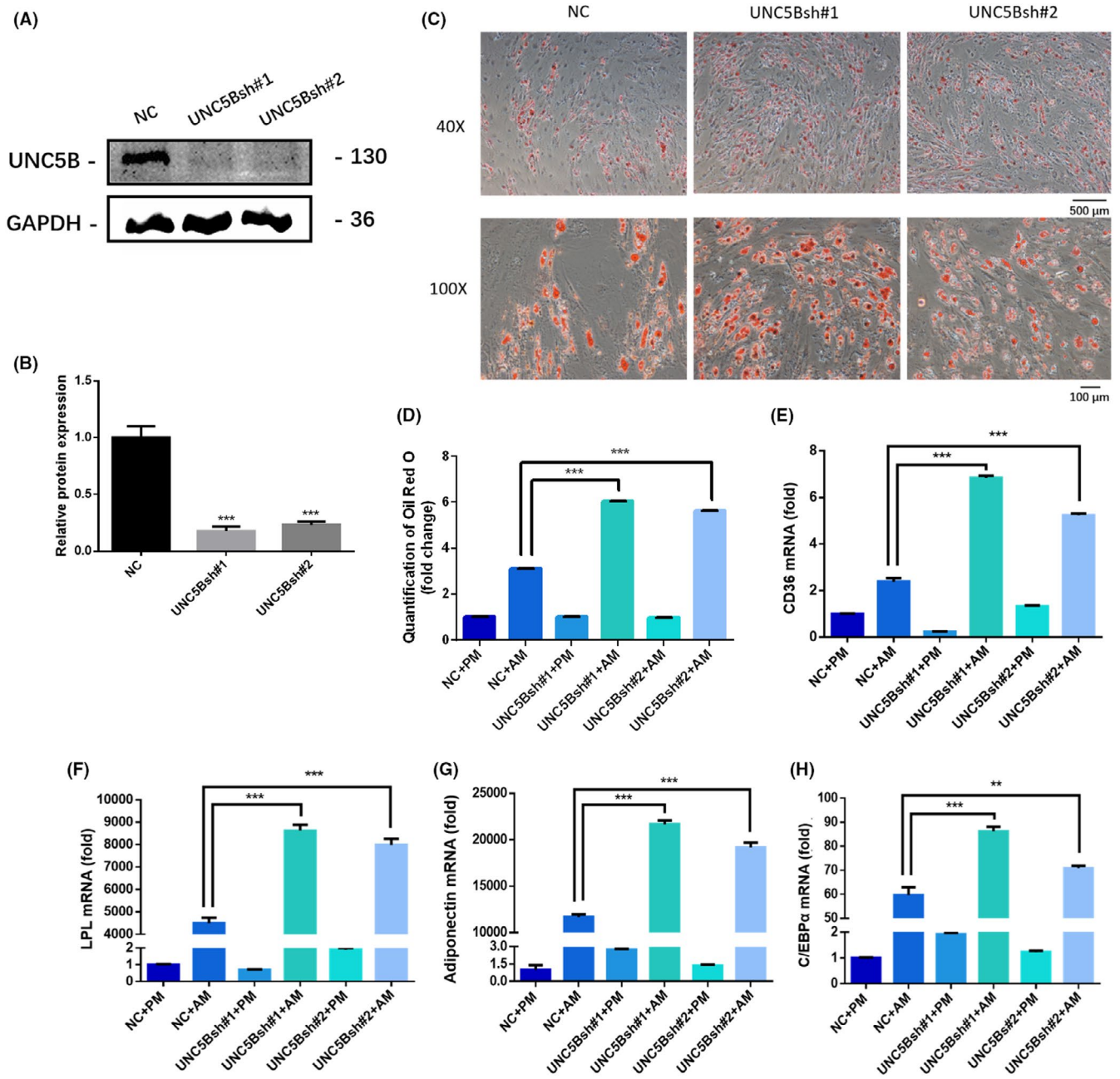

FIGURE 2 Knockdown of *UNC5B* promotes adipogenic differentiation in vitro. (A-B) Knockdown of *UNC5B* was validated by Western blotting (A) and quantification (B). (C-D) Oil Red O staining (C) and quantification (D) of cells at day 21 after adipogenic induction. Scale bar, 500 μm (up panel) and 100 μm (down panel). (E-H) Quantitative real-time PCR analysis of *CD36* (E), *LPL* (F), *adiponectin* (G) and *C/EBPα* (H) expression in transfected hASCs. All data were shown as the mean ± SD, n = 3. ***$P < .001$ and **$P < .01$. AM, adipogenic medium; NC, control cells; PM, proliferation medium; UNC5Bsh, *UNC5B*-knockdown cells [Colour figure can be viewed at wileyonlinelibrary.com]

during sprouting angiogenesis of mice. When combined with netrin-1, UNC5B could inhibit sprouting angiogenesis.[20] Moreover, UNC5B is able to induce apoptosis and suppresses tumour formation.[21] UNC5B-induced apoptosis is limited by netrin-1 in pluripotent ES cells, which may have influence on pluripotency maintenance of stem cells.[22] In addition, our previous study revealed that *UNC5B* knockdown significantly suppressed osteogenesis of hASCs through bone morphogenetic protein signalling.[9]

The present study revealed the role of UNC5B in adipogenesis of hASCs. Endogenous expression of UNC5B was increased during adipogenic differentiation, which indicated that UNC5B may be involved in adipogenesis. Oil Red O staining revealed that *UNC5B* knockdown significantly enhanced adipogenic differentiation in

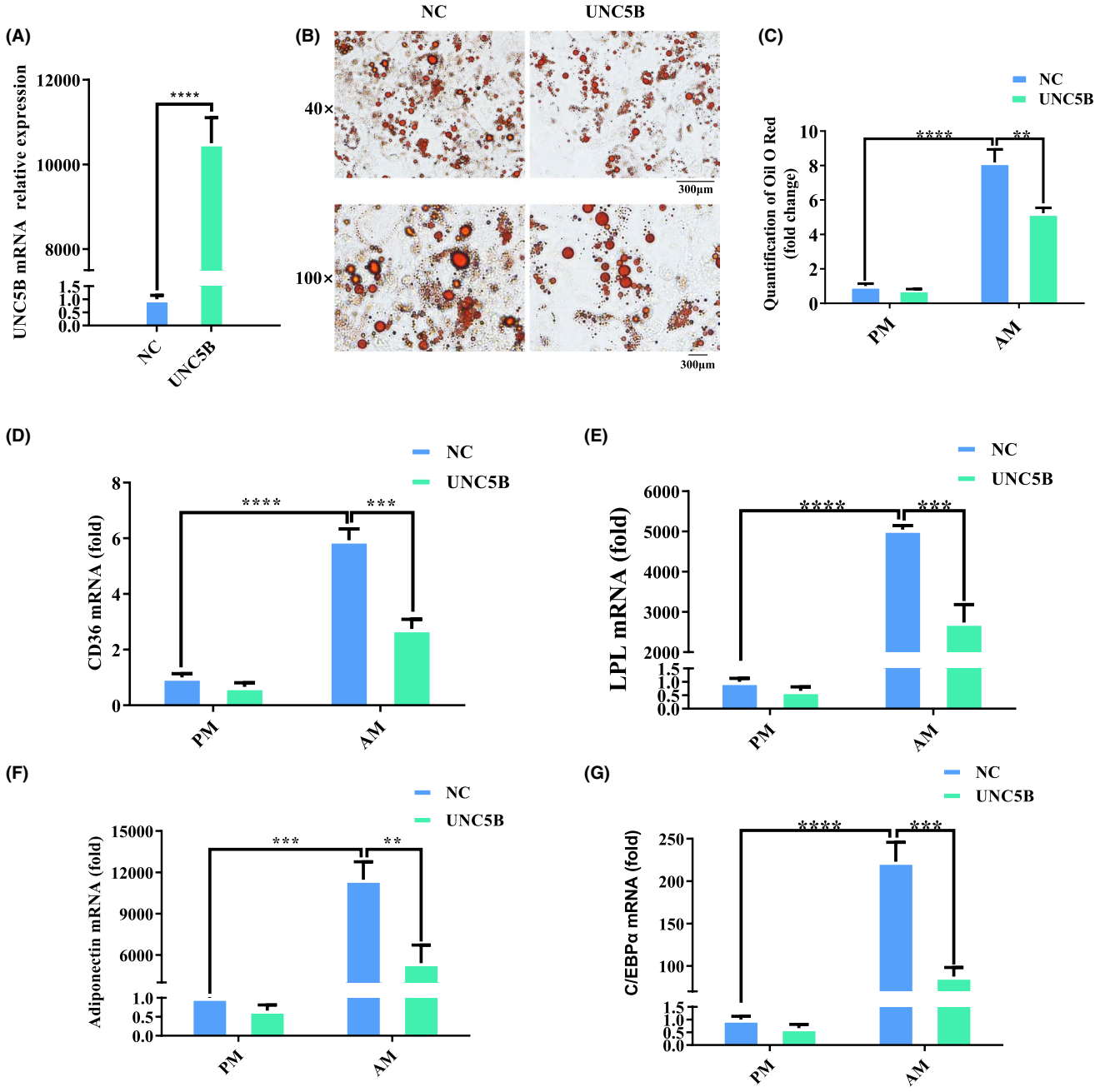

FIGURE 3 Upregulation of *UNC5B* inhibits adipogenesis in vitro. A, Upregulation of *UNC5B* was validated by quantitative real-time PCR analysis. (B-C) Oil Red O staining (B) and quantification (C) of cells at day 14 after adipogenic induction. Scale bar, 300 μm. (D-G) Quantitative real-time PCR analysis of *CD36* (D), *LPL* (E), *adiponectin* (F) and *C/EBPα* (G) expression in transfected hASCs. All data were shown as the mean ± SD, n = 3. *****P* < .0001, ****P* < .001, ***P* < .01. AM, adipogenic medium; NC, control cells; PM, proliferation medium; UNC5B, *UNC5B*-overexpressed cells [Colour figure can be viewed at wileyonlinelibrary.com]

vitro. In addition, *UNC5B* overexpression significantly impaired adipogenic differentiation in vitro. Transplantation into nude mice confirmed the role of UNC5B in adipogenesis in vivo. Furthermore, Western blotting analyses showed that phosphorylation of JNK in *UNC5B*-knockdown hASCs was increased after adipogenic induction, while total protein level of JNK was not affected. The specific inhibitor of JNK signalling (SP600125) reversed the promotion of adipogenesis induced by *UNC5B* knockdown. In general, our study clarified that UNC5B could positively regulate osteogenic differentiation and negatively regulate adipogenic differentiation of hASCs.

While UNC5B expression was significantly increased during adipogenic differentiation, UNC5B played a negative role in adipogenesis. It may be interpreted that UNC5B is a critical regulator for preventing excessive adipogenic differentiation and obesity. hASCs were considered as a novel clinical modality for the treatment of dysregulated metabolism induced by obesity.[23] During

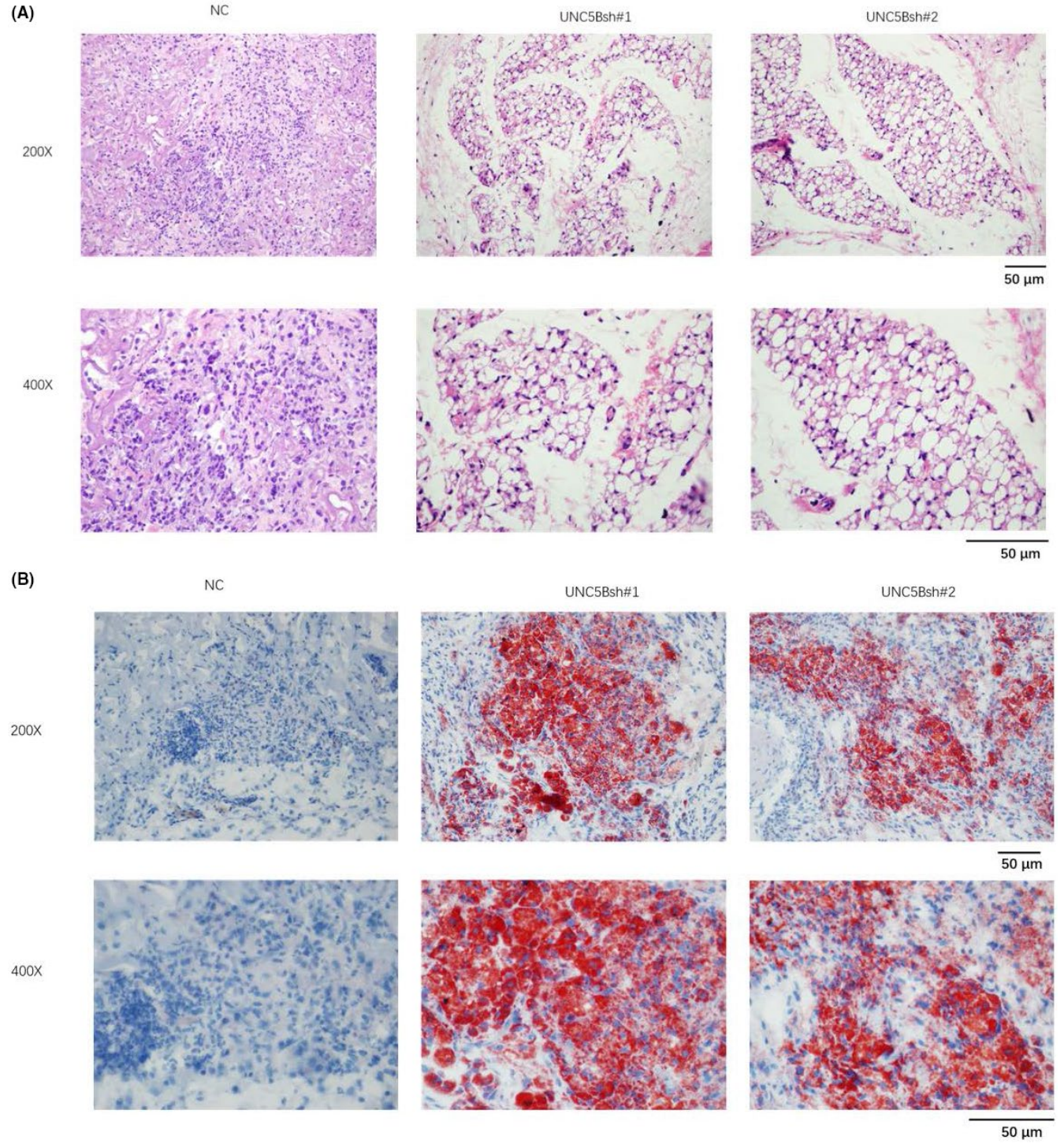

FIGURE 4 Knockdown of *UNC5B* promotes adipogenic differentiation in vivo. A, H&E staining of histological sections from implanted hASC-scaffold hybrids in NC, *UNC5Bsh1* and *UNC5Bsh2* groups. Scale bar: 50 μm. B, Oil Red O staining in NC, *UNC5Bsh1* and *UNC5Bsh2* groups. Scale bar: 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

adipogenesis, UNC5B expression of hASCs was upregulated, which inhibited adipogenic differentiation and formed a negative feedback loop. However, the relationship among UNC5B, hASCs and obesity and its potential implications remain unclear. Further investigation is necessary for hASC-based treatments for obesity.

Our previous work indicated that knockdown of *UNC5B* inhibited osteogenesis of hASCs effectively.[9] Collectively, our study clarified that UNC5B was a critical regulator for the lineage commitment of hASCs, which may benefit adipose or bone tissue engineering.

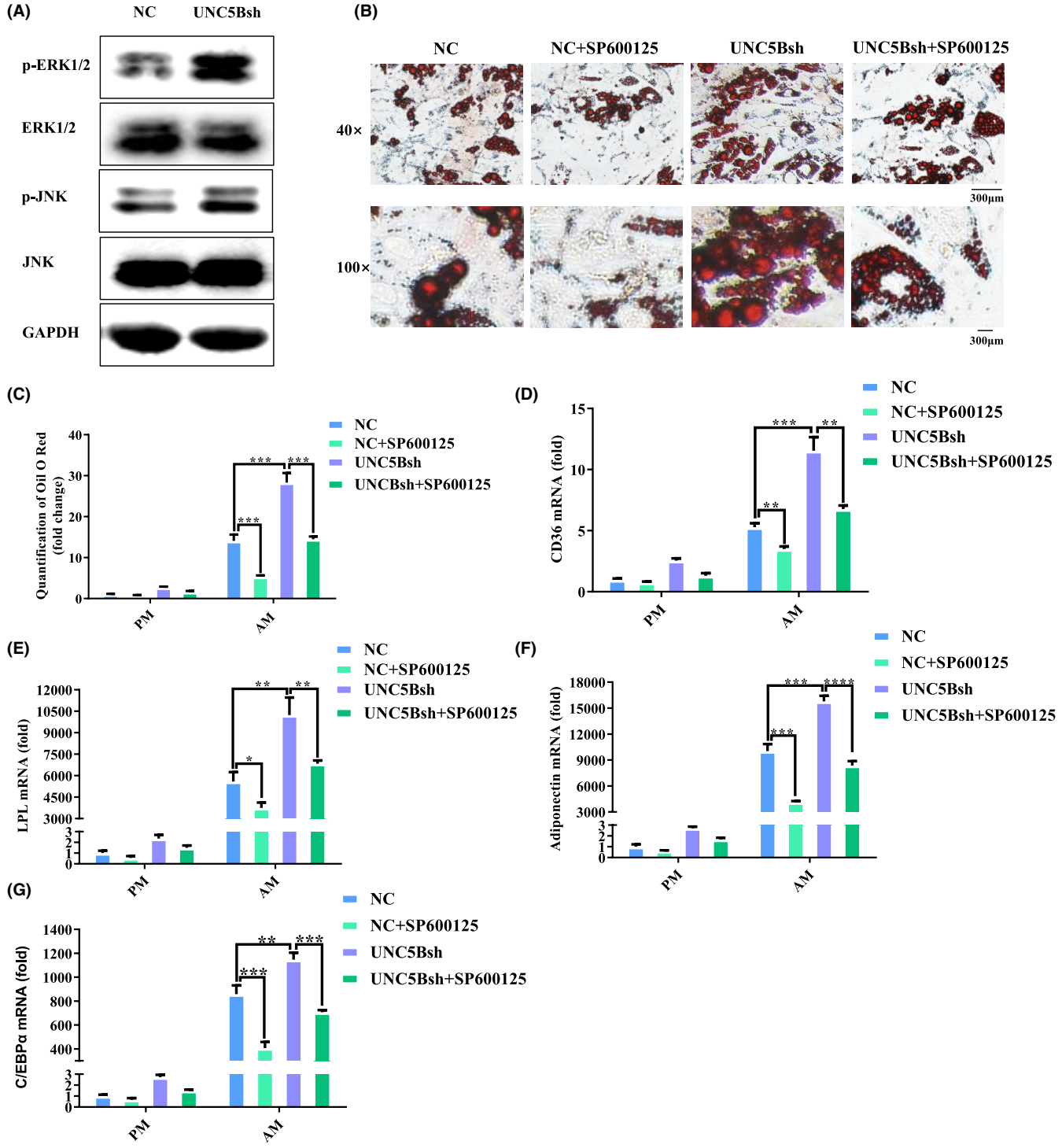

FIGURE 5 *UNC5B* regulates adipogenic differentiation through the JNK signalling pathway. A, Western blotting of p-ERK1/2, p-JNK, ERK1/2 and JNK expression in *UNC5B*-knockdown cells. (B-C) Control or *UNC5B*-knockdown cells were treated with the inhibitor of JNK signalling (SP600125, 40 μmol/L), which reduced the promotion of adipogenesis in Oil Red O staining (B) and quantification (C). Scale bar, 300 μm. (D-G) SP600125 treatment reduced the expression of *CD36* (D), *LPL* (E), *adiponectin* (F) and *C/EBPα* (G) in *UNC5B*-knockdown cells as determined by quantitative real-time PCR. All data were shown as the mean ± SD, n = 3. ***$P < .001$, **$P < .01$, *$P < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

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CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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