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Knockdown of *ARL4C* inhibits osteogenic differentiation of human adipose-derived stem cells through disruption of the Wnt signaling pathway

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ABSTRACT

ADP-ribosylation factor-like 4C (ARL4C) has been shown to play an important role in cholesterol secretion, microtubule dynamics, and cell morphological changes. However, its role in osteogenesis has not been explored. In this study, we found that *ARL4C* is downregulated during the osteogenic differentiation of human adipose derived stem cells (hASCs). Knockdown of *ARL4C* suppresses osteogenesis of hASCs *in vitro* and *in vivo*. We demonstrate that *ARL4C* knockdown likely attenuates osteogenesis of hASCs through inhibition of the Wnt signaling pathway. These results provide new insights into the mechanisms of osteogenic differentiation and provide a potential molecular target for bone tissue engineering.

1. Introduction

Bone defects are a serious problem, with the associated loss of function considerably impairing the quality of life of affected patients. Bone tissue engineering is a relatively young, but rapidly evolving and innovative research field. To successfully develop tissue substitutes, there are three crucial components that constitute the key focus of tissue engineering: stem cells that produce or replace lost tissue, a biocompatible scaffold, and tissue-inducing substances that will induce specific cell phenotypes [1–3].

ADP-ribosylation factor-like 4C (ARL4C) is a member of the ADP-ribosylation factor family of GTP-binding proteins that was first isolated through a search of the expressed sequence tags database and performing 5′ rapid amplification of cDNA ends. ARL4C is reported to modulate the dynamics of microtubule polymerization and depolymerization [4–6]. Previous studies have shown that ARL4C in an LXR target gene that stimulates cholesterol efflux through the high-density lipoprotein (HDL)-mediated reverse cholesterol transport (RCT) pathway [5,7,8]. ARL4C is implicated in tumorigenesis in colon and pancreatic carcinoma, and may promote proliferation, migration and invasion of cancer cells [5]. Moreover, ARL4C is considered to be a positive regulator of epithelial tube formation by stimulating motility and proliferation of epithelial cells during the formation of tube-like structures [6].

We have previously reported that *ARL4C* is downregulated during osteogenic differentiation of human adipose derived stem cells (hASCs), based on microarray analysis. Other studies have described a relationship between *ARL4C* and the Wnt signaling pathway. However, to date, there are no reports on the role of *ARL4C* in osteogenic differentiation. Here, we examined *ARL4C* expression during osteogenic differentiation to establish whether the Wnt signaling pathway is regulated by *ARL4C* expression.

2. Materials and methods

2.1. Cell culture and reagents

The hASCs were purchased from ScienCell (San Diego, CA, USA). Stem cells were grown in Dulbecco's Modified Eagle's medium, containing 10% fetal bovine serum (FBS) and 1% penicillin/

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streptomycin. Cells were maintained in an incubator at 37 °C in 5% CO_2. Osteogenic differentiation was induced with osteogenic medium (OM) containing 10% FBS, 100 IU/mL penicillin/streptomycin, 100 nM dexamethasone, 200 μM ascorbic acid and 10 mM β-glycerophosphate. All *in vitro* cell based experiments were repeated at least twice.

2.2. Lentivirus infection

Viral packaging and infection was performed as described previously [9]. Transfection of hASCs was performed by incubating cells with dilutions of the viral supernatant in the presence of polybrene (5 μg/mL) for 24 h, then supplanting with fresh medium. Beginning at 72 h post transfection, puromycin (10 μg/mL) was used to select the stably transfected cells. The shRNA target sequences were as follows:

sh*ARL4C*-1:GCTCAAGTTCAACGAGTTCGT1
sh*ARL4C*-2:GATGATCCTGAAACGCAGGAA

2.3. Alkaline phosphatase activity of hASCs

The hASCs were seeded in 6-well plates with same cell density, and alkaline phosphatase (ALP) activity assays performed on the 7th and 14th days of osteoinduction. ALP staining was performed with a nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining kit (CoWin Biotech, China) according to the manufacturer's instructions. For quantification of ALP activity, cells seeded in 6-well plates were rinsed twice with phosphate-buffered saline (PBS), and activity measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute). The total protein content of each sample was determined by the BCA method using a Pierce Protein Assay Kit (Thermo Fisher Scientific).

2.4. Alizarin red S staining and mineralization assays

The hASCs were seeded in 6-well plates, briefly rinsed with PBS, then fixed in 70% ethanol for at least 1 h. To monitor mineralization, cells were rinsed twice with PBS, stained with 40 mM filtered Alizarin red S (ARS) and rinsed five times with PBS to remove unbound ARS. To quantify matrix mineralization, ARS-stained cells were incubated in 100 mM cetylpyridinium chloride for 1 h to solubilize and release calcium-bound ARS into the solution. Prepared solution above for absorbance measurement at 562 nm, using an ARS standard curve in the same solution.

2.5. Real-time qRT-PCR

Total RNA was extracted from hASCs cultured in proliferation or differentiation medium for 14 days, using TRIzol Reagent (Invitrogen), and used for first strand cDNA synthesis with the Reverse Transcription System (Takara Bio). Differential gene expression was examined by qRT-PCR using a Power SYBR Green PCR Master Mix and an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA), with GAPDH used as a reference gene. Primers used in this study are listed in Table 1.

2.6. Western blot analysis

For evaluation of osteogenesis, total protein was extracted at 7 and 14 days following osteoinduction. Briefly, transfected cells were harvested and washed with PBS. Cells were lysed in radioimmunoprecipitation buffer containing 2% proteinase inhibitor, and lysates clarified by centrifugation at 14000 rpm for 30 min at 4 °C. Samples were resolved by SDS-PAGE and transferred onto membranes. Primary antibodies stostes against ARL4C (Abcam), RUNX2, WNT5A, WNT11, LRP6, P-LRP6 and GAPDH (Huaxingbio) were diluted 1:2000 with Tris -Hcl buffer solution (TBS-T) and incubated with the membranes at 4 °C overnight. Afuter three times of washes with TBS-T 5min, horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Cell Signaling) were diluted 1:10000 and incubated with the membranes at room temperature for 1 h. For analysis, the background was subtracted and the signal of each target band was normalized to that of the GAPDH band.

Table 1
List of primers used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ARL4C	ATCCCGGCCACCACCTATCA	GTCACCAGTCCGCTTCTTCTTCT
ALP	ATGGGATGGGTGTCTCCACA	CCACGAAGGGGAACTTGTC
OCN	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
RUNX2	CCGCCTCAGTGATTTAGGGC	GGGTCTGTAATCTGACTCTGTCC
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

2.7. In vivo implantation of hASCs and ectopic bone formation

This study was approved by the Institutional Animal Care and Use Committee of the Peking University Health Science Center (LA2014233) and all animal experiments were performed in accordance with the institutional animal guidelines.

Lentivirus-infected hASCs carrying control shRNA or *ARL4C* shRNA were incubated with Bio-Oss Collagen scaffolds for 1 h at 37 °C. The hASCs-seeded scaffolds were implanted into the dorsal subcutaneous space of six-week-old female nude mice. Each mouse was implanted with two scaffolds carrying either control hASCs or *ARL4C* knockdown hASCs. Eight weeks after implantation, animals were sacrificed and specimens were taken as a whole then decalcified for four weeks in 10% EDTA (pH 7.4). Osteogenesis was evaluated by immunohistochemical analysis.

2.7.1. Statistical analysis

Data were analyzed using SPSS Statistics 20.0 software (IBM). Differences between two groups were assessed by a two-tailed Student's *t*-test. A p value of <0.05 was considered to be statistically significance. Data shown represents the mean ± standard deviation.

3. Results

3.1. ARL4C is involved in osteogenic differentiation of hASCs

We previously conducted transcriptome profiling by microarray of hASCs following osteoinduction, and found that *ARL4C* may play a role in osteogenic differentiation of hASCs. To validate this result, we examined the expression of *ARL4C* by qRT-PCR at days 7 and 14 of hASCs osteogenesis (Fig. 1A). The results show that *ARL4C* is downregulated during osteogenic differentiation of hASCs and remains at a low level. The expression levels of *RUNX2*, *ALP*, and *OCN* are upregulated during osteogenic differentiation (Fig. 1B–D). Western blot analysis reveals a similar trend for ARL4C protein expression (Fig. 1E–F).

3.2. Validation of ALR4C knockdown

To explore the role of ALR4C in osteogenic differentiation, we established *ALR4C* knockdown hASCs using a lentivirus vector expressing shRNA. The transfection efficiency was estimated to be

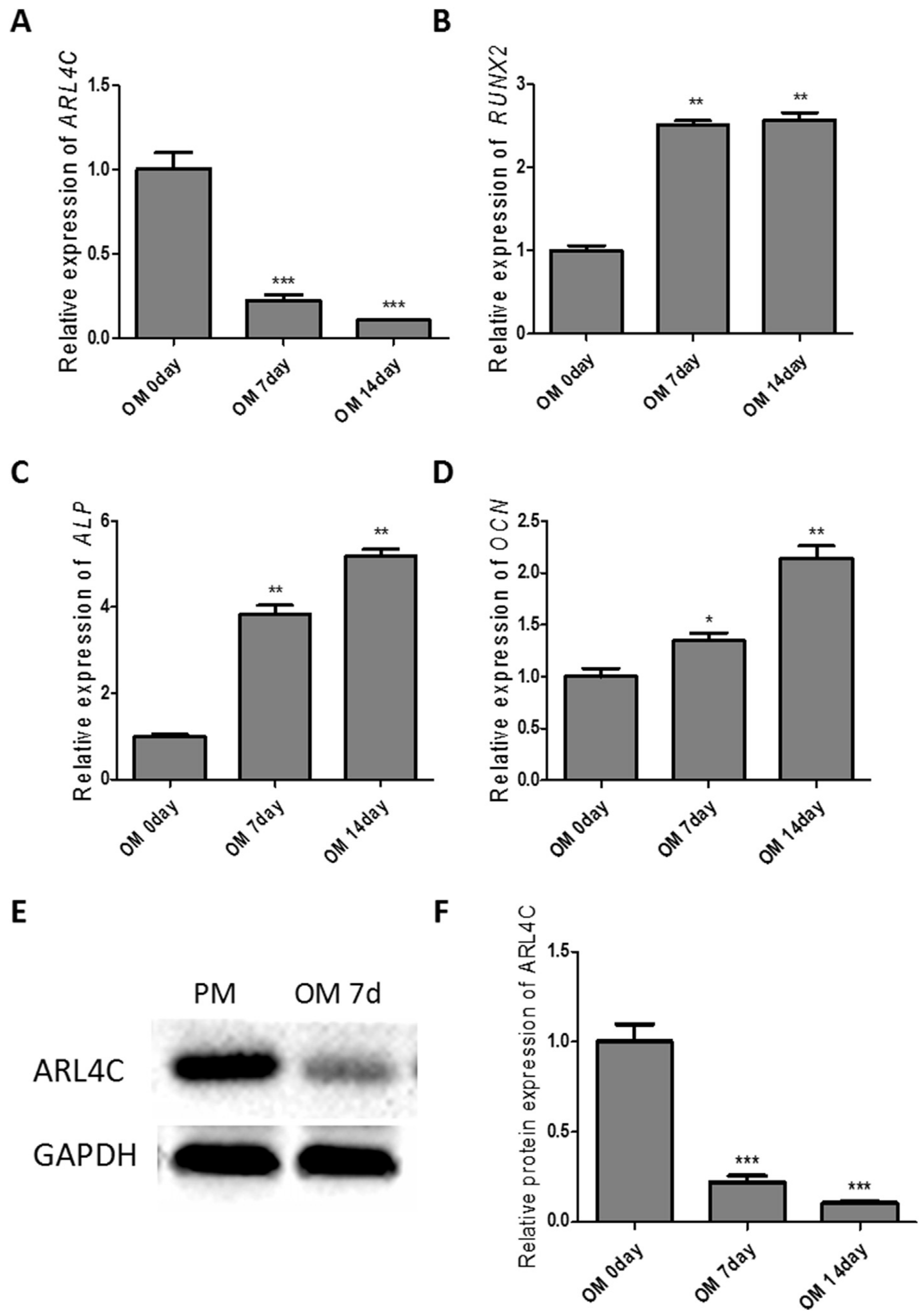

Fig. 1. Downregulation of *ARL4C* during osteogenic differentiation of hASCs. Relative mRNA expression of *ARL4C* (A) and the osteogenic markers *RUNX2* (B), *ALP* (C), and *OCN* (D) at days 0, 7 and 14 during osteogenic differentiation of hASCs as determined by qRT-PCR. Western blot (E) and quantification (F) of ARL4C protein expression on days 0 and 7 during osteogenic differentiation of hASCs. GAPDH served as a loading control. All data are presented as mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with day 0).

approximately 90% as determined by fluorescent microscopy (Fig. 2A). Analysis of *ARL4C* expression in transduced cells by qRT-PCR analysis confirmed a 70%–80% decrease in expression in the *ARL4C* knockdown group compared with the control group (Fig. 2B). Protein levels are also decreased in the ARL4C knockdown cells, as determined by Western blot (Fig. 2C–D).

3.3. Knockdown of ALR4C inhibits osteogenic differentiation in vitro

ALP staining and quantification shows that knockdown of *ARL4C* inhibits osteogenic differentiation of hASCs cultured in proliferation medium (PM) or osteogenic medium (OM) on day 7 (Fig. 3A–B). The ARS staining and quantification on day 14 displays outcomes similar to those of ALP assays (Fig. 3C–D). The suppression of *ARL4C* remarkably attenuates the expression level of *RUNX2* (Fig. 3E), *ALP* (Fig. 3F) at day 7, and *OCN* (Fig. 3G) at day 14.

3.4. ARL4C promotes osteogenic differentiation of hASCs in vivo

Next, hASCs stably expressing sh*ARL4C*-1, sh*ARL4C*-2 or control shRNA were loaded onto Bio-Oss Collagen scaffolds and implanted in the subcutaneous space of nude mice (six mice per group). After eight weeks, we harvested the implantation samples and performed analysis. H&E staining revealed little newly formed bone in the sh*ARL4C*-1/2 group (Fig. 4A). Collagen organization, shown in blue color by Masson's trichrome staining, was lower in the

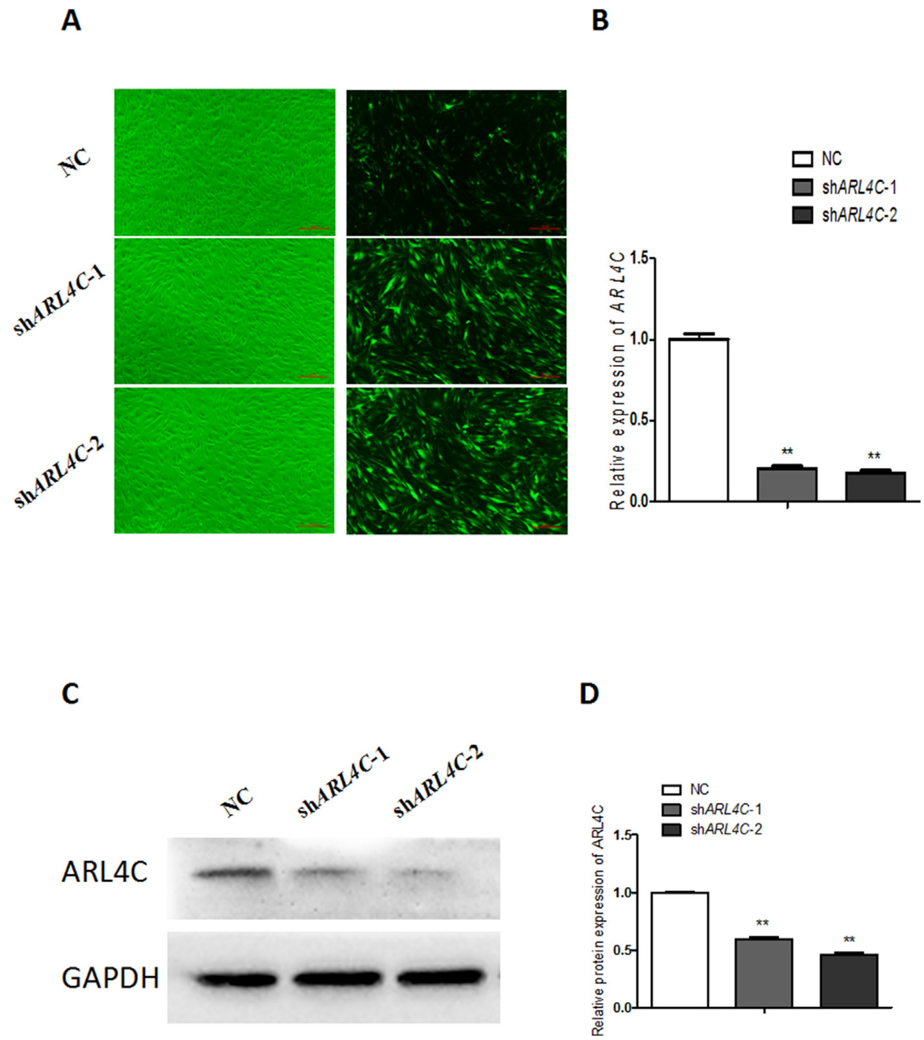

Fig. 2. Knockdown of *ARL4C* by shRNA. The hASCs were transfected with lentivirus expressing sh*ARL4C*-1, sh*ARL4C*-2, or the non-targeting control shRNA (NC). (A) Fluorescent micrographs show the efficiency of lentivirus transfection (>90%). Scale bar = 500 μm. (B) Relative mRNA expression of *ARL4C* in NC and sh*ARL4C* cells as determined by qRT-PCR. (C) Western blot showing ARL4C levels in sh*ARL4C*-1, sh*ARL4C*-2, or NC transfected cells. (D) Quantification of Western blot.

sh*ARL4C*-1/2 group (Fig. 4B).

3.5. ARL4C knockdown inhibits Wnt signaling

Wnt signaling is an important regulator in osteogenesis, therefore, we examined whether *ARL4C* regulates osteogenesis through Wnt signaling. The mRNA and protein expression levels of WNT5A, WNT11, LRP6 and P-LRP6 were analyzed. *ARL4C* knockdown inhibits the accumulation of WNT5A, WNT11, LRP6, and P-LRP6 in total cell lysates during osteogenesis as shown by Western blot (Fig. 5A–E). Expression levels of *WNT5A*, *WNT11*, and *LRP6* measured by qRT-PCR show a similar result (Fig. 5F–H).

4. Discussion

The ADP-ribosylation factor (ARF), a type of small GTP-binding protein, belongs to the Ras superfamily. It has been found to be involved in vesicular transport, organelle structure, membrane trafficking, and cytoskeletal remodeling [10]. The ARF-like (ARL) family, which including eight proteins, is a subgroup of the ARF family of proteins [11]. The *ARL4C* gene, also named ARF-like 7 (ARL7) was first isolated from a lymphokine-activated T-killer (T-LAK) cell subtraction library, encodes a GTP-binding protein which was identified as a member of the ARL family [5]. ARL4C and two closely related proteins, ARL4A and ARL4D, have unique characteristics among ARLs including their rapid nucleotide exchange through their C-terminal polybasic clusters which function as a nuclear localization signal [12]. Previous work identified the intrinsic rapid GTPase activity and a GDP restricted mutant is mainly distributed in the cytoplasm [13,14].

In the present study, we found that *ARL4C* is downregulated during the osteogenic differentiation of hASCs. We constructed *ARL4C* knockdown hASCs by lentivirus transfection, and found that knockdown of *ALR4C* inhibits osteogenic differentiation *in vitro and vivo*. RNA expression of *LRP6* was downregulated with in *ARL4C* knockdown cells. Western blot analysis shows that LRP6 levels are significantly decreased in sh-*ARL4C* cells after osteogenic culturing. *ARL4C* silencing also significantly decreased the protein expression levels of P-LRP6. It is well established that both the canonical and the noncanonical Wnt signaling pathways play a substantial role in the regulation of bone and mineral metabolism [15]. Wnt proteins are a large family of highly conserved secreted signaling molecules that mediate essential biological processes like embryogenesis, organogenesis, and tumorigenesis [18–21]. LRP6 serves as co-receptors for the Frizzled family of Wnt receptors [18], is required for optimal osteoblast function [15]. Mutations in LRP6 are

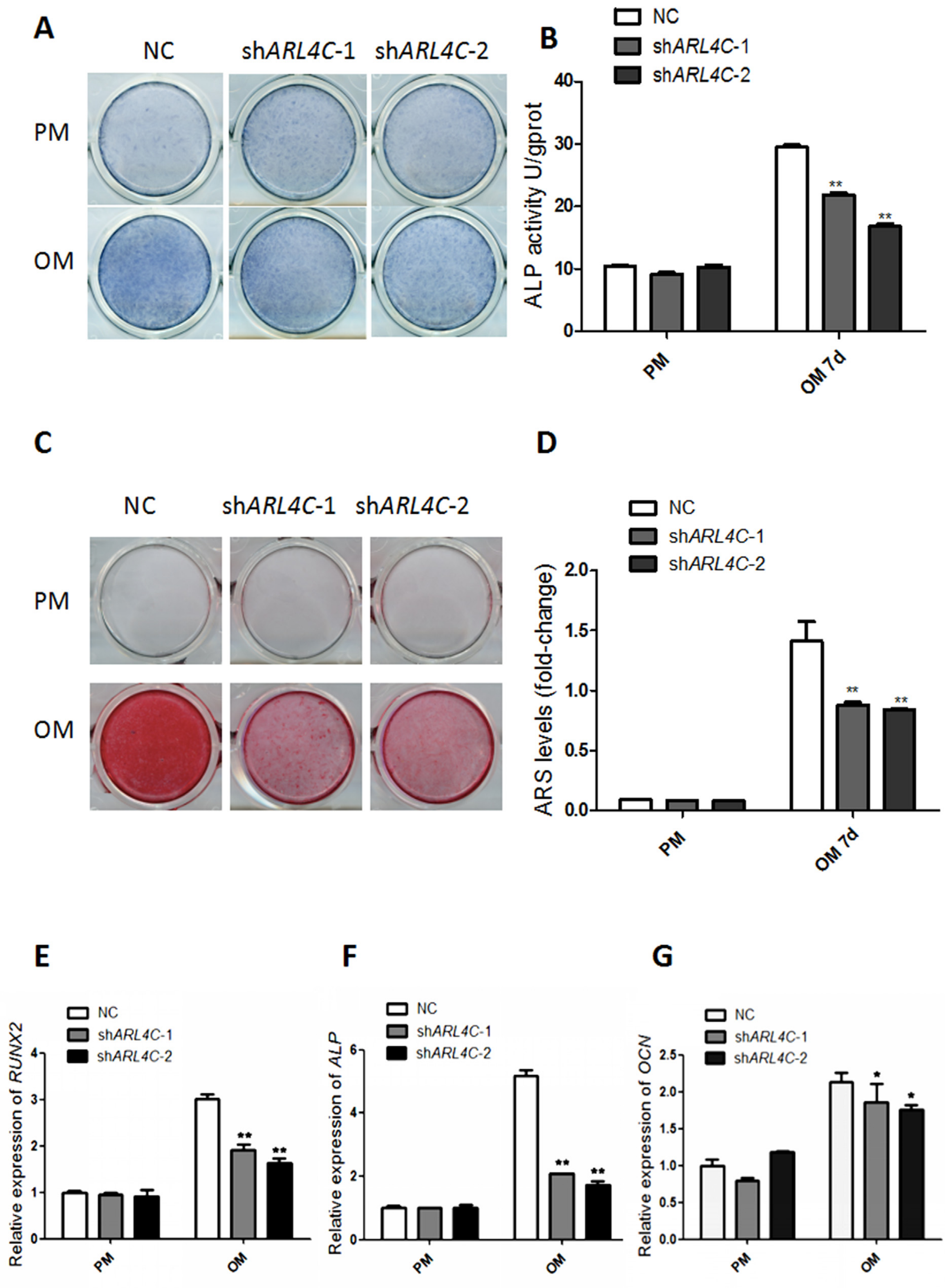

Fig. 3. Knockdown of *ARL4C* prevents osteogenesis of hASCs *in vitro*. ALP staining (A) and quantification (B) of cells at day seven after osteogenic induction. ARS staining (C) and quantification (D) of cells at day 14 after osteogenic induction. Relative mRNA expression of the osteogenic markers *RUNX2* (E), and *ALP* (F) assessed by qRT-PCR at day 7, and *OCN* (G) assessed by qRT-PCR at day 14 after osteogenic induction. Results are presented as mean ± SD (*$P < 0.05$, **$P < 0.01$, compared with NC).

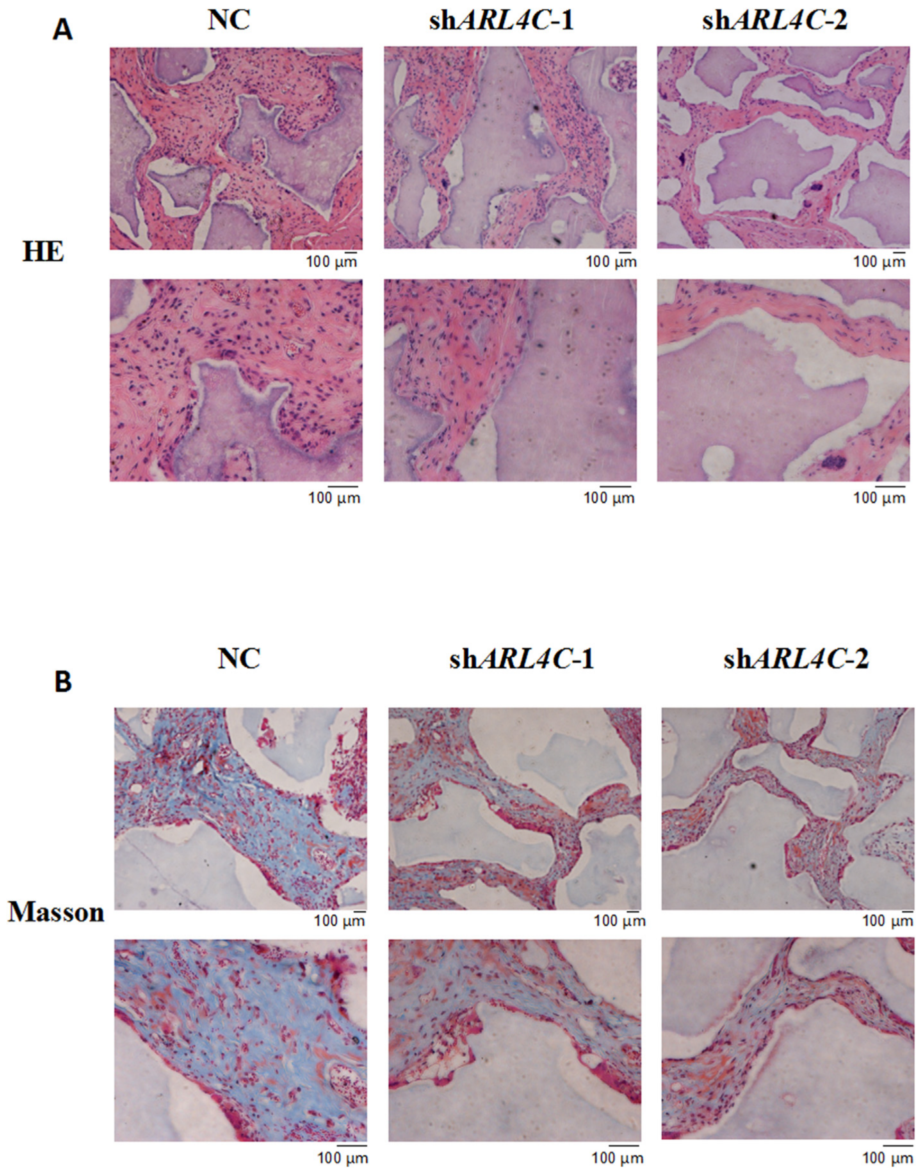

Fig. 4. Knockdown of *ARL4C* prevents osteogenesis of hASCs *in vivo*. H&E staining (A) and Masson staining (B) in NC, sh*ARL4C*-1, and sh*ARL4C*-2 transfected cells. Scale bar = 100 μm.

associated with several bone-related diseases in humans, and are a key genetic contribution toward the pathogenesis of vertebral segmentation defects and osteoporosis [16,17]. Extracellular Wnts can bind to the Frizzled and LRP5/6 co-receptors on the cell membrane, resulting in phosphorylation of LRP5 or LRP6 and creation of a binding site for the Axin protein. Normally, Axin is part of an intracellular protein complex that facilitates the phosphorylation of β-catenin, thereby targeting β-catenin for ubiquitin-dependent proteolytic degradation. In the presence of Wnt ligands, stabilization of β-catenin results in increased levels of β-catenin in cytoplasmic and nuclear [22,23]. In addition to canonical signaling, several Wnt proteins activate noncanonical pathways that do not target β-catenin [24]. We found a correlation between the decreases in *ARL4C* and non-canonical pathway components *WNT5A* and *WNT11*. The mRNA levels of *WNT5A* and *WNT11*, which have been shown to activate one or more of the noncanonical pathways, decrease with *ARL4C* knockdown in osteogenesis [25–27].

Together, our findings suggest that *ARL4C*, which is downregulated during osteoblast differentiation of hASCs, functions as a positive regulator of osteogenic differentiation. Downregulating of *ARL4C* inhibits osteogenesis of hASCs by suppressing the canonical and noncanonical Wnt signaling pathway.

Authors' contributions

W.W. conceived the experiments, performed the experiments, collected and analyzed the data, prepared the figures, and wrote the main manuscript text. S.W., X.L., R.G. and Y.Z. performed the experiments. Y.L. and P.Z. supervised the work, and edited the manuscript. Y.Z. conceived the experiments. All authors approved the final version of the manuscript.

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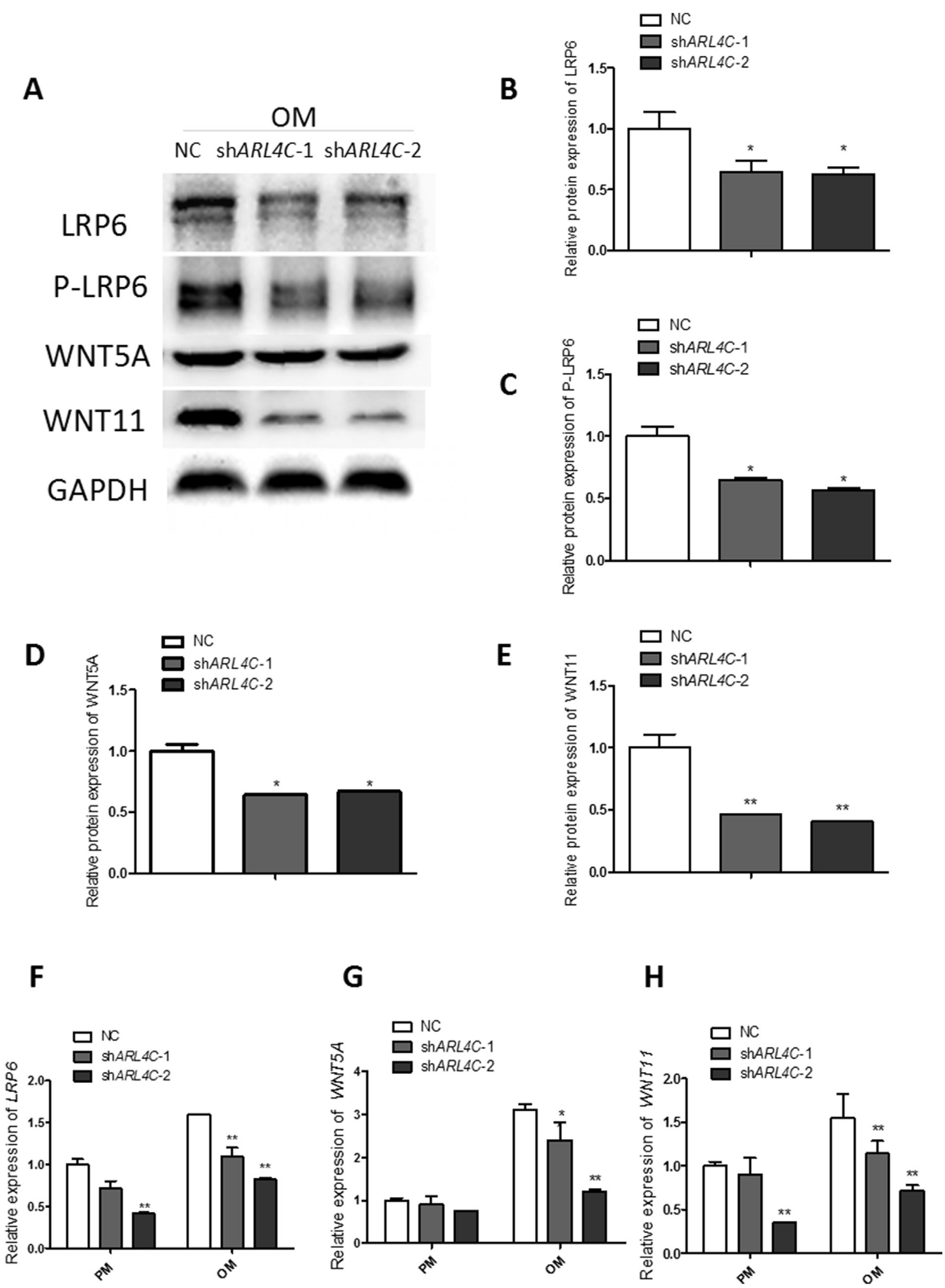

Fig. 5. *ARL4C* knockdown disrupts the Wnt signaling pathway.

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Competing financial interests

The authors declare no competing financial interests.

Relative mRNA expression of *LRP6*, a coreceptor involved in Wnt signaling (A) and non-canonical Wnt pathway markers *WNT5A* (B), *WNT11* (C) assessed by qRT-PCR at day 14 after osteogenic induction. Western blot (D) showing *ARL4C* knockdown downregulates the protein levels of LRP6 (E), P-LRP6 (F), WNT5A (G), WNT11 (H). Results are presented as mean ± SD (**$P < 0.01$).

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