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

Wensi Wang, Siyi Wang, Xuenan Liu, Ranli Gu, Yuan Zhu, Ping Zhang, Yunsong Liu, Yongsheng Zhou

Department of Prosthodontics, Peking University School and Hospital of Stomatology, Beijing 100081, China
National Engineering Lab for Digital and Material Technology of Stomatology, Peking University School and Hospital of Stomatology, Beijing 100081, China

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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/
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 PCR 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 radio- immunoprecipitation buffer containing 2% protease 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 stoxes against ARL4C (Abcam), RUNX2, WNT5A, WNT11, LRP6, P-LRP6 and GAPDH (Huaxiangbio) were diluted 1:2000 with Tris-HCl buffer solution (TBS-T) and incubated with the membranes at 4 °C overnight. After 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.

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 injected 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 significant. 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 ARL4C knockdown

To explore the role of ARL4C in osteogenic differentiation, we established ARL4C knockdown hASCs using a lentivirus vector expressing shRNA. The transfection efficiency was estimated to be...
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 ARL4C 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 shARL4C-1, shARL4C-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 shARL4C-1/2 group (Fig. 4A). Collagen organization, shown in blue color by Masson’s trichrome staining, was lower in the
shARL4C-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 showed 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 process. We constructed ARL4C knockdown hASCs by lentivirus transfection, and found that knockdown of ARL4C inhibits osteogenic differentiation in vitro and vivo. RNA expression of LRP6 was downregulated with 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).
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).

References


