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ORIGINAL ARTICLE

ALX1-transcribed LncRNA AC132217.4 promotes osteogenesis and bone healing via IGF-AKT signaling in mesenchymal stem cells

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Abstract

The osteogenic potential of bone marrow mesenchymal stem cells (BMSCs) is critical for bone formation and regeneration. A high non-/delayed-union rate of fracture healing still occurs in specific populations, implying an urgent need to discover novel targets for promoting osteogenesis and bone regeneration. Long non-coding (lnc)RNAs are emerging regulators of multiple physiological processes, including osteogenesis. Based on differential expression analysis of RNA sequencing data, we found that lncRNA AC132217.4, a 3'UTR-overlapping lncRNA of insulin growth factor 2 (IGF2), was highly induced during osteogenic differentiation of BMSCs. Afterward, both gain-of-function and loss-of-function experiments proved that AC132217.4 promotes osteoblast development from BMSCs. As for its molecular mechanism, we found that AC132217.4 binds with *IGF2* mRNA to regulate its expression and downstream AKT activation to control osteoblast maturation and function. Furthermore, we identified two splicing factors, splicing component 35 KDa (SC35) and heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), which regulate the biogenesis of AC132217.4 at the post-transcriptional level. We also identified a transcription factor, ALX1, which regulates AC132217.7 expression at the transcriptional level to promote osteogenesis. Importantly, in-vivo over-expression of AC132217.4 essentially promotes the bone healing process in a murine tibial drill-hole model. Our study demonstrates that lncRNA AC132217.4 is a novel anabolic regulator of BMSC osteogenesis and could be a plausible therapeutic target for improving bone regeneration.

Keywords Long non-coding RNA · Bone marrow mesenchymal stem cells · Osteogenic differentiation · IGF2 · Bone repair

Introduction

Mesenchymal stem cells (MSCs) derived from adult tissues, such as bone marrow and umbilical cord blood, can differentiate into various cell types, including osteoblasts, chondrocytes, and adipocytes [1–5]. The osteogenic potential of MSCs is critical for bone formation and regeneration, since osteoblasts are the principal cells that generate bone matrix [6–8]. Delay or failure of bone repair results in pain and physical disabilities, which severely impair the life quality of the patients [9, 10]. Current strategies to accelerate bone regeneration include applying growth factors, bone marrow aspirates, and scaffolds locally to the fracture sites [2, 9–11]. However, a significant non-union or delayed union rate still occurs, especially in 10–25% in aging populations (10–25%) [9] and diabetic patients (43%) [12, 13]. Thus, developing a more efficient treatment for bone regeneration is desperately needed.

LncRNAs, typically > 200 bp, were initially annotated as non-functional spurious RNAs. A closer examination of lncRNA properties reveals their functionality and diversity [14–17]. A few lncRNAs have been reported to participate in MSC differentiation, and some specifically regulate the osteogenesis of MSCs or fracture healing [18–23]. However, the functions of most lncRNAs during MSC osteogenic differentiation remain largely unknown. Therefore, elucidating the role of novel lncRNAs in regulating the osteogenesis of

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MSCs might facilitate the development of novel treatments for bone diseases. Compared to mRNAs, the expression of lncRNAs are more specific in a developmental stage- or disease status-specific manner, and genome sequences of lncRNA usually contain weaker transcription and splicing signals, indicating that their biogenesis requires more tight regulations [24–26]. However, the regulatory mechanism of lncRNA is barely studied in bone.

This study found that lncRNA AC132217.4 is a novel osteogenic factor in MSC. AC132217.4 was an IGF2 3'UTR overlapped lncRNA and has been shown to facilitate oral squamous cell carcinoma metastasis [27]. However, the role of AC132217.4 in bone was not previously reported. We found that AC132217.4 expression is highly upregulated during MSC osteogenesis and demonstrated that it enhances osteogenic differentiation through the IGF-AKT signaling axis. To explore the biogenesis mechanism of AC132217.4, we performed RNA-pull down and promoter analysis and found that AC132217.4 expression is cooperatively controlled by the transcriptional factor, ALX1, and two splicing factors, SC35 and HNRNPA1. Importantly, we also showed that AC132217.4 overexpression in mice promotes bone healing. Our findings might offer new treatment options for bone repair.

Materials and methods

Isolation and osteogenic induction of hBMSCs

hBMSCs were isolated from whole bone marrow samples collected from healthy donors at the Second Affiliated Hospital, Zhejiang University, after obtaining informed consent, as previously described [28]. Cells at the third passage were plated at 60–70% confluence and cultured in the osteogenic differentiation medium (L-Dulbecco's modified Eagle's medium [Life Technologies Inc., China] supplemented with 10% fetal bovine serum, 50 μg/mL L-ascorbic acid, and 10 mM β-sodium glycerophosphate [Sigma Inc., China]).

RNA–protein pull-down

Biotin-labeled AC132217.4 and AC132217.4 pre-RNA were transcribed in vitro using the MEGAscript T7 kit and purified lithium chloride. Purified RNA was incubated with the protein lysis product, and RNA pull-down was conducted using Pierce™ magnetic RNA–protein pull-down kit (Life Technologies Inc., China). The combined proteins and pre-RNA of AC132217.4 were confirmed using silver staining with the Silver staining kit (Beyotime Biotech Inc., China), mass spectrometry (MS), and western blotting.

Tibial drill-hole murine model

As previously described, the tibial drill-hole murine model for studying bone repair was established [29, 30]. Briefly, mice were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium solution (75 mg/kg body weight), and the right hindlimb was shaved and sterilized. Afterward, a 1-mm diameter hole was made in the cortical bone of the anterior proximal tibial of 3-month-old male C57BL/6 mice using an electronic drill. The muscle and skin were closed with layered sutures.

The control and experimental groups were administered 50 μL lentivirus (109 TU/mL), carrying green fluorescent protein (GFP) or AC132217.4 near the cortical hole for three consecutive days post-surgery. Each group comprised eight mice euthanized for tissue harvesting 7 and 14 days after the surgery. All the mice were housed at 20–22 °C on a 12 h light/dark cycle in the institutional animal facility of the Zhejiang University. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed following the Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using the Student's *t* test or two-way analysis of variance followed by post hoc Bonferroni correction, and significance was set at p value < 0.05.

More details of materials and methods are available in the supplemental files.

Results

LncRNA AC132217.4 promotes osteogenic differentiation of hBMSCs

To identify novel lncRNA regulators of osteogenic differentiation of hBMSCs, the lncRNA expression profiles of primary and osteogenic-differentiated hBMSCs were compared using RNA sequencing analysis. The data are deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus and are accessible through GEO Series accession number GSE114117 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). We observed a dramatic increase in lncRNA AC132217.4 among these altered lncRNAs during osteogenic differentiation of hBMSC (Figs. 1a, S1A), which was subsequently confirmed using qRT-PCR analysis with primers specific to AC132217.4 (Fig. 1b).

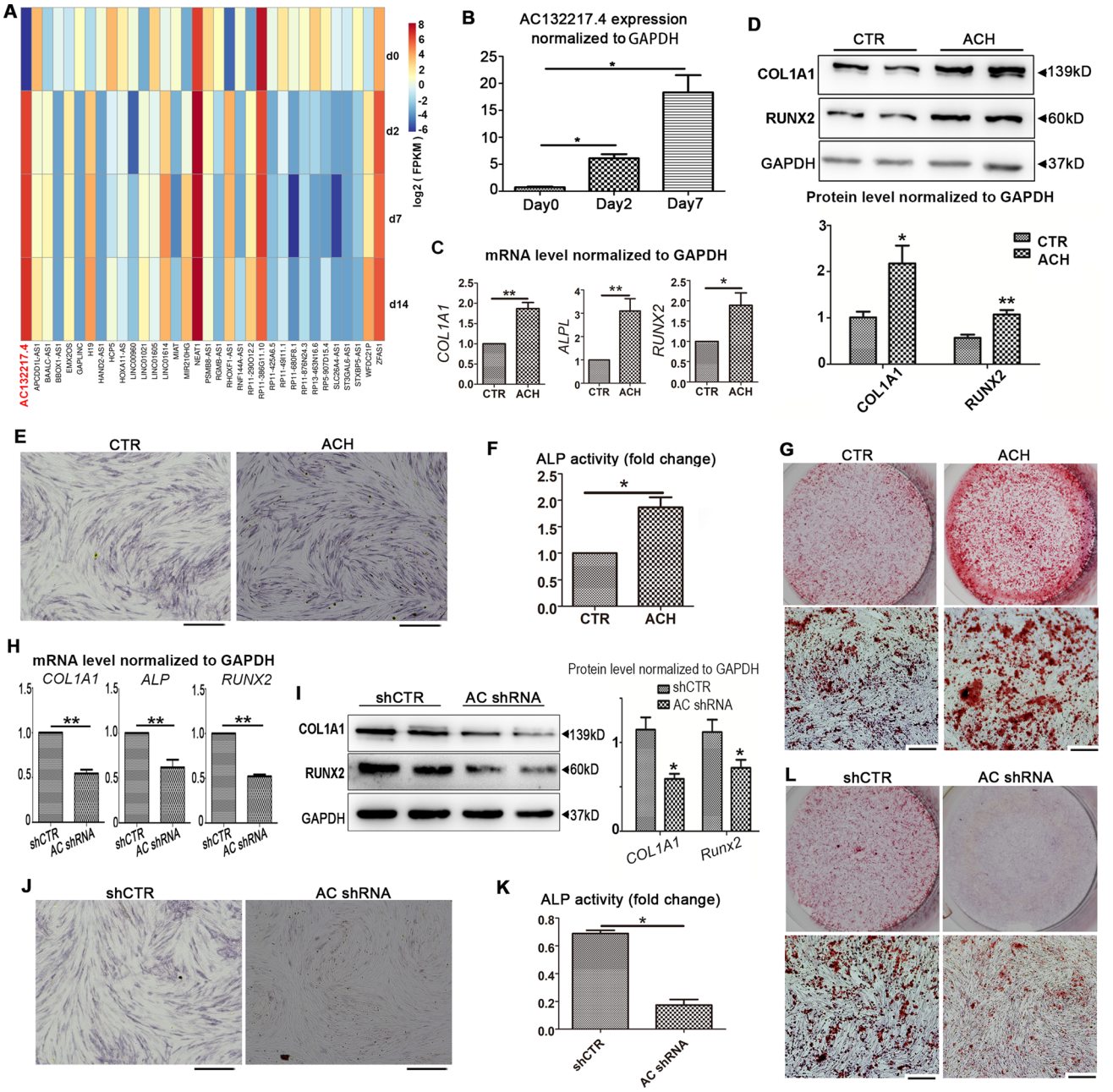


Fig. 1 AC132217.4 promoted osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs). **a** Differential expression of LncRNAs during the process of hBMSCs osteogenesis. **b** Expression of AC132217.4 increased with osteogenic induction. **c**, **d** Expression of osteoblast marker genes in osteogenic differentiated hBMSC with or without AC132217.4 overexpression is detected by **c** quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and **d** western blotting. **e**–**g** Osteoblast maturation and mineralization of osteogenic differentiated hBMSC with or without AC132217.4 overexpression are analyzed by alkaline phosphatase (ALPL) stain (**e**), quantification of ALPL activity (**f**), and Alizarin Red stain (**g**). **h**, **i** Expression of osteoblast marker genes in osteogenic differentiated hBMSC with or without AC132217.4 knockdown is detected by **h** qRT-PCR and **i** western blotting. **j**–**l** Osteoblast maturation and mineralization of osteogenic differentiated hBMSC with or without AC132217.4 knockdown are analyzed by ALPL stain (**j**), ALPL activity quantification (**k**), and Alizarin Red stain (**l**). Quantification of protein level from western blot data is co-presented in its lower panel (**d**) or right panel (**i**). *CTR* control hBMSC, *ACH* AC132217.4 over-expressing hBMSC, *shCTR* control-shRNA transfected BMSCs, *AC shRNA* AC132217.4-shRNA transfected hBMSCs. Scale: 50 μm. Data are expressed as mean ± SD; $N \geq 3$; $^{*}p < 0.05$, $^{**}p < 0.01$

To validate the role of AC132217.4 during osteogenic differentiation of hBMSCs, we over-express and knockdown AC132217.4 expression in hBMSCs, respectively. Osteogenic differentiation was induced in control and AC132217.4-overexpressing hBMSCs for 7 days (Fig. S1B). AC132217.4 overexpression

upregulates osteoblast marker gene expression, indicating that AC132217.4 promotes osteogenesis. AC132217.4-overexpressing cells showed increased *ALPL, COL1A1*, and *RUNX2* mRNA levels (Fig. 1c), as detected by qPCR, and elevated COL1A1 and RUNX2 protein levels (Fig. 1d), as shown by western blot. The ALPL and alizarin red stain consistently showed osteoblast maturation and mineralization increased in AC132217.4-overexpressing cells (Fig. 1e–g). Conversely, knocking down of AC132217.4 in hBMSCs decreased *ALPL, COL1A1,* and *RUNX2* mRNA expression (Figs. 1h, S1C), reduced COL1A1 and RUNX2 protein levels (Fig. 1i), and impaired ALPL activity and mineralized nodule formation (Fig. 1j–l).

AC132217.4 promoted osteogenic differentiation of hBMSCs via IGF2-AKT signaling

To investigate the mechanism underlying AC132217.4 regulating osteogenic differentiation of hBMSCs, we performed RNA pull-down coupled with MS using synthesized AC132217.4 (Fig. 2a–c). Unfortunately, none of the five proteins interacting with AC132217.4 seem to function in osteogenic differentiation (Fig. 2c). Thus, we speculated that lncRNA AC132217.4 might exert its role by interacting with nucleotides rather than proteins (Figs. 2a–c, S2A). To determine whether AC132217.4 binds to DNA or RNA, we examined the cellular location of AC132217.4. As observed in the FISH analysis and cell fractionation, we found that its distribution was restricted to the cytoplasm rather than nuclear compartments (Fig. 2d, e). Therefore, it is likely that AC132217.4 is binding to RNA rather than DNA in the cytoplasm. Grounded on this assumption, we predicted potential targeted mRNA by AC132217.4 using Starbase (Fig. S2B), and the *IGF2* mRNA showed the highest align score (39.0).

To test whether AC132217.4 regulates the expression of IGF2 to mediate osteogenesis, we first compared the expression pattern of IGF2 to that of lncRNA AC132217.4 in our RNA-seq data set. We found that they were similar (Fig. S2C). As confirmed by qPCR and western blot, the expression of IGF2 in hBMSCs subjected to osteogenic induction was consistently upregulated (Fig. 2f, g). Then we test whether AC132217.4 over-expression or knockdown impacts IGF2 abundance during osteogenic differentiation (Fig. 2h–j). As expected, the level of IGF2 mRNA was remarkably upregulated in AC132217.4-overexpressing cells and down-regulated in AC132217.4-knockdown cells. Then we examined whether AC132217.4 affects IGF2 mRNA stability after transcription. hBMSCs induced osteogenesis for 7 days were treated with actinomycin D (ActD) to block the de novo mRNA synthesis to examine IGF2 mRNA stability. The IGF2 mRNA stability increased in AC132217.4 overexpressing cells (Fig. 2k). IGF1 promotes osteoblast formation and bone development through AKT signaling [31, 32]. While IGF2's function was less known, the previous study also supports its anabolic roles in osteogenesis [33–35]. To confirm the role of IGF2 in osteogenic differentiation, we knock down IGF2 expression in MSCs, using lentivirus carrying the shRNA targeting IGF2 (sh-IGF2) (Fig. S2D). sh-IGF2 downregulates osteoblast marker gene expression at both RNA and protein levels, as shown by qPCR and western blot (Fig. 3a, b). sh-IGF2 also inhibits osteoblast maturation and mineralization, as shown by ALPL and Alizarin red stains, respectively (Fig. 3c–e). We also overexpressed IGF2 in MSCs and found it improves osteogenesis, as shown by more osteoblast marker gene and protein expression (Fig. S3A) and increased ALPL stain (Fig. S3B) as well as Von kossa stain (Fig.S3C, D) intensity. Those data prove that IGF2 promotes osteogenic differentiation just like AC132217.4. Significantly, IGF2 knockdown can suppress the overwhelming osteogenesis induced by AC132217.4 overexpression, as demonstrated by osteoblast marker gene expression (Fig. 3f, g), as well as ALPL and Alizarin red stains (Fig. 3h–j). These data indicate that IGF2 is the critical downstream effector of AC132217.4 in the osteoblast. We next investigated the possible downstream mechanisms underlying the AC132217.4-IGF2 axis. AKT phosphorylation increased with AC132217.4 over-expression, whereas IGF2 knockdown ablated the increase (Fig. 3k, l). In addition, we administrated 500 nM AKT inhibitor VIII (AKTiVIII) (MedChemExpress) for 7 days or 14 days in the AC132217.4 overexpressing hBMSCs during osteogenesis. Antagonizing AKT activation could also compromise the osteogenic function of AC132217.4 overexpression (Figs. S4, 3M, N). AKTiVIII brings down osteoblast marker gene mRNA (Fig. 3m) and protein level (Fig. 3n), ALPL stain intensity (Fig. S4A), and Von kossa stain intensity (Fig. S4A, B) to a similar level of control cells. Collectively, our data suggest that AC132217.4 confers bone anabolic properties through AKT signaling downstream of IGF2.

HNRNPA1 and SC35 promoted lncRNA AC132217.4 splicing and expression

AC132217.4 is a novel, 3′UTR-overlapping lncRNA of *IGF2*, spliced from an 844 bp gene segment in the *Ensembl database* (Fig. S5A). RT-PCR using flanking primers gives out a band indicating a spliced product of the expected size (4A, B). To confirm the splice sites of AC132217.4, we mutated them in its pre-RNA (Fig. S5B) and overexpressed the wild-type and mutated pre-RNA in hBMSCs. Overexpression of site-mutated pre-RNA leads to much less production of AC132217.4 compared to the wild-type pre-RNA (Fig. 4c). Then all existing splicing sites in the pre-RNA of AC132217.4 were predicted in silico using the NetGene2. Notably, compared with the highest ranked splice site, the AC132217.4 splice site yields a much lower splice

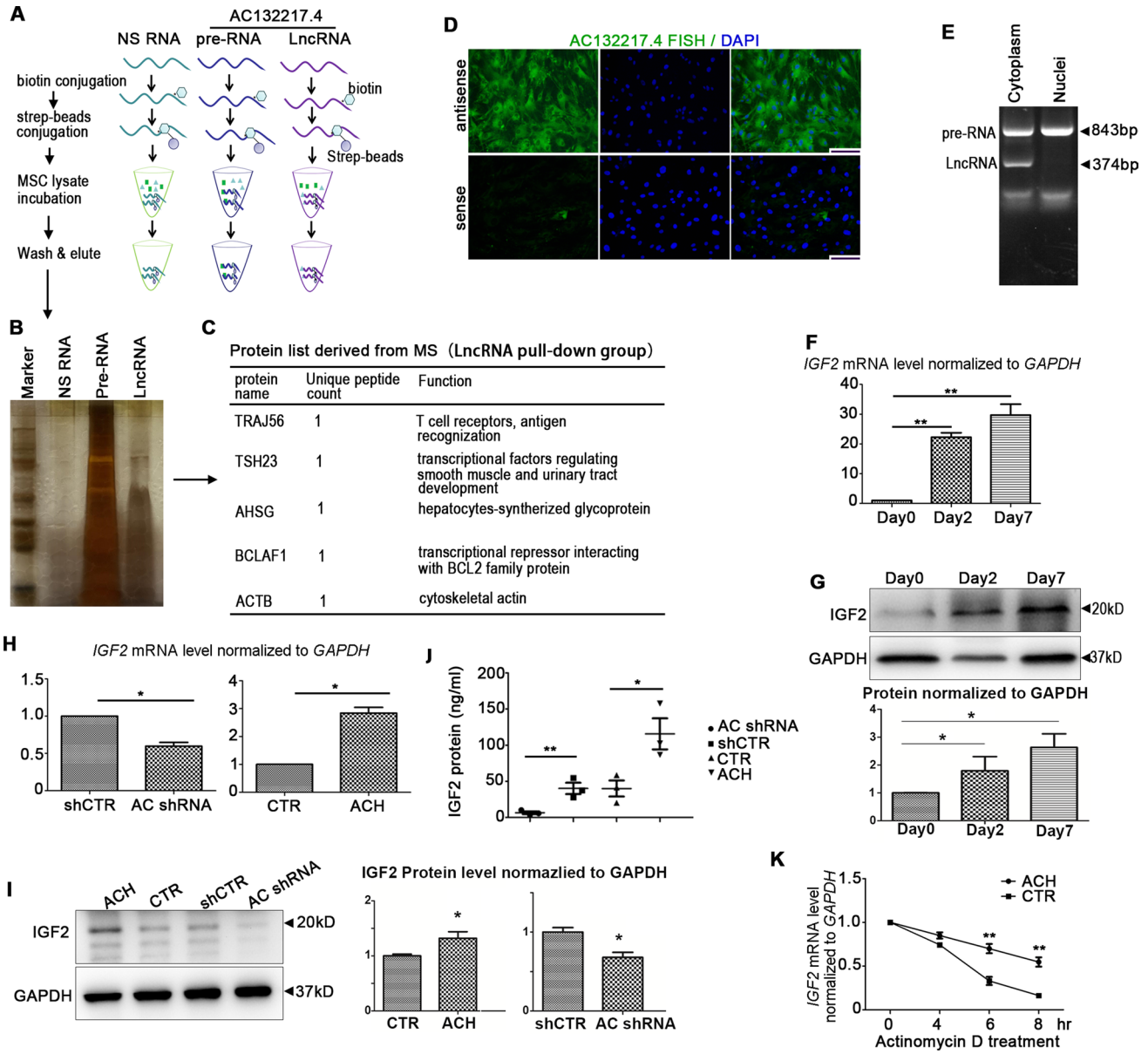
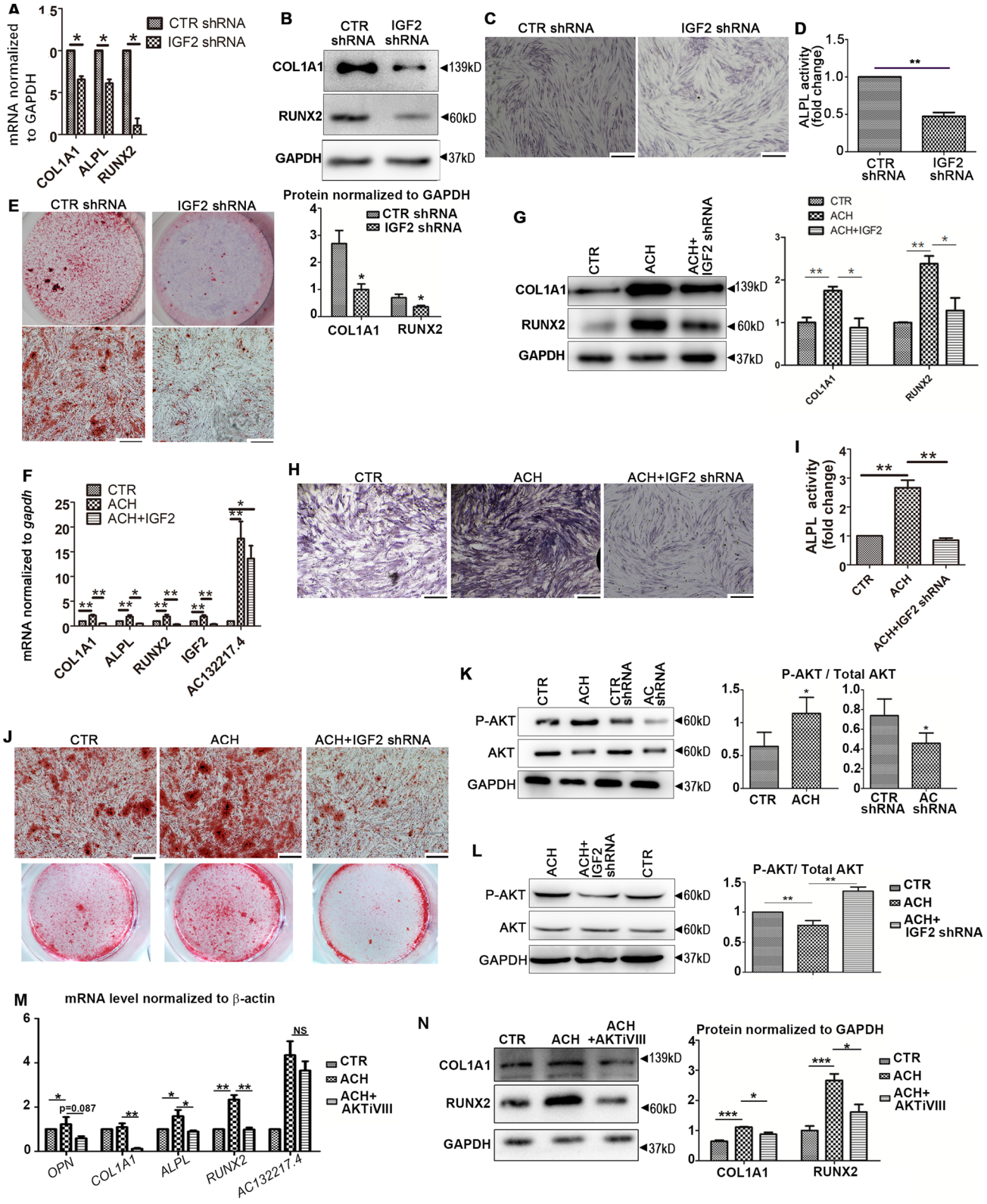


Fig. 2 AC132217.4 increased insulin growth factor 2 (IGF2) expression. **a** RNA pull-down was performed using synthesized biotin-conjugated AC132217.4 pre-RNA or lncRNA and cell lysates from osteogenic differentiated hBMSCs. **b**, **c** Proteins obtained from RNA pull-down in a were further analyzed using silver stain on the SDS PAGE gel (**b**) and mass spectrometry (**c**). All proteins binding with AC132217.4 LncRNA found by MS were listed in **c**. **d** Fluorescence in situ hybridization (FISH) and **e** cell fractionation analysis of AC132217.4 subcellular location. **f**, **g** Expression of IGF2 during osteogenic induction was analyzed using **f** quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and **g** western blotting. **h**, **i** Expression of IGF2 in osteogenic differentiated hBMSC with or w/o AC132217.4 over-expression or knockdown was determined by qPCR (**h**) and western blot (**i**). **j** Concentration of secreted IGF2 in control, AC132217.4 over-expressing, or AC132217.4 knockdown hBMSC was determined by Elisa. **k** Expression of IGF2 mRNA in osteogenic differentiated hBMSC with or w/o 5 μg/ml actinomycin D (ActD) treatment for the indicated time. Quantification of protein level from western blot data is co-presented in its lower panel (**g**) or right panel (**i**). CTR, Control hBMSC; ACH, AC132217.4 over-expressing hBMSC; shCTR, control-shRNA transfected BMSCs; AC shRNA, AC132217.4-shRNA transfected hBMSCs. Scale: 20 μm. Data are expressed as mean ± SD; $N \geq 3$; $^{*}p < 0.05$, $^{**}p < 0.01$

site value (0.43 compared with 0.66) (Fig. S5C). Nevertheless, AC132217.4 is still a major product spliced from its pre-RNA; this indicates that alternative splicing mechanisms alter the splice site preference to favor the production of AC132217.4. The protein list derived from RNA pull-down using AC132217.4 pre-RNA (Fig. 2a) found that two splice regulators, HNRNPA1 and SC35, obtained the highest score (Fig. 4d). The donor and acceptor splice sites of


A
mRNA normalized to GAPDH
CTR shRNA
IGF2 shRNA
COL1A1
ALPL
RUNX2
B
CTR shRNA
IGF2 shRNA
COL1A1
RUNX2
GAPDH
139kD
60kD
37kD
Protein normalized to GAPDH
C
CTR shRNA
IGF2 shRNA
D
ALPL activity (fold change)
E
F
mRNA normalized to gapdh
CTR
ACH
ACH+IGF2
IGF2
AC132217.4
G
ACH+ IGF2 shRNA
H
ACH+IGF2 shRNA
I
J
K
P-AKT
AKT
P-AKT / Total AKT
CTR shRNA
AC shRNA
L
P-AKT/ Total AKT
M
mRNA level normalized to β-actin
p=0.087
NS
OPN
ACH+ AKTiVIII
N
ACH +AKTiVIII
Protein normalized to GAPDH

◂**Fig. 3** AC132217.4 promoted osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs) through insulin growth factor 2 (IGF2)/AKT signaling. **a**, **b** Expression of osteoblast marker genes in osteogenic differentiated hBMSC with or w/o IGF2 knockdown is detected by **a** quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and **b** western blotting. **c**–**e** Osteoblast maturation and mineralization of differentiated hBMSC with or w/o IGF2 knockdown are analyzed by alkaline phosphatase (ALPL) stain (**c**), quantification of ALPL activity (**d**), and Alizarin Red stain (**e**). **f**, **g** IGF2 knockdown compromised the promoting effect of AC132217.4 overexpression on the expression of osteoblast marker genes, as detected by **f** qRT-PCR and **g** western blotting. **h**–**j** IGF2 knockdown compromised the promoting effect of AC132217.4 overexpression on osteoblast maturation and function, as detected by **h** ALPL stain, **i** quantification of ALPL activity, and **j** Alizarin red stain. **k** Western blot to analyze AKT phosphorylation in AC132217.4 overexpressed and knockdown cell. **l** Knockdown of IGF2 abolished the effect of AC132217.4 over-expression on AKT phosphorylation, as detected by western blot. **m**, **n** qRT-PCR and western blot analysis to detect osteoblast marker gene expression in both mRNA (**m**) and protein (**n**) levels in osteoblasts derived from control, AC132217.4 overexpressing, and AC132217.4 overexpressing and AKTiVIII treated hBMSCs. Quantification of protein levels from western blot data is co-presented in its lower panel (**b**) or right panel (**g**, **k**, **l**, **n**). *CTR* Control hBMSC, *ACH* AC132217.4 over-expressing hBMSC, *shCTR* control-shRNA transfected BMSCs, *AC shRNA* AC132217.4-shRNA transfected hBMSCs. *IGF2 shRNA* IGF2-shRNA transfected hBMSCs. Scale: 50 μm. Data are expressed as mean ± SD; $N \geq 3$; $^{*}p < 0.05$, $^{**}p < 0.01$

AC132217.4 in its pre-RNA were also consistent with the binding motif of HNRNPA1 and SC35 (Fig. S5D). RNA pull-down and western blotting results confirmed the binding of HNRNPA1 and SC35 with the pre-RNA of AC132217.4 (Fig. 4e). To confirm that HNRNPA1 and SC35 bind with AC132217.4 pre-RNA and are functionally involved in the splicing of lncRNA AC132217.4, we knockdown SC35 or HNRNPA1 expression, respectively, in the BMSCs using shRNA (Fig. 4f), and both markedly reduced the expression of AC132217.4 (Fig. 4g).

ALX1-regulated osteogenic differentiation of hBMSCs by promoting AC132217.4 expression

To uncover the molecular mechanism that controls the increased transcription of AC132217.4 during osteogenic differentiation of hBMSCs, we searched for transcription factors with a similar expression pattern as AC132217.4. Our RNA-seq data set found 12 upregulated and 17 downregulated transcription factors (TFs) (Fig. 5a). To determine which of the 29 candidates are involved in the transcription of AC132217.4, we analyzed the binding partners on the AC132217.4 promoter, a ~ 3000-bp genomic region upstream of AC132217.4, using PROMO. Among those differentially expressed TFs, ALX1 possesses the most abundant predicted binding sites within the promoter region of AC132217.4 (Fig. 5b, c; Table S1). These results indicate that ALX1 could be the transcription factor regulating the expression of AC132217.4. To confirm this, we knocked down ALX1 expression using two independent shRNAs, which efficiently reduced the mRNA and protein levels of ALX1 in hBMSCs. Compared to the control, ALX1 knockdown significantly decreased the expression of AC132217.4 and IGF2 (Fig. 5d, e). Conversely, ALX1 over-expression markedly elevated AC132217.4 and IGF2 expression (Fig. 5f, g). We further showed that ALX1 drives luciferase expression under the control of the promoter region of AC132217.4, whereas this effect was abolished by mutation of the ALX1 consensus sequences in the promoter region (Fig. 5h, i). This data indicated that ALX1 could drive AC132217.4 at the transcriptional level. Overexpression of ALX1 also increased IGF2 expression (Fig. 5j), which could be reversed by AC132217.4 knockdown (Fig. 5k). These data imply that the ALX1 controls AC132217.4 transcription, regulating IGF2 expression.

Importantly, we found ALX1 overexpression promotes osteoblast differentiation, which could be compromised by knockdown of either AC132217.4 or IGF2 (Fig. 6). ALX1 overexpression promoted osteoblast differentiation and maturation, as confirmed by ALPL and alizarin red stains, respectively (Fig. 6a, b). ALX1 overexpression promotes osteoblast gene expression, as detected by qPCR and western blot, which could also be compromised by knockdown of either AC132217.4 or IGF2 (Fig. 6c, d).

AC132217.4 promoted bone repair in mice

To evaluate the effects of AC132217.4 on osteoblast differentiation in vivo and bone regeneration, we manipulated AC132217.4 expression using lentivirus carrying AC132217.4 in a tibia drill-hole murine model (Fig. 7a) [29, 30]. Since the lentiviral vectors comprise a GFP-expressing element, we confirmed their infection rate by tracing GFP expression (Fig. 7b). A natural healing process goes through several stages, including inflammation, cartilaginous callus formation, bony callus formation, and bone remodeling [36]. To analyze bone healing at different stages, we harvested tissues 1- and 2-week post-surgery.

As shown by μ-CT, 1-week post-surgery, both bone fraction (BV/TV) and bone mineral density (BMD) were significantly higher in AC132217.4 over-expressing bone healing site, 2.675% ± 0.48%, and 1.5% ± 0.32%, respectively, as compared to control (Fig. 7c, d). Two-week post-surgery, AC132217.4 overexpression still elevated BV/TV and BMD (1.995% ± 0.08% and 1.9125% ± 0.385%, respectively), albeit not significantly (Fig. 7c, d). As shown by reconstructed three-dimensional images (Fig. 7c) and H&E

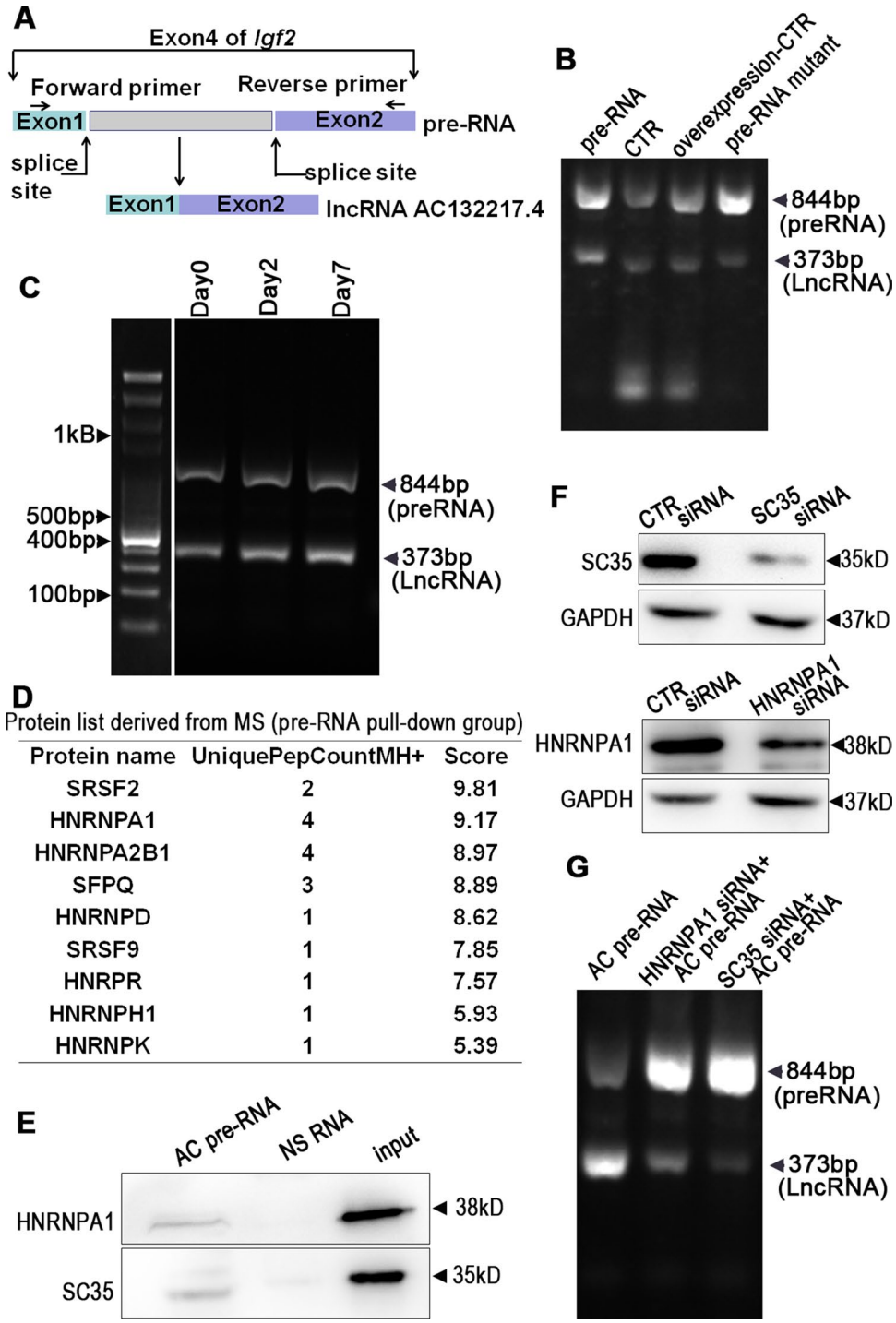


Fig. 4 SC35 and heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) regulated AC132217.4 splicing. **a**, **b** Flanking primers to identify AC132217.4 pre-RNA and mature LncRNA were indicated in AC132217.4 splicing-mode diagram (**a**) and confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (**b**). **c** RT-PCR to detect AC132217.4 expression in hBMSC overexpressing wild-type or splicing site mutated AC132217.4 pre-RNA. **d** List of proteins was identified binding with AC132217.4 pre-RNA using mass spectrometry coupled with RNA pull-down. **e** RNA pull-down coupled with western blotting verified the binding of AC132217.4 pre-RNA with HNRNPA1 and SC35. **f** Western blotting confirms HNRNPA1 and SC35 knockdown in hBMSCs by their siRNA. **g** Mature AC132217.4 lncRNA produced from its pre-RNA is decreased in the presence of HNRNPA1 or SC35 siRNA, as detected by RT-PCR

stain (Fig. 7e), control bone marrow at the healing site was stuffed with woven bones 2-week post-surgery (Fig. 7d). On the contrary, at the AC132217.4-overexpressing healing site, new bones were refined in the cortical area; Woven bones were already remodeled and cleared, with a reasonable reduction of callus tissue volume at the same stage. These data indicate that AC132217.4 promotes the transition from soft callus to hard callus and accelerates bone remodeling.

The histological analysis (Fig. 7e–i) confirmed the abovementioned findings. As shown by H&E (Fig. 7e) and Safranin O staining (Fig. 7f, h), 1-week post-surgery, AC132217.4-overexpressing bone healing sites were

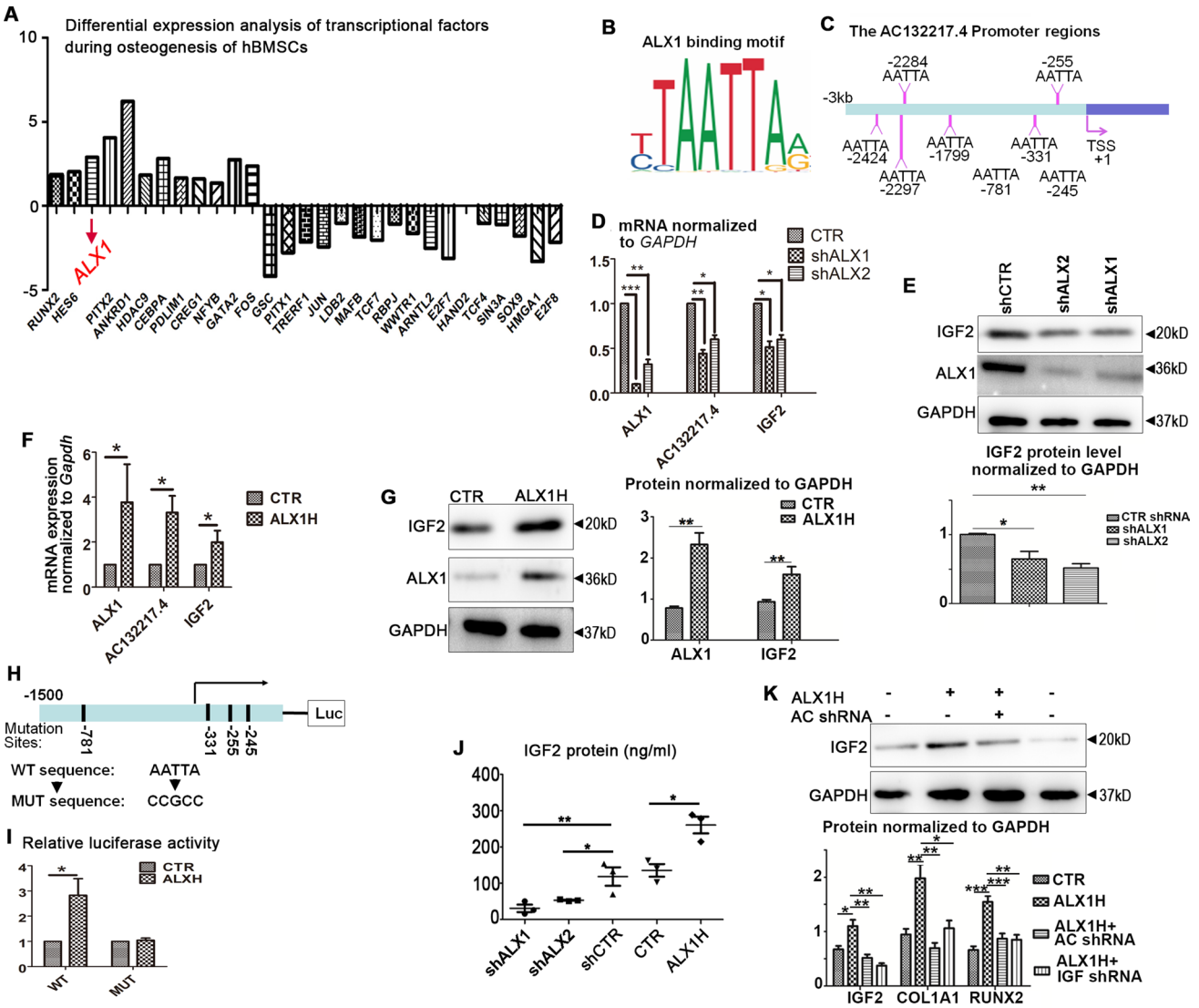


Fig. 5 ALX1 promoted IGF2 production by enhancing AC132217.4 gene transcription. **a** Differential expression analysis of transcription factors in human bone mesenchymal stem cells (hBMSCs) before and after osteogenic differentiation. **b**, **c** Prediction of ALX1 binding sites on the AC132217.4 promoter using PROMO. **d**, **f** mRNA level of ALX1, AC132217.4, and IGF2 in control, ALX1-knockdown (**d**) and ALX1-overexpressing (**f**) hBMSC, as detected by quantitative reverse transcription-polymerase chain reaction (q-RT-PCR). **e**, **g** Western blot analysis of IGF2 and ALX1 protein level of control, ALX1-knockdown (**e**), and ALX1-overexpressing (**g**) hBMSCs. **h** Wild-type (WT) or mutated (MUT) AC132217.4 promoter was constructed before a firefly luciferase CDS in the pGL3-basic vector. **i** Firefly luciferase activity driven by WT or MUT AC132217.4 promoter, with or w/o ALX1 overexpression, was detected and normalized with Renilla luciferase activity. **j** ELISA DETECTED THE secreted IGF2 concentration in control, ALX1-knockdown, and ALX1-overexpressing hBMSC. **k** AC132217.4 knockdown abolishes the effect of ALX1 overexpression on IGF2 production, as detected by western blot hBMSCs. Quantification of protein level from western blot data is co-presented in its lower panel (**e**, **k**) or right panel (**g**). *ALX1i-1/2* ALX1-shRNA transfected hBMSC, *CTR shRNA* control-shRNA transfected hBMSCs, *CTR* control hBMSCs, *ALX1H* ALX1-overexpressing hBMSCs, *AC shRNA* AC132217.4-shRNA transfected hBMSCs. Scale: 50 μm. Data are expressed as mean ± SD; $N \geq 3$; $^{*}p < 0.05$, $^{**}p < 0.01$

predominantly bony callus, whereas the control group is still in the cartilaginous callus formation stage. Two-week post-surgery, the cartilaginous callus ratio remains much higher in the control group (Fig. 7e, f, h). TRAP staining showed more osteoclast in the AC132217.4-overexpressing group than in the control group 1-week post-surgery (Fig. 7g, i). Increasing osteoclast number was not observed in the control group until 2-week post-surgery (Fig. 7g, i),

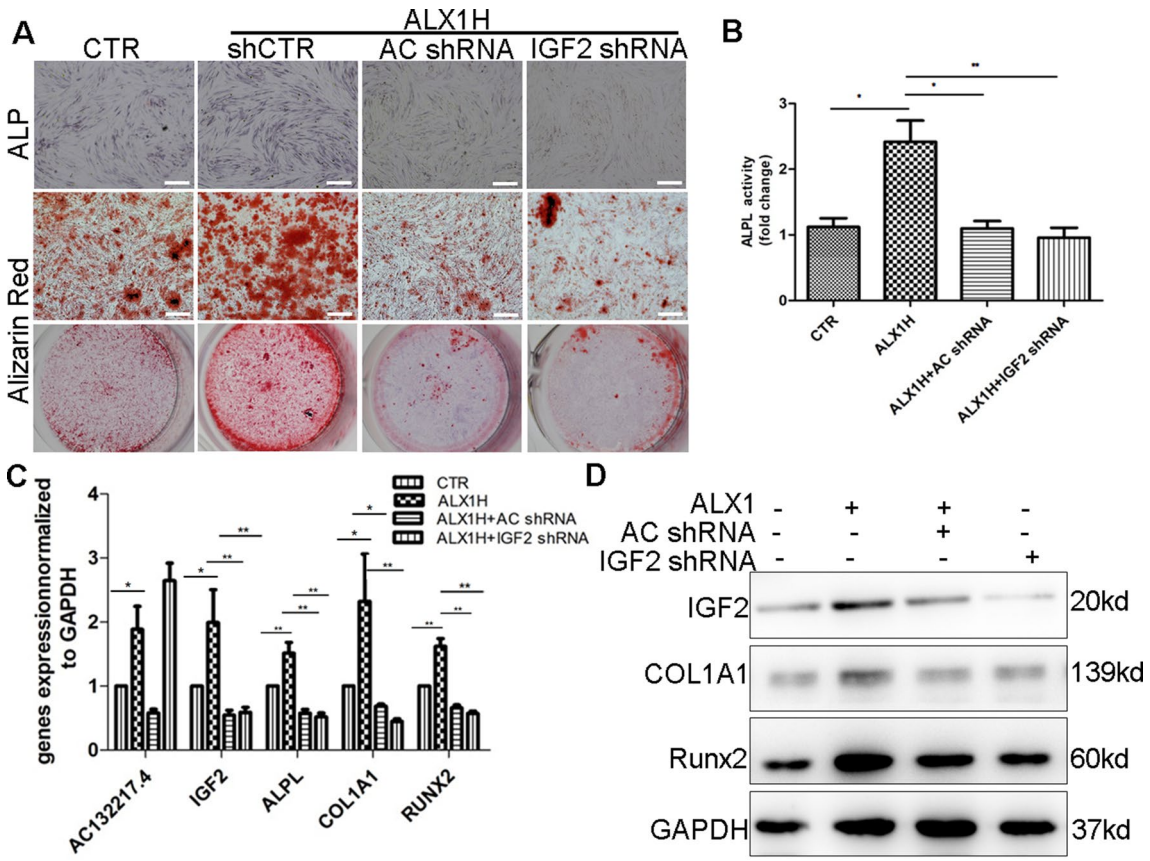


Fig. 6 ALX1 promoted osteoblast differentiation by AC132217.4-IGF2 signaling. **a**, **b** Osteoblast differentiation and maturation of control and ALX1 over-expressed hBMSCs with or without AC132217.4 or IGF2 knockdown is detected by ALPL stain (**a**), alizarin red stain (**a**), and ALPL activity (**b**). **c**, **d** Osteoblast marker gene expression in control and ALX1 over-expressed hBMSCs with or without AC132217.4 or IGF2 knockdown was detected by qRT-PCR (**c**) and western blot (**d**)

indicating its remodeling stage is 1 week lagged compared to the AC132217.4-overexpressing group. Overall, these results demonstrate AC132217.4 overexpression largely fastens multiple processes during bone regeneration.

Discussion

AC132217.4 is a novel lncRNA regulator of MSC osteogenesis

Many lncRNAs were identified to be functional during the last decades, while evidence is still lacking to support the functionality of most of them. To explore novel lncRNA regulators during osteogenesis, we performed differential expression analysis of RNA-seq data derived from hBMSCs before and after osteogenic induction. AC132217.4 stands out as the most altered lncRNA (100-fold change), which was later confirmed by qRT-PCR, indicating its potential role in osteogenesis. LncRNA AC132217.4 is spliced from an 844-bp gene segment overlapped with 3′UTR of the *Igf2* genome. Li et al. reported that AC132217.4 promotes oral squamous cell carcinoma metastasis [27], which is the solely prior publication addressing the biological function of AC132217.4. We found that AC132217.4 over-expression promoted and knockdown inhibited osteoblast differentiation of primary hBMSCs. Furthermore, gain-of-function of AC132217.4 in vivo promoted the formation of new bone in a murine drill-hole bone healing model. Therefore, we identified lncRNA AC132217.4 as a novel anabolic regulator for osteogenic differentiation in vivo and in vitro (Fig. 8).

Biogenesis of AC132217.4 is under cooperative promotion by multiple factors

The biogenesis of lncRNAs is considered to be more precisely controlled than mRNAs, based on their time- and spatial-specific expression patterns [24–26, 37]. Their genome also contains weaker transcription or splicing signals, rendering their low expression in an 'un-stimulated' status [24–26, 37]. Consistent with the notion, splicing sites for AC132217.4 also yield a relatively low score based on

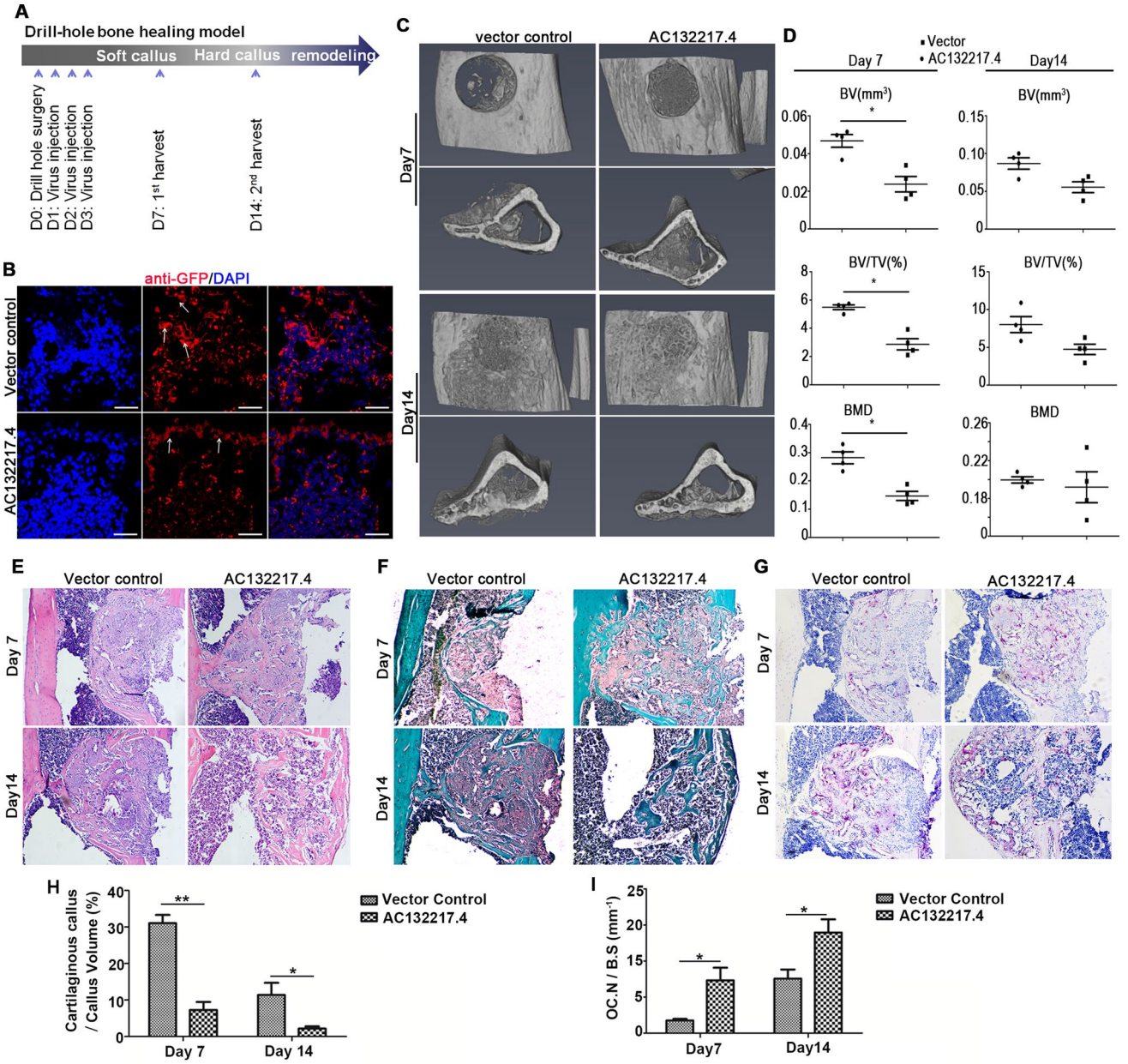


Fig. 7 AC132217.4 overexpression promoted bone healing in mice. **a** Timeline of the generation of a murine drill-hole model and the local injection of the lentivirus overexpressing GFP or GFP + AC132217.4. **b** Tracing of in-vivo lentivirus transfection using immunofluorescence staining with a GFP antibody. Scale: 25 μm. **c** Reconstructed three-dimensional images from μ-CT analysis of the bone healing sites in 1- and 2-week post-surgery (**d**). Quantification of bone volume (BV, mm3), bone volume/total volume (BV/TV, %), and bone mineral density (BMD), based on μ-CT analysis in **c**. **e**–**g** Hematoxylin–eosin (H&E) stain (**e**), Safranin O stain (**f**), and TRAP stain (**g**) of the paraffin sections prepared from the injured bone 1- and 2-week post-surgery. **h** Quantification of Cartilaginous callus volume per total callus volume in **f**. **i** Quantification of osteoclast surface (OC.S) per bone surface (B.S) in **g**. **e**–**g** Scale: 50 μm

bioinformatic prediction, indicating it might require extra regulators to increase its expression level. Using RNA-pull-down assay coupled with MS analyses, we identified two splicing factors, HNRNPA1 and SC35, bind to AC132217.4 pre-RNA and cooperatively produce the mature lncRNA. Either HNRNPA1 or SC35 knockdown leads to reduced expression of AC132217.4. Combining bioinformatics and experimental analysis, we also identified that the transcription factor ALX1 regulates AC132217.4 expression. Our study reveals that multiple factors (ALX1, HNRNPA1, SC35) are involved in the biogenesis of AC132217.4 in MSCs. This regulatory pattern might be applicable to other

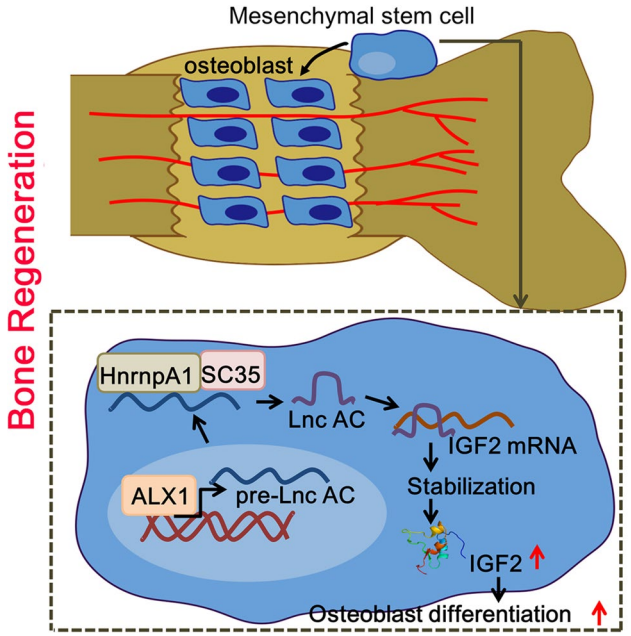


Fig. 8 AC132217.4 promotes osteogenesis and bone healing. A novel lncRNA in mesenchymal stem cells, AC132217.4, is identified to promote osteogenesis and bone regeneration. During osteogenesis of mesenchymal stem cells, the transcription factor ALX1 and the splicing factors HnrnpA1 and SC35 enhance the production of AC132217.4. Afterward, AC132217.4 binds to *IGF2* mRNA and stabilizes its expression to enhance IGF2/AKT signaling and osteoblast formation

lncRNAs important in MSC differentiation. It might also be an option to modulate their specific biogenesis machinery for therapies designed to target lncRNAs.

ALX1 was characterized as an evolutionarily conserved and crucial regulator of skeletogenesis. The echinoderm phylum is among the most ancient murine organisms first developing endoskeleton; Interestingly, ALX1 is the pivotal transcription factor controlling the skeletogenesis program throughout the echinoderm phylum [38, 39]. Furthermore, ALX1 was also demonstrated to control embryonic skeleton morphogenesis, especially craniofacial bone development, in higher organisms, such as mice, cats, and zebrafish [40–44]. In humans, missense mutations of the ALX1 gene cause type three frontonasal dysplasia (FND3), a rare congenital disorder characterized by abnormality of ocular and craniofacial components [42, 45, 46]. However, the role of ALX1 in MSC osteogenesis remains unclear due to prenatal lethality of ALX1 deficiency in mice. Our study reveals that ALX1 is among the highly upregulated transcription factors in MSCs during osteogenesis. ALX1 overexpression in MSCs promotes osteoblast differentiation and mineralization. Our study suggests a potential role of ALX1 in postnatal bone formation. Moreover, the downstream effector and signaling pathways of ALX1 in vertebrates remain to be studied, which is vital for understanding the molecular mechanism underlying ALX1's physiological role. Our study also elucidates that ALX1 regulates IGF-AKT signaling by targeting AC132217.4.

lncRNA AC132217.4 targets IGF-AKT signaling and promotes bone healing

Combining informatics and experimental analysis, we demonstrate that AC132217.4 promotes osteogenic differentiation through upregulating IGF2-AKT signaling. We found that AC132217.4 directly increases IGF2 expression at its mRNA level. LncRNA exerts its biological function in different ways, e.g., interacting with DNA, RNA, or proteins [14–17]. Our study showed that AC132217.4 is localized in the cytoplasm but does not bind with proteins, which indicates that AC132217.4 might regulate IGF2 expression through functioning on its mRNA. This hypothesis is confirmed by the fact that AC132217.4 overexpression compensates for the effect of RNA synthesis inhibitor on reducing *IGF2* expression.

IGFs (IGF1 and IGF2) are well-known for their role in the 'somatotropic axis' regulating body growth and bone mass. In this axis, growth hormones induce IGF production by the liver and locally, which play synergistic roles with the growth hormone [31, 32]. IGF1 regulating bone growth and integrity has been well-characterized in human and mouse models [31, 32], while IGF2 is much less studied. In general, currently documented evidence supports the promotional role of IGF2 in skeletal development and osteogenic differentiation. Loss-of-function mutation or decreased transcription of IGF2 in humans is related to Silver Russel Syndrome [47, 48], which is characterized by dwarfism and delayed bone maturation and might be associated with the risk of lower bone mass (NIH GARD website). Down-regulating IGF2 expression in mice the human disease phenotype, resulting in retarded growth and lower bone mass, with reduced osteoprogenitors [34]. Some other studies also showed a promoting role of exogenous IGF2 in osteogenesis in vitro and bone regeneration in vivo [34, 49–52]. Like IGF1, IGF2 also binds to the IGF1 receptor (IGF1R) or insulin receptor (INSR), which transduces signaling through AKT activation [32]. AKT activation promoting osteoblast differentiation and bone healing is extensively studied and well-documented [51, 53–55]. Thus, it is reasonable that IGF2 promotes osteoblast differentiation, while its physiological role in adult bone remains to be explored. In this study, we confirmed the anabolic function of IGF2 in hBMSC osteogenesis through loss-of-function (IGF2 knockdown) and gain-of-function (application of exogenous IGF2) studies. Significantly, IGF2 knockdown also impairs the anabolic role of AC132217.4 in osteogenesis. This data

further confirms that IGF2 is the indispensable effector of AC132217.4 to regulate osteoblast differentiation.

Moreover, both IGFs are highly expressed in the fracture callus [56, 57]. Altered IGF signaling has also been implicated in poor fracture healing [58–62]. Besides osteogenesis, IGF also promotes agiogenesis [63], another crucial physiological process during fracture healing. Moreover, locally delivered IGFs have a positive effect in multiple fracture models [50, 64–69]. Collectively, IGFs signaling is a critical local regulator to enhance bone repair. Our study showed that AC132217.4, which regulates IGF-2 expression, also possesses the ability to accelerate bone healing in mice.

In conclusion, our study identified a novel bone anabolic regulator, lncRNA AC132217.4, in vivo and in vitro. We also revealed that AC132217.4 regulates hBMSC osteogenic differentiation by interacting with IGF2 mRNA to stabilize its expression, which successively induces AKT activation (Fig. 8). Our study also showed that ALX1, SC35, and HNRNPA1 cooperatively regulate AC132217.4 expression (Fig. 8). Our finding indicates that AC132217.4 might be a novel pharmaceutical target for accelerating bone repair and treating bone disorders.

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Author contributions Conceptualization, MW, JW and CZ; methodology, MW, JW and CZ; investigation, CZ, SW, EC; resources, MW and CZ writing—original draft, MW, SW and CZ; writing—review and editing, MW and CZ; funding acquisition, MW YL and CZ; supervision, MW and JW.

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Data available statement RNA-seq data of hBMSCs during osteogenesis are deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE114117 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). All other data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest There are no competing financial interests related to the work described.

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