Original Article

Effects of Leukaemia Inhibitory Factor Receptor on the Early Stage of Osteogenic Differentiation of Human Bone Marrow Mesenchymal Cells

(leukaemia inhibitory factor receptor / osteogenic differentiation / human bone marrow mesenchymal stem cells)

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Abstract. Leukaemia inhibitory factor (LIF) has a wide variety of biological activities. While recent studies have focused on the role of LIF in osteoblast differentiation, the exact role of LIFR during the early stage of osteogenic differentiation remains unclear. We observed that LIFR expression gradually decreased during the early stage of osteogenic differentiation of hMSCs. To evaluate how LIFR regulates osteogenic differentiation in greater depth, we transfected hMSCs with LIFR overexpression and siRNA lentiviral plasmids. Cells were divided into four groups: a negative overexpression control group, a LIFR overexpression group, a negative siRNA control group, and a LIFR siRNA group. On different days (0, 3, and 6) of the osteogenic differentiation of hMSCs, alkaline phosphatase (ALP) activity was assayed with an ALP staining and activity assay kit. Cells were harvested to assess the mRNA and protein expression of LIF, LIFR, and osteogenesis-related factors (ALP; RUNX2; osteonectin) by qRT-PCR and western blot analyses, respectively. In addition, culture supernatants were tested for the LIF content by ELISA. Our results showed that overexpression of LIFR significantly suppressed the osteoblast differentiation of hMSCs. In contrast, LIFR siRNA markedly improved this osteoblast differentiation as determined by ALP staining and activity measurements. Moreover, RUNX2, ALP, and ONN expression was also significantly changed by altering LIFR expression. We further analysed the expression of LIF and LIFR, revealing consistent LIF and LIFR trends during the osteogenic differentiation of hMSCs. Together, these results suggested that LIFR may be a novel negative regulator during the early stage of hMSC osteogenic differentiation.

Introduction

Human mesenchymal stem cells (hMSCs) from bone marrow aspirates have the capacity to undergo self-renewal and multipotential differentiation (Pittenger et al., 1999). In the human body, hMSCs can be directed to differentiate into osteoblasts and adipocytes (Barry et al., 2001; Arinzeh, 2005; Helder et al., 2007; Rosen et al., 2012). If the homeostatic balance between these two cell types is disrupted such that there is a relative reduction in osteoblasts and an increase in adipocytes, this will lead to decreased bone mass, which may be a key process regulating the development of osteoporosis (Scheideler et al., 2008). During the development and differentiation of hMSCs, specific genes are activated and suppressed in a particular order to regulate the different stages of osteogenic differentiation (Steward and Kelly, 2015). The early stage of hMSC differentiation are particularly important, as they determine the future

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Abbreviations: ALP – alkaline phosphatase, hMSCs – human mesenchymal stem cells, HRP – horseradish peroxidase, LIF – leukaemia inhibitory factor, LIFR – leukaemia inhibitory factor receptor, MOI – multiplicity of infection, ONN – osteonectin, qRT-PCR – quantitative real-time PCR, PVDF – polyvinylidene difluoride, RUNX2 – runt-related transcription factor 2.

development of cells (Park et al., 2013; Martino et al., 2014). As such, the regulatory mechanisms governing the early osteogenic differentiation of hMSCs have been a major area of recent research. hMSCs that have been expanded in culture thus represent an ideal model for use in the exploration of the molecular events that trigger human osteogenic differentiation (Okolicsanyi et al., 2015).

The leukaemia inhibitory factor receptor (LIFR) consists of the LIFR α subunit (gp190) and β subunit (gp130) (Pan et al., 2006; del Valle et al., 2013). When leukaemia inhibitory factor (LIF) exerts its biological effects, it must combine with the LIF receptor α subunit of the target cell membrane and then form heterologous dimers with the β subunit, leading to protein phosphorylation to further initiate intracellular signalling (Plun-Favreau et al., 2003; Huang et al., 2012; Hwang et al., 2015). LIF is known to induce differentiation of murine myeloid leukaemia cell line M1 (Piekorz, 1998). LIF has also been demonstrated to have multiple effects on the regulation of osteogenic differentiation and bone formation (Malaval et al., 1995; Sims and Johnson, 2012).

Interestingly, to date there has been little focus on the role of LIFR in osteogenesis, with most research instead focused on LIF and leaving the role of LIFR in this process unclear. Early in our studies, we found that LIFR expression gradually decreases during the early stage of osteogenic differentiation of hMSCs, suggesting that LIFR may play a regulatory role in this complex process.

To evaluate the role of LIFR in the regulation of osteoblast differentiation in a more in-depth manner, LIFR overexpression and siRNA lentiviral plasmids were used to transfect hMSC cells. We further assessed the expression of LIF and LIFR over the course of osteogenic differentiation of hMSCs. Our findings suggest that LIFR may be a novel negative regulator during the early stage of osteogenic differentiation of hMSCs.

Material and Methods

Cell culture and osteogenic differentiation

Human bone marrow mesenchymal stem cells (hMSCs) (HUXMA-01001, Cyagen Biosciences, China) were confirmed to be positive for CD29, CD44, and CD105 (> 70 %) and negative for CD14 and CD45 (< 5 %) by flow cytometry. The hMSCs were plated to a density of 5×10^4cells/cm^2 and cultured in OriCellTM Human Mesenchymal Stem Cell Growth Medium (HUXMA-90011, Cyagen Biosciences) containing 10 % (v/v) foetal bovine serum, glutamine, and antibiotics penicillin and streptomycin under 5 % (v/v) CO_2 at 37 °C and 95% air humidity. The cells were passaged every 3 or 4 days with 0.25% trypsin-EDTA solution (Invitrogen, Carslbad, CA). The hMSCs between passages 3 and 6 were used in all experiments.

When cells were grown to 70% confluence, they were subsequently subjected to osteogenic differentiation for 6 days using media containing 50 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 10 mM β-glycerophosphate (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich). This differentiation medium was replaced every 3 days.

Lentiviral infection and hMSC selection

Lentivirus-mediated LIFR overexpression and siRNA expressing constructs were prepared by Shanghai Genechem Co., Ltd. Cells were divided into four groups: a negative overexpression control group, a LIFR overexpression group, a negative siRNA control group, and a LIFR siRNA group. The lentiviral titre was determined via serial dilution. hMSCs were then seeded into 6-well plates, grown to 20–30% confluence, and infected with 1×10^8 TU/ml lentivirus (10 µl; multiplicity of infection (MOI) = 5), 5 µg/ml polybrene, and complete medium. Cells were incubated in a 5% CO_2 environment at 37 °C for 10 h. Media was then refreshed, and cells were cultured for an additional 72 h. Media containing 0.5 µg/ml puromycin was then used for selection after 48 h, and was replaced every 1–2 days to maintain selective pressure for a total of 6 days until surviving cells began to proliferate. Before the osteogenic differentiation of hMSCs, LIFR was analysed by qRT-PCR to confirm the effectiveness of the lentiviral transduction.

On different days (0, 3, and 6) of the osteogenic differentiation of hMSCs, alkaline phosphatase (ALP) staining was carried out, cells were harvested to assess mRNA and protein expression of LIF, LIFR, and osteogenesis-related factors (runt-related transcription factor 2 (RUNX2), osteonectin (ONN), and ALP), and culture supernatants were collected to test the LIF levels.

ALP staining

ALP activity was assayed with an ALP staining kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, cells were washed with phosphate-buffered solution (PBS) twice and fixed in 4% formalin for 20 min. The cells were equilibrated using ALP buffer (0.1 M NaCl, 0.1 M Tris-HCl, 50 mM $MgCl_2.6H_2O$, pH 9.5) for 5 min twice, and were then incubated with ALP substrate solution (5 µl BCIP and 10 µl NBT in l ml ALP buffer) at room temperature in the dark for 30 min, after which the reaction was stopped with distilled water. Finally, the cells were observed under a microscope (Olympus, Tokyo, Japan).

ALP activity measurement

ALP activity was measured using a commercial ALP Detection Kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Ltd., Nanjing, China). Briefly, cells were freeze-thawed from –20 °C to room temperature four times to release the ALP. These lysates were transferred to 96-well plates and incubated with ALP substrate at 37 °C for 30 min. Reactions were then stopped via addition of a stop buffer. The p-nitrophenol product formed by the enzymatic hydrolysis of

Table 1. Primer sequences used for real-time quantitative PCR

Gene symbol	Forward primers	Reverse primers	Length (bp)
RUNX2	5‘-GGACGAGGCAAGAGTTTCACC-3‘	5‘-GGTTCCCGAGGTCCATCTACT-3‘	161
ONN	5‘-TCTTCCCTGTACACTGGCAGTTC-3‘	5‘-AAGCGGGTGGTGCAATGC-3‘	124
ALP	5-‘CCCCGTGGCAACTCTATCTTT-3‘	5-‘GCCTGGTAGTTGTTGTGAGCATAG-3‘	161
LIFR	5‘-AGCCTCAAGCAAAACCAGAA-3‘	5‘-TTGGCCTGAGGTCTGTAACC-3‘	144
LIF	5‘-CTGTTGGTTCTGCACTGGAA-3‘	5‘-CCCCTGGGCTGTGTAATAGA-3‘	154
β-Actin	5‘-GCGAGAAGATGACCCAGATCATGT-3‘	5‘-TACCCCTCGTAGATGGGCACA-3‘	160

the p-nitrophenyl phosphate substrate was measured at 520 nm using a microplate reader (Biorad, Hercules, CA).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted with the Trizol Reagent according to the manufacturer’s instructions (Invitrogen). First-strand cDNA was obtained using the Reverse Transcription System and Oligo(dT), following the manufacturer’s instructions (Thermo Scientific, Waltham, MA). Quantitative real-time PCR (qPCR) was performed using the SYBR Premix Ex Taq kit (Toyobo Co., Osaka, Japan) in a 7300 Real-Time PCR System (ABI, Foster, CA), and relative quantification via the ($2^{-\Delta\Delta CT}$) method was used to analyse the data. Endogenous β-actin mRNA was used as a reference control for mRNA quantification. Sequences of all primers are shown in Table 1.

Western blot analysis

Cells were lysed on ice using RIPA buffer (50 mM Tris (pH7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin). Proteins were then boiled in 5 × SDS sample buffer for 5 min, separated by electrophoresis in SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA). After this transfer, membranes were blocked with skim milk and probed with primary antibodies. Mouse anti-LIFR antibody (1 : 1,000; Cat. No. ab89792; Abcam, Cambridge, UK), rabbit anti-RUNX2 antibody (1 : 1,000; Cat. No. ab23981; Abcam), rabbit anti-ONN antibody (1 : 500; Cat. No. ab55847; Abcam) and mouse anti-β-actin (1 : 2,000; Cat. No. ab173838; Abcam) were used. Anti-mouse horseradish peroxidase (HRP)-conjugated IgG (1 : 5,000; Cat. No. 7076P2) and anti-rabbit HRP-conjugated IgG (1 : 5,000; Cat. No. 7074P2; both from Cell Signaling Technology, Inc., Beverly, MA) were used as secondary antibodies. The immune-stained protein bands were detected by chemiluminescence.

Quantification of LIF concentrations

LIF protein in cell culture supernatants was collected and LIF concentrations were determined using an ELISA kit (Senxiong Biotech, Shanghai, China). The assays were conducted according to the manufacturer’s instructions. Absorbance was read at 450 nm and was background corrected. LIF concentrations were determined using a reference standard curve.

Statistical analysis

All data are presented as mean ± SD from three independent measurements. SPSS v16.0 was used for all statistical analyses via one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

LIFR expression during the early stage of osteogenic differentiation

As shown in Fig. 1, the expression of LIFR was found to gradually decrease during the early stage of osteogenic differentiation of hMSCs, suggesting that LIFR may play a role in osteogenic differentiation.

Effectiveness of lentiviral transduction

On day 6 of selection, the surviving puromycin-resistant cells were successfully transfected, proliferating and showing good growth. To verify the effectiveness of the lentiviral transduction, transduction efficiency was assessed by qRT-PCR analysis. The results revealed a greater than 2.5-fold increase in LIFR expression in the overexpression group compared with the negative control group. Similarly, the LIFR siRNA group showed a

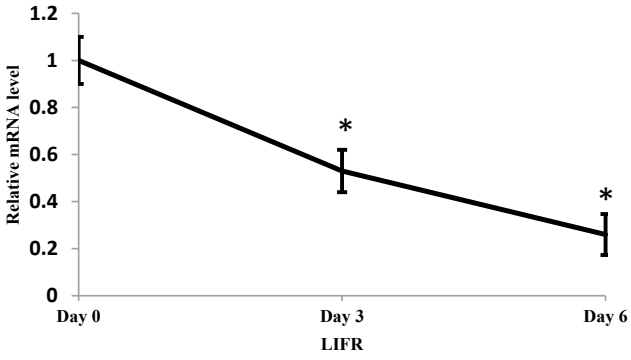

Fig. 1. The expression of LIFR during osteogenesis analysed by qRT-PCR at the indicated time points
All values are expressed as mean ± SD (X ± SD, N =3). *P < 0.01 vs. Day 0.

greater than 3-fold decrease in LIFR expression compared with the negative control group (Fig. 2).

ALP staining and activity

The osteogenic differentiation of hMSC cells was assessed on days 0, 3, and 6 based on ALP staining. The results showed that cells were stained with blue-violet (positive cells). The LIFR-overexpressing cells showed weaker ALP staining and colour (Fig. 3A). In contrast, the LIFR siRNA group exhibited a strongly positive staining and a deeper colour (Fig. 3B).

The activity of intracellular ALP was also investigated at these same time points, revealing a significant decrease in ALP activity in the LIFR overexpression cells relative to controls, with a corresponding significant increase in the LIFR siRNA group relative to controls. These findings supported the above ALP staining results (Fig. 4).

Expression levels of key osteogenesis-related factors, LIF, and LIFR during osteogenic differentiation

As shown in Fig. 5, the expression of osteogenesis-related factors (RUNX2, ALP, and ONN), LIF, and LIFR was assessed in each group by qRT-PCR. LIFR overexpression significantly suppressed osteogenic differentiation, and the expression levels of RUNX2, ONN, and ALP were also inhibited. There was also an upward trend in the level of LIF mRNA. LIFR siRNA, in contrast, significantly enhanced osteogenic differentiation, with increased expression levels of RUNX2, ONN, and ALP. In these cells, there was also a downward trend in LIF mRNA consistent with the decreased LIFR levels.

Protein expression of key osteogenesis-related factors, LIF, and LIFR during the early stage of osteogenic differentiation

The mRNA levels of RUNX2, ONN, LIF and LIFR were all elevated in each group. Consistent with this, western blotting showed comparable increases in the corresponding protein expression levels (Fig. 6). In each group, LIF concentrations were also determined by ELISA (Fig. 7).

Discussion

In post-menopausal osteoporosis and senile osteoporosis, the intrinsic properties of hMSCs are thought to be disturbed (Kim et al., 2016; Casado-Díaz et al., 2017). Studies showed that the osteogenic differentiation potential of hMSCs decreases over time, which is an important factor in the development of osteoporosis (Scheideler et al., 2008; Benisch et al., 2012). Therefore, the study of osteogenic differentiation of hMSCs is warranted in order to better understand how to treat osteoporosis and promote fracture healing.

It is well known that the early stage of cell differentiation determines the future development of cells (Park et al., 2013; Martino et al., 2014; Steward and Kelly, 2015). We initially observed that LIFR expression trended downward during the early stage of osteogenic differentiation of hMSCs (Fig. 1). This result implied that LIFR may play a regulatory role in osteogenesis. To confirm our hypothesis, we then constructed stable LIFR overexpression and siRNA lentiviral plasmids and used them to transduce hMSCs (Fig. 2), maintaining elevated or decreased expression of LIFR, respectively.

Subsequently, ALP staining and activity measurements were carried out in the early stage of osteogenic differentiation of hMSCs. As shown in Figs. 3A and 4A, LIFR-overexpressing cells exhibited weaker ALP staining and activity. In contrast, cells transduced with LIFR siRNA exhibited significantly enhanced ALP staining and activity (Figs. 3B and 4B). ALP is known to be linked to the promotion of calcification, and its activity is also one of the earliest signs of osteogenesis (Pinero et al., 1995). These findings thus suggested that LIFR emerges as a negative regulator of osteogenesis.

To further explore the effects of LIFR on the osteogenic differentiation of hMSCs, osteogenesis-related factors (RUNX2, ONN, and ALP) were analysed at the

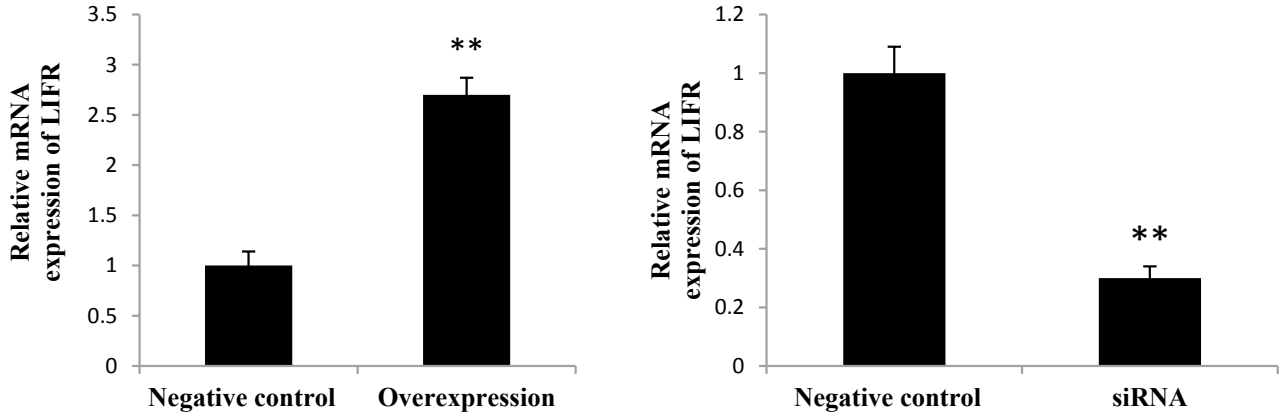

Fig. 2. Detection of LIFR expression by qRT-PCR
All values are expressed as the mean ± SD (X ± SD, N = 3). **P < 0.01 vs. negative control group.

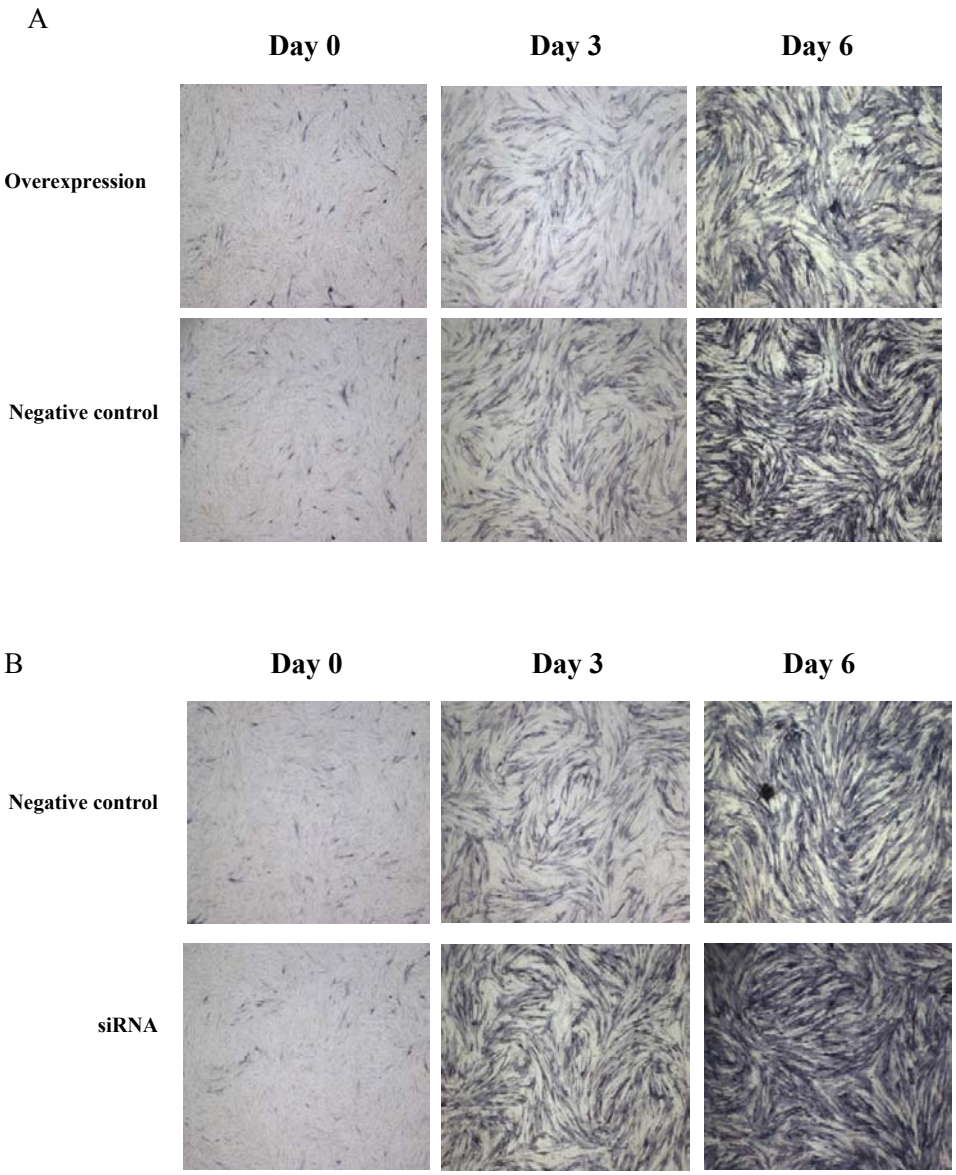

Fig. 3. Osteogenic differentiation of hMSCs identified on days 0, 3, and 6 by ALP staining
(A) Cells overexpressing LIFR showed weaker ALP staining. (B) Cells expressing LIFR siRNA groups showed stronger ALP staining.

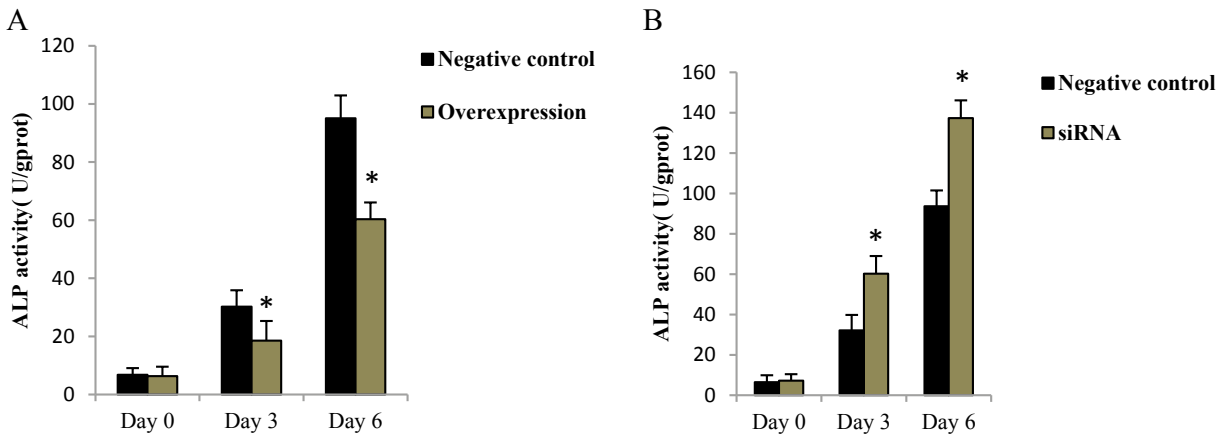

Fig. 4. Quantification of ALP activity on days 0, 3, and 6 during osteogenic differentiation of hMSCs
All values are expressed as the mean ± SD (X ± SD, N = 3). *P < 0.01 vs. negative control group.

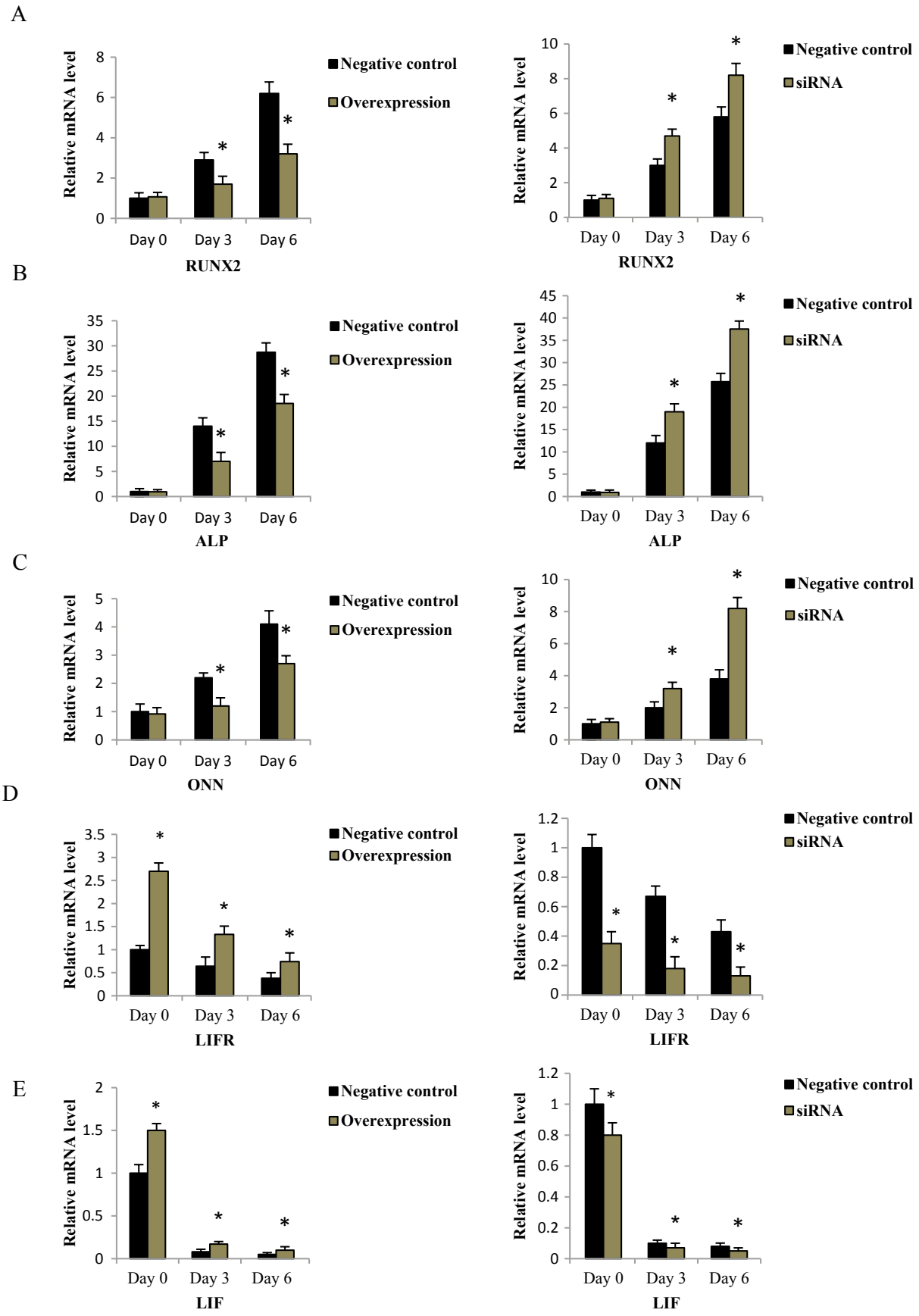

Fig. 5. qRT-PCR analysis of the expression of RUNX2 (A), ALP (B), ONN(C) , LIFR (D) and LIF (E) in each group All values are expressed as the mean ± SD (X ± SD, N =3) *P < 0.05 vs. negative control group.

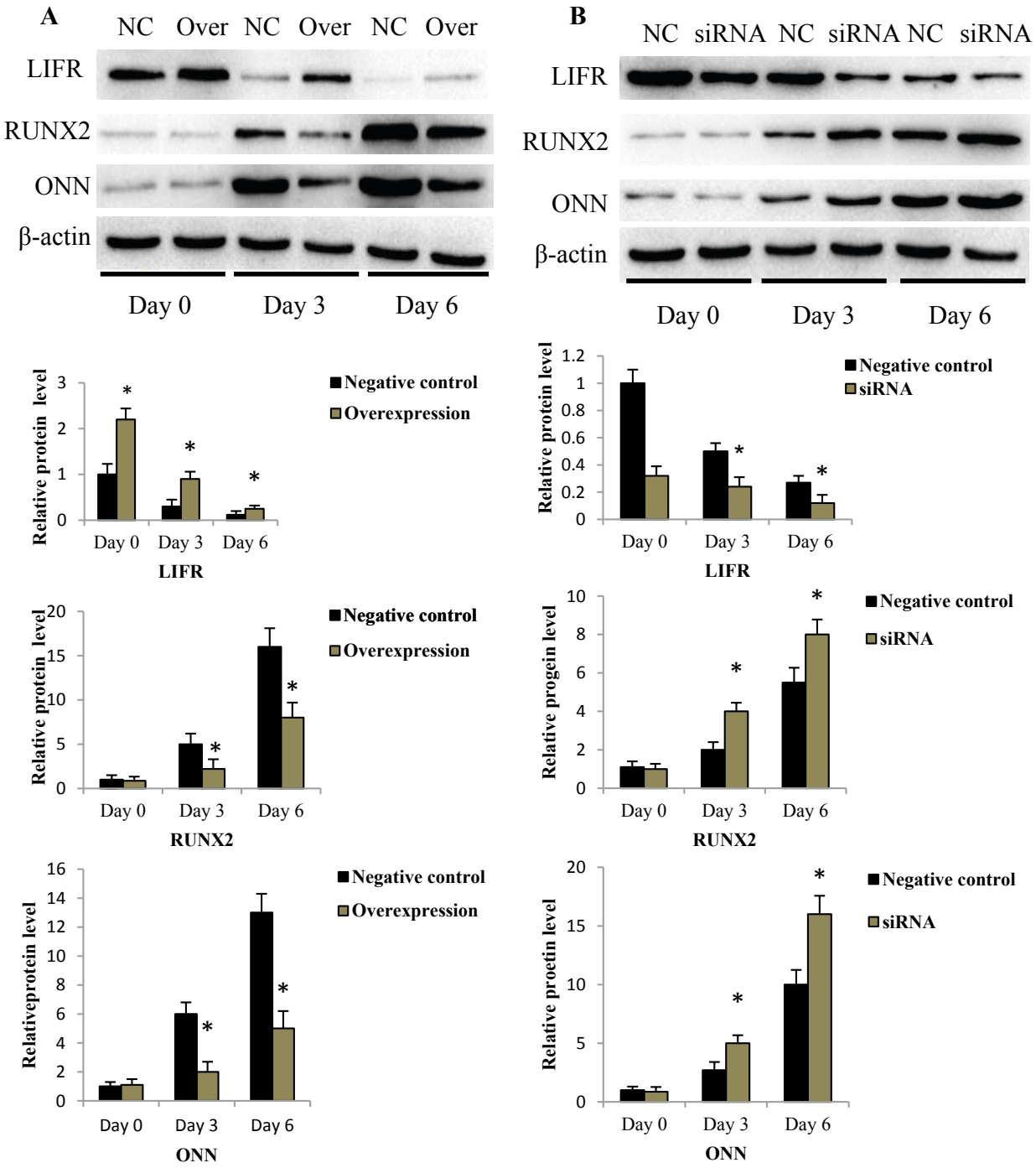

Fig. 6. Protein expression levels of osteogenesis-related factors and LIFR during osteogenic differentiation
A: The protein expression levels of osteogenesis-related factors decreased significantly in the LIFR overexpression group relative to negative controls. **B**: The protein expression level of osteogenesis-related factors and LIFR increased significantly in cells expressing LIFR siRNA compared to negative controls. β-Actin was used as a loading control. All values are expressed as the mean ± SD (X ± SD, N =3) *P < 0.05 vs. negative control group.
NC (negative control); Over (overexpression group); siRNA (siRNA group).

mRNA and protein levels. After LIFR overexpression and siRNA-mediated knockdown in hMSCs, the expression levels of these osteogenesis-related factors were clearly altered during the early stage of osteogenic differentiation (Figs. 5 and 6). RUNX2 has been shown to be necessary for osteogenic differentiation and bone formation in mesenchymal stem cells (Sun et al., 2012; Meng et al., 2016). RUNX2 directly activates the osteoblast-specific expression of ONN, triggering the formation of bone matrix proteins early in the process of osteogenic differentiation (Liu et al., 2008). Our experimental results showed that altering LIFR expression can lead to changes in the expression of these three osteogenesis-related factors, thereby affecting the early osteogenic

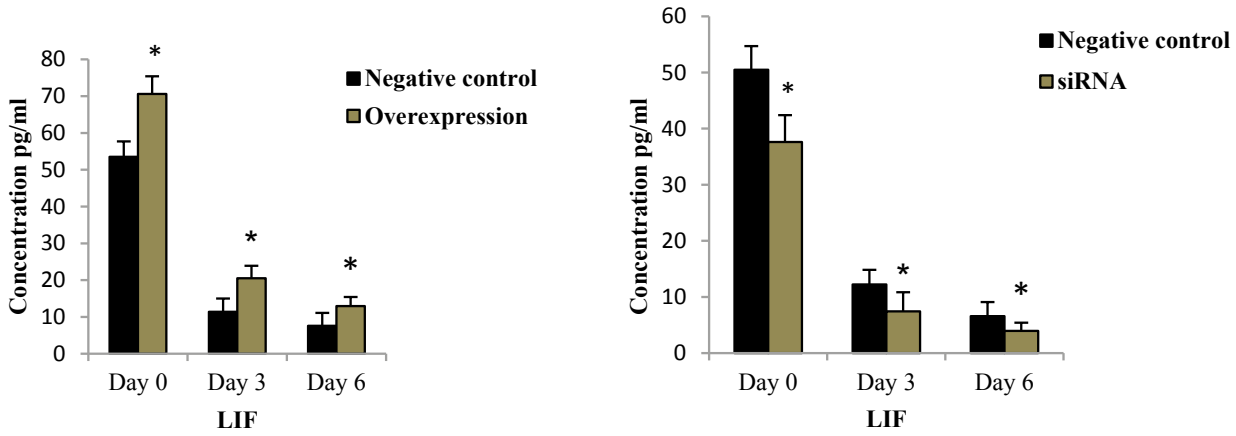

Fig. 7. Quantification analysis of LIF concentrations
All values are expressed as the mean ± SD (X ± SD, N =3). *P < 0.05 vs. negative control group.

differentiation of hMSCs. In addition, existing research indicates that RUNX2, ONN, and ALP are closely related to osteoporosis (Dalle Carbonare et al., 2009). Our results thus confirmed a possible inherent association between LIFR and these diseases related to abnormalities in osteogenic differentiation.

When studying LIFR, it is important to consider the role of LIF. LIF plays a functional biological role via signalling through LIFR. At present, most studies have focused on LIF but not on LIFR, leading us to explore the relationship between the expression of both LIF and LIFR during the early stage of osteogenic differentiation. In our study, we observed consistent changes in both LIF and LIFR expression during the osteogenic differentiation of hMSCs. After initiation of differentiation, LIF expression sharply decreased and LIFR expression gradually decreased. Interestingly, LIF expression was also increased upon LIFR overexpression and decreased upon LIFR knockdown (Fig. 5D and E; Figs. 6 and 7), suggesting that LIFR and LIF synergistically suppress osteogenic differentiation of hMSCs.

It is well known that a basic function of LIF is to inhibit differentiation of mouse embryonic stem cells (ES) (Natesh et al., 2015; Cherepkova et al., 2016). Some studies have also shown that LIF can inhibit osteoblast proliferation and activity in murine MC3T3-E1 pre-osteoblasts (Hakeda et al., 1991; Kozawa et al., 2002; Liu and Jiang, 2017). These related studies thus indirectly and strongly support our results. In addition to binding LIF, LIFR can also bind to a variety of cytokines, including tumour suppressor protein M, ciliary neurotrophic factor, and myocardial nutrients, thereby initiating a variety of downstream signalling pathways (Plun-Favreau et al., 2003; Wagener et al., 2014; Natesh et al., 2015). Many of these signalling pathways are closely related to osteogenic differentiation, including MAPK and JAK-STAT signalling (Plun-Favreau et al., 2003; Huang et al., 2012; Hwang et al., 2015; Luo et al., 2017). Clearly, LIFR serves as a pivotal link between cytokines and signalling pathways. These studies thus further support that LIFR plays an important role in the early stage of osteogenic differentiation.

Our study thus revealed an important role for LIFR in regulating hMSCs during the early stage of osteogenic differentiation, suggesting that LIFR may be a novel negative regulator of osteogenic differentiation.

Disclosure of conflict of interest

The authors declare no financial or commercial conflict of interest.

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