Interleukin-35 controls the balance between osteogenic and adipogenic differentiation of progenitor cells

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Research Article

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Abstract

Aims: Mesenchymal stem cells (MSCs) are multipotent stem cells that are able to differentiate into several cell types, including cartilage, fat, and bone. It has been reported that the decision process of MSCs into fat and bone cells is competing and reciprocal. Interleukin (IL)-35 is an important effector protein in the Wnt/β-catenin signaling pathway that acts as a bone metabolism regulator. However, it is unclear whether IL-35 is also important for regulating MSCs differentiation to fat and bone.

Methods: In current study, we evaluated the role of IL-35 in C3H10T1/2 cells, which are a good cell model for investigating osteogenesis and adipogenesis in bone-marrows. The role of IL-35 on MSCs proliferation and apoptosis were assessed using cell counting kit -8 assay and flow cytometry, respectively. Extracellular matrix mineralization and lipid accumulation were measured by Alizarin red S staining and Oil-red O staining, respectively. The most important transcription factor of the process of osteogenesis Runx2 and Wnt/β-catenin signaling pathway components β-catenin and Axin2 were investigated in response to IL-35 treatment. Furthermore, the adipogenic markers PPAR-γ and C/EBPα were also investigated.

Results: Our observations showed that IL-35 could promote the proliferation of MSCs and inhibit the apoptosis of MSCs. We found that IL-35 treatment resulted in a dramatic stimulation of osteogenesis and inhibition of adipogenesis. Moreover, IL-35 enhanced Wnt/β-catenin pathway key component β-catenin as well as Axin2 expression during MSCs differentiated to osteoblasts.

Conclusions: Our findings suggested that IL-35 might control the balance between osteogenic and adipogenic differentiation of progenitor cells through Wnt/β-catenin-PPARγ signaling pathway, suggesting its potential application in providing an intervention in osteoporosis and obesity.

Introduction

The lineage commitment of mesenchymal stem cells (MSCs), which is a common progenitor, plays a crucial role in the maintenance of bone homeostasis[1]. MSCs can differentiate into different cell types. However, the competitive differentiation of MSCs to adipocytes and osteoblasts has been drawn much attention[2]. The dysregulation between osteogenic and adipogenic differentiation is often observed in multiple human diseases, such as osteoporosis and obesity[3]. Thus, it is important to explore the mechanisms that control the balance between osteoblasts and adipocytes and the commitment to differentiate to either lineage. Osteogenesis and adipogenesis are mediated through multiple signal pathways and a harmonious cascade of downstream transcription factors. Previous studies have demonstrated that Wnt/β-catenin signaling, β-catenin and Runt-related transcription factor 2 (Runx2) are closely linked to osteogenesis, peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) are closely related to adipogenesis.

Interleukin (IL)-35 is a heterodimer which exhibits growth factor-like activities on diverse cell types, including osteoblasts, osteoclasts, and synovial fibroblasts[4–6]. IL-35 was a new member of the IL-12 family which was identified as an immunosuppressive and anti-inflammatory cytokine and was observed to mitigate collagen-induced arthritis (CIA) in mice[7]. IL-35 is main produced by Tregs, which played a crucial role in exerting the maximal immune-suppressive function of Tregs. Tregs exerted immune-stimulatory function mainly via EBI3, which could stimulate macrophages to synthesize macrophage inflammatory protein-1 and further commit B and T lymphocytes to inflammatory local sites[8]. Further study showed that IL-35 could stimulate Tregs proliferation in turn, which named iTR35. This type of iTR35 exhibited inhibition of inflammation by switching initial T cells into a new type of Foxp3-Treg[9].

Recent research has demonstrated that IL-35 plays physiological and pathophysiological roles in different cells and tissues including osteoblasts, osteoclasts, liver, intestine and lung[5, 10–13]. IL-35 is necessary for in vitro bone formation and bone resorption. In MC3T3E1 cells line, IL-35 could upregulate expression of osteoprotegerin (OPG) and downregulate the expression of receptor activator of nuclear factor-κB ligand (RANKL) through Wnt/β-catenin signaling pathway, the pre-osteoblasts cells line[10]. In RAW264 mouse monocytic cells line, IL-35 collaborated with RANKL to stimulate osteoclastogenesis via signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase pathway. Furthermore, IL-35-MSCs exerted a stronger immunosuppressive function and further decreased the percentage of Th17 cells, increased the proportion of CD4 + Foxp3 + T cells, and controlled Th1/Th2 balance in heart transplant mice. To date, it is unknown whether and how IL-35 regulates osteogenic and adipogenic differentiation from mesenchymal progenitor cells.

However, it has not yet fully been investigated whether IL-35 have an impact on MSCs. In present study, the biological changes in C3H10T1/2 cells after IL-35 treatment are measured, which are a good cell model for investigating osteogenesis and adipogenesis in bone-marrows. We demonstrated that IL-35 could promote osteogenesis and inhibit adipogenesis in MSCs in vitro, which is likely contribute to the activation of the crosstalk between Wnt/β-catenin signaling pathway and PPARγ signaling pathway.

Materials And Methods

Cells

C3H10T1/2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). For osteoblast differentiation, C3H10T1/2 cells of 80% confluence were exposed to osteogenic medium (a-MEM containing 10% FBS, 50 μg/mL ascorbic acid, and 5 mM β-glycerophosphate) for 14 days followed by 21 days for alizarin red staining. For adipogenic differentiation, C3H10T1/2 cells of 100% confluence were exposed to adipogenic medium (a-MEM containing 10% FBS, 0.5μM dexamethasone, 0.25mM methylisobutylxanthine, 5μg/mL insulin, and 50μM indomethacin) for 3 days, then with insulin alone for an additional 2 days followed by oil-red O staining. The medium was refreshed every 3 days.

CCK-8 assay

Effect of IL-35 on cell proliferation was measured by Cell Counting Kit-8(CCK-8) assay. Briefly, C3H10T1/2 cells were seeded in 96-well plates at a density of 3×10^3 cells per well. Then, cells were treated with IL-35 at various concentrations (0, 25, 50 and 100 ng/mL) for 0、24、48 and 72h. Then 10ul CCK-8 was added to each well and the mixture was incubated for 4 h at 37°C. After shaking for 15 min, absorbance was measured on microplate reader at 450 nm.

Flow cytometry

Flow cytometry was used to detect apoptosis frequency in MSCs. Briefly, 1×10^6 MSCs were seeded into 6 well plates. Briefly, after incubation with IL-35 at various concentrations (0, 25, 50 and 100ng/mL) for 48h, MSCs were collected by trypsin digestion. Discard the supernatant and gently resuspend the cells by adding 195μL of Annexin V-FITC binding solution. Then 5μL Annexin V-FITC and 10μL propidium iodide staining solution were added to each well and mix gently 15 min in darkness at room. The MSCs apoptosis frequency was analyzed by flow cytometer.

Alizarin red staining

After 14 days culture of MSCs in the presence of osteogenic medium, the differentiated osteoblasts were gently washed twice with PBS and fixed in 4% paraformaldehyde for 15 minutes. For alizarin red staining, MSCs were cultured in the presence of osteogenic medium for 21 days, then washed twice with PBS buffer, fixed with 70% ethanol, and incubated for 1 hour at 37℃. Then, the cells were stained with 40 mM alizarin red S for 30 minutes. The calcium salt nodules were visualized by microscopy.

Oil-red O staining

Fully differentiated adipocytes were gently washed twice with PBS and fixed in 4% paraformaldehyde for 15 minutes. The samples were washed twice with deionized water and then stained for 5 minutes with 0.3% oil-red O in 60% saturated isopropanol. The stained fat droplets in the adipocytes were visualized under light microscope and photographed. Spectrophotometric analysis of the stain was performed by re-dissolving the stained lipid droplets with isopropanol and measuring the light absorbance at 520 nm.

Quantitative RT-PCR

RNA was extracted from cells using a total RNA isolation kit. The reverse transcription was performed by using MMLV reverse transcriptase. The quantitative real-time PCR was implemented by using a SYBR Green fluorescence PCR kit on a real-time PCR cycler with gene specific primers. The cycling scheme consisted of 40 cycles (95℃ for 10 seconds, 57℃ for 10 seconds, and 72℃ for 10 seconds) after an initial denaturing step (95℃ for 2 minutes). The expression levels were normalized against β-actin and measured by the $^{\Delta\Delta}$Ct method. The primers used are listed as follows:

β-catenin forward: GCAACCCTGAGGAAGAAGA;

β-catenin reverse: GGATGAGCAGCGTCAAACT;

Runx2 forward: GCAGCACTCCATATCTCTACT;

Runx2 reverse: TTCCGTCAGCGTCAACAC;

Axin2 forward: CAACGACAGCGAGTTATCC;

Axin2 reverse: GTTCCACAGGCGTCATCT;

PPARγ forward: ACCACTCGCATTCCTTT;

PPARγ reverse: CACAGACTCGGCACTCA;

C/EBPα forward: GAGGAGGACGAGGCGAAGCA;

C/EBPα reverse: CGATCTGGAACTGCAAGTGGG;

β-actin forward: CTGTGCCCATCTACGAGGGCTAT;

β-actin reverse: TTTGATGTCACGCACGATTTCC.

Western blot analysis

Cells were lysed using RIPA buffer, supplemented with protease inhibitors and phosphatase inhibitors, and proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride membranes. The membranes were probed overnight with primary antibodies including rabbit mAbs by Abcam (Cambridge,Massachusetts): anti-β-catenin, anti-Runx, anti-Axin2, anti-PPARγ and anti-C/EBPα. Finally, enhanced chemiluminescence reagent was used to visualize the results. β-actin was used as a loading control.

Statistical analysis

All data were analyzed by SPSS 17.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6 software. All values were presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between groups were assessed by Student's t-tests and One way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons post-test. Differences of $P < 0.05$ were considered significant.

Results

IL-35 promoted the proliferation of MSCs

To identify the effect of IL-35 on MSCs growth, we tested the MSCs viability rate using CCK-8 assay. As shown in Fig. 1, the results of CCK-8 assay showed that IL-35 promoted the proliferation of MSCs in a time and a dose dependent manner.

IL-35 inhibited apoptosis of MSCs

MSCs apoptosis in each group was measured by flow cytometry. In the control group, the frequency of apoptotic MSCs was 8.43 ± 1.06%. In the 100 ng/ml IL-35 group, the frequency of apoptotic MSCs was only 2.03 ± 0.35%, which was significantly lower (all $p < 0.05$) than that in the 50ng/ml IL-35 group (4.01 ± 0.54%) and 25ng/ml IL-35 group (5.71 ± 0.57%) (Fig. 2). This result suggested that IL-35 could inhibit MSCs apoptosis in a dose dependent manner.

IL-35 regulates osteogenic-adipogenic gene expressions in progenitor cells

First, we examined the effect of IL-35 on Runx2 expression, which was the most important transcription factor of the process of osteogenesis. The C3H10T1/2 cells initiated their differentiation to osteoblasts upon treatment with osteogenic medium. At the same time, Wnt/β-catenin signaling components was changed. Interestingly, we found that the IL-35 treatment resulted in a significant increase in the β-catenin and Axin2 expression (Fig.3a, 3b, 3c).Next, to explore whether IL-35 influences the adipogenesis of MSCs, we investigated the effect of IL-35 on adipocyte differentiation. The C3H10T1/2 cells initiated their differentiation to adipocytes upon treatment with adipogenic medium. Compared to control cells, the expression levels of the adipogenic markers PPAR-γ and C/EBPα were also reduced in matureadipocytes in response to the treatment with IL-35 (Fig.3d, 3e).

Figure 3. (a, b, c) Osteogenic transcription factor Runx2 and Wnt/β-catenin component including β-catenin and Axin2 mRNA expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100 ng/mL) when MSCs were cultured in osteogenic medium; (d, e) The two master regulators of adipocyte differentiation including PPARγ and C/EBPα mRNA expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100 ng/mL) when MSCs were cultured in adipogenic medium. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group (IL-35(0 ng/mL). Comparison between two groups was conducted by Student t-test. P-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

IL-35 regulates osteogenic-adipogenic protein expressions in progenitor cells

Consistent with real time-PCR results, western blot analysis showed that exogenous IL-35 increased the protein levels of osteogenic transcription factors and Wnt/β-catenin component, including β-catenin, Runx2 and Axin2 after 14 days of osteogenic induction dose dependently. However, exogenous IL-35 decreased the protein levels of the two master regulators of adipocyte differentiation including PPARγ and C/EBPα dose dependently (Fig. 4).

IL-35 promoted mineralization and inhibited lipid accumulation in C3H10T1/2 Cells

Last, we explored what effect of IL-35 on matrix mineralization and lipid accumulation in MSCs. C3H10T1/2 cells at confluence were treated with IL-35 for 48h. Alizarin red staining showed that IL-35 could increase the area and the density of mineralized nodules in MSCs. Furthermore, oil red O images revealed that IL-35 could enhance the lipid accumulation in a dose dependent manner in MSCs (Fig. 5).

Discussion

IL-35 is a novel member of the IL-12 family, which is composed of a p35 subunit and an Epstein-Barr virus-induced gene 3 (EBI3) subunit. IL-35 is an immune-suppressive cytokine, mostly derived from CD3 + CD4 + CD25 + Foxp3 + regulatory T cells (Tregs). Previous studies have demonstrated that IL-35 was significantly associated with the multiple autoimmune diseases including rheumatoid arthritis (RA)[14, 15], periodontitis[16], ulcerative colitis[12] as well as idiopathic inflammatory myopathies[17]. Additionally, elevated IL-35 levels in allografts may reduce the occurrence of acute graft-versus-host disease after allogeneic haematopoietic stem cell transplantation[18]. Cell-based remedy using MSCs has great promising prospect in immunosuppression. These multipotent cells have immunomodulatory and paracrine properties, which have shown promise in treatment of multiple diseases such as multiple sclerosis[19] and graft-versus-host disease. MSCs can differentiate into different cell types including adipocytes, osteoblasts, myocytes and chondrocytes[20]. Several signaling pathways have been confirmed to be involved in the lineage commitment and differentiation of MSCs, including the cooperation of Wnt, BMP, Notch, Hedgehog, PPAR[21]. Previous study demonstrated that marrow fat volume was increased in animal models of ovariectomy and in aging in humans[22]. This phenomenon exists partly because MSCs have a decreased capacity to differentiate into osteoblasts and an increased capacity to differentiate into adipocytes[23]. This observation might suggest that the differentiation pathways of osteoblasts and adipocytes are mediated jointly and indicated that an inverse relationship occurs between the two lineages.

In present study, IL-35 promoted MSCs proliferation and inhibited MSCs apoptosis. These results may suggest that IL-35 exerts its immunosuppressive functions through increasing the number of MSCs. MSCs have been demonstrated to act is by proliferating circulating Treg cells population[24]. IL-35-transduced Ad-MSCs could promote expansion of CD4 + CD25 + Tregs and refrain the function of effector T cells including T helper (Th) 1, Th2 as well as Th17 cells and may delay the development of allograft rejection[25]. In bone metabolism, the role of IL-35 is widely investigated. IL-35 could promote the expression of OPG and inhibited the expression of RANKL in osteoblasts through Wnt/β-catenin signaling pathway[10]. In this study, IL-35 could promote the expression of Runx2, which was the most important transcription factor of the process of osteogenesis. At the same time, IL-35 suppressed the expression of PPARγ, which was the best known for its crucial role in mediating adipogenic differentiation in MSCs. MSCs derived from many adult tissues have the potential to differentiate into cells of different lineages, including osteoblasts, chondrocytes, adipocytes and myocytes[26]. Among these plausible fates, differentiation to the osteoblasts and adipocytes lineages has specific effect on the maintenance of normal bone metabolism. Previous study demonstrated that a shift in MSC differentiation to favor the

adipocytes lineage over the osteoblasts lineage can lead to imbalances in bone formation and resorption, finally contribute to bone loss. The reduction in bone volume related to age-related osteopenia and osteoporosis was followed with a rise in marrow adipose tissue[27]. Therefore, another mechanism on osteoblastic roles of IL-35 could be explained that IL-35 could control the balance between osteogenic and adipogenic differentiation of progenitor cells.

At the same time, we also linked this phenomenon to the increased expression of the members of the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling components such as β-actin and Axin2 were also elevated in response to IL-35. The activation of β-catenin can be mediated when expression and/or phosphorylation of the Wnt signaling components was changed. Interestingly, we found that the IL-35 treatment resulted in a significant increase in the β-catenin and Axin2 expression. Furthermore, we also found that IL-35 treatment down-regulated the expression of PPAR-γ and C/EBPα, which are considered as the crucial factors in the adipogenesis of MSCs. Previous studies demonstrated that the activation of PPAR-γ led to a decrease of β-catenin expression during adipogenesis; this reduction of β-catenin is involved via a proteasome dependent degradation mechanism[28]. In current study, the IL-35 treatment resulted in an increase of β-catenin and Axin2 with a concomitant suppression of adipogenesis, indicating that activation of Wnt/β-catenin signaling pathway and the inhibition of PPARγ signaling pathway are not parallel and independent events caused by IL-35. Thus, it is likely that the IL-35-induced activation of osteogenesis contributes to the inhibition of adipogenesis in MSCs. However, it is not clear whether Wnt/β-catenin signaling and PPARγ signaling pathway exist “cross-talk” and what the specific interaction factor during the IL-35-induced promotion of osteogenesis and inhibition of adipogenesis during MSCs differentiation. Molecular study during the past decades has confirmed that the Wnt/β-catenin signaling components act as negative mediator of adipogenesis. In our study, Axin2 was upregulated by IL-35 in MSCs. We further screened out PPARγ that may be transcriptionally activated by Axin2 according to the JASPAR database (supplementary Fig. 1.). We could speculate that IL-35 stimulate osteogenesis and inhibit adipogenesis via PPARγ-Axin2-Wnt/β-catenin crosstalk during MSCs differentiation. However, further studies are needed to be verified.

Conclusion

The balance between adipogenesis and osteogenesis within the bone marrow microenvironment offers a promising target for medical intervention. We confirmed that IL-35 could promote MSCs proliferation and inhibit MSCs apoptosis. Furthermore, IL-35 is the key regulator of adipo-osteogenic differentiation in MSCs. Our findings suggested that IL-35 might prevent the development of bone loss through shifting adipo-osteogenic differentiation, indicating a promising treatment target for bone loss in the future.

Abbreviations

MSCs
Mesenchymal stem cells
IL

Interleukin
Runx2
Runt-related transcription factor 2
PPARγ
peroxisome proliferator activated receptorγ
C/EBPs
CCAAT/enhancer binding proteins
CIA
collagen-induced arthritis
OPG
osteoprotegerin
RANKL
receptor activator of nuclear factor-κB ligand
FBS
fetal bovine serum
CCK-8
Cell Counting Kit-8
SD
standard deviation
ANOVA
One way analysis of variance

Declarations

Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

Yuxuan Li, Xiaofei Wang, Jing Lu declare that they have no conflicts of interest.

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Authors' contributions

YX L, J L participated in the design of the study and performed the statistical analysis. XF W, J L conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

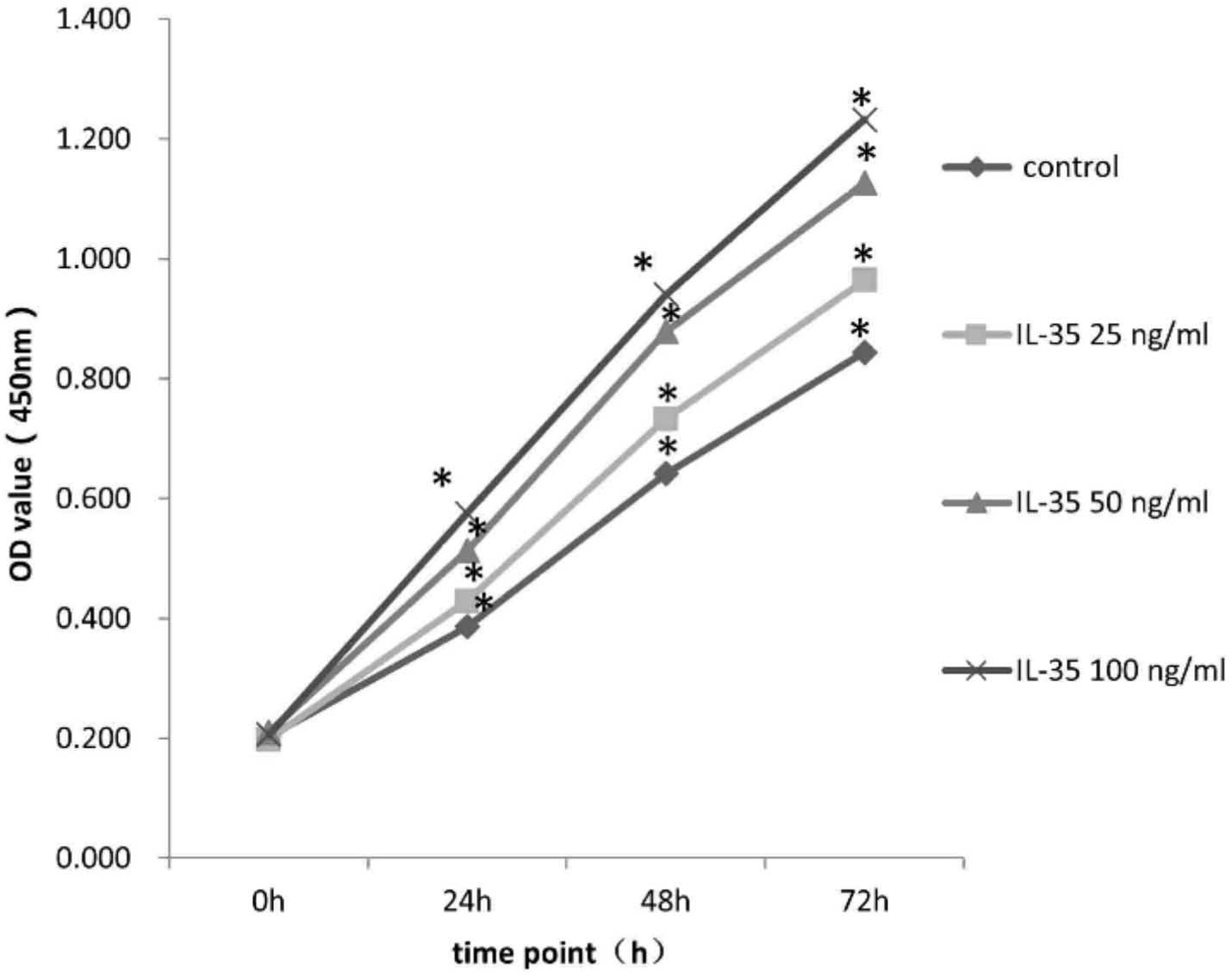

Figure 1

The effects of IL-35 at different concentration (0, 25, 50, 100 ng/mL) for 0, 24, 48 and 72h on the proliferation of MSCs. *p<0.05 vs. control group (IL-35(0 ng/ml)). P-value were estimated using one-way ANOVA.

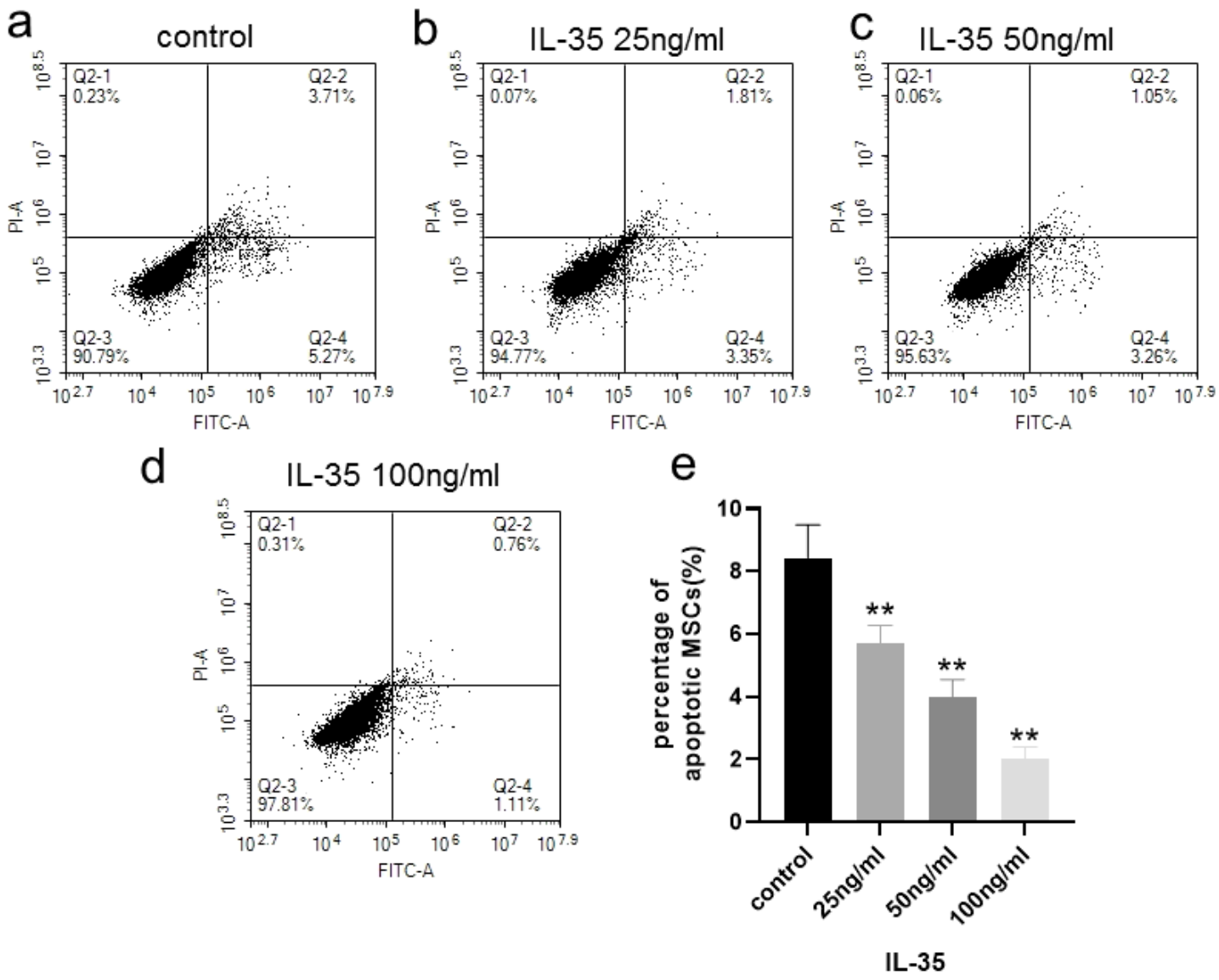

Figure 2

The effects of IL-35 at different concentration (0, 25, 50, 100 ng/mL) for 48h on the percentage of apoptotic MSCs. *p<0.05 vs. IL-35(0 ng/ml). (a, b, c, d) Representative flow cytometric analysis of FLS apoptosis. (e) Percentages of apoptotic FLS after IL-35 treatment. Data are expressed as mean±SD, n=3. **p < 0.01 versus control group. The right upper quadrant represents the late stage of MSCs apoptosis, and the right lower quadrant represents the early stage of MSCs apoptosis).

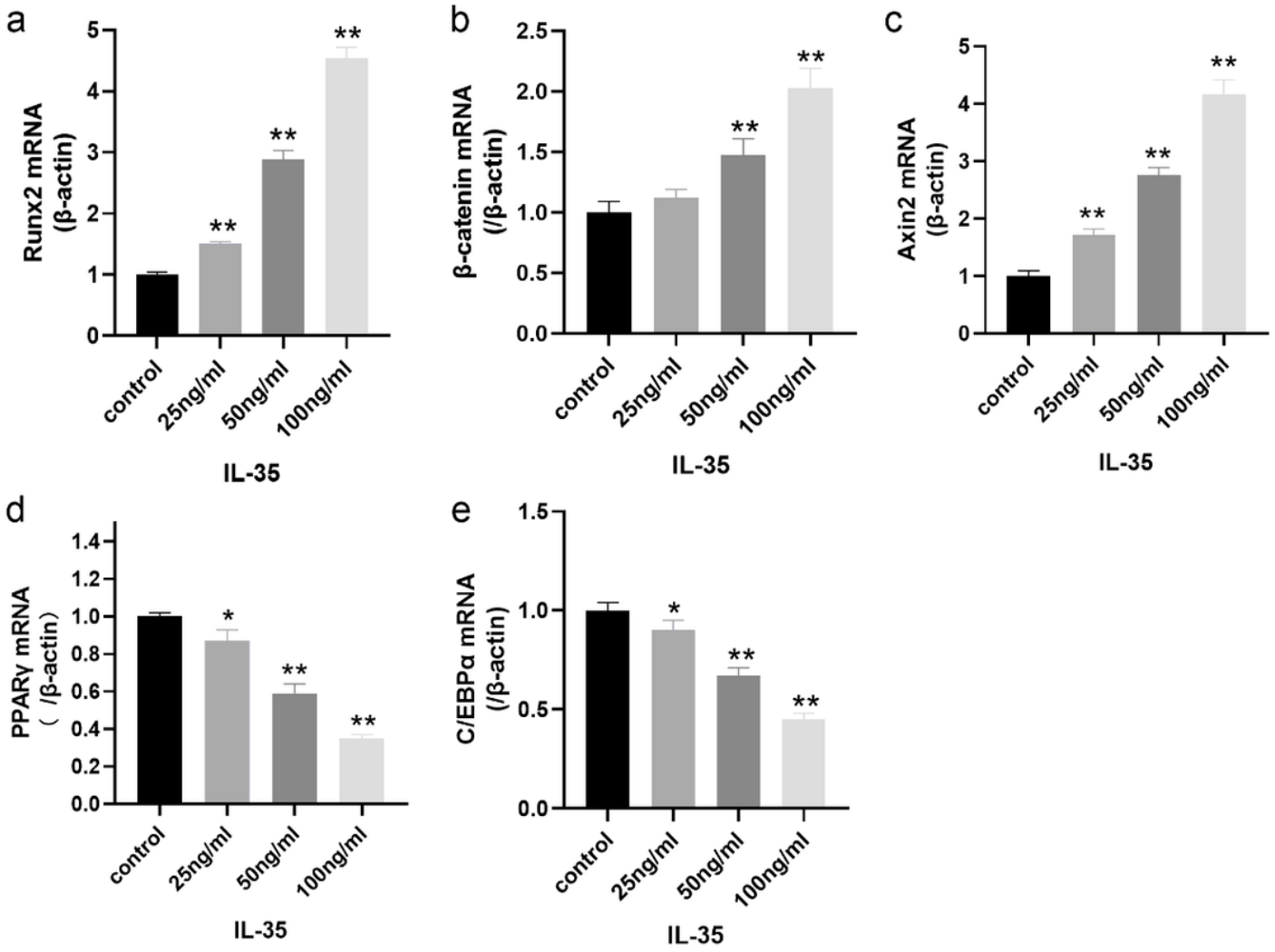

Figure 3

(a, b, c) Osteogenic transcription factor Runx2 and Wnt/β-catenin component including β-catenin and Axin2 mRNA expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100 ng/mL) when MSCs were cultured in osteogenic medium; (d, e) The two master regulators of adipocyte differentiation including PPARγ and C/EBPα mRNA expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100 ng/mL) when MSCs were cultured in adipogenic medium. $^{*}p<0.05$, $^{**}p<0.01$ vs. control group (IL-35(0 ng/mL). Comparison between two groups was conducted by Student t-test. P-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

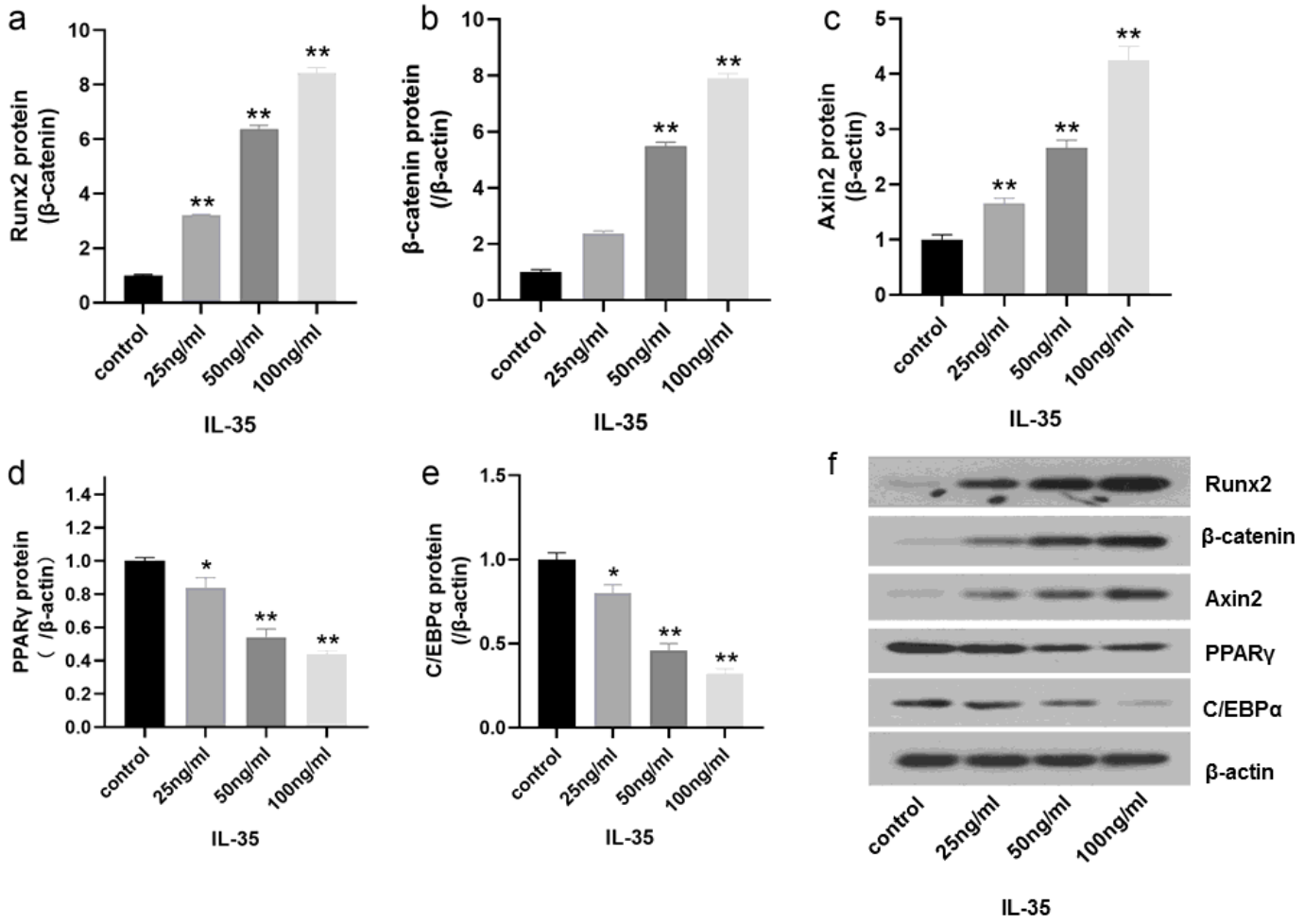

Figure 4

(a, b, c) Osteogenic transcription factors and Wnt/β-catenin component including Runx2、β-catenin and Axin2 protein expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100 ng/mL) when MSCs were cultured in osteogenic medium; (d, e) The two master regulators of adipocyte differentiation including PPARγ and C/EBPα protein expression in MSCs in response to IL-35 at different concentrations (0, 25, 50 and 100ng/mL) when MSCs were cultured in adipogenic medium. $^{*}p<0.05$, $^{**}p<0.01$ vs. control group (IL-35(0ng/mL). Comparison between two groups was conducted by Student t-test. P-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

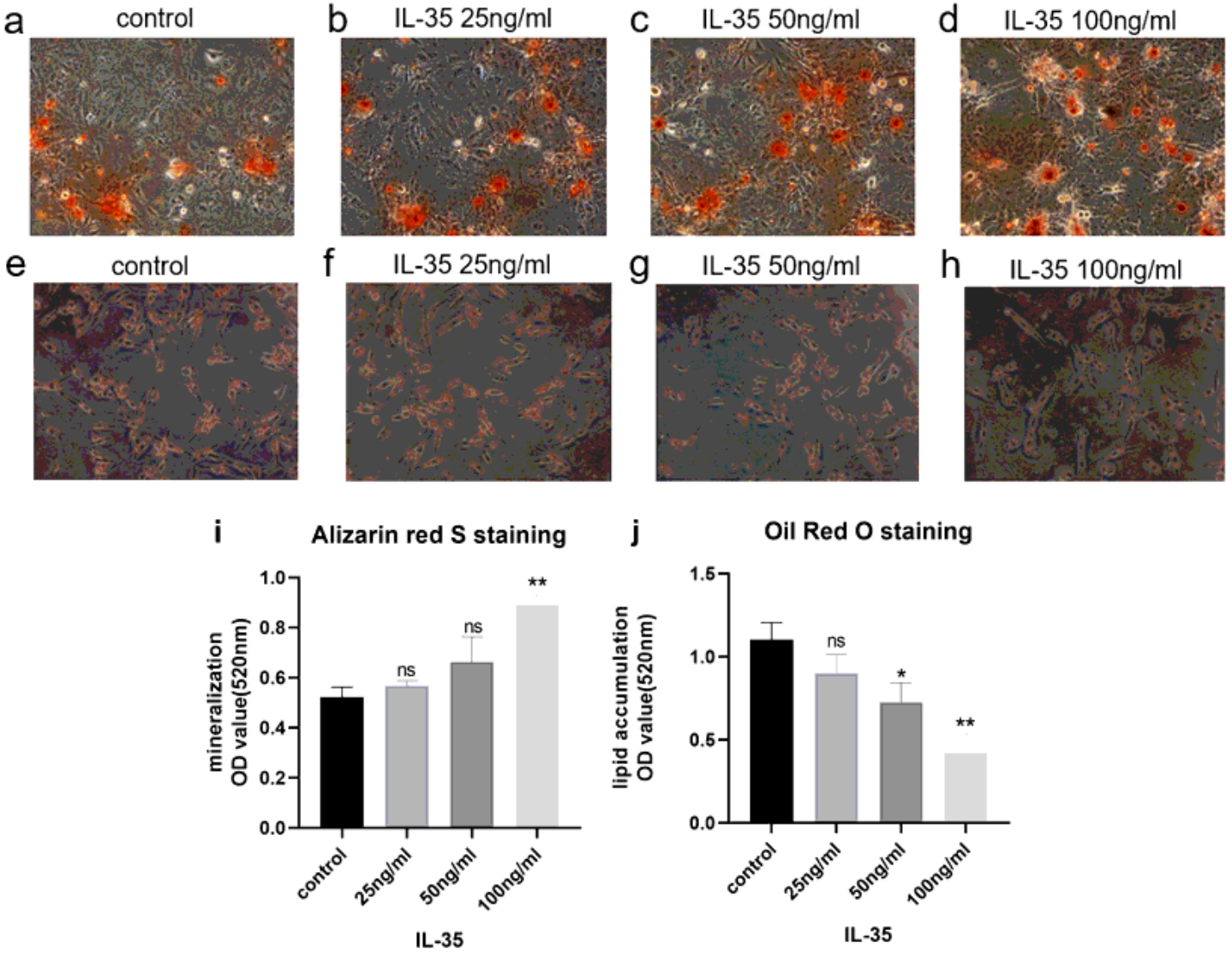

Figure 5

Effect of IL-35 on lipid accumulation and mineralization in MSCs. (a-d) With the increased concentration of IL-35, the area and the density of mineralized nodule in MSCs was enhanced; (e-h) With the increased concentration of IL-35, lipid droplet accumulation in MSCs was prohibited; (i, j) Evaluation of MSCs matrix mineralization and lipid accumulation by Alizarin Red S and Oil Red O staining, respectively, using microplate reader at 520nm. $^{*}p<0.05$, $^{**}p<0.01$ vs. control group (IL-35(0 ng/mL)); ns, non-significant. Comparison between two groups was conducted by Student t-test. P-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

Supplementary Files

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- Onlinefloatimage6.png