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Original Article

Role of RUNX2 in osteogenic differentiation of mesenchymal stem cells induced by BMP9

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Abstract: This study aimed to explore the role of RUNX2 on osteogenic differentiation of C3H10T1/2 mesenchymal stem cells, induced by bone morphogenetic protein 9 (BMP9). First, the methods of reverse transcription polymerase chain reaction (RT-PCR) and western blot were carried out to investigate the effect of BMP9 on RUNX2 from the aspects of gene and protein expression. Afterwards, alkaline phosphatase (ALP) activity assay, staining and calcium deposition assay were used to show RUNX2 overexpression. The mRNA and protein levels of RUNX2 in C3H10T1/2 cells treated with BMP9 were significantly higher than that in the blank control group (C3H10T1/2 cells without any treatment) and C3H10T1/2 cells treated with green fluorescent protein delivered via adenovirus (Ad). BMP9-induced gene expression of osteocalcin and distal-less homeobox 5 was strongly increased by Ad-RUNX2 in C3H10T1/2 cells. ALP activity assay and staining showed that overexpression of RUNX2 increased BMP9-induced ALP activity compared with BMP9 plus red fluorescent protein group. After two weeks of culture, Alizarin Red S staining revealed that overexpression of RUNX2 enhanced the deposition of BMP9-induced calcium salt nodules. These results indicated that RUNX2 can promote osteogenic differentiation of C3H10T1/2 mesenchymal stem cells induced by BMP9.

Keywords: RUNX2, bone morphogenetic protein 9, mesenchymal stem cells, osteogenic differentiation

Introduction

Mesenchymal stem cells (MSCs) have the abilities of multi-directional differentiation and self-replication, and differentiation into many connective tissue cell types that include osteoblasts, chondrocytes, adipocytes, and myoblasts. MSCs have become an important source of osteoblasts in bone tissue engineering research [1]. Bone morphogenetic protein 9 (BMP9) can induce osteogenic differentiation of mesenchymal stem cells [2]. However, BMP9 is not yet used clinically and the mechanism of osteo-induction remains unclear [3].

RUNX2 is an important downstream regulatory factor of BMPs, and plays an important role in inducing bone regeneration [4, 5]. RUNX2 expression is regulated by many factors involved in osteogenic differentiation. Of these, BMPs can up-regulate RUNX2 expression through SMADs, while TGF-β inhibits RUNX2 expression and osteogenic differentiation [5]. Moreover, RUNX2 can also promote osteogenic differentiation of BMP2-induced MSCs [6]. However, the mechanism of BMP9, as a new factor that induces osteogenic differentiation, and may regulate the differentiation of MSCs into osteoblasts remains unidentified.

Therefore, in this study, the role of RUNX2 on osteogenic differentiation induced by BMP9 was explored.

Materials and methods

Reagents

BMP9 was delivered via adenovirus (Ad-BMP9), along with the controls in which adenovirus delivered red fluorescent protein (Ad-RFP) or green fluorescent protein (Ad-GFP) were provided by Beijing BioLab Technologies Co., Ltd. RUNX2 was delivered via adenovirus

(Ad-RUNX2), and RUNX2 luciferase reporter plasmid p(6OSE)-Luc were provided by Kangwei Century Biotechnology Co., Ltd. The C3H10T1/2 mouse MSC line was purchased from Shanghai Xin Yu Biotechnology Co., Ltd. Naphthol AS-MX phosphate Alkaline Solution was provided by Sigma-Aldrich. Alizarin red S was purchased from Beijing Hua Yueyang Biotech. Vitamin C was purchased from Shanghai Fahrenheit Pharmaceutical Co., Ltd. β-phosphoglycerol was purchased from Shanghai Guangrui Biotechnology Co., Ltd. RUNX2 antibody was purchased from Shanghai Anken Trading Co., Ltd. β-actin antibody was purchased from Wuhan Amy McNair Technology Co. TRIZOL RNA extraction reagent was provided by Shanghai Amico Biotechnology Co., Ltd. High glucose Dulbecco's modified Eagle's medium (DMEM) was provided by Bioengineering (Shanghai) Co., Ltd. High quality fetal bovine serum was purchased from Shanghai Ha Ling Biotechnology Co., Ltd.

C3H10T1/2 cell culture and alkaline phosphatase (ALP) quantification and staining

The C3H10T1/2 cells were cultured in the high glucose DMEM, consisting of 100 units/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. When the density of these cells was approximately 30%, they were seeded into wells of 24-well plates. Once they were adherent, Ad-RFP and Ad-RUNX2 were used to infect the cells. After being infected for 36 h, BMP9 conditioned medium was added. ALP activity assay and staining was performed after 1 week in accordance with the manufacturers' instructions.

Calcium salt deposition experiment

The treatment of the C3H10T1/2 cells was similar as the above description. BMP9 conditioned medium and osteogenic medium were added. After 2 weeks of continuous culture, the culture medium was removed and the cells were washed once using phosphate buffered saline. Subsequently, the washed cells were fixed with 200 μl/well of 0.05% glutaraldehyde for 10 min and then washed once with deionized water. Alizarin Red S (0.04%, 250 μl/well) was then added. When the accumulation of red material was observed, we removed the staining solution. Then the cells were washed with deionized water and imaged under a microscope.

Preparation of conditioned media

C3H10T1/2 cells were cultured with high glucose DMEM in a 10-cm dish. Ad-BMP9 and Ad-GFP were added at approximately 70% density. Then the culture medium was collected and centrifuged at 24 h for Ad-BMP9, and at 48 h for Ad-GFP, respectively.

Western blot

When the C3H10T1/2 cells were adherent to the dish, different factors were used to treat them with for 48 h. Afterwards, 12% SDS-PAGE gel electrophoresis was performed to separate the lysate of cells. The resolved proteins were transferred to PVDF membrane and 5% skim milk was used to block the membrane for 1 h. Then the pre-processed protein was incubated overnight with primary antibodies of RUNX2 (ab76956, 1:1000; Abcam, MA, USA), DLX5 (ab64827, 1:1000; Abcam), OCN (ab13420, 1:1000; Abcam) at 4°C. Subsequently, the mixture was conjugated with secondary antibody with horseradish peroxidase at 37°C for 1 h. The binding of the antibodies was visualized using standard technique and the membrane was photographed.

RT-PCR

The treatment of C3H10T1/2 cells is described above. First, the C3H10T1/2 cells were inoculated into 35 cm^2 cell culture flasks at an approximate density of 30%. Once the cells were adherent, Ad-BMP9 was added. After 48-h of culture, total RNA of the cells was extracted and cDNA was prepared by reverse transcription reaction, then agarose gel electrophoresis was performed. The following specific primer sequences were used: MusRUNX2 F: 5'-GGTGAAACTCTTGCCTCGTC-3'; R: 5'-AGTCCCAACTTCCTGTGCT-3'; MusDLX F: 5'-TGTCTCCTTCTCCCATGTCC-3'; R: 5'-GAACTGATGTAGGGGCTGGA-3'; MusOCN F: 5'-TGACTGCATTCTGCCTCTG-3', R: 5'-CGGAGTCTATTCACCACCTTAC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-GGCTGCCCAGAACATCAT-3'; R: 5'-CGGACACATTGGGGGTAG-3'.

Statistical analysis

The data were all analyzed by SSPS 21.0 software. One-way analysis of variance (ANOVA)

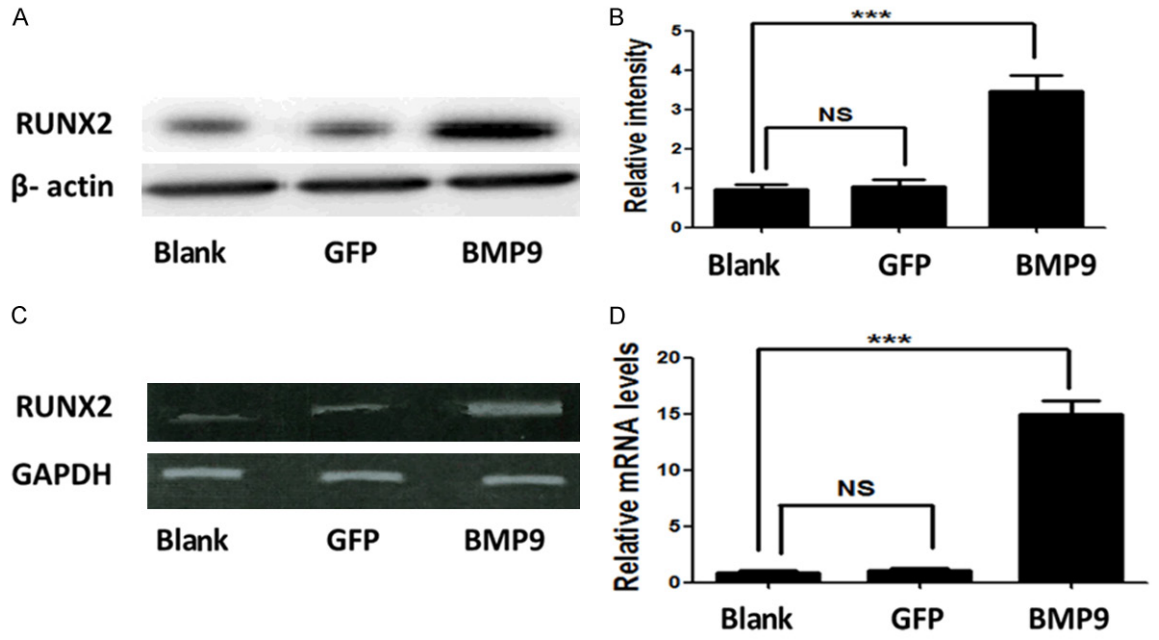

Figure 1. The expression level of RUNX2 in C3H10T1/2 cells regulated by BMP9. Western blot was used to detect the protein level of RUNX2 between Ad-BMP9 infection and control Ad-GFP for 48 h (A and B). The mRNA expression level of RUNX2 was measured by RT-PCR (C and D). *P<0.05 represented a significant difference compared with black group. NS, Not significant.

was used to compare the differences among multiple groups. The Student's *t*-test was utilized to compare the difference between two groups. *P*-value <0.05 represented a statistically significant difference.

Results

Effect of BMP9 on RUNX2 expression

The mRNA and protein expression of RUNX2 in the C3H10T1/2 cells treated with BMP9 was both significantly higher than those in blank control group (untreated C3H10T1/2 cells) and C3H10T1/2 cells treated with Ad-GFP (**Figure 1**).

Effect of RUNX2 on BMP9-induced protein and mRNA expression levels of pivotal osteogenic markers

Western blot and PCR analysis showed that gene expression of osteocalcin and distal-less homeobox 5 induced by BMP9 were strongly enhanced by Ad-RUNX2 (**Figure 2**). These results demonstrated that RUNX2 may regulate the levels of pivotal osteogenic marker proteins and mRNA expression in BMP9-induced C3H10T1/2 cells.

Role of RUNX2 overexpression in osteogenic differentiation induced by BMP9

As shown in **Figure 3**, compared with BMP9 plus RFP group, the overexpression of RUNX2 increased the activity of ALP induced by BMP9. After 2 weeks of culture, the overexpression of RUNX2 was found to increase BMP9-induced deposition of calcium-rich nodules by Alizarin Red S staining (**Figure 4**). These results indicated that overexpression of RUNX2 could increase the ALP activity and calcium deposition induced by BMP9.

Discussion

Remodeling of the skeleton is a continuous process during a human's life. In order to replace damaged bone and meet the metabolic needs of the body, the remodeling process requires the coordination of bone resorption and bone formation [7]. Furthermore, bone remodeling or turnover is regulated by the subtle balance between osteoclasts and the quantity and activity of osteoblasts. Increasing evidence has shown that many important signaling molecules have essential effects on the regulation of MSC differentiation into osteoblasts.

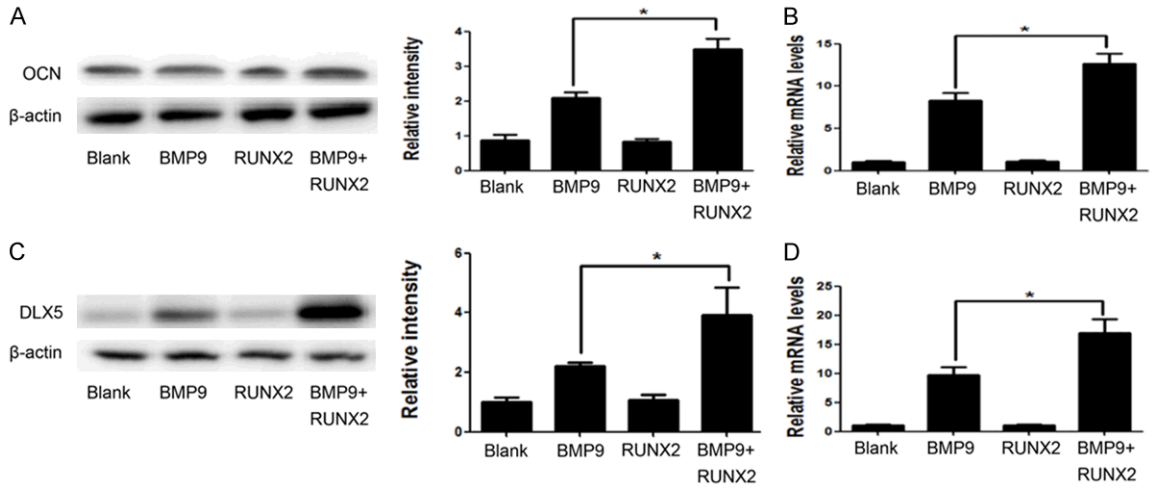

Figure 2. The effect of RUNX2 on protein and mRNA expression levels of pivotal osteogenic markers induced by BMP9. RUNX2 overexpression enhanced the mRNA and protein levels of BMP9 induced osteocalcin (A and B). RUNX2 overexpression promoted protein and mRNA expression levels of distal-less homeobox 5 (DLX5) (C and D).

Furthermore, the BMP signaling pathway has been reported to be important for promoting osteogenesis and osteoblast differentiation [8, 9]. The understanding of the function of MSCs in bone repair and bone regeneration is hampered by the lack of clarity concerning the induction of the MSCs osteogenic differentiation. In addition, BMP9 is an induction factor that is more powerful than BMP2 and BMP7 in osteogenesis [10, 11]. However, the exact mechanism of BMP9 is not yet known and further studies are needed before it can be used clinically.

RUNX1, RUNX2 and RUNX3 are three members of the RUNX transcription factor family that have different roles during development. For instance, RUNX1 is essential for the later stage of hematopoiesis, and RUNX3 is involved in the process of neurogenesis. However, RUNX2, an osteoblast-related transcription factor downstream of BMPs, is extremely important in the development of the skeletal system. In several studies, where rat calvarial cells were cultured in an RUNX2 depleted condition, no osteogenesis occurred. BMP2 was added to induce the formation of osteoblasts, but no cartilage formation was observed, indicating that RUNX2 was indispensable in the development of osteogenesis [12, 13]. However, it is not clear how RUNX2 plays roles in osteogenic differentiation induced by BMP9-derived MSCs [14, 15].

In the current study, the expression of RUNX2 with BMP9 treatment was significantly higher than that in other groups, which indicated that RUNX2 could regulate protein and mRNA expression levels of pivotal osteogenic markers. These findings suggest that RUNX2 is likely involved in osteogenic differentiation of BMP9-induced MSCs. Therefore, RUNX2 overexpressed was used to observe its function in osteogenic differentiation of BMP9-induced MSCs. The overexpression of RUNX2 strengthened the ALP activity of the early osteogenic index induced by BMP9 and late-stage indicator calcium deposition. Our results demonstrated that RUNX2 may act as a potential biomarker that plays a key role in osteogenic differentiation.

In conclusion, we have provided the first demonstration that the RUNX2 transcription factor can promote osteogenic differentiation of BMP9-induced C3H10T1/2 cells. In addition, the process of osteogenic differentiation is extremely complicated, with a variety of transcription factors and signaling pathways involved. Future studies will explore the specific molecular mechanisms and signal transduction pathways associated with RUNX2.

Acknowledgements

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Disclosure of conflict of interest

None.

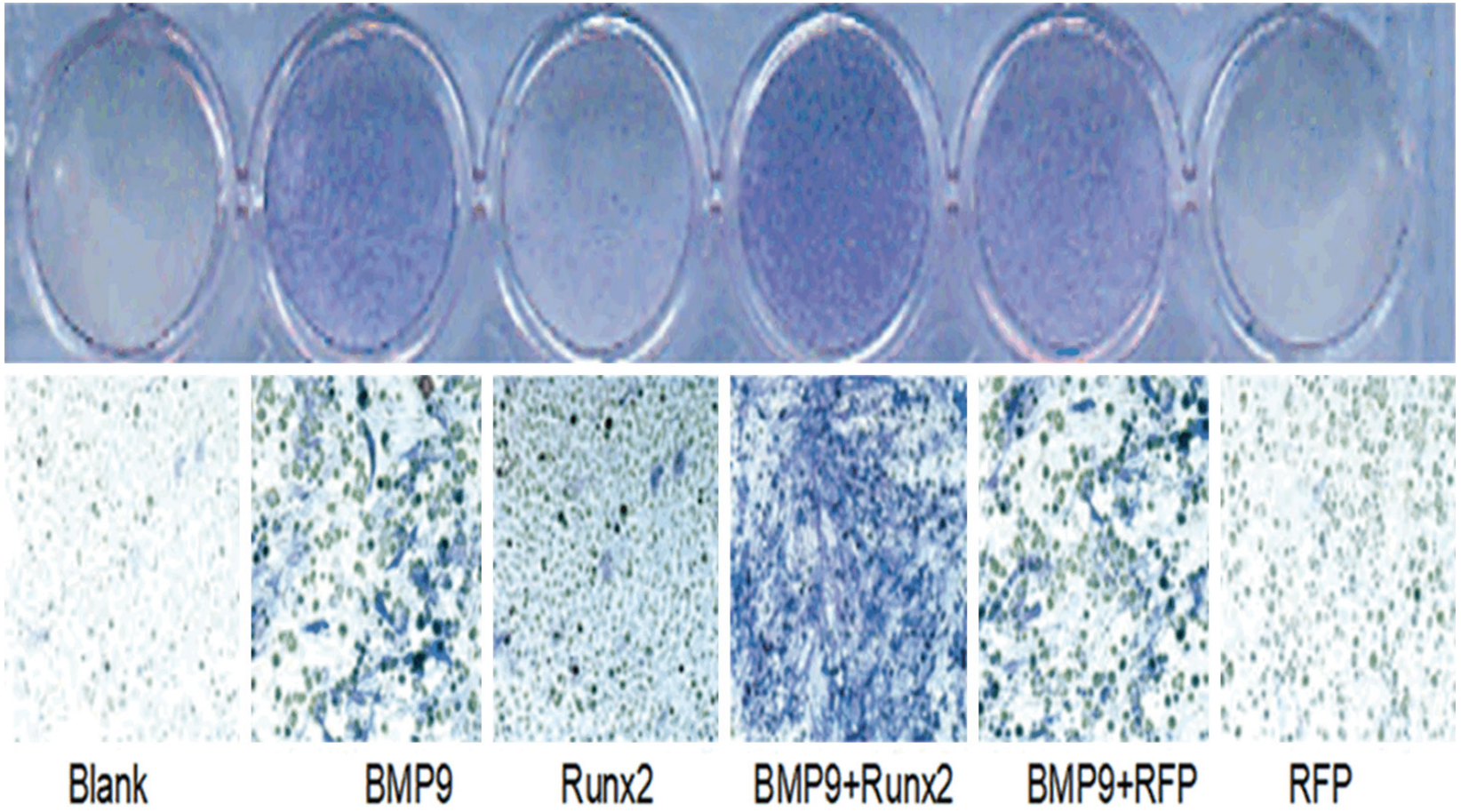

Figure 3. The effect of RUNX2 on BMP9-induced late osteogenic differentiation in C3H10T1/2 cells.

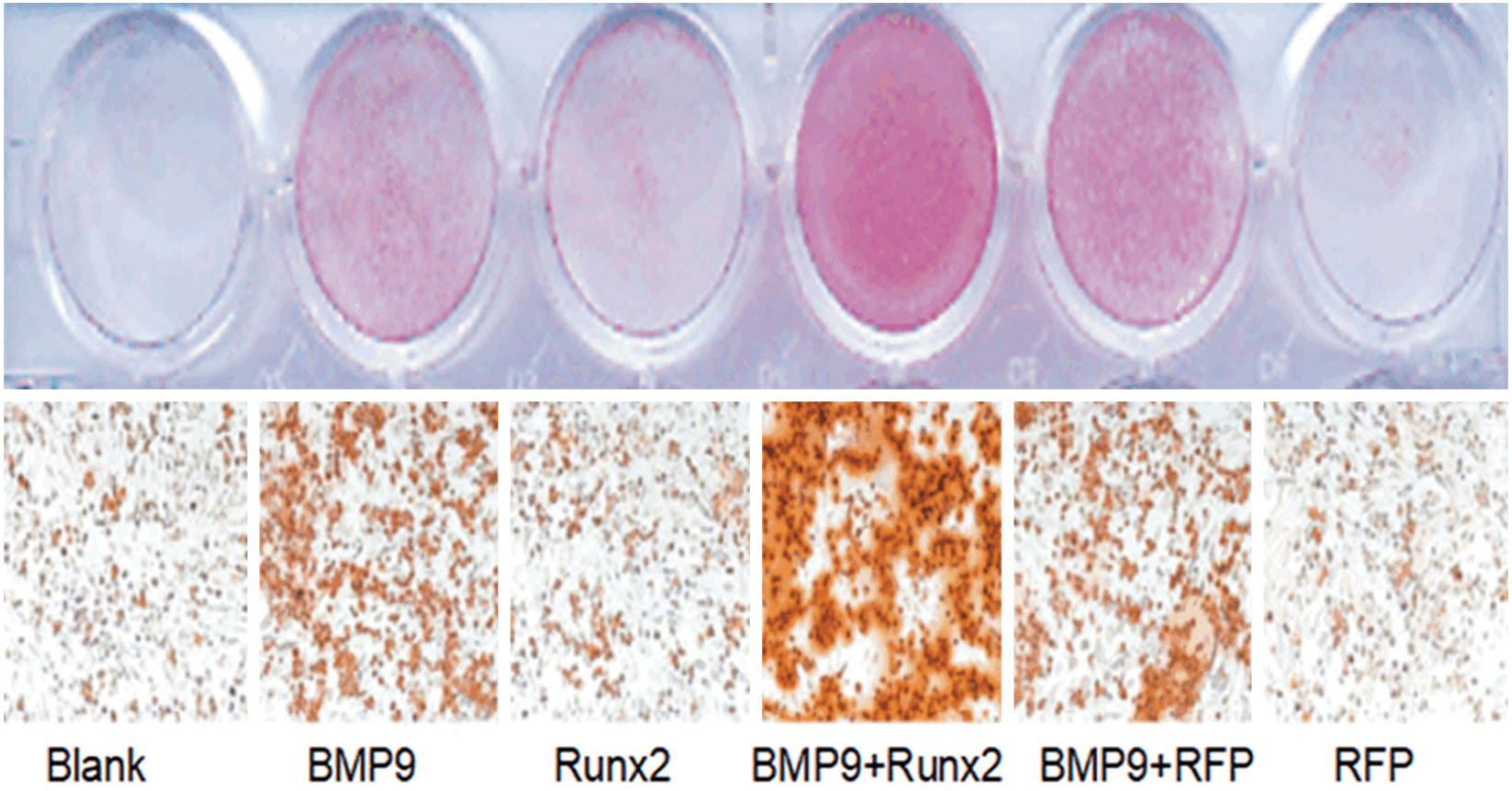

Figure 4. Runx2 enhances BMP9-induced late osteogenic differentiation in C3H10T1/2 cells.

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