

GABP β 2 expression during osteogenic differentiation from human osteoblast-like Saos-2 cells

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Abstract: The E26 transformation-specific (ETS) family of transcription factors plays an important role in osteogenic differentiation. Whether GA-binding protein β 2 (GABP β 2), a member of the ETS family, is involved in osteogenic differentiation has not been previously reported. In the present study, directed differentiation of human osteoblast-like Saos-2 cells was induced and validated by examining alkaline phosphatase (ALP) activity, presence of mineralized nodule and other phenotypic characteristics of the cells on days 0, 3, 6 and 9, thus establishing their osteogenic potential. Real-time PCR revealed that similarly to the bone-specific transcription factor *Runx2*, the expression of *Gabpb2* in Saos-2 cells also peaked on day 3 and was significantly reduced on days 6 and 9. Immunocytochemical staining showed that changes in the immunoreactivity of GABP β 2 also exhibited a similar trend to that of *Runx2*. Initially, *Runx2* was predominantly localized in the nuclei, while GABP β 2 was relatively diffuse. Both exhibited a significant increase in immunoreactivity on day 3, with presence in both the nuclei and cytoplasm. By day 6, both showed a significant decrease in immunoreactivity and were mainly localized in the nuclei. Therefore, we surmise that GABP β 2, as an ETS family member, may play a regulatory role in early osteoblastic differentiation and potentially act in synergy with *Runx2*. (*Folia Histochemica et Cytobiologica* 2014, Vol. 52, No. 3, 225–231)

Key words: GABP β 2; *Runx2*; human osteoblast-like Saos-2 cells; osteogenic differentiation; IHC; QPCR

Introduction

The E26 transformation-specific (ETS) family of transcription factors (TFs) is one of the largest TFs families, comprising 27 ETS genes in humans, all sharing a conserved winged helix-turn-helix DBD of ~85 amino acids. Additionally, all ETS genes contain an evolutionarily conserved DNA-binding ETS domain found in target genes which binds to a consensus DNA sequence containing a core 5'-GGAA/T-3' motif [1–4]. The ETS family regulates a variety of physiological and pathological processes in many tissues such as development, cell proliferation, differentiation, apoptosis, migration, invasion and metastasis [5–8].

ETS transcription factors are expressed ubiquitously or in tissue-specific patterns [9, 10] and have been strongly implicated in osteoblast differentiation [11]. Some ETS TFs are reported to regulate bone development. *Ets1*, the founding member of the ETS family which was originally discovered as part of avian E26 retrovirus genome, is expressed in proliferating preosteoblastic cells, whereas *Ets2* is expressed in terminally differentiated osteoblasts [11]. Myeloid elf-1-like factor (MEF), a member of the ETS family which was originally isolated from a human megakaryocytic leukemia cell line, is highly expressed in the early differentiation phase of MC3T3-E1 osteoblasts, with expression levels reduced by bone morphogenetic protein 2 (BMP2) treatment [12]. *Runx2* (runt-related transcription factor 2) has been found to interact with MEF and binds to the promoters of genes of the osteoblast markers such as alkaline phosphatase (ALP), osteopontin (Opn), and osteocalcin (Oc). These findings would imply that osteoblast differentiation and bone formation can be

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increased by activating MEF to elicit the synergistic effect of Runx2 and MEF [13]. While Runx2 is a key transcriptional regulator of osteogenesis [14], basal Runx2 gene transcription is regulated by dynamic interactions between Sp1 transcription factor (SP1) and ETS-like factors (e.g. Ets1 and ELK1) during osteogenesis [15].

GA-binding protein (GABP), a member of the ETS family of transcription factors, consists of two subunits, GABP α and GABP β , and is known to critically regulate cell cycle, protein synthesis, and cellular metabolism [16]. In comparison with extensive studies on GABP α [17–19], the role of the GABP β subunit has not been investigated. The most studied GABP β isoforms are GABP β 1L and GABP β 1S, which are splice variants encoded by the *gabpb1* allele. A third GABP β isoform, GABP β 2, is encoded by *Gabpb2* gene [20–22]. Currently, the function of GABP β 2 is still unclear, with no prior implications made pertaining to osteoblast differentiation. In this study, intracellular localization and expression of GABP β 2 and Runx2 has been examined in human osteoblast-like Saos-2 cells in order to provide evidence for the involvement of GABP β 2 in osteoblast differentiation.

Material and methods

Cell culture and osteogenic differentiation. Human osteoblast-like Saos-2 cells were purchased from the cell bank of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in McCoy's 5a medium (Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum (FBS), 50 μ g/ml penicillin and 50 μ g/ml gentamicin under the atmosphere of 5% CO₂, at 37°C. The osteogenic culture medium was prepared on the basis of the above medium by adding 0.1 μ M dexamethasone, 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) were added.

Morphological examination with ALP staining. Saos-2 cells in the logarithmic growth phase were seeded in culture plates, with osteogenic differentiation induced after 24 h. Cells on days 0, 3, 6 and 9 were examined: the medium was discarded, cells were rinsed with phosphate-buffer saline (PBS, pH 7.3), fixed with 4% paraformaldehyde for 30 min, rinsed in PBS and stained with an Alkaline Phosphatase (ALP) staining kit (Burstone azo coupling method, Sigma-Aldrich) according to the manufacturer's protocol. The morphology of cultured cells was examined under the macroscopic and microscopic views.

Alizarin red staining. Saos-2 cells in the logarithmic growth phase were seeded in culture plates and osteogenic differentiation induced after 24 h. On days 6 and 9, the medium was

discarded; the cells were fixed with 95% ethanol for 10 min and rinsed several times with distilled. 0.1% Alizarin red (Sigma, USA) dye solution (prepared using Tris-HCl, pH 8.3) was added, samples were incubated at 37°C for 30 min and rinsed for several times with distilled water before imaging.

Quantitative real-time PCR (qRT-PCR). Saos-2 cells in the logarithmic growth phase were seeded in culture plates, and osteogenic differentiation was induced after 24 h. On days 0, 3, 6 and 9, total RNA was extracted using a RNA extraction kit (TIANGEN Biotech, Beijing, China). For each time point, equal amounts of RNA were used to examine Runx2, GABP β 2 and the internal reference gene GAPDH. For each time point, three parallel reaction tubes were used. SYBRGreen Real-time PCR Master mix kit (TOYOBA, Osaka, Japan) was prepared according to the manufacturer's protocol and the fluorescence quantitative PCR analyzer (ABI7500, Applied Biosystems, Inc. Foster, CA, USA) protocol set for a total of 40 cycles (pre-denaturation at 95°C for 30 sec; main loop 95°C for 15 sec, 60°C for 34 sec). Expression levels were determined using the comparative Ct value method (2^{- $\Delta\Delta$ Ct}). Specific amplification primers were synthesized by Invitrogen Corp. (Invitrogen, Shanghai, China), and their sequences were as follows: *Gabpb2* upstream primer: 5'-CCAAACTTCAATCCCTACT-3', downstream primer: 5'-TGGTTTCTTTGTTAGTGGC-3', *Runx2* upstream primer 5'-TGTTTCAGCTCCTTCCTGATTC-3', downstream primer 5'-CTTCTTACACTTCCACCATAC-3', *Gapdh* upstream primer: 5'-AGAAGGCTGGGGCTCATTG-3', downstream primer: 5'-AGGGGCCATCCACAGTCTTC-3'.

Immunocytochemical staining of Saos-2 cells with avidin-biotin complex (ABC) method. Saos-2 cells in the logarithmic growth phase were seeded in culture plate climbing slides and osteogenic differentiation was induced after 24 h. On days 0, 3, 6 and 9, the medium was discarded, cells were fixed with 4% paraformaldehyde for 30 min and rinsed with PBS (pH 7.3) prior to immunocytochemical (IHC) staining. The main IHC steps were as follows: (1) rabbit anti-Runx2 and anti-GABP β 2 antibodies (both 1:100, Santa Cruz, Dallas, TX, USA) were added to the wells of culture plates for incubation at room temperature for 24 h; (2) biotinylated goat anti-rabbit IgG (1:200, Vector, Burlingame, CA, USA) was added for incubation at room temperature for 2 h; (3) horseradish peroxidase-labeled streptavidin (1:100, Vector, USA) was added for incubation at room temperature for 2 h; (4) visualization step with diaminobenzidine (DAB/H₂O₂) at room temperature for 30 min. After the reaction, the climbing slides were dehydrated, cleared and coverslipped.

Statistical analysis. ALP, Alizarin red and IHC stainings were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA), with integrated optical density (IOD) and mean optical density (MOD) of positive

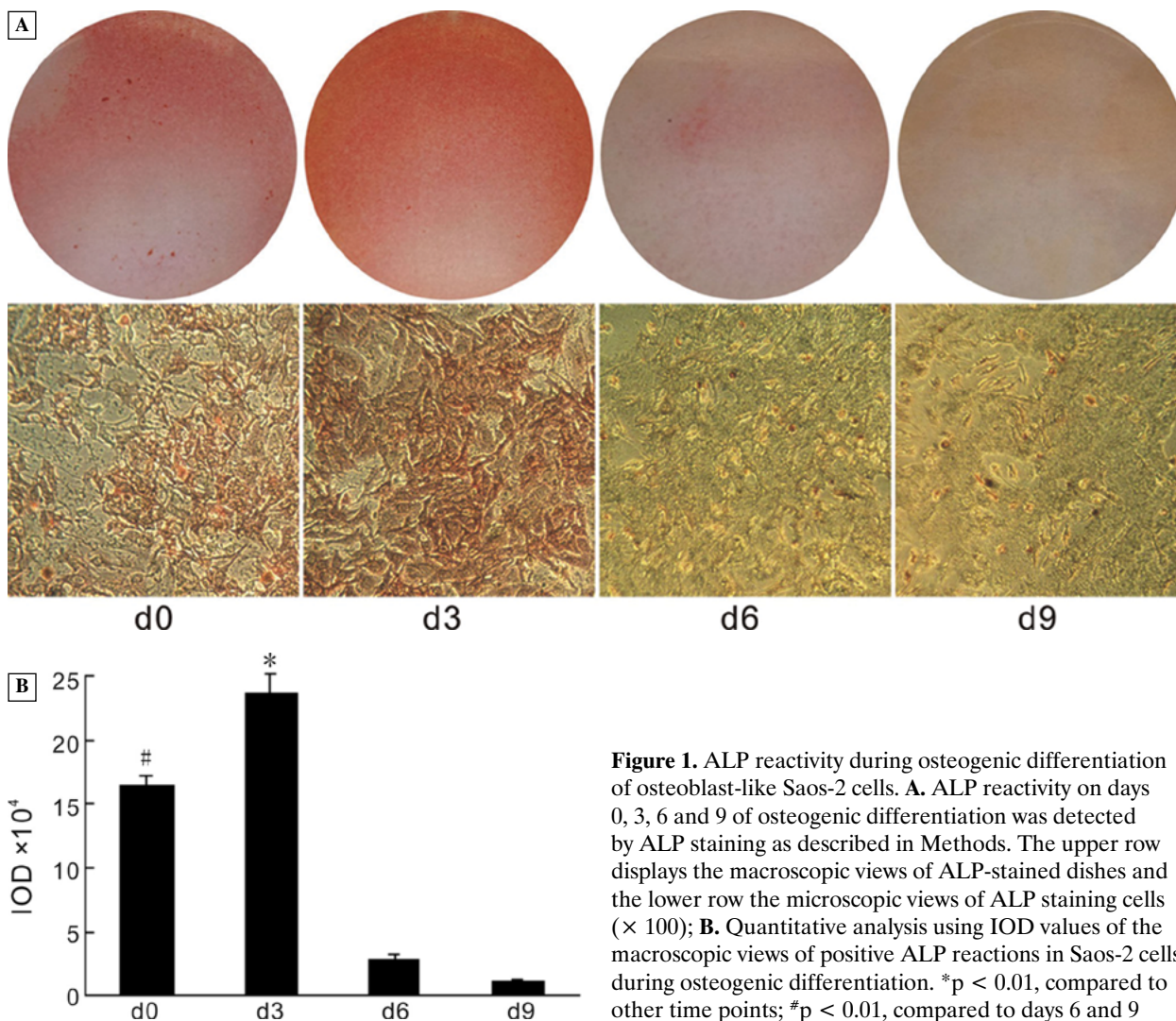


Figure 1. ALP reactivity during osteogenic differentiation of osteoblast-like Saos-2 cells. **A.** ALP reactivity on days 0, 3, 6 and 9 of osteogenic differentiation was detected by ALP staining as described in Methods. The upper row displays the macroscopic views of ALP-stained dishes and the lower row the microscopic views of ALP staining cells (× 100); **B.** Quantitative analysis using IOD values of the macroscopic views of positive ALP reactions in Saos-2 cells during osteogenic differentiation. *p < 0.01, compared to other time points; #p < 0.01, compared to days 6 and 9

reactions in each well measured. All data are expressed as means ($\bar{x} \pm SD$) from at least 3 independent experiments, with comparisons between groups performed using a *t* test and statistical analysis performed utilizing the SPSS13.0 software.

Results

ALP reactivity during osteogenic differentiation of human osteoblast-like Saos-2 cells

ALP reactivity in Saos-2 cells on days 0, 3, 6 and 9 was examined. Positive ALP staining under the macroscopic views was the highest on day 3, with a notable reduction observed on days 6 and 9 (Figure 1A). Samples were quantified using IOD values and the Image-Pro Plus software to further confirm the highest ALP activity to be on day 3 (p < 0.01; Figure 1B). Examination under the light microscope showed there were the largest number of ALP-positive cells and

the strongest positive reactivity on day 3, with only few ALP-positive cells on days 6 and 9 (Figure 1B).

Mineralized nodules in human osteoblast-like Saos-2 cells during osteogenic differentiation revealed by Alizarin red staining

Osteogenic differentiation led to the generation of mineralization nodules as demonstrated by an increasing trend in Alizarin red staining from day 0 to day 9 under the macroscopic views (Figure 2A). Sample quantification reflected by IOD values obtained by measurements with Image-Pro Plus software showed the highest level of Alizarin red staining on day 9 (p < 0.01; Figure 2B). Examination under the light microscope revealed no notable red nodule-like structures on days 0 and 3, with a small number of nodules observed on day 6 and a large number on day 9 (Figure 2A). These results suggest that terminal osteogenic differentiation of the Saos-2 cells was reached between days 6 to 9.

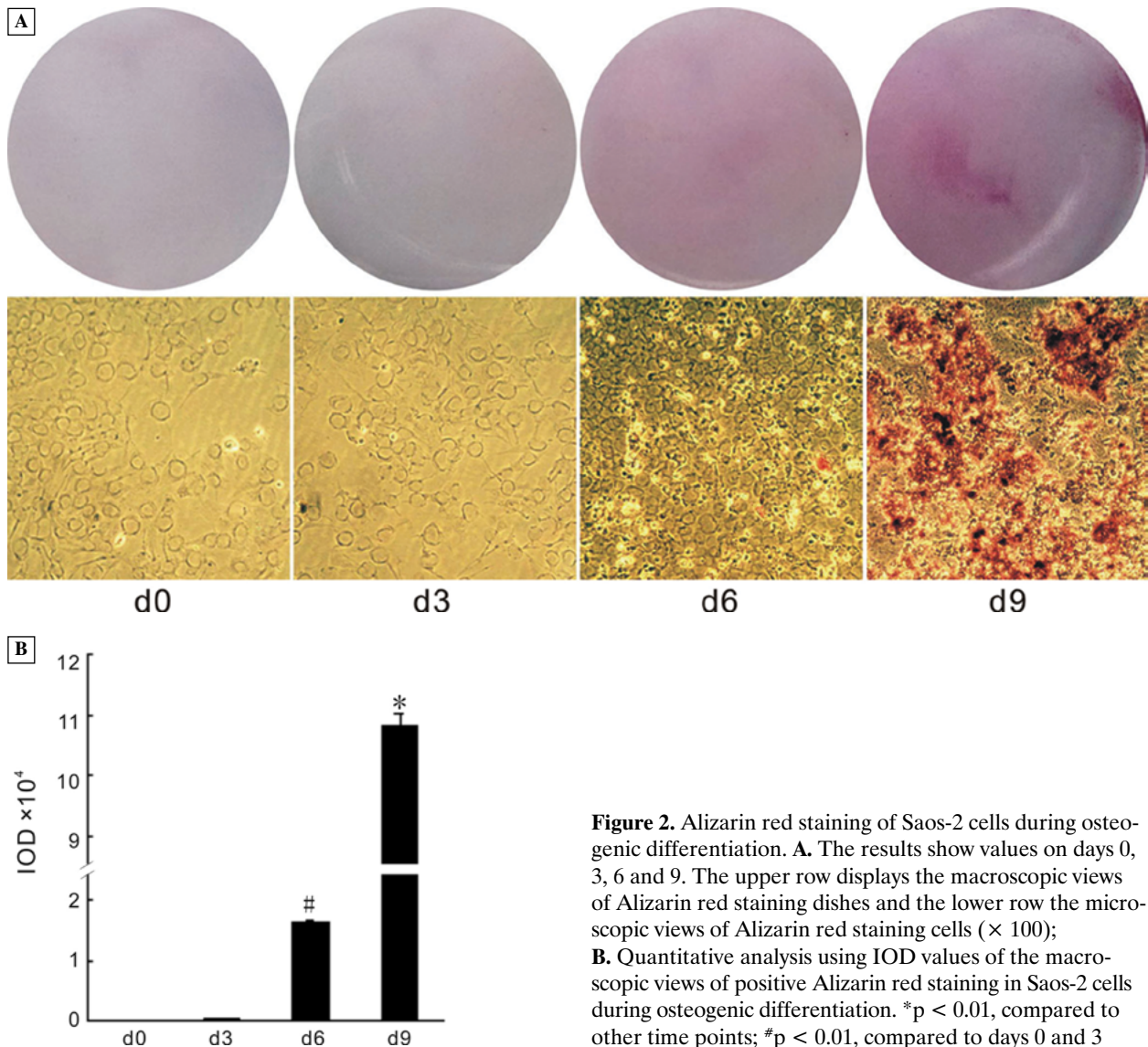


Figure 2. Alizarin red staining of Saos-2 cells during osteogenic differentiation. **A.** The results show values on days 0, 3, 6 and 9. The upper row displays the macroscopic views of Alizarin red staining dishes and the lower row the microscopic views of Alizarin red staining cells ($\times 100$); **B.** Quantitative analysis using IOD values of the macroscopic views of positive Alizarin red staining in Saos-2 cells during osteogenic differentiation. * $p < 0.01$, compared to other time points; # $p < 0.01$, compared to days 0 and 3

Runx2 and Gabpb2 expression in human osteoblast-like Saos-2 cells during osteogenic differentiation

qRT-PCR result showed that on day 3 of osteogenic differentiation, *Runx2* gene expression was the highest and was significantly higher than on days 0, 6, and 9 ($p < 0.01$; Figure 3A). *Gabpb2* expression showed similar changes as *Runx2* expression during osteogenic differentiation, with the highest expression levels noted on day 3 relative to days 0, 6, and 9 ($p < 0.05$; Figure 3B).

Intracellular localization of Runx2 and GABP β 2 during osteogenic differentiation

Immunocytochemistry revealed that changes in Runx2 and GABP β 2 protein expression levels during osteogenic differentiation were consistent with the corresponding gene expression. Upon exa-

mination under the light microscope, Runx2 was found to be localized mainly in the nuclei of Saos-2 cells. On day 3 of induced osteogenic differentiation, Runx2 immunoreactivity was notably enhanced, and its expression in both nuclei and cytoplasm was substantially increased. On day 6, Runx2 immunoreactivity was significantly reduced, and was again predominantly localized in the nuclei (Figure 4A). GABP β 2 immunoreactivity was diffusely distributed in osteoblast-like Saos-2 cells. On day 3, GABP β 2 immunoreactivity was notably enhanced, with a substantially increased expression in the nuclei, whereas on day 6 was notably decreased, with its expression predominantly localized in the nuclei (Figure 4A). Additionally, MOD values for both Runx2- and GABP β 2-immunoreactive cells were significantly higher on day 3 relative to other time points ($p < 0.05$; Figure 4B).

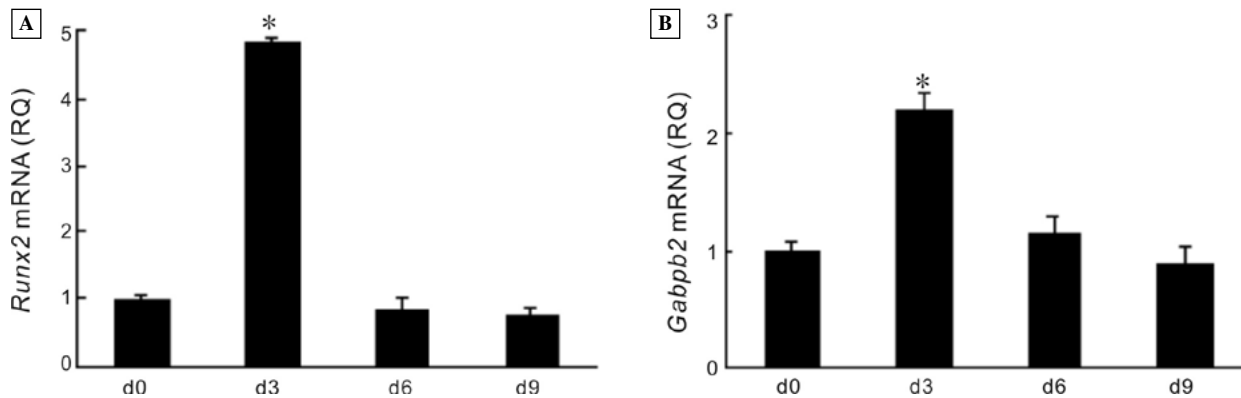


Figure 3. RQ (relative quantity) of *Runx2* and *Gabpb2* mRNA expression in Saos-2 cells on day 0, 3, 6 and 9 of osteoblastic differentiation. **A.** *Runx2*; **B.** *Gabpb2*. *p < 0.01, compared to other time points

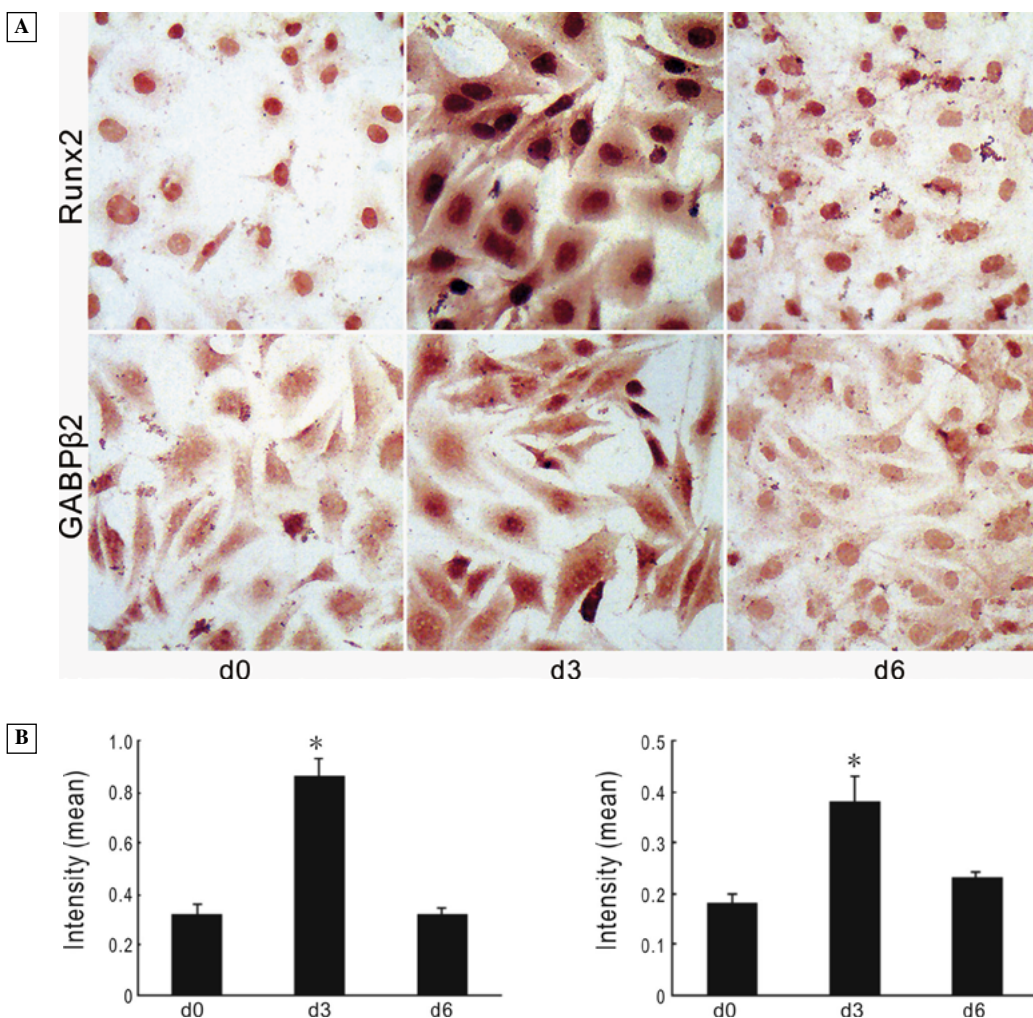


Figure 4. Immunohistochemical localization of Runx2 and GABPβ2 in Saos-2 cells during osteogenic differentiation. **A.** The localization of Runx2 and GABPβ2 on days 0, 3 and 6 (× 200); **B.** Quantitative analysis of MOD values of Runx2 (left panel) and GABPβ2 (right panel) immunoreactive cells during osteogenic differentiation. *p < 0.05, compared to other time points

Discussion

Osteogenic differentiation is crucial for bone development, remodeling under normal physiological conditions and bone damage repair under pathological conditions. Abnormal osteogenic differentiation can lead to serious pathologic states including osteoporosis and multiple myeloma. Thus, elucidation of the mechanisms regulating osteogenic differentiation can provide new therapeutic targets for treating such pathologies [23, 24]. *In vitro* studies of osteogenic differentiation and its regulation mostly employ directed induction of human mesenchymal stem cells (hMSCs) or murine MC3T3-E1 preosteoblast cell lines [25–27]. hMSCs and human osteoblasts can be limited by availability, passage number, cell purity, proportion of induced cells and other factors, with MC3T3-E1 cells also subjected to certain restrictions in basic research.

Saos-2 cells lines are derived from human osteosarcomas and are also known as human osteoblast-like cells. Saos-2 cells exhibit phenotype characteristics of osteoblasts, express alkaline phosphatase and can undergo osteogenic differentiation producing mineralized nodules. ALP is a specific marker for early osteogenic differentiation that is synthesized and secreted by osteoblasts and can promote bone matrix calcification. Mineralized nodules generated from bone matrix calcification are important markers for late stage osteogenic differentiation [28]. Thus, Saos-2 cells can serve as a research tool to study osteogenic differentiation [29]. In the present study, a hMSC osteogenic induction system [25] was used to induce osteogenic differentiation in Saos-2 cells, thus providing a consistent differentiation mechanisms with those previously used to characterize osteogenesis in hMSCs.

By inducing directed differentiation of human osteoblast-like Saos-2 cells and examining changes in ALP activity, presence of mineralized nodules and expression of the bone-specific transcription factor Runx2, Saos-2 cells were demonstrated to possess good osteogenic capability. These results indicated that changes in the phenotype characteristics of Saos-2 cells during induced osteogenic differentiation were similar to that of hMSCs [25]. In addition, Saos-2 cells are easily cultured and show fast *in vitro* proliferation, making it possible to complete induction in a relatively short time. Saos-2 cells also have a high differentiation rate, providing a high density of mature osteoblasts. Obviously, the establishment of an effective method for the induced differentiation of Saos-2 cells will provide a basis for investigating the mechanisms of

osteogenic differentiation and diseases related to osteogenic differentiation using this cell line.

Runx2, also known as Cbfa1, is a runt family transcription factor and acts as a master regulator in the commitment of osteoblast differentiation [30]. Runx2 was first detected in preosteoblasts, with an increased expression seen in immature osteoblasts, and a reduced expression noted during osteoblast maturation [31, 32]. *Runx2*-deficient (*Runx2*^{-/-}) mice completely lack bone formation due to an absence of osteoblasts, which reveals that Runx2 is essential for both endochondral and membranous bone formation [33]. Furthermore, Runx2 induces ALP activity, expression of bone matrix protein genes and mineralization in osteoblasts *in vitro* [34].

In this study, the change in Runx2 expression during osteogenic differentiation in Saos-2 cell in the present study was consistent with previous findings reported by other authors [31, 32]. More importantly, we found *Gabpb2* expression to follow a similar trend to that of *Runx2* gene, with both exhibiting significantly higher expression on day 3. Changes in GABP β 2 immunoreactivity were also found to be similar to those of Runx2, but GABP β 2 immunopositivity was first observed as a diffuse one and, in the course of osteogenic differentiation of the Saos-2 cells, gradually moved to the nuclei. This observation is in contrast with the predominantly nuclear Runx2 localization, which suggests that GABP β 2 may play different roles at the different stages of osteoblast differentiation.

It is well known that GABP β 2 is one of the GABP β isoforms and was found to have both redundant and distinct roles in the immune system, with the manipulation of GABP β 2 expression as a potentially useful approach to modulate B cell responses without interfering with normal B cell development [21]. However, to our best knowledge, there were almost no reports on the function of GABP β 2 outside of the immune system. Our results revealed that during osteoblast differentiation, GABP β 2 expression exhibited a similar expression trend to Runx2 which implicates its potential involvement in early osteoblast differentiation.

In conclusion, GABP β 2 expression during osteogenic differentiation of human osteoblast-like Saos-2 cells shows a novel marker that may be related to the osteoblastic function in bone formation, especially in early osteogenic differentiation. As an ETS family member, GABP β 2 may regulate downstream target genes and regulate early osteoblast differentiation in synergy with Runx2. The elucidation of the regulatory role of GABP β 2 in osteogenesis needs further investigations.

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