PI3K/AKT Pathway Involvement in the Osteogenic Effects of Osteoclast Culture Supernatants on Preosteoblast Cells

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This study aimed to investigate the ability of osteoclasts during bone resorption activities to regulate the differentiation and calcification of osteoblast precursor cells. The bone resorption model was established using *in vitro* cortical bone slices and mouse RAW264.7 cells, which were differentiated into osteoclasts by stimulation with the receptor activator of nuclear factor-κB ligand and macrophage colony-stimulating factor. Tartrate-resistant acid phosphatase (TRAP) staining, reverse transcriptase–polymerase chain reaction (RT-PCR), and scanning electron microscopy (SEM) were used to detect osteoclast differentiation. The osteoblast precursor cell line MC3T3-E1 was cultured with the bone resorption supernatant (BRS). Involvement of the phosphatidylinositol 3-kinase (*PI3K*)/*AKT* pathway in osteogenesis was evaluated by Western blotting, RT-PCR, and ELISA analysis of markers of the early (runt-related transcription factor-2 and alkaline phosphatase) and late (osteocalcin [OCN]) stages of osteogenesis, and Alizarin Red S staining of matrix mineralization. TRAP staining, RT-PCR, and SEM analysis demonstrated the successful establishment of the bone resorption model. Osteoclast BRS effectively increased the differentiation and calcification of MC3T3-E1 cells. Western blot analysis indicated that the BRS enhanced AKT and p-AKT expression levels in MC3T3-E1 cells. Following *AKT2* knockdown and treatment with the PI3K/AKT pathway inhibitor LY294002, the expression of OCN in MC3T3-E1 cells was decreased ($p < 0.05$), as was the calcification area ($p < 0.05$). The data obtained in this study indicated that the osteoclast bone resorption medium promoted the differentiation and calcification of MC3T3-E1 cells and that the *PI3K/AKT* pathway played a role in this process.

Introduction

PERIODONTITIS IS A chronic inflammatory disease characterized by alveolar bone loss leading to tooth movement, and ultimately, to tooth loss.[1] Reconstruction of the lost bone tissue is the main goal of periodontal treatment. Bone tissue-engineering techniques, including guided tissue regeneration, autografting, and allografting are novel methods currently being evaluated in the clinic that may yield beneficial clinical outcomes. However, these techniques are in their infancy and currently have poor clinical predictability.

Bone tissue-engineering techniques require three key factors: cells, scaffolds, and growth factors (GFs),[2] delivery of the latter being the most feasible for use in clinical applications. Several GFs such as the bone morphogenetic protein,[2] transforming growth factor (TGF)-β,[3] insulin-like growth factor (IGF)-1,[3] and platelet-derived growth factor (PDGF),[4] have been shown to promote bone regeneration through induction of osteoblast precursor cell differentiation and mineralization. However, since GFs are normally produced

by healthy cells, the mechanism by which normal bone tissue formation occurs and the molecular signaling pathways involved remain to be elucidated.

The homeostatic balance of the bone system is based on the communication between osteoblasts and osteoclasts. Bone resorbing osteoclasts are multinucleated cells derived from hematopoietic cells of monocyte/macrophage lineage,[5] whereas bone-forming osteoblasts are derived from the mesenchymal lineage.[6] Osteoblasts regulate the differentiation of osteoclasts via the plasma membrane-bound receptor activator of nuclear factor-κB ligand (RANKL) and secreted osteoprotegerin.[7] However, the influence of osteoclasts on osteoblasts remains controversial. In the transition phase of the bone remodeling cycle, osteoblast precursors are recruited to the resorbed surface followed by differentiation, mineralization, and new bone formation.[8] The reason why the resorption of bone by osteoclasts is followed by the differentiation and activity of osteoblasts[9] and how osteoblasts are recruited to the resorbed bone surface are still unknown. In 2005, Martin *et al.* reported the involvement of some GFs

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(IGF-I and II and TGF-β,[3] in particular) released from the bone matrix as a result of osteoclastic bone resorption in this phenomenon, although this theory is still not clear enough to resolve some key issues in the bone remodeling stage. Since the transition phase occurs in a bone resorption microenvironment, we speculate that osteoclasts have the ability to recruit and regulate the osteogenic activity of osteoblasts in this microenvironment. Previous studies on the osteogenic effects of GFs on osteoblasts are far from being comprehensive. Therefore, we established a bone resorption model *in vitro* for the analysis of the osteogenic effects of the bone resorption supernatant (BRS), which may contain GFs released from the bone matrix and by osteoclasts.

The phosphatidylinositol 3-kinase (*PI3K*) pathway is an important promoter of cellular growth, metabolism, differentiation, proliferation, survival, and angiogenesis.[10] Recently, it has been demonstrated that PI3K and its downstream target AKT serine/threonine kinases play a significant role in the osteoblast differentiation of progenitor cells into mature osteoblasts.[11] The AKT family consists of three separate genes (*AKT1-3*) encoding serine/theronine protein kinases. *AKT1* and *AKT2* are believed to have specific roles in bone. Furthermore, *AKT2* has been shown to be required for osteoblast differentiation.[12]

In this study, we investigated osteogenic effects of BRS-containing complex GFs produced in the bone resorption microenvironment and the possible involvement of the PI3K/AKT pathway in the osteogenic process by using RNA interference to silence the *AKT2* gene in the mouse osteoblastic cell line MC3T3-E1, which has been well studied and has the capacity to form calcified bone tissue *in vitro*.[13]

Materials and Methods

Experimental design

We chose the RAW264.7 cell line here as osteoclast precursor cells, which were induced with RANKL and macrophage colony-stimulating factor (M-CSF) and plated on cortical bone slices to establish a bone resorption model *in vitro*. The culture medium of RAW264.7 cells was then collected to culture MC3T3-E1 cells with or without the PI3K/AKT pathway inhibitor LY294002 (5, 10, and 20 μM) and *AKT2* gene knockdown cells. We examined the following parameters: osteoblastic gene expression, osteocalcin (OCN) expression, and cell calcification.

Establishment of in vitro bone resorption model

Details of the *in vitro* bone resorption model establishment and collection of the BRS are provided in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea).

Cell culture

MC3T3-E1 cells (purchased from Chinese Academy of Sciences cell library) were cultured in α-MEM (Gibco) as described for the culture of RAW264.7 cells. Exponentially growing cells were plated in six-well plates (6×10^3 cells/well) for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, in 24-well plates (1×10^4 cells/well) for OCN detection and in six-well plates (1×10^4 cells/well) for Alizarin Red S staining and Western blot analysis. After incubation in the regular medium for 24 h, cells were transferred to a medium containing 25% (V/V) BRS. Groups N0, N1, N2, and N3 (negative controls) were incubated in a normal culture medium plus the PI3K/AKT pathway inhibitor LY294002 (5, 10, and 20 μM). Groups S0, S1, S2, and S3 were treated with a medium containing 25% BRS plus LY294002 (5, 10, and 20 μM). Groups SH1, 2, 3, and NS were MC3T3-E1 cells transfected with *AKT2* RNAi plasmids *AKT2-1*, *AKT2-2*, *AKT2-3* and scrambled plasmids, respectively. Group WT referred to untransfected cells.

BRS selection test

The biological activity of the BRS collected on different days (day 5, 7, 10, 12, 14) was evaluated using the OCN ELISA kit (R&D).

RT-PCR evaluation of early stage of cell differentiation

Early stage differentiation of MC3T3-E1 cells collected on days 7 and 14 was evaluated by analysis of alkaline phosphatase (*ALP*), runt-related transcription factor-2 (*RUNX-2*) mRNA expression levels using the RT-PCR conditions described previously with the exception of annealing at 51°C for 30 s and extension at 72°C for 30 s for 35 cycles. The primer sets used were *ALP* (forward: TCAGGGCAATG AGGTCACATC; reverse: CACAATGCCCACGGACTTC), *RUNX-2* (forward: TTTAGGGCGCATTCCTCATC; reverse: TGTCCTTGTGGAT TAAAAGGACTTG), *β-actin* (forward: TGGGTATGGAATC CTGTGGC; reverse: CCAGACAGCACTGTGTTGGC).

AKT2 knockdown

Knockdown of *AKT2* (NCBI reference sequence: NM_001110208.1) was achieved by RNA interference. Three pairs of *AKT2*-specific RNAi oligos were designed and synthesized (Table 1) and used to generate three different *AKT2*-specific RNAi plasmids, which were transfected into MC3T3-E1 cells. *AKT2* mRNA expression was detected by real-time PCR (using a previously described method[14]) to validate the knockdown effect and to select the most effective *AKT2*-specific RNAi plasmid. The primer sets used were *AKT2* (forward: GCCG CCTGCCATTCTACAACC; reverse: GCCGAGCCTCTGCTTT GGGT), and mouse *18s rRNA* (forward: CGGACACGGAC

TABLE 1. SEQUENCES OF THREE *AKT2*-SPECIFIC RNAI OLIGOS AND SCRAMBLE OLIGOS

Plasmid	Oligo sequence (5′–3′)
AKT2-1	CCGGGCTCATTCTTATGGAGGAGATCTCGAGATCTCCTCCATAAGAATGAGCTTTTTG
AKT2-2	CCGGGATGGATCTTTCATTGGGTATCTCGAGATACCCAATGAAAGATCCATCTTTTTG
AKT2-3	CCGGTGACCATGAATGACTTCGATTCTCGAGAATCGAAGTCATTCATGGTCATTTTTG
Scramble	CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCCTTAACCTTAGGTTTTTG

Underline indicates the *AKT2*-specific sequence.

AGGATTGACA; reverse: CCAGACAAATCGCTCCACCAA CTA) as the house keeping gene. MC3T3-E1 cells were then transfected with the most effective *AKT2*-specific RNAi plasmid. Negative control group cells were transfected with a scrambled plasmid. Relative *AKT2* mRNA expression was analyzed by $2^{\Delta Ct} \times 10^6$.

Analysis of OCN in culture medium

To assess the terminal differentiation of MC3T3-E1 cells stimulated by the BRS and the link with the PI3K/AKT pathway, OCN secreted into the cell culture medium was determined. Mediums from different groups were collected on day 14. Then, measure the OCN value in the culture medium following the instructions of the osteocalcin ELISA kit (R&D). The cells were lysed in the radio-immunoprecipitation assay buffer (RIPA; Beyotime) and centrifuged at 14,000 rpm for 15 min at 4°C. The concentration of the protein was determined by the BCA protein assay kit (Pierce). The OCN level was normalized against the total protein content.

Analysis of cell calcification

Calcification of MC3T3-E1 cells was assessed by Alizarin Red S staining of matrix mineralization as described previously.[15] MC3T3-E1 cells were prepared as previously described, and then cultured with a mineralizing medium (10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ mL ascorbic acid; Sigma-Aldrich) containing 25% BRS. The medium was replaced every 2 days. After 2 weeks, the medium was removed and the cells were washed with phosphate-buffered saline (×3) and fixed in 10% paraformaldehyde (10–30 min, 4°C). Cells were stained in a solution of 0.1% Alizarin Red S/Tris-HCl (pH 8.3) (30 min, 37°C), washed three times with distilled water, and air-dried before being photographed (5 images per well) using a NIS-Elements F2.20 (NikonEclipse 80i). Images were analyzed using Photoshop CS3. The mean percentage of the calcified area was normalized against the total protein content determined as previously described. This experiment was repeated three times.

Western blot analysis

The extraction of protein and determination of the concentration of total protein was the same as described above in the Analysis of OCN in culture medium section. Protein samples (60 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% skimmed milk (1 h, room temperature) and incubated with primary antibodies (overnight, 4°C). Membranes were then washed (×3, 10 min) and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000; Sigma) (room temperature, 1 h). Immunoreactive bands were visualized using the ECL kit (Pierce). Membranes were washed and reprobed with the anti-GAPDH antibody (Santa Cruz) to ensure equal protein loading.

Statistical analysis

Differences between the means of OCN and RT-PCR were calculated using one-way ANOVA plus the Dunnett's T3 test. Analysis of calcified areas was conducted using one-way ANOVA

plus the LSD-t multiple comparison test. The significance level was set at $p < 0.05$. Data are expressed as the mean ± SD.

Results

Osteogenic effect of osteoclast excreted factors on MC3T3-E1 cells

MC3T3-E1 cells were cultured with the BRS collected on days 5, 7, 10, 12, and 14. Increased OCN production was detected in the supernatants collected at day 10, 12, and 14 time-points, with the most significant increase detected on day 10 ($p < 0.05$) (Fig. 1). Therefore, supernatants collected on day 10 were used in all experiments to detect the osteogenic effects of osteoclast secreted factors on MC3T3-E1 cells.

ALP and RUNX-2 mRNA expression

ALP and *RUNX*-2 are the key genes required for the early differentiation of osteoblasts.[16,17] Expression of *RUNX*-2 and *ALP* mRNA was not detected in negative controls (MC3T3-E1 cells cultured in α-MEM alone) (Fig. 2A). However, expression of *RUNX*-2 and *ALP* mRNA was detected in MC3T3-E1 cells treated with the BRS at days 7 and 14, although there was no difference between the levels detected at these time points ($p > 0.05$) (Fig. 2B).

Confirmation of the reduced p-AKT expression in MC3T3-E1 cells mediated by LY294002 and RNAi knockdown

MC3T3-E1 cells were transfected with three different *AKT2*-specific RNAi plasmids and negative plasmids (Fig. 3A) to analyze the effects of *AKT2* knockdown on the osteogenic effects of osteoclasts on osteoblast differentiation. *AKT2* mRNA expression in MC3T3-E1 cells transfected with *AKT2*-1, *AKT2*-2, *AKT2*-3, a scrambled plasmid, and in untransfected cells was analyzed by RT-PCR (Fig. 3B). *AKT2* mRNA expression was 8.70 ± 0.12, 30.16 ± 2.29, 29.50 ± 1.43, 44.16 ± 3.26, and 57.42 ± 3.58 in each of these groups, respectively. The greatest inhibition was detected following transfection with *AKT2*-1 ($p < 0.05$) (Fig. 3B). Therefore, *AKT2*-1 was used in all subsequent experiments.
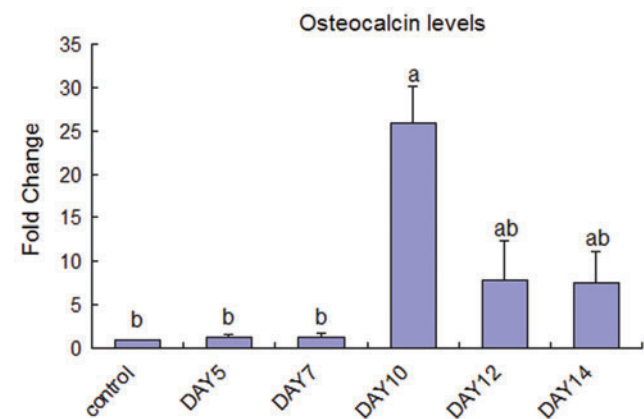

FIG. 1. Osteocalcin (OCN) levels in MC3T3-E1 cells treated without (control) or with the bone resorption supernatant (BRS) were determined by ELISA. BRS were collected days 5, 7, 10, 12, and 14 ($n = 6$). The OCN level of each group was normalized to the total protein level, and then compared to that of the control ([a]$p < 0.05$ vs. control; [b]$p < 0.05$ vs. day 10). Color images available online at www.liebertpub.com/tea
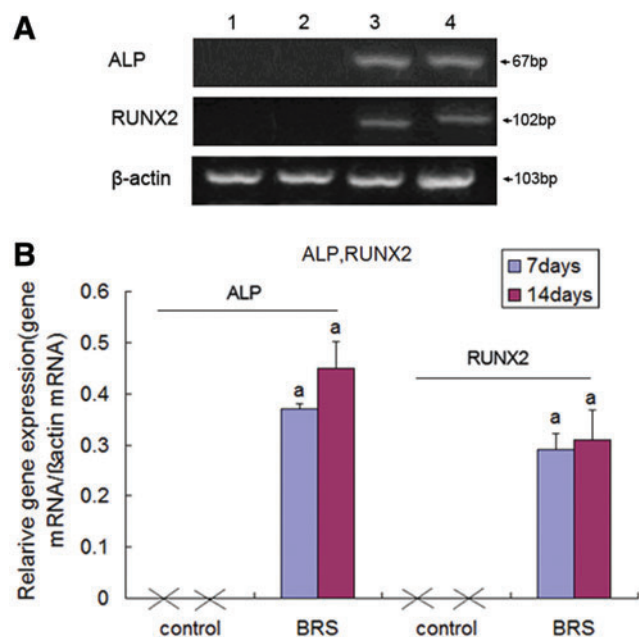
FIG. 2. Reverse transcriptase–polymerase chain reaction (RT-PCR) detection of alkaline phosphatase (*ALP*) and runt-related transcription factor-2 (*RUNX-2*) expression. **(A)** Representative images of three repeated experiments. Lane 1: negative control, MC3T3-E1 cells treated without BRS for 7 days; lane 2: negative control, 14 days; lane 3: MC3T3-E1 cells treated with BRS for 7 days; lane 4: 14 days. **(B)** Densitometric quantification of *RUNX-2* and *ALP* mRNAs expressed as the ratio of *β-actin* expression in the same sample ($^a p < 0.05$ vs. control). Color images available online at www.liebertpub.com/tea

Western blot analysis showed that the expression of p-AKT in MC3T3-E1 cells was upregulated by treatment with the BRS for 14 days. This effect was inhibited in a dose-dependent manner by LY294002 treatment. BRS and LY294002 treatments used in isolation had no effect on the expression of *AKT*. Low-level p-AKT2 and AKT2 were detected in *AKT2* knockdown cells (Fig. 3C, D)

OCN expression

OCN has recently been identified as an osteoblast-secreted hormone suitable for use as an indicator of terminal differentiation of osteoblasts.[18] A dose-dependent decrease in OCN was detected following treatment of MC3T3-E1 with the PI3K/AKT-specific inhibitor LY294002 ($p < 0.05$). Furthermore, the lowest OCN expression was detected in *AKT2* knockdown cells treated with the BRS (Fig. 4).

Cell matrix mineralization

After 14 days in culture, the matrix mineralization level of MC3T3-E1 cells was detected by Alizarin Red S staining. Compared with MC3T3-E1 cells cultured with the BRS (S0), very low levels of calcified deposits were detected in MC3T3-E1 cells cultured in α-MEM alone plus LY294002 (N0-N3). In contrast, a significant and dose-dependent decrease in the calcification area of MC3T3-E1 cells incubated with the BRS was detected following treatment with different concentra-

tions of LY294002 (S0–S3) ($p < 0.05$). Furthermore, the *AKT2* knockdown cells almost abolished the ability of MC3T3-E1 cells to generate calcified deposits (Fig. 5).

Discussion

Osseous repair in periodontitis and similar bone defects remains a challenge. The repair methods, such as autografting and allografting currently used in the clinic are associated with various limitations. Investigation of the potential of GFs to induce bone repair for the purposes of reconstruction has become a focus of research in the field of bone tissue engineering. For example, IGF-1 and TGF-β, which are released from the bone matrix as a consequence of resorption, are responsible for the events involved in bone formation.[3,19,20] Furthermore, it has been observed that osteoclasts release factors that mediate subsequent cellular events when bone resorption ceases.[21] For instance, sphingosine 1-phosphate (S1P) is a bioactive phospholipid that can stimulate osteoblast migration and survival[22] and the B polypeptide chain PDGF homodimer (PDGF BB) is a potent mitogenic factor that may increase osteoblast proliferation[23] as well as suppress the genetic program of osteoblast differentiation and even completely abrogate mineralization *in vitro*.[4]

The aim of this study was to investigate the hypothesis that complex GFs produced during bone resorption have the potential to regulate the differentiation and calcification of osteoblast precursor cells in bone resorption activities. In this study, an *in vitro* bone resorption model analogous to the *in vivo* process was established based on the culture of mouse RAW264.7 cells with cortical bone slices prepared from fresh bovine femur in the presence of RANKL and M-CSF, which are essential for osteoclastogenesis.[24] RAW264.7 cells were induced to differentiate into tartrate-resistant acid phosphatase (TRAP)-positive, multinucleate cells from day 3 following stimulation with RANKL and M-CSF. After 7 days, bone resorption lacunae were detected on the bone cortical slices. Furthermore, positive TRAP staining, detection of the TRAP activity, and the expression of *TRAP* and *CtsK* gene expression indicated the successful establishment of a bone resorption model *in vitro*.

In this study, the expression of *ALP*, *RUNX-2*, and *OCN* were detected as the markers of osteoblast precursor cell differentiation.[16–18] RUNX-2, which potently initiates the expression of major bone matrix protein genes, and ALP are markers of the early stage of osteoblast differentiation.[16,25] OCN, which was recently identified as an osteoblast-secreted hormone regulating insulin secretion and sensitivity, is a terminal marker of osteoblast differentiation.[26] Osteoclast supernatants (BRS) collected on day 10 had the highest OCN biological activity and were used in all subsequent experiments. Expression of *ALP* and *RUNX-2* mRNA was detected in MC3T3-E1 cells cultured in the presence of BRS for 7 days, and this level remained unchanged by 14 days, which is probably because *ALP* and *RUNX-2* are early osteoblast differentiation markers.[16,17] These data suggested that the BRS induced MC3T3-E1 cell differentiation at both the early and late stages.

β-glycerophosphate and ascorbic acid are widely used osteodifferentiation factors *in vitro*.[27,28] The former promotes the calcium deposition, while the latter boosts the synthesis of collagen and matrix mineralization of osteoblasts. In this

FIG. 3. Results of *AKT2* gene knockdown in MC3T3-E1 cells. **(A)** Fluorescence photograph of MC3T3-E1 cells transfected with *AKT2* RNAi plasmids. **(a)** *AKT2-1*; **(b)** *AKT2-2*; **(c)** *AKT2-3*; **(d)** cells transfected with scrambled RNAi; **(e)** normal MC3T3-E1 cells; **(f)** normal MC3T3-E1 cells detected under optical microscope. **(B)** RT-PCR analysis of *AKT2* mRNA expression in MC3T3-E1 cells transfected with NS, scrambled plasmids; WT, untransfected cells; *AKT2* RNAi plasmids *AKT2-1*, *AKT2-2*, and *AKT2-3* ($^a p < 0.05$ vs. NS). **(C)** Western blot analysis of p-AKT, AKT, p-AKT2, and AKT2 protein expression in cells. **(a)** AKT and p-AKT protein expression in MC3T3-E1 cells treated with different concentrations of LY294002 with or without BRS; **(b)** AKT2 and p-AKT2 expression in *AKT2* knockdown MC3T3-E1 cells; NS: scrambled plasmids; WT: untransfected cells. **(D)** The relative intensity of p-AKT, AKT, p-AKT2, and AKT2 protein in cells. **(a)** The relative intensity of p-AKT and AKT protein in the N0 and S0 group ($^* p < 0.05$ vs. N0). **(b)** The relative intensity of the p-AKT and AKT protein in the S0, S1, S2, and S3 group ($^a p < 0.05$ vs. S0, $^b p < 0.05$ vs. S1, $^c p < 0.05$ vs. S2). **(c)** The relative intensity of the p-AKT and AKT protein in the N0, N1, N2, and N3 group ($^a p < 0.05$ vs. N0, $^b p < 0.05$ vs. N1, $^c p < 0.05$ vs. N2). **(d)** The relative intensity of the p-AKT2 and AKT2 protein in the WT, NS, and AKT2-1 group ($^\# p < 0.05$ vs. WT). Color images available online at www.liebertpub.com/tea
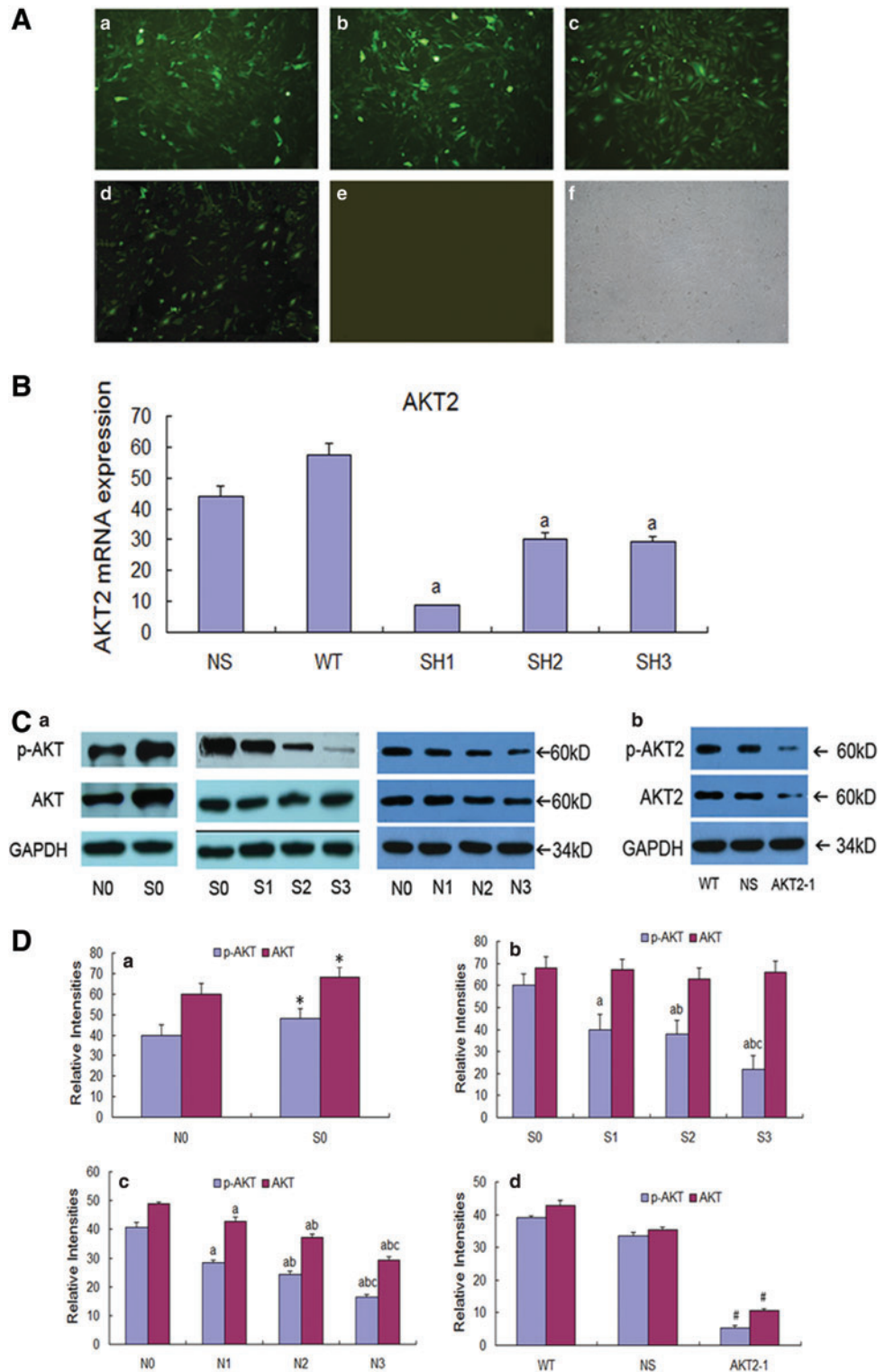
study, Alizarin Red S staining of MC3T3-E1 cells showed the presence of calcified deposits following culture for 7 days, which was more apparent after 14 days, thus indicating that the BRS induced matrix mineralization of MC3T3-E1 cells.

Subsequently, the capacity of the BRS to regulate the osteogenic activity of MC3T3-E1 cells via the PI3K/AKT pathway was investigated. In 2006, Zhao and colleagues demonstrated the effect of bidirectional ephrinB2-EphB4

signaling in osteoclasts and osteoblasts in mouse models.[29] They observed that forward signaling through EphB4 enhances osteoblast differentiation.[29] We have confirmed upregulation of the EphB4 protein and gene expression in MC3T3-E1 cells treated with the BRS (unpublished data). The ephrin family, of which EphB4 is a member, represents the largest family of receptor tyrosine kinases (RTKs).[30] Activation of RTKs by phosphorylation, in turn, activates the PI3K
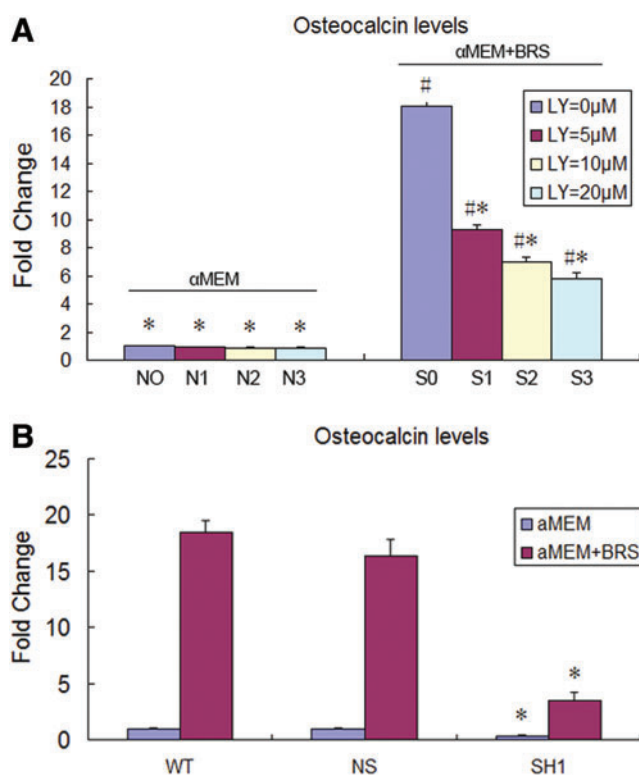
FIG. 4. ELISA analysis of OCN expression. **(A)** MC3T3-E1 cells treated with the BRS (S) and without BRS (N) in the presence of LY294002 (0, 0 μM; 1, 5 μM; 2, 10 μM; and 3, 20 μM). Values represent the mean ± SD (*$p < 0.05$ vs. S0; #$p < 0.05$ vs. N0). **(B)** MC3T3-E1 cells transfected with *AKT2* RNAi plasmids. WT: normal MC3T3-E1 cells; NS: cells transfected with scrambled RNAi; SH1: *AKT2-1*. Values represent the mean ± SD (*$p < 0.05$ vs. NS). Color images available online at www.liebertpub.com/tea
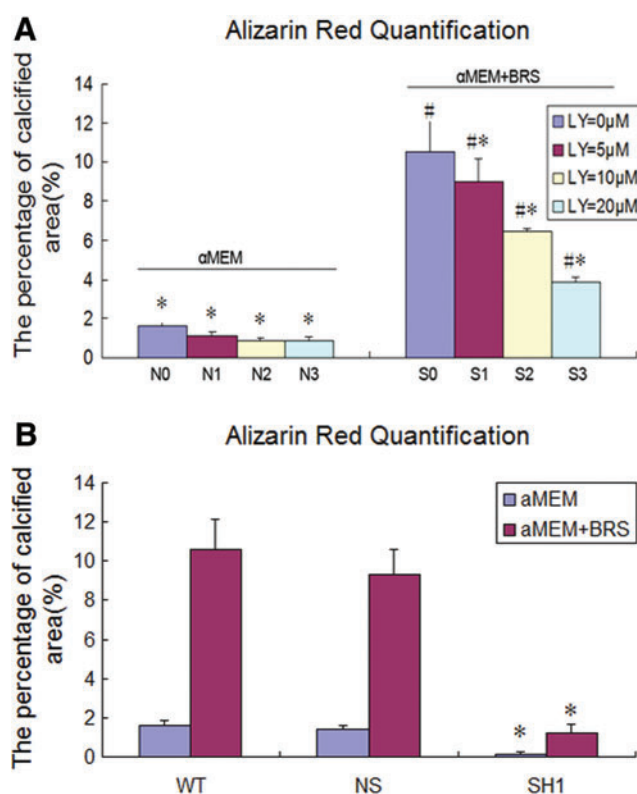
FIG. 5. Analysis of matrix mineralization of MC3T3-E1 cells by Alizarin Red S staining. **(A)** MC3T3-E1 cells treated with the BRS (S) and without BRS (N) in the presence of LY294002 (0, 0 μM; 1, 5 μM; 2, 10 μM; and 3, 20 μM). Values represent the mean ± SD (*$p < 0.05$ vs. S0; #$p < 0.05$ vs. N0). **(B)** MC3T3-E1 cells transfected with *AKT2* RNAi plasmids. WT: normal MC3T3-E1 cells; NS: cells transfected with scrambled RNAi; SH1: *AKT2-1*. Values represent the mean ± SD (*$p < 0.05$ vs. NS). Color images available online at www.liebertpub.com/tea

pathway.[31] To investigate the role of the PI3K pathway in the effects of BRS on the osteogenic activity of MC3T3-E1 cells, we used the PI3K/AKT inhibitor LY294002 and an RNA interference approach to mediate *AKT2*-specific knockdown in MC3T3-E1 cells. Western blot confirmed decreased expression of p-AKT in MC3T3-E1 cells after treatment with LY294002 and very low expression of AKT2 and p-AKT2 in *AKT2* knockdown cells. The level of the AKT level was not changed, while the BRS was shown to activate p-AKT. These data indicate that the BRS, which increased EphB4 expression in MC3T3-E1 cells, activated the PI3K/AKT pathway, and that this activity was inhibited in the presence of LY294002 or following *AKT2* gene knockdown. Similarly, analysis of OCN expression and cell matrix mineralization were also shown to decline. These observations indicate that the PI3K/AKT pathway plays a fundamental role in the differentiation and calcification of MC3T3-E1 cells induced by the BRS. However, other signaling pathways may be associated with the induction of osteoblastic activity by the BRS. RTKs are key regulators of cell-signaling pathways involved in proliferation, differentiation, migration, invasion, and cell death.[32] However, RTKs can also activate other signaling pathways such as the MAPK pathway.[33] These preliminary data obtained in our study do not define signaling pathways and the specific GFs responsible for the osteogenic activity of the

BRS. Thus, future investigations are required to analyze the effective component through mass spectrometry and its relative proportion in the BRS, to advance our knowledge of the potential of GFs for tissue engineering.

Disclosure Statement

No competing financial interests exist.

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