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Receptor for advanced glycation end products inhibits proliferation in osteoblast through suppression of Wnt, PI3K and ERK signaling

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ABSTRACT

Expression of receptor for advanced glycation end products (RAGE) plays a crucial role in bone metabolism. However, the role of RAGE in the control of osteoblast proliferation is not yet evaluated. In the present study, we demonstrate that RAGE overexpression inhibits osteoblast proliferation in vitro. The negative regulation of RAGE on cell proliferation results from suppression of Wnt, PI3K and ERK signaling, and is restored by RAGE neutralizing antibody. Prevention of Wnt signaling using Sfrp1 or DKK1 rescues RAGE-decreased PI3K and ERK signaling and cell proliferation, indicating that the altered cell growth in RAGE overexpressing cells is in part secondary to alterations in Wnt signaling. Consistently, RAGE overexpression inhibits the expression of Wnt targets cyclin D1 and c-myc, which is partially reversed by RAGE blockade. Overall, these results suggest that RAGE inhibits osteoblast proliferation via suppression of Wnt, PI3K and ERK signaling, which provides novel mechanisms by which RAGE regulates osteoblast growth.

1. Introduction

Normal bone homeostasis is achieved by balancing the number and activity of bone forming osteoblasts and bone resorbing osteoclasts [1]. Elucidation of the molecular mechanisms that control osteoblast proliferation and survival is therefore of major importance for a better understanding of the regulation of bone formation and bone mass [2]. Recently, canonical Wnt signaling has emerged as an important regulator of bone formation and bone mass [3–5]. Wnt proteins are a family of secreted glycoproteins and integral regulators of embryogenesis, cell proliferation, and differentiation [6]. When Wnt ligands bind to the heterodimeric receptor molecules, the Frizzled family and low density lipoprotein receptor-related proteins 5 and 6 (Lrp5/6), Wnt signaling is triggered [7]. Then a series of events occur in which Wnt proteins bind to cell-surface receptors of the Frizzled family to activate Dishevelled family proteins and downregulates GSK-3β activity, which ultimately results in stabilization of β-catenin. Stabilized β-catenin then accumulates in the nucleus, where it interacts with the transcription factors lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to activate target gene transcription [8]. Wnt proteins could also regulate kinase signaling pathways. Canonical Wnt3a increases PI3K/Akt activity, resulting in increased free β-catenin levels [9]. Moreover, Wnt3a activates ERK1/2 by direct signaling and posttranscriptional activation via the β-catenin/Tcf4 complex [10], suggesting that these kinases may act as important mediators of Wnt signaling.

Wnt signaling is particularly important for bone homeostasis as revealed in human disease [11]. Patients with loss-of-function mutations in the receptors Lrp5 [12] or Lrp6 [13] are characterized with low bone mineral density (BMD) and skeletal fragility. In contrast, patients with gain-of-function mutations in Lrp5 (reduced affinity of LRP5 for Dkk1) have high bone mass [14–16]. Animal studies [17,18] further support the importance of Wnt signaling in osteoblast differentiation and bone formation. In vitro, Wnt signaling positively controls osteoblast proliferation and differentiation by activating the Wnt/LRP5/β-catenin/LEF-TCF/Runx2 signaling cascade [19]. Moreover, Wnt signaling was found to prevent apoptosis in uncommitted osteoblast progenitors and more mature osteoblasts [20]. However, prevention of apoptosis in uncommitted osteoblasts and mature osteoblasts by Wnt proteins may also occur through activation of Src/ERK and PI3K/Akt pathways [20], indicating that multiple pathways are involved in the control of osteoblast proliferation and apoptosis by Wnt proteins.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell-surface molecules expressed in a range of cell types, including smooth muscle cells [21], fibroblasts [22], osteoblasts [23], and osteoclasts [24]. Previous studies indicate that RAGE plays a crucial role in bone metabolism. Mice lacking RAGE had increased bone mass and BMD and decreased bone resorptive activity [24] although the detailed mechanisms are not fully understood. Zhou et al. also

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showed that impaired osteoclast terminal differentiation and reduced integrin-mediated cell adhesion signaling in bone marrow macrophages (BMMs) and prefusion osteoclasts (pre-OCs) derived from RAGE mutant mice, indicating that RAGE is an essential factor in the regulation of osteoclast maturation and function [24]. However, the role of RAGE in the control of osteoblast proliferation remains unknown. In the present study, we investigated the molecular mechanisms involved in the control of osteoblast growth by RAGE. We provide here novel evidence that RAGE acts as a negative regulator of cell proliferation in osteoblasts via suppression of Wnt, ERK and PI3K/Akt signaling pathways.

2. Materials and methods

2.1. Reagents

α-Minimal essential medium (α-MEM), penicillin–streptomycin (5000 U/ml penicillin, 5000 U/ml streptomycin) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (NY, USA). Wnt3a-conditioned medium (CM) was prepared as previously described [25]. Recombinant human Sfrp1 was from R&D (MN, USA), and blocking RAGE antibody and pharmacologic inhibitors of PI3K (wortmannin) and MEK (U0126) were from Sigma (USA). Antibodys for p-ERK, p-PI3K, β-catenin, RAGE, c-Myc, Cyclin D1, and β-actin were obtained from Santa Cruz Inc. (USA). An ECL kit (Pierce) was purchased from Thermo Co. Ltd. (USA).

2.2. Cell culture

Osteoblastic MC3T3-E1 cells (ATCC, CRL-2593, USA) were cultured in regular growth medium (α-MEM with 10% FBS and 1% penicillin/streptomycin) at 37 °C in a 5% CO_2 incubator. They were subcultured every 72 h using 0.2% trypsin plus 0.02% EDTA. For experiments, cells were cultured for 24 h to obtain monolayers containing 3 ml α-MEM with 10% FBS.

2.3. Lentivirus transfection

Murine RAGE cDNA was cloned by PCR. Then RAGE cDNA or RAGE siRNA (Santa Cruz, catalog# sc-36375, USA) was subcloned into lentiviral vectors pPRIME-CMV-GFP-FF3 carrying GFP as reporter. The recombinant lentivirus was produced by co-transfection of 293T cells with plasmids pPRIME-CMV-RAGE, pLP1, pLP2, and pLP/VSVG with lipofectamine 2000 (Invitrogen, USA). When the MC3T3-E1 cells were at ~60% confluence, the lentivirus was added to cell dishes. Viral infection was carried out at 37 °C and 5% CO_2 at MOIs of 10 for 12 h. Three days after viral infection, cells were harvested and examined for the level of RAGE by using western blot.

2.4. Cell proliferation and cell cycle analysis

For analysis of cell replication, cells were plated at 3000 cells/dish in 96 wells, treated as indicated and cell replication was determined using the BrdU ELISA assay (Roche, France) and cell number. For analysis of cell cycle, cells were suspended in 1 ml solution containing 0.4 mM sodium citrate, 25 μg/ml propidium iodide (PI), and 50 μg/ml RNase. The stained cells were analyzed in a FACScan flow cytometer (BD Biosciences, USA) using the ModFit LT program (BD Biosciences, USA).

2.5. Luciferase reporter assay

For luciferase reporter assays, 20 ng of pCMV-β-galactosidase (β-Gal) was added to the transfection mix (90 ng of TCF and 30 ng of TopFlash or FopFlash). In some experiments, luciferase activity was determined in the presence of RAGE siRNA or control siRNA (2 μg/30,000 cells; Santa Cruz, USA). Luciferase activity was determined by using a luciferase assay kit (Promega) and a β-Gal gene reporter assay kit (Roche, Meylan, France).

2.6. Western blot

Approximately 3×10^6 cells were collected and lysed in RIPA lysis buffer. Samples were centrifuged for 20 min at 12,000 rpm at 4 °C and clear supernatants were collected. The protein concentration in the supernatant was measured using the Miro BCA kit. Fifty micrograms of protein obtained from each sample was loaded into 10% or 12% SDS–PAGE gels and then transferred to a polyvinylidene difluoride (PVDF) membrane. The non-specific proteins were blocked with 5% non-fat dried milk for 1 h. The membranes were incubated with the primary antibodies anti-RAGE, anti-p-ERK, anti-p-PI3K, anti-β-catenin, anti-c-Myc, anti-Cyclin D1, and anti-β-actin overnight at 4 °C, and with secondary antibody (HRP-conjugated IgG) for 1 h. HRP-conjugated secondary antibodies were used in conjunction with an ECL chemiluminescence detection system. Western blots were repeated 3–5 times and qualitatively similar results were obtained.

2.7. Statistical analysis

The experiments were repeated 3 times with at least six replicates per experiment. Data are expressed as mean ± SD and analyzed using the statistical package super-ANOVA (Macintosh, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Enforced expression of RAGE suppresses cell proliferation in MC3T3-E1 cells

To investigate the effect of RAGE on osteoblast cell proliferation, we overexpressed RAGE in murine MC3T3-E1 osteoblasts by transduction with lentivirus (Fig. 1A). As shown in Fig. 1B, RAGE overexpression decreased cell proliferation compared to control (EV) cells. This effect was in part related to a 40% decrease in cell replication, as shown by the BrdU assay (Fig. 1C). Since cell proliferation is a result of cell cycle progression, we next examined the functional effect of RAGE on the cell cycle of MC3T3-E1 using propidium iodide (PI) staining and flow cytometry. We observed that RAGE overexpression induced cell cycle arrest in G2/M phase and decreased S phase as compared with control cells (Fig. 1C). These results show that increasing RAGE expression in osteoblasts results in decreased cell proliferation in vitro.

3.2. Enforced expression of RAGE decreases Wnt/β-catenin signaling

To investigate whether RAGE overexpression negatively regulates Wnt/β-catenin signaling in osteoblasts, we examined the response to Wnt3a in RAGE-overexpressing osteoblasts. As shown in Fig. 2A, Wnt3a increased cell proliferation 1.9-fold in control cells and only 1.3-fold in RAGE overexpressing cells, indicating that forced expression of RAGE decreases the response to Wnt signaling in osteoblasts. In addition, the neutralizing RAGE antibody could reverse this negative effect, indicating that the decreased cell proliferation induced by RAGE overexpression results from alteration of Wnt signaling (Fig. 2A). To examine whether endogenous RAGE affects cell proliferation in osteoblasts, we knocked down RAGE using lentivirus-mediated expression of siRNA. As shown in Fig. 2B, RAGE silencing increased cell proliferation in the presence

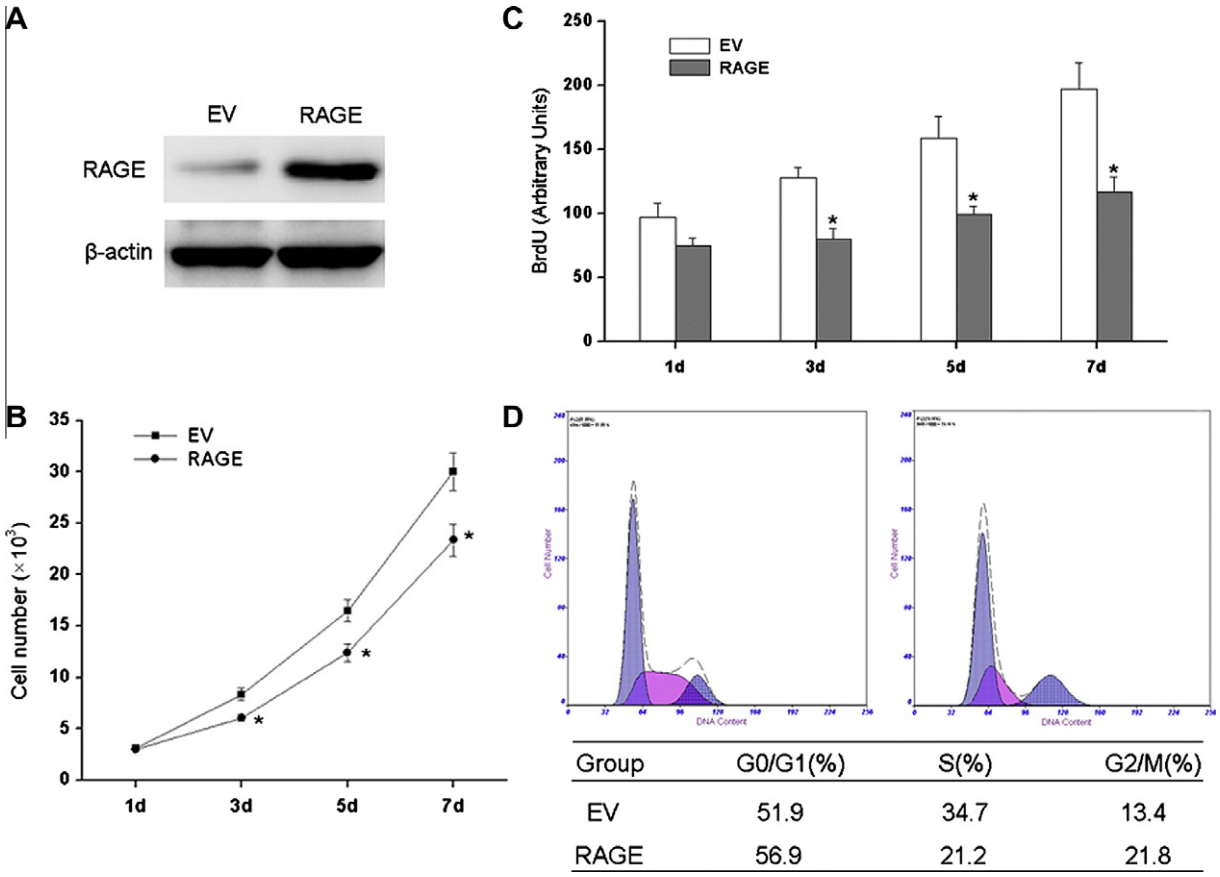

Fig. 1. RAGE overexpression decreases cell proliferation and cell cycle progression in MC3T3-E1 cells. (A) MC3T3-E1 osteoblasts were transfected with lentivirus overexpressing RAGE, and total cellular protein were extracted. Western blot was used to analyse them for RAGE protein expression. (B and C) RAGE overexpression decreased cell number and replication compared to control cells. (D) RAGE overexpression induced cell cycle arrest in G2/M phase and decreased S phase as compared with control cells (*$P < 0.05$ compared to control cells) EV, empty vector.

or absence of Wnt. These results show that RAGE overexpression as well as endogenous RAGE inhibits cell proliferation in osteoblasts.

We then investigated the mechanism by which RAGE may suppress Wnt signaling in osteoblasts. We found that Wnt3a increased TCF/LEF transcriptional activity in control cells (Fig. 2C). In contrast, Wnt3a induced low TCF/LEF transcriptional activity in RAGE-overexpressing cells (Fig. 2C), indicating that the canonical Wnt signaling pathway is altered by RAGE overexpression. To further confirm the implication of Wnt signaling in the altered cell proliferation, we analyzed the expressions of c-myc and cyclin D1 in RAGE cells. As shown in Fig. 2E, both c-myc and cyclin D1 protein levels were markedly decreased in RAGE cells compared to control cells. RAGE antibody increased decreased c-myc and cyclin D1 levels whereas the Wnt antagonist Sfrp1 had opposite effects.

3.3. RAGE overexpression decreases ERK and PI3K signaling

To investigate the molecular mechanism underlying the suppression of cell proliferation induced by RAGE, we examined PI3K and ERK pathways which are most important signaling pathways involved in cell growth. As shown in Fig. 3A, p-PI3K and p-ERK levels were suppressed in RAGE overexpressing cells compared to control cells. Wnt3a increased p-PI3K and p-ERK levels, and this effect was inhibited by the Wnt inhibitor Sfrp1 (Fig. 3A). To confirm this finding, cells were treated with the RAGE neutralizing antibody. The RAGE antibody increased p-PI3K and p-ERK levels in both control cells and RAGE cells (Fig. 3B), indicating that RAGE negatively regulates ERK and PI3K signaling. Moreover, RAGE silencing increased ERK and PI3K phosphorylation, indicating that endogenous RAGE negatively regulates these pathways (Fig. 3C). We then examined the effects of PI3K and ERK inhibitors on cell proliferation induced by Wnt. As shown in Fig. 3C, the positive effect of Wnt3a on cell growth was abolished by wortmannin (10 mM) and U0126 (10 mM) which are inhibitors of PI3K and MEK, respectively, in both control and RAGE cells. These results indicate that the inhibition of PI3K and ERK signaling pathways by RAGE leads to the suppression of cell proliferation in osteoblasts.

3.4. Inhibition of Wnt signaling abolishes PI3K and ERK signaling restored by RAGE blockade

To determine the implication of canonical Wnt signaling in the altered ERK and PI3K signaling, cells were treated with the neutralizing RAGE antibody and the cells were then transfected with the Wnt inhibitor DKK1. As shown in Fig. 4A, the RAGE antibody increased p-PI3K and p-ERK levels in control and RAGE cells. Transient transfection with DKK1 effectively increased DKK1 protein levels and abolished the restoration of ERK and PI3K activation induced by the RAGE antibody, indicating that the altered ERK and PI3K signaling in RAGE cells results in large part from suppression of Wnt signaling. Next, we investigated the functional role of Wnt signaling in the altered cell proliferation induced by RAGE. As shown in Fig. 4B, transient transfection with DKK1 decreased cell proliferation in control cells. RAGE antibody increased cell growth

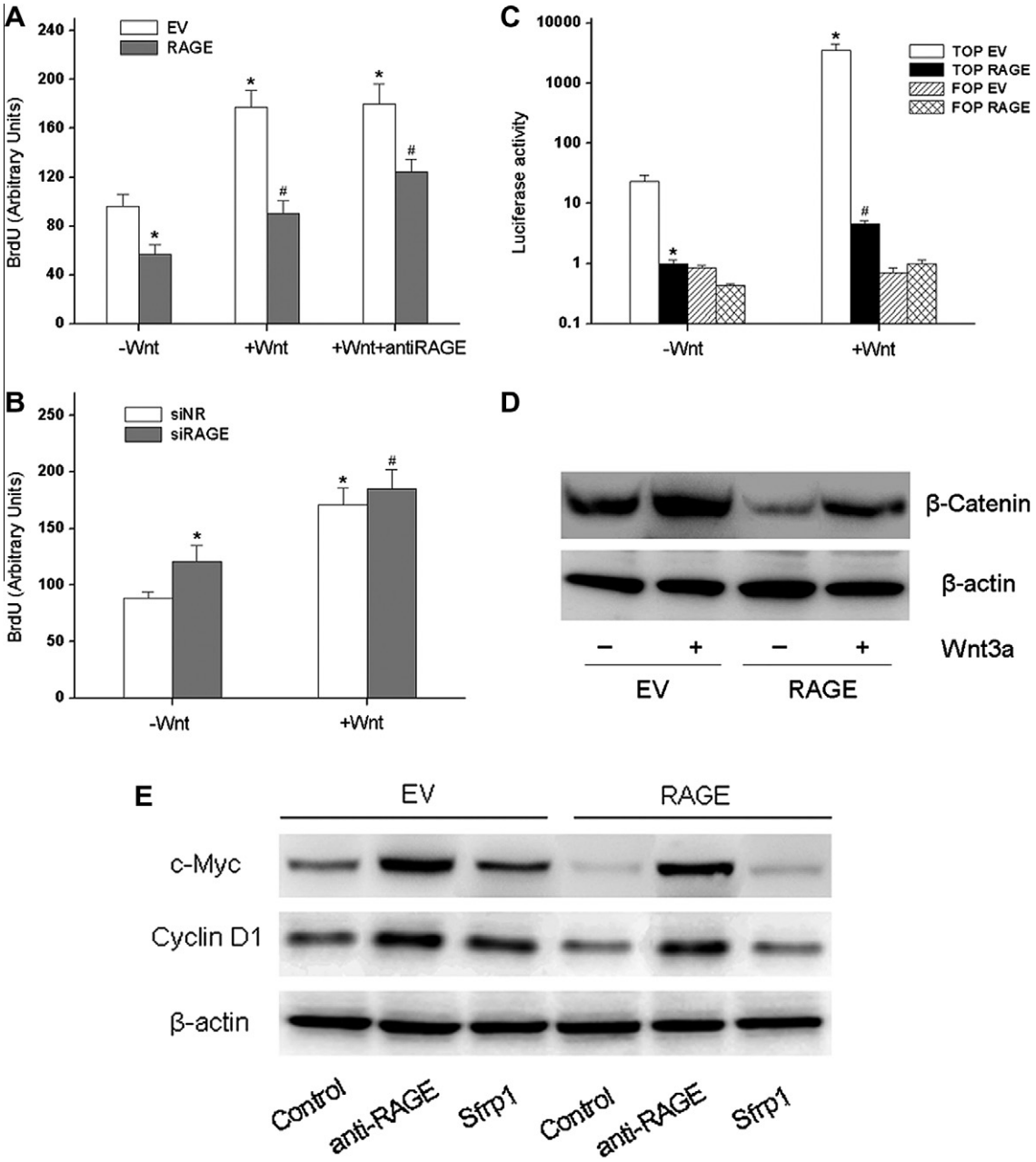

Fig. 2. RAGE overexpression inhibits cell proliferation via suppression of Wnt/β-catenin signaling. (A) Cell proliferation is restored in response to Wnt3a or blocking RAGE antibody in RAGE cells. RAGE and control cells were treated with Wnt3a CM or RAGE antibody for 24 h, and cell replication was determined ($^{*}P < 0.05$ compared to untreated EV cells; $^{\#}P < 0.05$ compared to EV treated with Wnt3a cells). (B) RAGE silencing increases osteoblast proliferation. Control cells were transfected with a lentivirus-mediated RAGE siRNA or siNR and treated with Wnt3a CM for 24 h and cell replication was determined ($^{*}P < 0.05$ compared to untreated siNR cells; $^{\#}P < 0.05$ compared to untreated siRAGE cells). (C) RAGE overexpression reduces TCF/LEF transcriptional activity in basal conditions and in response to 15% Wnt3a-CM ($^{*}P < 0.05$ compared to untreated EV cells; $^{\#}P < 0.05$ compared to untreated RAGE cells). (D) RAGE overexpression reduces total β-catenin levels in response to Wnt3a-CM. RAGE cells or control cells were treated with Wnt3a-CM for 24 h, and total cell lysates was analyzed by Western blot using anti-β-catenin. (E) RAGE overexpression reduces c-myc and cyclin D1 expression. Control and RAGE overexpressing cells were treated with the blocking RAGE antibody, control antibody (IgG) or the Wnt antagonist Sfrp1 for 24 h and the levels of the Wnt-responsive proteins c-Myc and cyclin D1 were analyzed by Western blot. EV, empty vector.

and this effect was reduced by DKK1 transfection in both control and RAGE cells (Fig. 4B). These results confirm that Wnt signaling is implicated in the altered ERK and PI3K signaling in these cells.

4. Discussion

RAGE, a multiligand receptor, contributes to the pathogenesis of multiple disorders, including diabetic complications, neuronal degeneration, and inflammatory disorders [26]. Moreover, RAGE has been shown to play a crucial role in bone metabolism. In this study, we present evidence for a role of RAGE in regulating osteoblast proliferation and apoptosis. We show that overexpression of RAGE inhibits cell proliferation in osteoblasts in vitro. Enforced expression of RAGE decreases Wnt, ERK and PI3K/Akt signaling, and this negative regulation is restored by RAGE neutralizing antibody. The data reported here provides novel mechanisms by which RAGE regulates osteoblast number.

Bone mass in adults is tightly regulated by the balance between bone formation and resorption [1]. Osteoblasts are the bone-forming cells that play an essential role in bone mass acquisition [2].

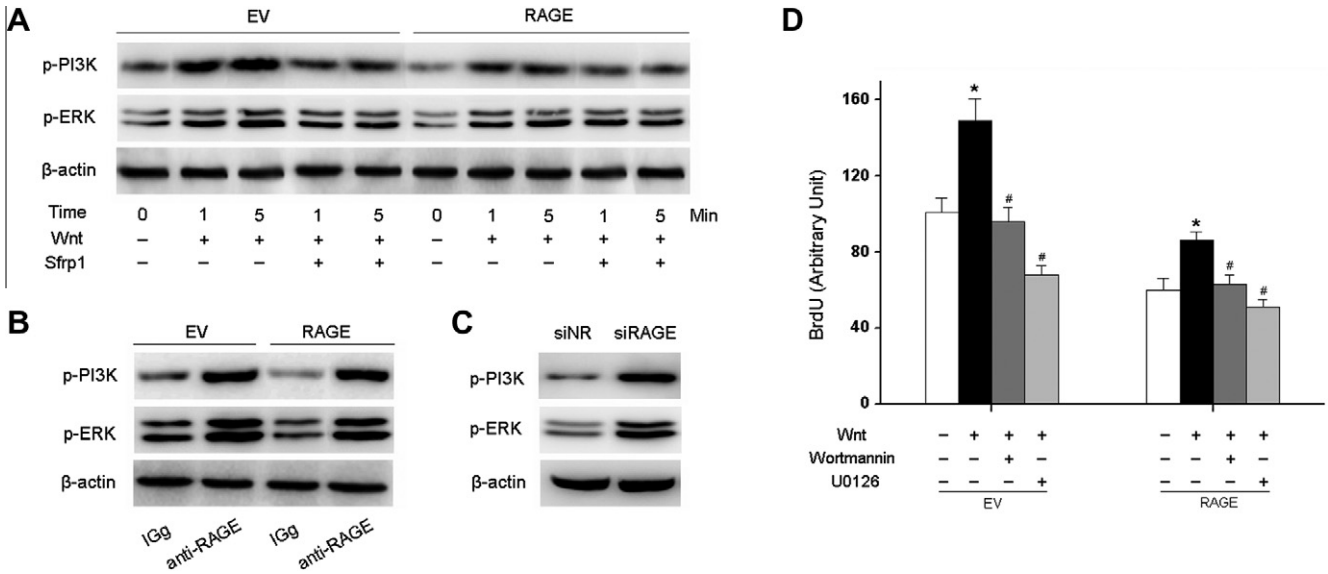

Fig. 3. RAGE overexpression decreases ERK and PI3K signaling. (A) Cells were treated with Wnt3a for 1 or 5 min and PI3K and ERK levels were analyzed by Western blot. (B) RAGE blockade restored cell signaling in RAGE osteoblasts. Control and RAGE cells were treated with RAGE antibody or control antibody (IgG) for 5 min, and PI3K and ERK signaling was analyzed by Western blot. (C) RAGE silencing increased ERK and PI3K signaling. Control cells were transfected with a specific RAGE siRNA or a non relevant siRNA (siNR) and p-ERK and p-PI3K levels were determined by Western blot. (D) Treatment with PI3K and MEK inhibitors (Wortmannin and U0126, respectively) abolished cell proliferation induced by Wnt3a in both control and RAGE cells at 24 h ($^{*}P < 0.05$ compared to untreated cells; $^{\#}P < 0.05$ compared to Wnt3a-treated cells). EV, empty vector.

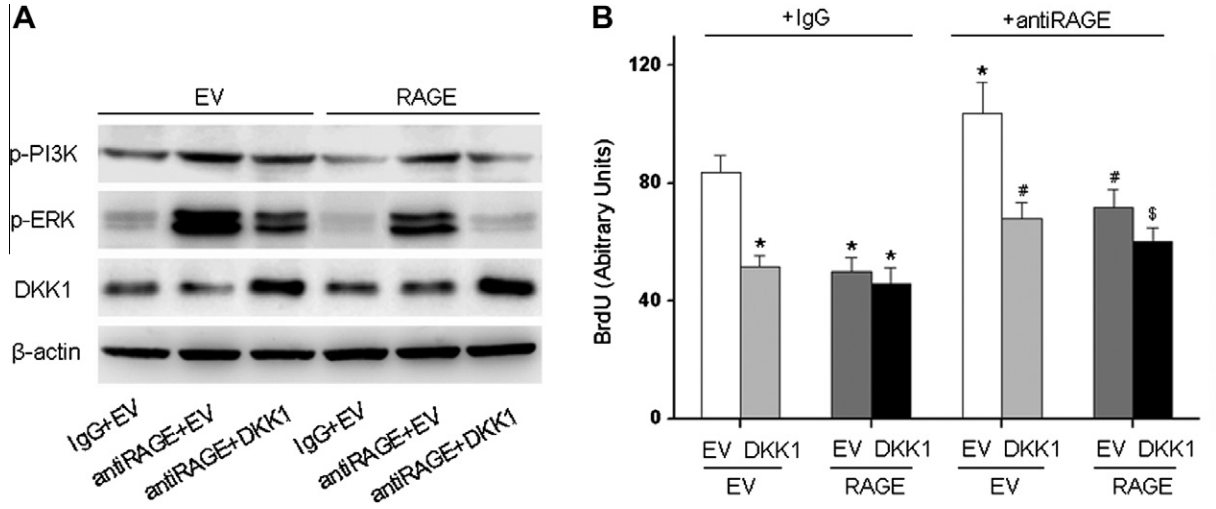

Fig. 4. The Wnt inhibitor DKK1 abolishes PI3K and ERK signaling restored by RAGE blockade. (A) RAGE and control cells transiently transfected with EV or DKK1 were treated with antiRAGE or IgG for 24 h and DKK1 levels and p-PI3K and p-ERK levels were analyzed by Western blot. (B) RAGE and control cells transfected with empty EV or DKK1 were treated with the antiRAGE or IgG for 24 h, and cell replication was determined ($^{*}P < 0.05$ compared to EV control cells; $^{\#}P < 0.05$ compared to antiRAGE treated EV control cells; $^{\$}P < 0.05$ compared to antiRAGE treated EV RAGE cells). EV, empty vector.

Elucidation of the molecular mechanisms that regulate osteoblast growth is therefore of major importance for a better understanding of the regulation of bone formation and bone mass [2]. There is compelling evidence that the co-ordinated activities of many different signaling pathways function to control osteoblast growth and activity. One such way is the Wnt pathway. Many studies have indicated that Wnt/β-catenin is crucial in regulating osteoblast commitment, proliferation and differentiation [27,28]. Here, we showed that RAGE overexpression inhibits osteoblast proliferation by decreasing Wnt/β-catenin signaling. Enforced expression of RAGE reduced TCF/LEF transcriptional activity in basal conditions, and the total β-catenin levels was also reduced. Wnt3a treatment could partly restored this negative regulation, suggesting that the alteration of canonical Wnt signaling contributes to the inhibition of RAGE on cell growth. Consistently, we showed that RAGE overexpression reduced the expression of Wnt-responsive genes such as cyclin D1 and c-myc which control cell growth.

In addition, Wnt signaling could also regulate kinase signaling pathways, such as PI3K and ERK signaling, which play a key role in the control of cell proliferation. We found that RAGE overexpression suppresses PI3K and ERK activity which mediates in part the negative effect of RAGE on osteoblast growth. The suppression of RAGE overexpression on ERK and PI3K pathways and cell growth was reversed by RAGE blockade. In addition, inhibition of Wnt signaling using Sfrp1 or DKK1 abolished the ability of RAGE blockade to restore p-PI3K and p-ERK levels and cell proliferation in RAGE overexpressing cells. Moreover, cell replication induced by Wnt3a was inhibited by pharmacological inhibition of ERK and PI3K, sug-

gesting that these kinases act downstream of Wnt3a to promote osteoblastic cell growth.

In conclusion, we provide evidence for a contribution of RAGE in the regulation of osteoblastogenesis in vitro. Our data indicate that RAGE inhibits osteoblast proliferation via suppression of Wnt, PI3K and ERK signaling pathways in osteoblasts. Our results suggest that RAGE may be an additional target for the design of therapeutic approaches to treat bone loss–related disorders.

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