

Activated Rap1A Induces Osteoblastic Differentiation and Cell Adhesion

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Abstract

Rap1 is a key regulator of cell adhesion and migration. Although increasing evidence indicates that the Rap1 signaling pathway is involved in the process of bone remodeling, the mechanism by which Rap1 regulates osteoblastic differentiation and cell adhesion remains unknown. Here, we investigated the morphological characteristics and osteoblastic differentiation of cells expressing constitutively activated form of Rap1A (Rap1ACA) or Rap1 GTPase activating protein Rap1GAP and found that activated Rap1 induces osteoblastic differentiation and cell adhesion as well as cell spreading. When osteoblastic differentiation was induced, Rap1ACA cells showed considerably higher levels of calcium deposits than the wild-type and Rap1GAP-overexpressing cells did. Rap1ACA cells showed increased spreading and size, as well as strong cell adhesion and significantly decreased growth rates. F-actin staining using phalloidin revealed several thin thread-like filopodia around the protrusions in Rap1ACA cells, which possibly contribute to the increased cell adhesion.

Keywords: Rap1A, filopodia, GTPase

1. Introduction

Bone is a crucial tissue that forms the skeleton, which provides structure and support to the body. It is the site of formation of hematopoietic cells and regulates calcium level in the blood^[1]. In order to maintain bone strength and structural integrity, continuous bone remodeling is required. Dysregulation of bone remodeling by various hormones and cytokines disrupts bone equilibrium and leads to bone-related diseases, such as osteoporosis, osteopetrosis, and osteogenesis imperfecta^[2]. Bone remodeling is mediated by osteoclasts and osteoblasts. Bone formation following bone resorption is initiated by apoptosis of osteoclasts and osteoblastic differentiation of preosteoblasts. Mature osteoblasts produce a variety of bone-related proteins and extracellular matrix components, including runt-related transcription factor-2, alkaline phosphatase, collagen type I α -1, oste-

ocalcin, osteopontin, osterix, and bone sialoprotein. Apoptosis of osteoclasts is induced through the Bim/caspase-3-dependent pathway or estrogen-induced Fas ligand, and osteoblastic differentiation of preosteoblasts is induced by Smad 1,5,8 phosphorylation by bone morphogenetic protein-2 or PI3K/Akt activation by transforming growth factor- β 1^[3-5].

A small GTP-binding protein of the Ras family, Rap1, is involved in a variety of biological processes, including cell adhesion, migration, phagocytosis, morphogenesis, and development^[6-8]. Rap1 mediates these diverse biological activities through regulation of actin cytoskeleton^[9-11]. Recently, it has been reported that Rap1 and Talin1 are critical for resorptive function of osteoclasts^[12]. Deletion of Rap1 in mature osteoclasts retarded pathological bone loss. The semaphorin signaling pathway is also reported to be interconnected with Rap signaling^[13]. Plexins, transmembrane receptors for semaphorins, which serve as guidance cues for neurite outgrowth, have been demonstrated to function as GAPs for Rap proteins rather than M-Ras or R-Ras. Semaphorin 3A, a member of the semaphorin protein family, facilitated bone regeneration in mice by simultaneously reducing bone destruction and increasing bone synthesis^[14]. Even though Rap1 is suggested to

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play important roles in bone formation by osteoblasts, it is not certain whether Rap1 regulates osteoblastic differentiation and cell morphology through regulation of actin cytoskeleton in osteoblasts. In this study, we prepared cells expressing constitutively activated form of Rap1A (Rap1ACA) or Rap1 GTPase activating protein (Rap1GAP) and investigated their morphological characteristics and osteoblastic differentiation.

2. Materials and Methods

2.1. Materials and Plasmids

α -MEM medium, 100 \times penicillin/streptomycin (p/s), and Fetal Bovine Serum (FBS) were obtained from Welgene Inc. (Daegu, Korea). TRITC conjugated-phalloidin was purchased from Sigma-Aldrich Inc (St Louis, MO, USA). The 2x myc-tagged human Rap1A G12V (Rap1ACA) was obtained from UMR cNDA Resource Center. The flag-tagged human Rap1GAP was obtained from Dr. Philip stork in UMR cDNA Resource center and Missouri-Rolla University^[7].

2.2. Cell Culture and Stable Cell Lines

MC3T3-E1 mouse preosteoblast cells were cultured in α -MEM medium (no ascorbic acid) with 10% heat-inactivated FBS and 1% p/s at 37°C in a concentration of 5% CO₂ gas. The culture medium was changed every 2 or 3 days. To establish stable cell lines, pRap1ACA and pRap1GAP were transfected into MC3T3-E1 cells using Lipofectamine²⁰⁰⁰ (Invitrogen). After transfection of 8 h, the medium was changed to growth medium containing 100 μ g/mL of G418 to select transfected cells.

2.3. Osteogenic Induction

1 \times 10⁵ cells were seeded onto 6-wells, and then their differentiation were induced by osteogenic medium containing 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate. Culture medium was replaced every 2 or 3 days.

2.4. Cell Adhesion Assay

Cell adhesion assay was performed as previously described^[15]. Confluent cells (80-90%) attached on plates were washed with PBS and then were incubated in media containing 2% FBS for 12 h. Cells were harvested with 0.6 mM EDTA and were resuspended at a

density of 4 \times 10⁵ cells/mL in the same media. The amount of 1 \times 10⁵ cells were placed onto 24-well plates. After 1 h incubation, unattached cells were washed out. The attached cells on plates were photographed for counting the number of total cells, and unattached cells were removed by washing twice with PBS. Attached cells on plates were photographed and counted. Adhesion cells were presented as a percentage of attached cells to total cells.

2.5. Cell Growth Rate Assay

Cells were plated onto 24-well plates at a density of 10,000 cells per well and were incubated for 5 days without changing media. Every 24 h, pictures of the plates were taken randomly, and the cells on the pictures were counted. The growth rate was calculated as the ratio of the number of the cells to wild-type cells.

2.6. Immunofluorescence

Cells were fixed in PBS containing 3.7% formaldehyde for 5 min, permeabilized in 0.1% Triton X-100 for 1 min, and blocked with 1% BSA in PBS for 1 h. The cells were stained with TRITC-phalloidin (Sigma-Aldrich), to label the F-actin, at room temperature for 40 min. Stained cells were observed in Fluoromount-G (Southern Biotech, Birmingham, AL, USA) mounting solutions using an inverted microscope (IX71; Olympus, Tokyo, Japan) with a camera (DS-Fil; Nikon, Tokyo, Japan).

2.7. Alizarin Red S Staining

For alizarin red staining, cells were fixed with 70% ethanol for 1 h, and rinsed two times with ddH₂O to remove ethanol completely. The cells were stained with 2% alizarin red stain solution (pH4.2) for 20 min to label the calcium deposits, and then were rinsed with ddH₂O five times to remove unbound Alizarin red S. The stained cells were photographed using an inverted microscope with a camera.

2.8. Statistical Analysis

The data were expressed as the mean \pm standard deviation (SD) or standard error of measurement (SEM). Data were collected from at least three independent experiments and were analyzed using Student's two-tailed t-test. * p <0.05, and ** p <0.01, were considered to be statistically significant.

3. Results and Discussion

3.1. Activated Rap1A Induces Osteoblastic Differentiation in Preosteoblasts

To determine whether the level of activated Rap1A is associated with osteoblastic differentiation of preosteoblasts, we established stable cell lines expressing Rap1ACA or Rap1GAP and examined the extent of their osteogenic differentiation (Fig. 1A). Cell differentiation was induced by replacing the normal culture medium with the differentiation-inducing medium, and then calcium accumulation, which is reflective of bone mineralization, was detected by alizarin red S staining. Calcium accumulation was observed 7 days after initiation of differentiation. While the wild-type and Rap1GAP-expressing cells displayed small calcium accumulates, Rap1ACA cells exhibited more and larger calcium accumulates and were stained intensely. These observations were more prominent at 15 days after initiation of differentiation. These results suggest that the level of activated Rap1 is associated with differentiation of preosteoblasts and activated Rap1 promotes progression of osteoblastic differentiation. These results were confirmed by morphological analyses. Differentiated osteoblasts exhibit adipocyte-like morphology^[16]. At 21 days after initiation of differentiation, most Rap1ACA cells showed differentiated adipocyte-like morphology, whereas Rap1GAP-overexpressing cells remained undifferentiated with a fibroblast-like morphology, compared to the wild-type and Rap1ACA cells (Fig. 1B).

These results are in agreement with those of previous reports^[4]. During osteoblastic differentiation, Rap1 expression increases, which in turn promotes differentiation^[4]. Although the mechanism for osteogenic differentiation remains unknown, Rap1 and PI3K/Akt signaling have been suggested to play important roles in the process^[5]. Our results suggest that the level of activated Rap1 correlates with the extent of osteoblastic differentiation. Further studies on the regulation of Rap1 activation by guanine nucleotide exchange factors (GEFs) or GAPs would provide valuable insights into the process of osteoblastic differentiation.

3.2. Activated Rap1A Stimulates Spreading of Preosteoblasts

Rap1 is involved in a variety of processes, including cell growth, differentiation, adhesion, phagocytosis, and

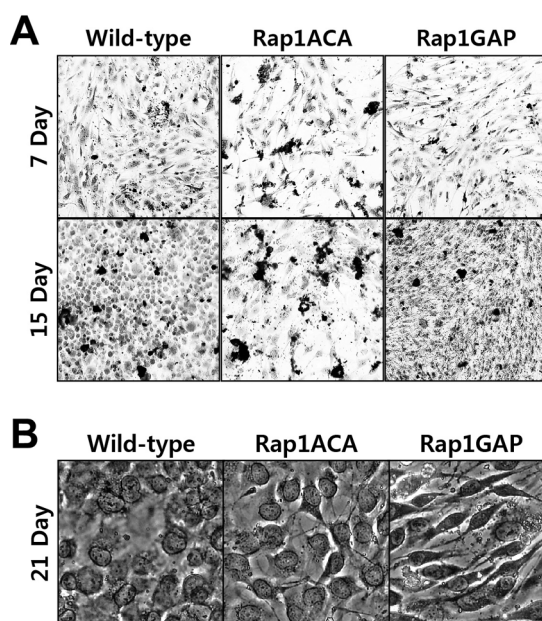


Fig. 1. Osteoblastic differentiation of cells expressing Rap1ACA and Rap1GAP. (A) Alizarin red S staining of the cells after induction of differentiation. Preosteoblastic cells were induced to differentiate into osteoblasts, and were stained with alizarin red S at the indicated days after differentiation. The representative images of the stained cells are presented. (B) Phase-contrast images of the cells 21 days after differentiation.

morphogenesis^[7,8,11,17]. We investigate the effects of Rap1A on cell morphology (Fig. 2). Rap1ACA cells showed more spreading and flatter morphology than the wild-type and Rap1GAP-overexpressing cells (Fig. 2A). Rap1GAP-expressing cells were elongated and thin. The size of Rap1ACA cells was 1.6-fold that of wild-type and Rap1GAP-expressing cells (Fig. 2B). Distribution analysis of cell sizes revealed that relatively bigger cells were more common in the Rap1ACA cell population, even though the highest number of cells were found in similar size of cells between wild-type and Rap1ACA cells. Compared with wild-type cells, Rap1GAP-expressing cells showed a greater number of smaller cells (Fig. 2C). These results suggest that activated Rap1A stimulates cell spreading, thereby increasing cell sizes.

Rap1 is associated with cell proliferation through Akt signaling^[18,19]. Expectedly, the growth rate of osteoblasts was associated with the level of activated Rap1.

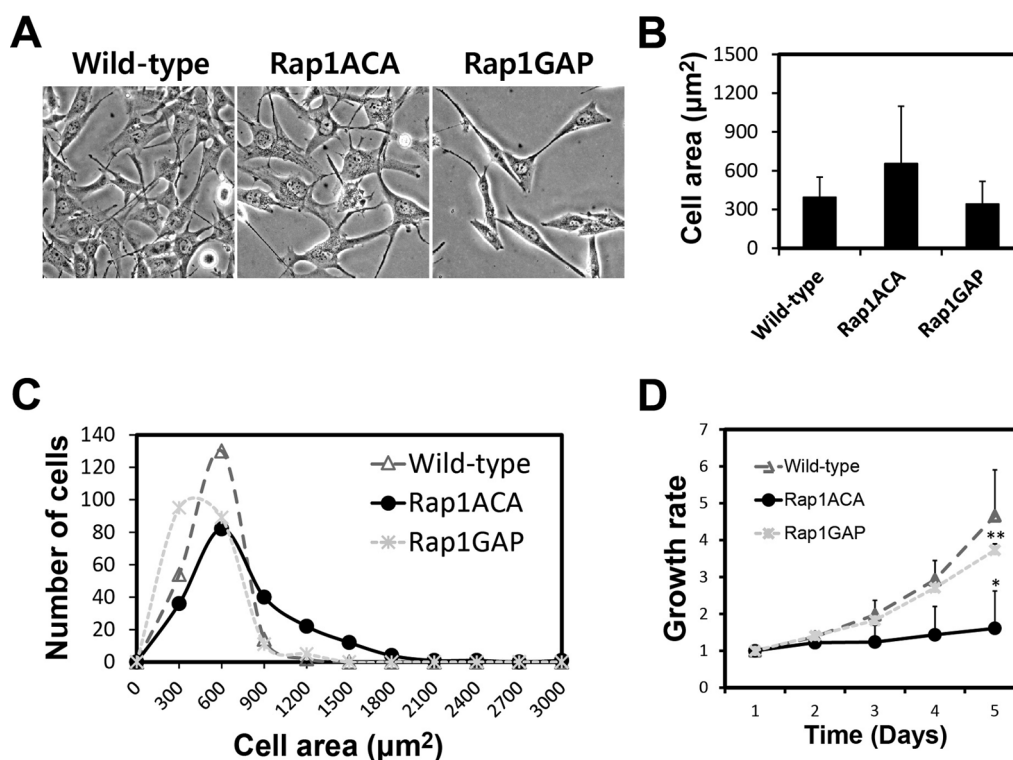


Fig. 2. Cell morphology of cells expressing Rap1ACA and Rap1GAP. (A) Morphology of the cells. Cells grown in 24-well plates were imaged. (B) Quantification of cell size. The sizes of the cells were measured by ImageJ software ($n=200$). The data were presented as the mean \pm SD. (C) Histogram of cell size. The number of cells was graphed against cell sizes. (D) Growth rates of the cells. Preosteoblastic cells were cultured in 24-well plates for 5 days. The number of the cells in the well was counted every 24 h. The data shown are the means \pm SEM. of three experiments (* $p<0.05$; ** $p<0.01$ compared to the control.)

While Rap1GAP-expressing cells showed similar growth rate as the wild-type cells, the growth rates of Rap1ACA cells was highly decreased (Fig. 2D). The doubling time of Rap1ACA cells was 2.8-fold that of wild-type cells. The decreased growth rate of Rap1ACA cells is likely the results of Akt signaling-mediated gene regulation by the elevated amount of activated Rap1.

3.3. Activated Rap1A Promotes Formation of Filopodia and Increases Cell Adhesion

Rap1 is a key regulator of cell adhesion, integrin-mediated cell adhesion, and the formation of cadherin-based cell-cell junctions^[20-22]. It was recently revealed that Rap1 directly interacts with Talin1 and mediates cell adhesion in osteoclasts^[12]. To examine the effects of Rap1 activation on preosteoblasts, we investigated

adhesion of Rap1ACA cells and Rap1GAP-overexpressing cells (Fig. 3A). Rap1ACA cells, expectedly, showed stronger adhesion, and Rap1GAP-overexpressing cells showed slightly lower adhesion than wild-type cells. F-actin staining using TRITC-phalloidin revealed that Rap1ACA cells had protrusions with several thin thread-like filopodia, which were not observed in wild-type and Rap1GAP-overexpressing cells (Fig. 3B and C). Wild-type and Rap1GAP-expressing cells formed smooth protrusions, and the protrusions of Rap1GAP-expressing cells were thinner and longer than those of wild-type cells. In addition, Rap1ACA cells showed ruffle-like structures, which have a high density of F-actin, along the cell cortex. The increased F-actin around the cell cortex and thin filopodia in Rap1ACA cells appear to contribute to increased cell adhesion.

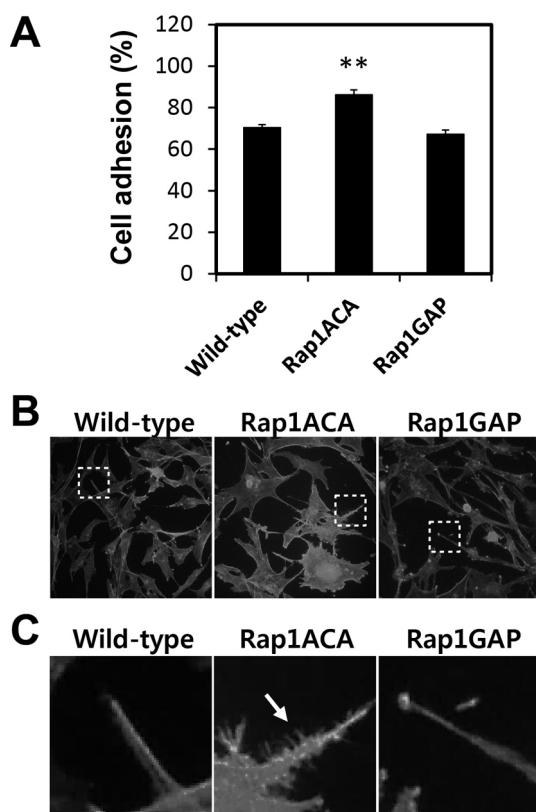


Fig. 3. Cell adhesion and F-actin in cells expressing Rap1ACA and Rap1GAP. (A) Cell adhesion of the cells. The data shown are the means \pm SEM. of three experiments (** p <0.01 compared to the control.). (B) F-actin staining with TRITC-labeled Phalloidin. (C) The enlarged images of the dotted box in figure 3B. The white arrow indicates thread-like thin filopodia in Rap1ACA cells.

4. Conclusion

We examined the morphological characteristics and osteoblastic differentiation of cells expressing Rap1ACA or Rap1GAP. Our results suggest that activated Rap1A induces osteoblastic differentiation, which was determined from the calcium depositions after induction of differentiation, and promotes cell adhesion, possibly through increased F-actin and thin thread-like filopodia around the cell cortex.

Acknowledgments

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