Biochemical and Biophysical Research Communications 400 (2010) 39–45

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Osteogenic differentiation of mouse mesenchymal progenitor cell, Kusa-A1 is promoted by mammalian transcriptional repressor Rbpj

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ARTICLE INFO

Article history:
Received 12 July 2010
Available online 4 August 2010

Keywords:
Bone marrow stromal cells
Rbpj
Notch signaling
Osteoblast differentiation

ABSTRACT

Pluripotent mesenchymal stem cells possess the ability to differentiate into many cell types, but the precise mechanisms of differentiation are still unclear. Here, we provide evidence that Rbpj (recombination signal-binding protein for immunoglobulin kappa j region) protein, the primary nuclear mediator of Notch, is involved in osteogenesis. Overexpression of Rbpj promoted osteogenic differentiation of mouse Kusa-A1 cells *in vitro* and *in vivo*. Transient transfection of an Rbpj expression vector into Kusa-A1 cells upregulated promoter activities of Runx2 and Ose2. Enhanced osteogenic potentials including high alkaline phosphatase activity, rapid calcium deposition, and increased calcified nodule formation, were observed in established stable Rbpj-overexpressing Kusa-A1 (Kusa-A1/Rbpj) cell line. *In vivo* mineralization by Kusa-A1/Rbpj was promoted compared to that by Kusa-A1 host cells. Histological findings revealed that expression of Rbpj was primarily observed in osteoblasts. These results suggest that Rbpj may play essential roles in osteoblast differentiation.

1. Introduction

Bone marrow stromal cells (BMSC) contain pluripotent mesenchymal stem cells, allowing them to differentiate into bone, cartilage, adipocytes, and hematopoietic supporting tissues [1]. Stem cell characters and the fact that BMSC can be manipulated *in vitro* and can subsequently form bone *in vivo* provide a powerful new model system for studying the basic biology of bone, and for development of strategies for gene/cell therapy of skeletal pathologies [1,2]. Many factors are involved in BMSC commitment to the osteogenic differentiation pathway, and their significance in regulating proliferation and differentiation has been well documented [3,4]. However, the molecular processes controlling lineage commitment and self-renewal remain to be elucidated.

Rbpj (recombination signal-binding protein for immunoglobulin kappa j region) [5], also known as CBF1 (Epstein–Barr virus (EBV) latency C promoter binding factor 1) protein, KBF2 (MHC enhancer κB binding factor), and LMP-2 (EBV latent membrane promoter binding protein), that we will refer to as Rbpj, is an evolutionarily conserved transcription factor which belongs to the CSL (Rbpj in mammalian cells, Suppressor of Hairless in *Drosophila*, Lag-1 in *Caenorhabditis elegans*) family of nuclear proteins. Rbpj has been shown to be the primary nuclear mediator of the membrane receptor Notch, which plays a fundamental role in cell fate decisions in both invertebrate and vertebrate developments by regulating local cell interactions in embryonic morphogenesis and tissue differentiation. Notch signaling occurs through the proteolytic release of its cytoplasm domain which subsequently translocates into the cell nucleus, and activates responsive promoters by interaction with Rbpj [6]. Deletion of Rbpj or Notch in *Drosophila melanogaster* leads to lethal expansion of neural tissues at the expense of epidermal tissues in the developing embryo. Rbpj knockout mice show severe growth retardation and embryonic lethality before 10.5 days of gestation [7].

Notch signaling mechanisms control an extraordinarily broad spectrum of cell fates and developmental processes. An accumulating body of research indicates that Notch signaling also mediates the generation of mesenchymal tissues such as in myogenesis and angiogenesis [8,9]. Expression of Notch and its related genes is also observed in cells that are recruited for cartilage and bone formation [10,11]. In bone marrow, Notch signaling is showed to maintain a pool of mesenchymal progenitors by suppressing osteoblast differentiation [12]. We previously constitutively transfected

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.07.133

active Notch1 (Notch intracellular domain, NICD) into two different osteoblastic mesenchymal cell lines, and found that Notch signaling suppressed osteoblastic differentiation of mesenchymal progenitor cells [13]. While in bone homeostasis Notch signaling shows potential dimorphic effects [14]. Notch signaling in osteoblasts is well-reviewed recently [15]. Gain-of-function and loss-of-function experiments reveal a suppressive effect of Notch in osteoblast and osteoclast differentiation in development and in the postnatal bone. These observations suggest that Notch signaling regulates growth and differentiation of osteogenic cells.

Rbpj was originally classified as a transcriptional repressor in vertebrates [16]. However, binding of the Notch intracellular domain was proposed to turn Rbpj into a transcriptional activator [17]. Although functions of Rbpj have been extensively investigated in many cell types [18–20], little is known about its role in osteogenesis. We now provide evidence that Rbpj is involved in osteogenesis, and overexpression of Rbpj promoted osteogenic differentiation in mouse mesenchymal progenitor Kusa-A1 cells *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

Kusa-A1, Kusa-O, and MC3T3E1 cells and NIH3T3 cells were maintained in α-modified minimum essential medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, respectively. The recombinant human BMP2 was purchased from Peprotech.

2.2. Transient transfection and luciferase activity assay

Reporter plasmids with the Runx2 promoter (Runx2-luc) and the Ose2 element (Ose2-luc) were provided by Sumitomo Pharmaceuticals Research Center. The Hes1 reporter plasmid (Hes1-luc) was a gift from R. Kageyama (Kyoto University, Japan). Transient cotransfections of pTagHA/Rbpj and reporter plasmids were conducted using FuGENE6 (Roche Molecular Biochemicals). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). All experiments were performed in triplicates, and firefly luciferase activity was normalized against cotransfected *Renilla* luciferase activity (pRL-EF, a gift from Y. Mochida, Boston University).

2.3. Establishment of stably Rbpj-expressing Kusa-A1 (Kusa-A1/Rbpj) cell line

The Flp-In mammalian expression system (Invitrogen) was used to generate the Kusa-A1/Rbpj cell line. Briefly, mouse Rbpj cDNA (RIKEN Gene Bank, courtesy of Dr. T. Honjo) was subcloned into pEF5/FRT/V5-D-TOPO mammalian expression vectors, and pEF5/FRT/V5/Rbpj recombinant and pOG44 expression plasmids were cotransfected into the Kusa-A1/host cell line, which were generated by pFRT/lacZeo target site vectors. For selection, 200 μg/ml hygromycin B was used.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from Kusa-A1/Rbpj cells using the Isogen RNA isolation kit (Nippon Gene). cDNA was converted from 2 μg total RNA using Superscript II (Invitrogen).

2.5. Western blot analysis

Cells were cultured on 90 mm dishes till confluence. The lysate was prepared using 300 μl cell lysis buffer (50 mM Tris, pH 7.8; 150 mM NaCl; 1% Nonidet P-40, and cOmplete® protease inhibitor cocktail (Roche Applied Science)). A 10% polyacrylamide gel was used for electrophoresis of proteins. Following electrophoresis, proteins were transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare). Protein detection was performed using anti-V5 antibody (Invitrogen) or rabbit anti-RANKL polyclonal antibody FL-317 (Santa Cruz Biotechnology).

2.6. Assays for alkaline phosphatase (ALP) and calcium deposition

ALP activity in Kusa-A1 cells was measured using the ALP K-test Wako kit (Wako Pure Chemical Industries). Cells in 24-well plates were passively lysed using TNTC buffer (100 mM Tris–Cl, pH 7.6; 150 mM NaCl; 1% Triton X-100; 1 mM $CaCl_2$). Fifty microliters of cell lysate was incubated with assay buffer at 37 °C, and absorbance of the solution was measured at 540 nm. Protein concentrations of samples were measured using a Protein Quantification Kit (Dojindo Molecular Technologies). Amount of calcium deposits on culture dishes was measured using a calcium measurement kit (Ca-E test; Wako Pure Chemical Industries).

2.7. In vitro mineralization assay

Cells were seeded in 24-well plates. For induction of mineralization, 0.2 mM L-ascorbic acid-2-phosphate and 10 mM of β-glycerophosphate were added to the medium after the cells reached confluence. Calcified nodules were stained with Alizarin Red S at 3, 5, and 7 days after confluence.

2.8. In vivo osteogenesis assays

One hundred microliters of cells (1×10^8 cells/ml) per mouse (C3H/HeN mice, male, 8-week-old) was subcutaneously injected into the abdomen of mice. Thirty days after inoculation, the animals were sacrificed, and soft X-ray photos were taken to examine calcification. After weighing, bone-like tissues originating from injected cells were dissected, and processed for histological analyses. Decalcified frozen sections were stained with ALP, TRAP (tartrate-resistant acid phosphatase), and HE (hematoxylin and eosin). All animal experiments were performed in accordance with the Animal Guidelines of Tokyo Medical and Dental University.

2.9. Real-time polymerase chain reaction and cDNA microarray

Real-time PCR was performed using the DNA Engine Opticon real-time system (Bio-Rad Laboratories) with Platinum SYBR Green qPCR supermix (Invitrogen). For quantification, target genes were normalized against the internal standard β-actin gene. Two GeneChip® mouse expression array set 430s (Affymetrix) were used to reveal expression profiles in Kusa-A1/host and Kusa-A1/Rbpj cells, respectively, using the single color hybridization method, in which RNA probes were labeled only with the Cy3 dye. Gene expression was analyzed using the GeneSpring software (Agilent Technologies).

2.10. In situ hybridization

Digoxigenin-labeled riboprobes for Rbpj were synthesized from EcoRI- or XbaI-digested Rbpj cDNA templates in pTagHA with T7 or T3 RNA polymerase (Roche Applied Science). Frozen sections were prepared from E17 C3H/HeN mouse embryos.

3. Results

3.1. Expression of Rbpj in adult mouse organs, cells, and embryo tissues

Quantitative RT-PCR analysis revealed ubiquitous expression of Rbpj mRNA in various organs and cell lines. Rbpj expression level

was high in muscles and kidneys, medium in the heart, dental pulp, thymus, liver, brain, and bone (Fig. 1A). Kusa-A1 cells, which were established from mouse bone marrow stromal cells [21], are highly committed to the osteoblastic lineage, and can form bone-like tissues both *in vitro* and *in vivo* [13,22,23], showed high Rbpj expression compared to Kusa-O cells, a cell line derived from bone marrow stromal cells just as Kusa-A1 cells, and compared to the typical osteoblastic cell line MC3T3E1 and the fibroblastic cell line NIH3T3 (Fig. 1B). In order to identify Rbpj-expressing cells in bone-forming tissues, claws of mouse embryos were collected for *in situ* hybridization. Although Rbpj expression was ubiquitous both in the epithelial and mesenchymal cells, extremely strong staining was found in mesenchymal cells surrounding the bone (Fig. 1C–H).

3.2. Expression of Rbpj and other Notch-related genes in Kusa-A1 cells following induction of differentiation

To observe the dynamics of Rbpj and expressions of other Notch-related genes during osteoblast differentiation, Kusa-A1 cells were cultured till confluence under mineralization-inducing conditions, and gene expression was examined by quantitative RT-PCR. Expression of Notch1, its ligands Delta1 and Jagged1, and its direct downstream effector Hes1 were all downregulated at 1 day following osteogenic induction. Then, their levels were maintained low, whereas Rbpj expression was largely unaltered, and was maintained at a similar level during all that period (Fig. 2A).

3.3. Effects of transient transfection of Rbpj into Kusa-A1 cells on promoter activities of Runx2 and Ose2

A luciferase assay revealed dose-dependent increase of Ose2 promoter activity (Fig. 2B). Runx2 promoter activity also increased in response to Rbpj transfection. In order to investigate the relation between Rbpj- and BMP-mediated Runx2 upregulation, Kusa-A1 cells cotransfected with Runx2-luc or Ose2-luc and Rbpj were treated with recombinant BMP2 protein, and luciferase activities were measured (Fig. 2C). In the presence of BMP2, promoter activities of Runx2 and Ose2 were both upregulated more than without BMP2. Rbpj acted synergistically on promoter activities of Runx2 and Ose2, resulting in highest levels of upregulation.

3.4. Generation of stable Rbpj-expressing transformants

We established the Kusa-A1 cell line that stably expresses Rbpj (Kusa-A1/Rbpj). Clone-dependent phenotypic variation was eliminated using the Flp-In system. Flp-In Kusa-A1 host cell line showed similar osteogenic potential as the original Kusa-A1 cells in terms of ALP activity, and ability of calcification (data not shown); therefore, the Kusa-A1/host cell line was used as control in the following experiments. Expression of integrated Rbpj gene was confirmed by RT-PCR and Western analysis (Fig. 2D). Rbpj mRNA expression level was 10-fold higher in Kusa-A1/Rbpj cells compared to Kusa-A1 host cells (data not shown). Kusa-A1/Rbpj cells were not morphologically different from Kusa-A1/host cells, and from the original Kusa-A1 cells. Kusa-A1/host and Kusa-A1/Rbpj cell lines showed the same proliferating rate, with a doubling time of about 20 h (data not shown). To examine effects of Rbpj on the Notch-Hes1 pathway, NICD and Hes1-luc were transiently cotransfected into Kusa-A1/host and Kusa-A1/Rbpj cells. The Hes1 promoter was activated in Kusa-A1/host cells, but effects of NICD were significantly attenuated in Kusa-A1/Rbpj cells (Fig. 2E), suggesting that Notch signal transduction to the Hes1 pathway was inhibited in Kusa-A1/Rbpj cells.

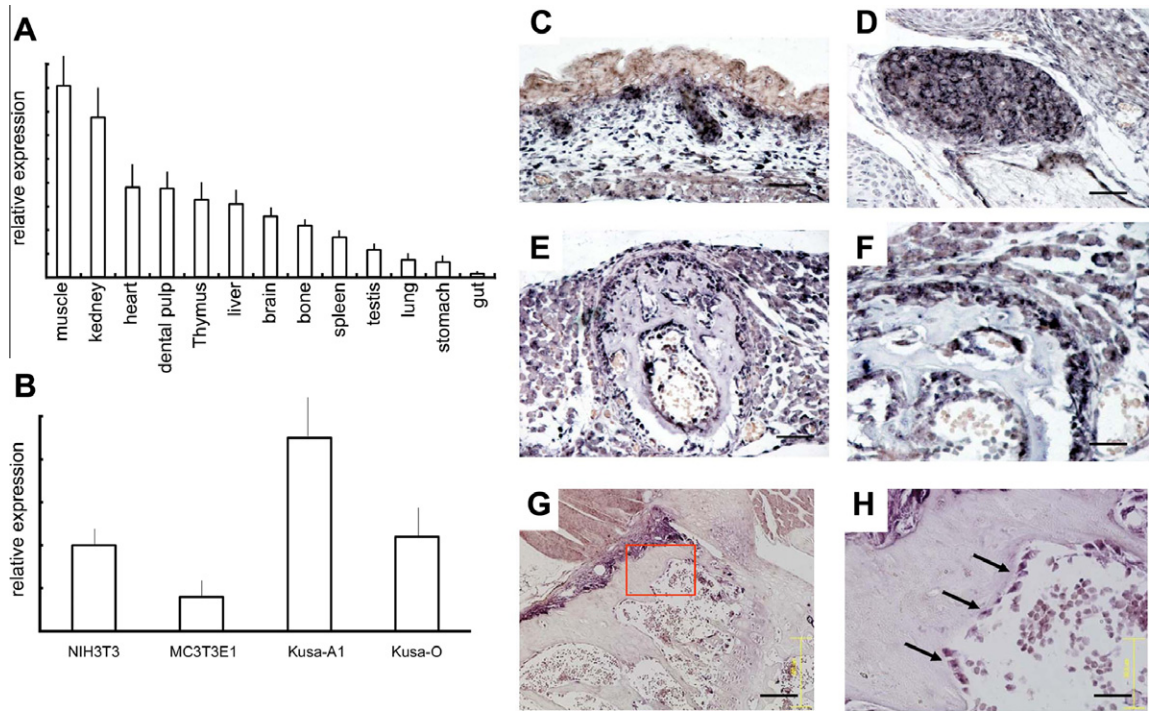
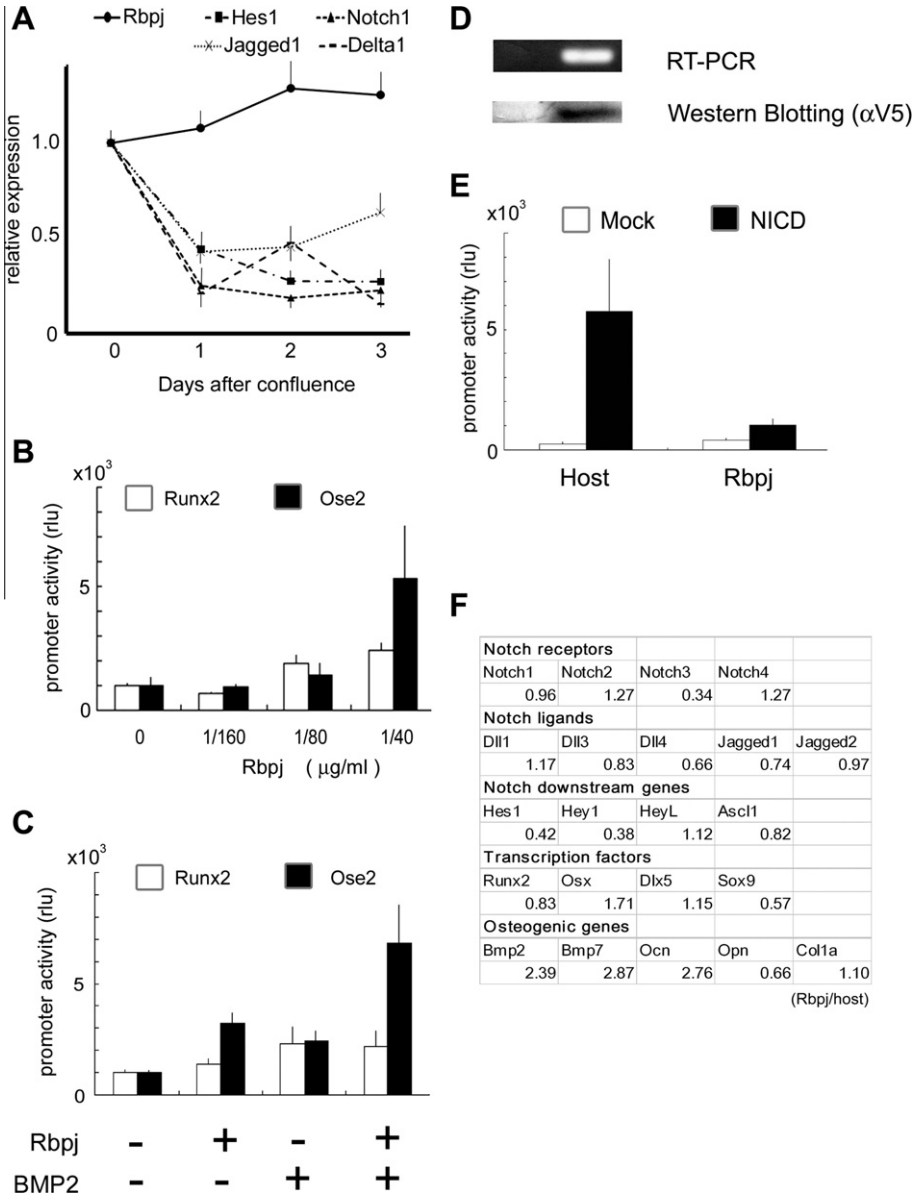


Fig. 1. Rbpj mRNA expression in different organs, cell lines, and embryos. (A) Rbpj expression level was evaluated by real-time PCR. High expression of Rbpj was observed in muscles and kidneys, and medium expression was observed in dental pulp, thymus, liver, brain, and bone. (B) Kusa-A1, a mesenchymal progenitor cell line derived from bone marrow stromal cells, showed high Rbpj expression. (C–H) *In situ* hybridization using Rbpj-specific DIG RNA probes revealed ubiquitous expression of Rbpj in skin (C), ganglions (D), and the spinal cord (E,F), but there was a relatively high expression of Rbpj in osteoblasts of the femur (G,H). Arrows: Rbpj-expressing cuboidal osteoblasts. Bars: 100 μm (C–E,G) and 25 μm (F,H).



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Notch receptors				
Notch1	Notch2	Notch3	Notch4	
0.96	1.27	0.34	1.27	
Notch ligands				
Dll1	Dll3	Dll4	Jagged1	Jagged2
1.17	0.83	0.66	0.74	0.97
Notch downstream genes				
Hes1	Hey1	HeyL	Ascl1	
0.42	0.38	1.12	0.82	
Transcription factors				
Runx2	Osx	Dlx5	Sox9	
0.83	1.71	1.15	0.57	
Osteogenic genes				
Bmp2	Bmp7	Ocn	Opn	Col1a
2.39	2.87	2.76	0.66	1.10

(Rbpj/host)

Fig. 2. Notch-related gene expression in Kusa-A1 cells, promoter assay of Runx2 and Ose2, and gene expression profile of stable Rbpj transformants. (A) Notch-related gene expression in Kusa-A1 cells under mineralization-inducing conditions. In contrast to the downregulation of *Hes1*, *Notch1*, *Jagged1*, and *Delta1*, expression of *Rbpj* was largely unaltered. (B,C) Promoter activities of Runx2 and Ose2 in Rbpj expression vector-transfected Kusa-A1 cells. Dose dependent upregulations of Runx2 and Ose2 promoter activities were observed in Rbpj expression vector-transfected Kusa-A1 cells (B). Ose2 promoter activity induced by transient Rbpj expression in Kusa-A1 cells was enhanced in the presence of BMP2 (C). (D) Establishment of stable Rbpj transformants (Kusa-A1/Rbpj) in Kusa-A1 cells. Strong expressions of Rbpj mRNA and tag protein (V5) were observed in Kusa-A1/Rbpj cells. (E) Hes1 promoter activity in Kusa-A1/Rbpj. Hes1 promoter activity, which was forcedly induced by transfection of NICD (Notch intracellular domain) expression vector into Kusa-A1 cells, was remarkably downregulated in Kusa-A1/Rbpj cells. (F) Expression profiles of notch related and osteogenic genes in Kusa-A1/Rbpj cells. Compared to Kusa-A1 host cells, downregulation of Notch downstream genes such as *Hes1* and *Hey1*, and upregulation of osteogenic genes such as *BMP2*, *BMP7*, and *OC*, were observed in Kusa-A1/Rbpj cells.

3.5. Gene expression in Kusa-A1/Rbpj cells

Gene expression was examined by microarray methods, and was compared between Kusa-A1/host and Kusa-A1/Rbpj cells (Fig. 2F). *Notch1* expression was largely unaltered, *Notch2* and *Notch4* were slightly upregulated, and *Notch3* was downregulated by Rbpj. Notch ligand expressions showed slight downregulation except for *Dll1*. The direct target genes of the Notch signaling pathway, *Hes1* and *Hey1* were significantly downregulated. *Runx2* expression was slightly downregulated, but *Osterix* (*Sp7*) expression was significantly upregulated. Expressions of *Bmp2* and *Bmp7* were more than 2-fold upregulated. *Osteocalcin* (*Ocn*) was also significantly upregulated, but *Osteopontin* (*Opn*) expression was downregulated.

3.6. Osteogenic potential of Kusa-A1/Rbpj cells in vitro

To evaluate osteogenic potential of Kusa-A1/Rbpj cells, *in vitro* calcium deposition, ALP activity, and amount of mineralized foci were examined under mineralization-promoting culture conditions. BMP2 promoted ALP activity, but no significant effects of Rbpj on ALP activity were detected (Fig. 3A and B). Calcium deposition in Kusa-A1/Rbpj cells reached its highest level 1 day after confluence, while it reached its highest level at 3 days after confluence in

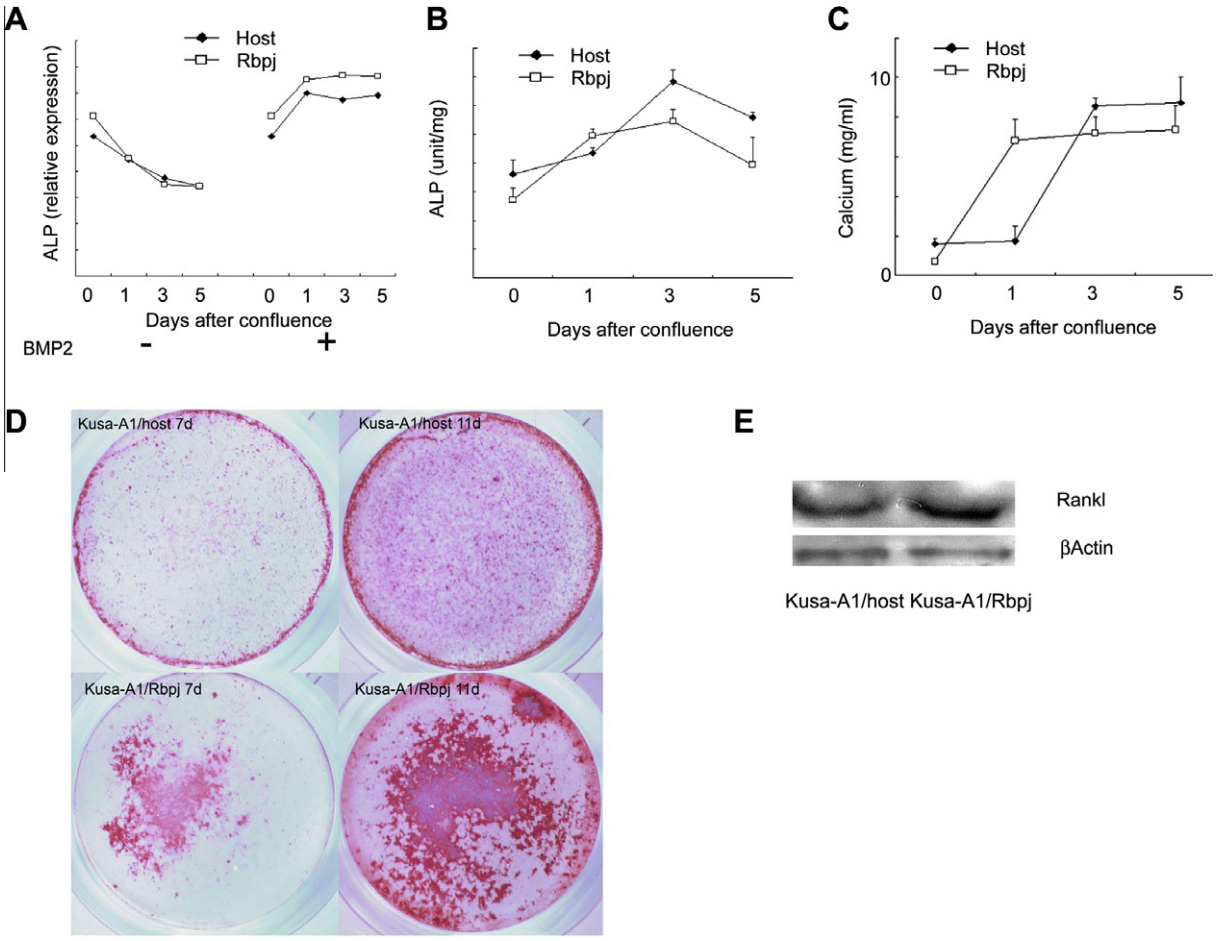

Fig. 3. ALP activity, calcium deposition, mineralized nodule formation, and *Rankl* expression in Kusa-A1/Rbpj cells. No typical changes in ALP mRNA expression and enzyme activities were observed in Kusa-A1/Rbpj in the presence or absence of BMP2 (A,B). Calcium deposition (C), mineralized nodule formation (D), and *Rankl* expression (E) were upregulated in Kusa-A1/Rbpj cells.

Kusa-A1/host cells (Fig. 3C), indicating that expression of Rbpj accelerated calcium deposition. Amount of calcified nodules was greatly increased in Kusa-A1/Rbpj cells (Fig. 3D). The receptor activator of NF-κB ligand (RANKL) plays an important role in osteoclastogenesis and bone resorbing activity. Twenty-four hours after confluence under mineralization-promoting culture conditions, Kusa-A1/Rbpj cells produced more RANKL than Kusa-A1/host cells (Fig. 3E).

3.7. Osteogenic potential in Kusa-A1/Rbpj cells in vivo

To assess effects of Rbpj on osteogenic potential of Kusa-A1 cells *in vivo*, we subcutaneously injected the cultured cells into the mouse abdomen, and examined them after 30 days. In all cases (12/12), injected cells proliferated, and formed nodular masses containing calcified tissues that were observed as radio-opaque foci (Fig. 4A and B). Histologically, although both Kusa-A1/Rbpj and Kusa-A1/host cells showed well-formed bone-like structures, Kusa-A1/Rbpj cells formed larger and more compact trabecular bone tissues compared to Kusa-A1/host cells. Bone-like tissues formed by Kusa-A1/Rbpj cells also exhibited more calcium deposits. Average size of masses formed by Kusa-A1/Rbpj cells was larger than those formed by Kusa-A1/host cells. Average weight of masses formed by Kusa-A1/Rbpj cells was 67 mg, which was twice heavier than those formed by Kusa-A1/host cells, with an average weight of 25 mg (Fig. 4C). ALP-positive osteoblasts were observed around the bone trabeculae both in Kusa-A1/Rbpj and Kusa-A1/host cells (Fig. 4D). Multinucleated TRAP-positive cells were observed adjacent to trabeculae in Kusa-A1/Rbpj cells, but were less frequently observed in Kusa-A1/host cells.

4. Discussion

Osteoblasts are derived from mesenchymal progenitor cells, and various factors are involved in their development/maturation. Here, we disclose that Rbpj is a potent positive regulator of osteoblast differentiation/maturation in pluripotent mesenchymal Kusa-A1 cells. Regarding osteoblast differentiation, Runx2 is only expressed in osteochondrogenic lineages, and is a critical regulator of commitment to the osteoblast lineage and osteoblastic differentiation [24]. We revealed that Rbpj increased luciferase activity of Runx2 promoter (Fig. 2B and C), indicating that Rbpj affected transcription of Runx2 in Kusa-A1 cells. Rbpj may be involved in osteoblast differentiation since the beginning of differentiation. Furthermore, Rbpj induced an increase in Ose2 promoter activity and *Ocn* mRNA expression, which are related to osteoblast maturation. Calcium deposition and mineralization *in vitro* (mineralized nodule formation) and *in vivo* (bone-like tissue formation) were enhanced by enforced expression of *Rbpj* in Kusa-A1 cells, and enlarged and mature bone-like hard tissues were formed by Kusa-A1/Rbpj compared to original Kusa-A1 cells. These results indicated that Rbpj was a potent modulator of osteoblast differentiation and maturation.

Generally, Rbpj is a modulator of Notch signaling, and NICD binds to Rbpj in the cell nucleus to activate expression of target

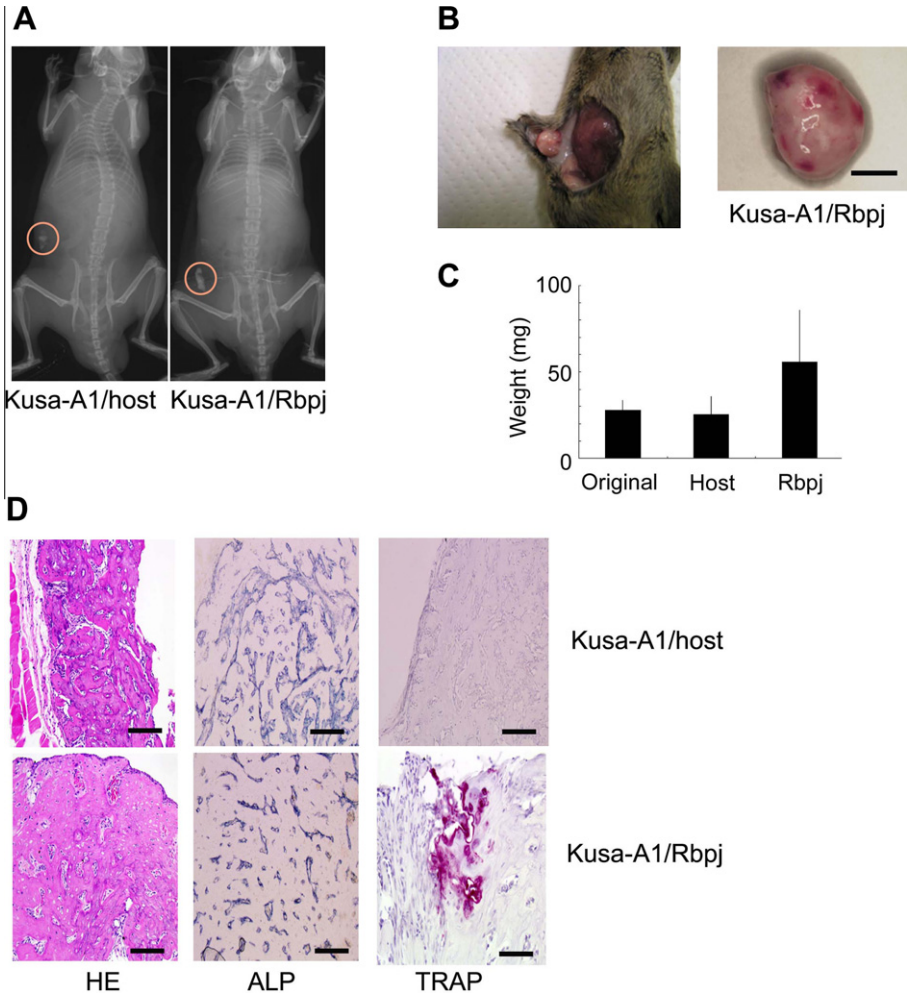

Fig. 4. *In vivo* mineralization in Kusa-A1/Rbpj cells. Radio-opaque hard tissues formed by Kusa-A1/Rbpj cells (circles in A), which were subcutaneously injected with culture medium, these were bigger than those formed by Kusa-A1/host cells (A–C). Bar indicates 5 mm (B). Histologically, hard tissues formed from Kusa-A1/Rbpj cells exhibited properties of mature bone tissues such as strong ALP activity and the presence of TRAP-positive cells, compared to those formed by Kusa-A1/host cells (D). Bar indicates 100 μm.

genes, although a Rbpj-independent Notch signaling has also reported [25]. In this study, Hes1 promoter activity was significantly attenuated by Rbpj, suggesting that Rbpj inhibited transcription of Hes1 in Kusa-A1 cells (Fig. 2E). Hes1 belongs to a basic helix–loop–helix (bHLH) transcriptional factor, and it is a typical downstream regulator of Notch signaling. It plays an essential role in differentiation of many cell types using the Notch-Hes1 pathway [26]. Hey1 is also an important downstream regulator of Notch signaling and tightly related to expression of *Rbpj* [27]. Hey1 has been reported to be a negative regulator of osteoblast differentiation/maturation [28]. GeneChip analysis revealed downregulation of not only *Hes1* but *Hey1* expressions in Kusa-A1/Rbpj cells (Fig. 2F). These data indicated that Rbpj was a negative modulator of Notch-Hes1/Hey1 signaling. Involvement of Notch signaling in osteoblast differentiation has been reported, and stable expression of the intracellular domain of Notch (NICD), which is a constitutive active form of the Notch receptor, in Kusa-A1 cells [13] and MC3T3E1 [29] induced downregulation of osteoblast differentiation/maturation. The major effects of Rbpj in Kusa-A1 cells are thought to occur from negative regulation of Notch signaling, especially of Notch-Hes1/Hey1 cascades. Several studies reported opposite functions for Rbpj compared to that of Notch signaling. Overexpression of Rbpj induces degradation of Ikkb in hepatic stellate cells, which is opposite to effects from overexpression of Notch [30]. Rbpj combined with SHARP results in recruitment of CtIP/CtBP corepressors to silence Notch target genes [31].

In conclusion, we revealed that Rbpj was a positive modulator of osteoblast differentiation/maturation, and the major function of Rbpj was related to downregulation of Notch-Hes1/Hey1 signaling, although it had possible direct effects on osteoblast differentiation/maturation. Importance of the Notch-independent activity of Rbpj was pointed out in development of socket cells [32].

Acknowledgments

This study was supported by Grants-in-Aid from the Japanese Society for the Promotion of Science #22390357 and #22659343 to N.K.

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