

Osteoblastic γ -Aminobutyric Acid, Type B Receptors Negatively Regulate Osteoblastogenesis toward Disturbance of Osteoclastogenesis Mediated by Receptor Activator of Nuclear Factor κ B Ligand in Mouse Bone*

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Yoshifumi Takahata¹, Takeshi Takarada¹, Eiichi Hinoi, Yukari Nakamura, Hiroyuki Fujita, and Yukio Yoneda²

From the Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Ishikawa 920-1192, Japan

The prevailing view is that signaling machineries for the neurotransmitter GABA are also expressed by cells outside the CNS. In cultured murine calvarial osteoblasts, mRNA was constitutively expressed for both subunits 1 and 2 of metabotropic GABA_B receptor (GABA_BR), along with inhibition by the GABA_BR agonist baclofen of cAMP formation, alkaline phosphatase (ALP) activity, and Ca²⁺ accumulation. Moreover, baclofen significantly inhibited the transactivation of receptor activator of nuclear factor- κ B ligand (RANKL) gene in a manner sensitive to a GABA_BR antagonist, in addition to decreasing mRNA expression of bone morphogenetic protein-2 (BMP2), osteocalcin, and osterix. In osteoblastic MC3T3-E1 cells stably transfected with GABA_BR1 subunit, significant reductions were seen in ALP activity and Ca²⁺ accumulation, as well as mRNA expression of osteocalcin, osteopontin, and osterix. In cultured calvarial osteoblasts from GABA_BR1-null mice exhibiting low bone mineral density in tibia and femur, by contrast, both ALP activity and Ca²⁺ accumulation were significantly increased together with promoted expression of both mRNA and proteins for BMP2 and osterix. No significant change was seen in the number of multinucleated cells stained for tartrate-resistant acid phosphatase during the culture of osteoclasts prepared from GABA_BR1-null mice, whereas a significant increase was seen in the number of tartrate-resistant acid phosphatase-positive multinucleated cells in co-culture of osteoclasts with osteoblasts isolated from GABA_BR1-null mice. These results suggest that GABA_BR is predominantly expressed by osteoblasts to negatively regulate osteoblastogenesis through down-regulation of BMP2 expression toward disturbance of osteoclastogenesis after down-regulation of RANKL expression in mouse bone.

In the mammalian CNS, GABA is the major inhibitory amino acid neurotransmitter with two major receptor subtypes categorized into ionotropic and metabotropic receptors on the

basis of homologous intracellular signals (1). The ionotropic GABA_A receptor is a heteromeric protein complex composed of a number of different receptor subunits, whereas the GABA_CR is derived from an assembly between various isoforms of the ρ subunit (2). The metabotropic GABA_BR belongs to a superfamily of the seven-transmembrane-domain receptors with high similarity to metabotropic receptors for the central excitatory amino acid neurotransmitter L-glutamic acid (3). The GABA_BR couples to adenylyl cyclase through trimeric G-proteins to negatively regulate intracellular cAMP formation, in addition to inhibiting voltage-sensitive Ca²⁺ channels and activating voltage-sensitive K⁺ channels, respectively. The GABA_BR is orchestrated by a heterodimer comprised of members of GABA_BR1 and GABA_BR2 subunits, neither of which is fully functional when individually expressed. Any GABA_BR1 subunits are unable to activate K⁺ channels alone (4), whereas heterodimerization between GABA_BR1 and GABA_BR2 subunits is required for the orchestration of fully functional GABA_BR at the cell surface in HEK293T cells (5). A view that GABAergic signaling machineries are functionally expressed by cells outside the CNS is now prevailing. For example, we have recently shown that functional GABA_BR is expressed in rat cultured calvarial osteoblasts toward negative regulation of osteoblastogenesis (6).

In bone, both formation and maintenance are sophisticatedly regulated by bone-forming osteoblasts and bone-resorbing osteoclasts (7–9). The osteoblast lineage is derived from primitive multipotent mesenchymal stem cells with potentiality to differentiate into bone marrow stromal cells, chondrocytes, muscles, and adipocytes, whereas osteoclasts are multinucleated cells derived from the fusion of mononuclear hematopoietic precursors. The development and differentiation of these two distinct cells are under tight regulation by a number of endogenous substances. These include growth factors, cytokines, and hormones, which are individually secreted through endocrine, paracrine/autocrine, and neurocrine systems essential for the delicate balancing between bone formation and resorption by the two different cells in the bone marrow microenvironment. An imbalance between these two cells leads to the pathogenesis and etiology of certain bone metabolic diseases, including osteoporosis and osteopetrosis (10). Several transcription factors are identified to be responsible for the regulation of cell differentiation and function as a master regu-

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¹ Both authors equally contributed to this work.

² To whom correspondence should be addressed: Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan. Tel./Fax: 81-76-234-4471; E-mail: yyoneda@p.kanazawa-u.ac.jp.

lator of osteoblastogenesis. These include runt-related transcription factor-2 (Runx2)³ and osterix. Runx2 is a cell-specific member of the runt family of transcription factors that plays a critical role in cellular differentiation processes during osteoblastogenesis. Runx2 is the most specific and crucial molecular marker of the osteoblast lineage during cellular maturation among different transcription factors identified to date (11–14). Runx2 expression is indispensable and sufficient to induce osteoblastic differentiation for subsequent regulation of expression of a variety of genes characteristic to the osteoblastic phenotype, including osteocalcin, bone sialoprotein, osteopontin, and type 1 collagen. By contrast, osterix is a downstream signal of Runx2 for sufficiently inducing osteoblastic differentiation and maturation (13). In the present study, therefore, we have further investigated the role of GABA_BR in mechanisms underlying skeletogenesis mediated by both osteoblastogenesis and osteoclastogenesis using stable transfectant of GABA_BR1 subunit in osteoblastic MC3T3-E1 cells and cells isolated from mice defective of GABA_BR1 subunit.

EXPERIMENTAL PROCEDURES

Materials—MC3T3-E1 cells were purchased from RIKEN Cell Bank (Saitama, Japan). *Taq* polymerase was obtained from Takara (Tokyo, Japan). Moloney murine leukemia virus reverse transcriptase, Lipofectamine, Plus reagent, and α -minimal essential medium (α -MEM) were supplied by Invitrogen. Recombinant mouse macrophage-colony stimulating factor (M-CSF) and recombinant mouse receptor activator of nuclear factor- κ B ligand (RANKL) were purchased from R & D Systems International (Minneapolis, MN). A Bio-Rad protein assay kit was provided by Bio-Rad Laboratories. Nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolylphosphatase *p*-toluidine salt was obtained from Roche Applied Science (Mannheim, Germany). Both ECLTM detection reagent and protein A-Sepharose were purchased from Amersham Biosciences (Buckinghamshire, UK). Anti-GABA_BR1 antibody was purchased from Chemicon (Temecula, CA). The dual luciferase assay system and the promoterless pGL-3 basic vector were provided by Promega (Madison, WI). GABA_BR1-null mice were kindly donated by Novartis Pharma AG (Basel, Switzerland). All other chemicals used were of the highest purity commercially available.

Preparation of Osteoblasts—The protocol employed here meets the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Osteoblasts were prepared from calvaria of 2–6-day-old neonatal GABA_BR1-null and WT mice of Balb/c strain by the sequential enzymatic digestion method as described previously (15). In

brief, mouse calvaria were gently incubated at 37 °C for 15 min with 0.2% (w/v) collagenase in α -MEM, followed by collection of cells in supernatants thus obtained. This incubation was consecutively repeated four times. Cells obtained during the last three digestion processes were altogether collected in α -MEM containing 10% (v/v) FBS and several antibiotics, followed by centrifugation at 250 \times g for 5 min. The pellets were suspended in α -MEM containing 10% FBS. The cells were plated at a density of 1 \times 10⁴ cells/cm² in appropriate dishes and then cultured at 37 °C for different periods under 5% (v/v) CO₂ with medium change every 3 days. Throughout the experiments, α -MEM containing 10% FBS, 50 μ g/ml ascorbic acid, and 5 mM sodium β -glycerophosphate were used for promotion of osteoblastic differentiation.

Preparation of Bone Marrow Stromal Cells—Bone marrows were isolated from tibias and femurs of GABA_BR1-null and WT Balb/c male mice at 8 weeks old and cultured in α -MEM containing 15% FBS for 24 h as described previously (16). Cultures were washed with PBS to remove nonadherent cells five times, and adherent cells were further cultured in α -MEM containing 15% FBS under subconfluent conditions. The cells were then treated with 0.25% trypsin and 0.03% EDTA for 5 min, followed by collection of floating cells in resultant supernatants for subsequent culture in α -MEM containing 15% FBS for an additional 24 h. Adherent cells were collected as bone marrow stromal cells. For promotion of osteoblastic differentiation, the cells were plated at 1 \times 10⁴ cells/cm² and cultured in α -MEM containing 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid, and 15% FBS for 3 days under 5% CO₂.

Preparation of Osteoclasts—Osteoclasts were prepared from bone marrows according to the procedures described previously (17). In brief, bone marrows were prepared from tibias and femurs of male GABA_BR1-null and WT mice at 4 weeks of age and cultured for 24 h with M-CSF at 10 ng/ml in α -MEM containing 10% FBS. After culturing for 24 h in the presence of M-CSF alone, supernatants were collected by gentle aspiration, followed by lamination of nonadherent cells collected in supernatants on Ficoll gradient and subsequent centrifugation at 500 \times g for 15 min. The cells fractionated in the monocyte fraction were defined as preosteoclastic cells throughout this study. These preosteoclasts were collected and suspended in α -MEM containing 10% FBS, 20 ng/ml M-CSF, and 20 ng/ml RANKL. The cells were then plated at a density of 1 \times 10⁵ cells/cm², followed by culturing in α -MEM containing 10% FBS, 20 ng/ml M-CSF and 20 ng/ml RANKL at 37 °C under 5% CO₂ for 5 consecutive days unless indicated otherwise. For tartrate-resistant acid phosphatase (TRAP) staining, cultured cells were fixed with 10% formalin in PBS for 10 min and subsequently fixed with ethanol-acetone (50:50, v/v) for 1 min at room temperature. The cells were then incubated in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate as a substrate and fast red violet LB salt as a dye in the presence of 50 mM sodium tartrate. TRAP-positive cells with more than five nuclei were scored as TRAP-positive multinucleated cells (MNCs).

Co-culture of Osteoclasts and Osteoblasts—Calvarial osteoblasts were seeded on a 48-well plate at the density of 1 \times 10⁴ cells/cm² and cultured for 24 h before the beginning of osteo-

³ The abbreviations used are: Runx2, runt-related transcription factor-2; ALP, alkaline phosphatase; α -MEM, alpha minimal essential medium; BMP2, bone morphogenetic protein-2; CRE, cAMP responsive element; EV, empty vector; GAT, γ -aminobutyric acid transporter; M-CSF, macrophage-colony stimulating factor; MNC, multinucleated cell; OPG, osteoprotegerin; OSE2, osteoblast specific element-2; RANKL, receptor activator of nuclear factor- κ B ligand; TRAP, tartrate-resistant acid phosphatase.

GABA_B Receptor in Osteoblasts

TABLE 1

Primers used for RT-PCR analysis in this study

Isoforms	Upstream (5'-3')	Downstream (5'-3')	Estimated Base pair
GABA _A R			
α1	CATTCTGAGCACTCTCTCGGGAAG	GTGATACGCAGGAGTTTATTGGGC	396
α2	AGGTGGTGCTGGCTAACATCCAA	AACGGAGTCAGAAGCATTGTAAGTCC	549
α3	CCAGCAGCCCCAACCAAGAA	GTTTGCAGATCATGCCCTTG	325
α4	CAAAACCTCTCCAGAAGTTCCA	ATGTTAAATGCCCAAATGTGACT	532
α5	TGACCCAAACCCTCCTTGCTTTCT	ACCGCAGCCTTTCATCTTTCC	300
α6	TGTTGCTTCTCCCCTGGCTCTTC	TCTCATCGGACAGTCAGCGTTG	473
β1	GTTTGGGGCTTCTCTTTTCT	AGTTACTGCTCCCTCTCCTCCATT	578
β2	CAGGTTCTTATCCCAGATGTCCC	GGTCCATCTTGTGACATCCAGG	408
β3	CTTTTCGGCATCTTCTCGG	TCCAGCCAGTAACAGCCTTG	587
γ1	TAGTAACAATAAAGGAAAACCACCAGA	CCAGATTGAACAAGGCAAAAGCT	296
γ2	TGGTGACTATGTGGTTATGTCCGTG	AGGTGGGTGGCATTGTTTCATT	423
γ3	TTCTCTCTGCCTGTTCTCGG	CGAAGGCGACTGTCTGTCCAG	304
GABA _B R			
1	GGCATGGATGTCCTGACTCT	CTCGTTGTTGTTGGTGGATG	394
2	TTGATGGTGTGGAGGCGTCT	GCTGTGGAGTCTTCCCTGAG	628
GABA _C R			
ρ1, ρ2	ATGTGCAACATGGACTTCAGC	TGATGGTGGACATGGTCAGCA	394
ρ3	TGTCCACAATCGTCACTGGT	CCTCCAGGGATTTTCTCT	362
GABA transporter			
m GAT1	CACGCTCCTGGCTTTTAGTC	CCAGAACAGAGTGGCAGTGA	552
m GAT2	GTCCACAGGCAAGGTTGTTT	ACTCTTTCGGAGCTGCTGAG	556
m GAT3	GAATTCAGAAAGGCCAATGA	AAGCCAGGATGGAGAAGAT	494
m GAT4	TGCTGAGGTGGCAGAATCAGGTCC	TGTTGTACTIONTGGCGGTTTG	480
Vesicular GABA transporter			
VGAT	CCTGGGGTGTTCCTCATCA	ACTTCCTTGGTCTCGTCGGC	649
Glutamic acid decarboxylase			
GAD65	CCTTCTGGTGAGTGCCACAGCTGGAACC	TTTGAGAGGCGGCTCTTCTCTCTTATTG	469
GAD67	CACAAACTCAGCGGCATAGA	GAGATGACCATCCGGAAGAA	532
Osteopontin	TCACCATTGCGATGAGTCTG	ACTTGTGGCTCTGATGTTCC	437
Osteocalcin	GACAAAGCCTTCATGTCCAAGC	AAAGCCGAGCTGCCAGAGTTTG	241
Cbfa1 (Runx2)	GGCAGTCACTAAGATCCCA	CCCAGACTCCATGGCTTTAC	289
Osterix	CCGCACGACAACCGCACCAT	CGCTCCGGCCCCACAAATCTC	563
Col I	CCTGGCTCTAGAGGTGAACG	GGATGCCCTGAGGACCCTA	300
Bmp2	GACGGACTGCGGTCTCCTAAAG	TCTGCAGATGTGAGAACTCGTC	472
RANKL	GGTCGGCAATTCTGAATT	GGGGAATTACAAAGTGCAGGA	814
OPG	ACCAGAAACTGGTCATCAGC	CTGCAATACACACTCATCACT	157
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA	452

clast culture. Osteoclasts were prepared from bone marrows derived from tibia and femur of male mice at 4 weeks, followed by lamination of cells in supernatants on Ficoll gradient and subsequent centrifugation at 500 × g for 15 min as described above. Cells fractionated in the monocyte fraction were collected and suspended in α-MEM containing 10% FBS and 10 nM 1,25-dihydroxycholecalciferol, followed by lamination of 1 × 10⁵ cells on the osteoblastic layer and subsequent culture at 37 °C under 5% CO₂ for 5 consecutive days.

Cell Line Culture—MC3T3-E1 cells were cultured in α-MEM containing 10% FBS. For differentiation, culture medium was replaced with α-MEM containing 50 μg/ml ascorbic acid and 5 mM sodium β-glycerophosphate. Culture medium was changed every 2–3 days.

RT-PCR—Cultured calvarial osteoblasts were superficially washed with PBS twice, followed by extraction of total RNA using ISOGEN (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions and subsequent synthesis of cDNA with 25 ng/μl oligo(dT)₁₈ primer, 0.5 mM deoxy nucleotide triphosphate mix, and Moloney murine leukemia virus reverse transcriptase. Reverse transcriptase reaction was run at 37 °C for 50 min, and an aliquot of synthesized cDNA was directly

used for PCR performed in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each of deoxy nucleotide triphosphate, 20 pmol of each primer set (Table 1), and 2 units of *Taq* DNA polymerase. Electrophoresis was run for an aliquot of PCR amplification products on a 1.5% agarose gel, followed by detection of DNA with ethidium bromide. In preliminary experiments, a clearly linear correlation was optimized with each primer set, respectively. Quantitative analysis was done at the cycle number with high linearity between mRNA expression and cDNA production using primers for the housekeeping gene GAPDH. Densitometry was done with the individual PCR products by using a densitograph, followed by calculation of ratios of expression of mRNA for each gene over that for GAPDH for normalization as described previously (18).

Quantification of cAMP—Osteoblasts cultured for 7 days were washed twice with PBS, followed by incubation in PBS containing 1 mM 3-isobutyl-1-methylxanthine for 20 min at 37 °C under 5% CO₂, and subsequent addition of 10 μM forskolin in either the presence or absence of the GABA_BR agonist (R)-baclofen at 1 mM and the antagonist 2-hydroxysaclofen at 1 mM in PBS containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37 °C under 5% CO₂ as described previously (6). The

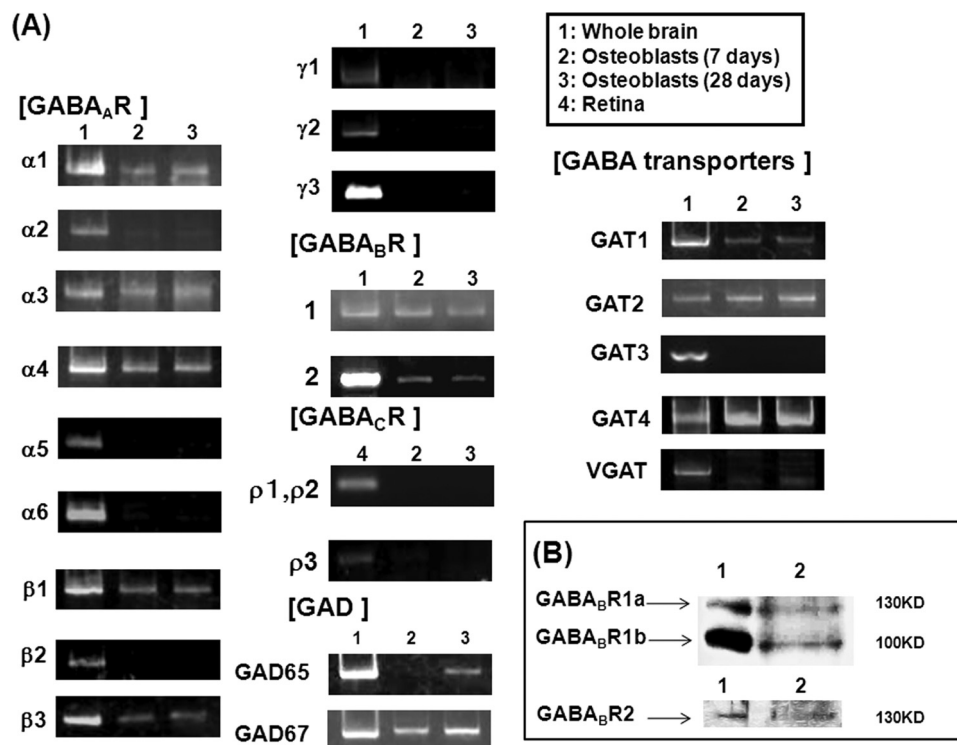


FIGURE 1. **GABAergic signaling molecules.** A, mouse calvarial osteoblasts were cultured for 7 or 28 days, followed by extraction of total RNA for subsequent RT-PCR analysis. B, mouse calvarial osteoblasts were cultured for 7 days for subsequent immunoblotting with the anti-GABA_BR1 and anti-GABA_BR2 antibodies. Typical pictures are shown in the figure with similar results in at least three different examinations. Mouse whole brain and retina were used as a positive control.

cells were sonicated in lysis buffer (0.25% solution of dodecyltrimethylammonium bromide in 0.05 M acetate buffer, 0.02% (w/v) bovine serum, albumin and 0.01% (w/v) preservative). Measurement of cAMP was conducted by the enzyme immunoassay according to the manufacturer's instructions.

Determination of ALP Activity—Calvarial osteoblasts were plated at a density of 1×10^4 cells/cm² in 24-well dishes and cultured for different days. The cells were washed twice with cold PBS and then sonicated in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% (v/v) Triton X-100. The assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM MgCl₂, and 10 mM *p*-nitrophenylphosphoric acid was added at a volume of 200 μl into 10 μl of cell suspensions, followed by reaction for 30 min at 37 °C and subsequent immediate determination of the absorbance of *p*-nitrophenol at 405 nm. Protein concentration was determined with a Bio-Rad protein assay kit, and ALP activity was linearly increased with incubation time up to 60 min under our experimental conditions.

Von Kossa Staining—The formation of bone nodules was analyzed by the von Kossa staining method. In brief, the cells were washed twice with PBS, followed by fixation with 10% formalin neutral buffer solution and subsequent incubation with 5% silver nitrate for 60 min under UV light. The dishes were rinsed with distilled water, and then the nodules formed were photographed using a phase contrast microscope.

Immunoblotting Analysis—Cell homogenates were prepared as described previously (19) in 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% 2-mercaptoethanol at a volume ratio of 1:4, followed by boiling at 100 °C for 10 min. Aliquots were subjected to electro-

phoresis on a 7.5% SDS-polyacrylamide gel at a constant current of 10 mA/plate for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skim milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20 (TBST), the membrane was incubated with a guinea pig polyclonal antibody against GABA_BR1 or GABA_BR2 subunit diluted with TBST containing 1% skim milk to 2000-fold and then incubated with the anti-guinea pig IgG antibody conjugated with horseradish peroxidase (1:40,000). Finally, the membrane was incubated with ECL detection reagent (Amersham Biosciences), followed by exposure to x-ray films for different periods to obtain most adequate blots.

Luciferase Reporter Assay—Both p6OSE2-luc and pOG2-luc were generous gifts from Dr. G. Karsenty (Baylor College of Medicine, Houston, TX). Cbfa1 promoter (P1 promoter of type 2 Runx2) from -2164 to +111 bp (20) was kindly donated by Dr. P. Ducy (Baylor College of Medicine, Houston, TX). A pcDNA3.1-Runx2 (21) was kindly provided by Dr. S. C. Bae (Chungbuk National University, Cheongju, Korea). Luciferase reporter plasmids were co-transfected with a SV40 *Renilla* luciferase construct in either the presence or absence of Runx2 expression vector and different test drugs into MC3T3-E1 cells using Lipofectamine and Plus reagent for 1 h in Opti-MEM. One day after transfection, the cells were lysed for determination of luciferase activity using specific substrates in a luminometer according to the manufacturer's protocol (Promega). Transfection efficiency was normalized by determining the activity of *Renilla* luciferase (19).

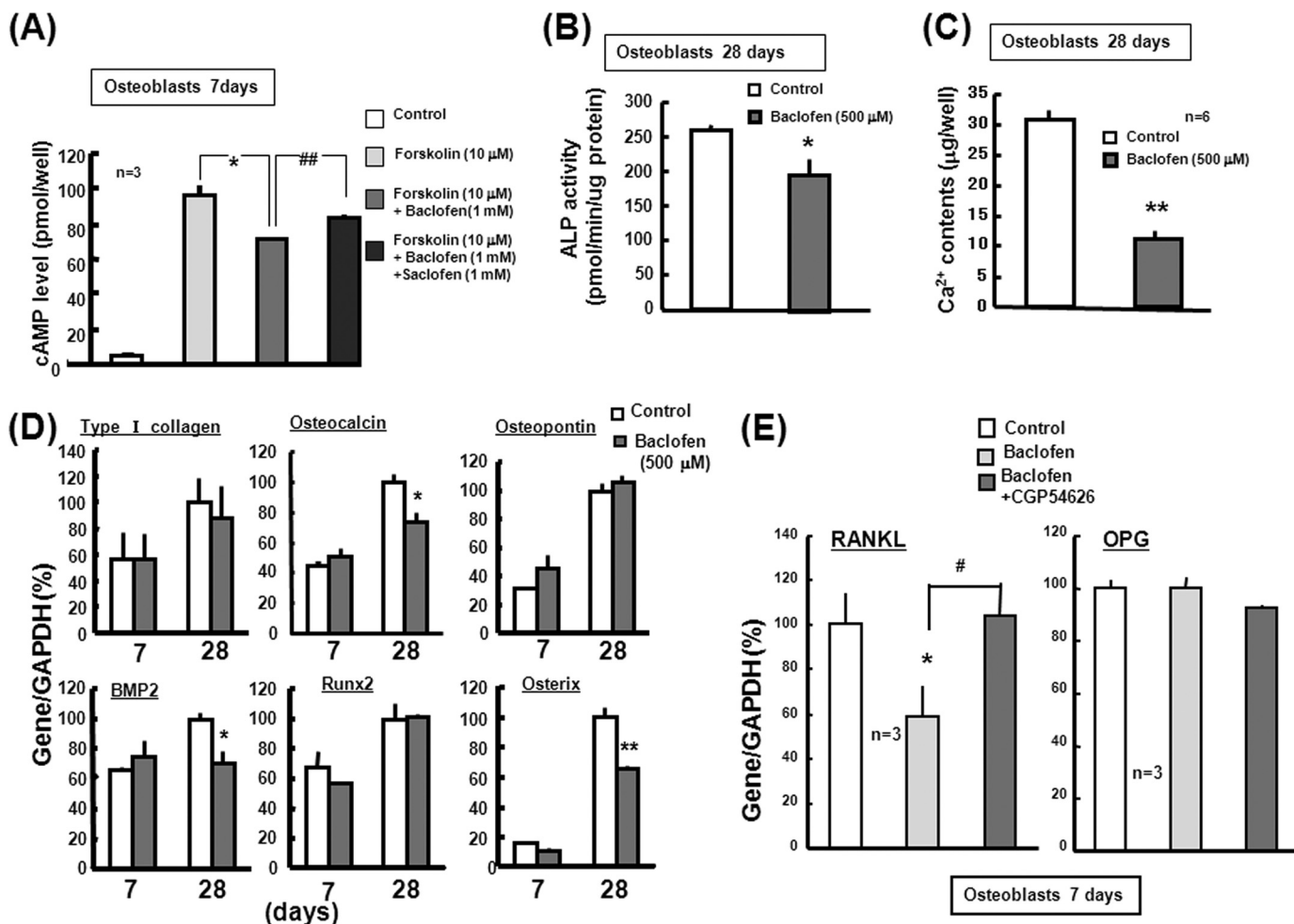


FIGURE 2. Effect of baclofen on osteoblastic differentiation. *A*, mouse calvarial osteoblasts were cultured for 7 days, followed by incubation with 10 μM forskolin in either the presence or absence of 1 mM baclofen and 1 mM 2-hydroxysaclofen for 10 min and subsequent determination of cAMP levels by enzyme immunoassay. *, $p < 0.05$, significantly different from the value obtained in the presence of forskolin alone; ##, $p < 0.01$, significantly different from the value obtained in the presence of both forskolin and baclofen. cAMP levels were as follows (pmol/well): control, 4.8 ± 0.6 ; forskolin, 96.7 ± 5.8 ; baclofen/forskolin, 71.4 ± 1.3 ; 2-hydroxysaclofen/baclofen/forskolin, 84.1 ± 1.4 . *B* and *C*, mouse osteoblasts were cultured in α -MEM in either the presence or absence of 500 μM baclofen for 28 days, followed by determination of ALP activity (*B*) and Ca²⁺ accumulation (*C*). *D*, total RNA was extracted from mouse osteoblasts cultured for different periods in either the presence or absence of 500 μM baclofen, followed by quantitative determination of mRNA expression of different markers. The values are the means \pm S.E. in three to six separate determinations. *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in the absence of baclofen. *E*, calvarial osteoblasts were cultured in either the presence or absence of 0.5 mM baclofen and 0.5 mM CGP54628 for 7 days, followed by determination of mRNA expression of both RANKL and OPG. *, $p < 0.05$, significantly different from each control value obtained in the absence of added ligand; #, $p < 0.05$, significantly different from the value obtained in the presence of baclofen alone.

Establishment of Stable Transfectants—MC3T3-E1 cells were plated at a density of 1.5×10^5 cells/cm². After 24 h, they were stably transfected with PCI-neo mammalian expression vector containing the full-length coding region of GABA_BR1 subunit (22), which was kindly donated by Dr. K. Kaupmann (Novartis Pharma AG, Basel, Switzerland), or with the empty vector (EV) using 2 μg of DNA and Lipofectamine Plus reagent (Invitrogen) in 10 ml of Dulbecco's modified Eagle's medium as described previously (17). After 24 h, and every 48 h thereafter for 2 weeks, media were replaced with fresh media containing 600 μg/ml of G418 (Sigma). Pools of clones between passages 2 and 5 were used for these experiments.

Determination of BrdU Incorporation—Cultured cells were treated with diluted BrdU labeling solution for 2 h at 37 °C, followed by immunohistochemical staining using a BrdU staining kit (Zymed Laboratories Inc.) according to the manufacturer's instructions.

Data Analysis—The results are all expressed as the means \pm S.E., and the statistical significance was determined by the two-tailed and unpaired Student's *t* test or the one-way analysis of variance with Bonferroni/Dunnnett post hoc test.

RESULTS

Expression of GABAergic Signaling Machineries in Mouse Osteoblasts—To confirm mRNA expression of different GABAergic signaling machineries as shown in rat calvarial osteoblasts (6), mouse calvarial osteoblasts were isolated and cultured for 7–28 days for determination of mRNA expression by RT-PCR analysis. Both mouse whole brain and retina were used as a positive control. In mouse osteoblasts cultured for 7 or 28 days, mRNA expression was seen for $\alpha 1$, $\alpha 3$, $\alpha 4$, $\beta 1$, and $\beta 3$ subunits of GABA_A receptor, GABA_BR subunits 1 and 2, glutamic acid decarboxylase 65 and 67 isoforms, and GABA transporter (GAT) 1, GAT2, and GAT4 isoforms, but not for $\alpha 2$, $\alpha 5$,

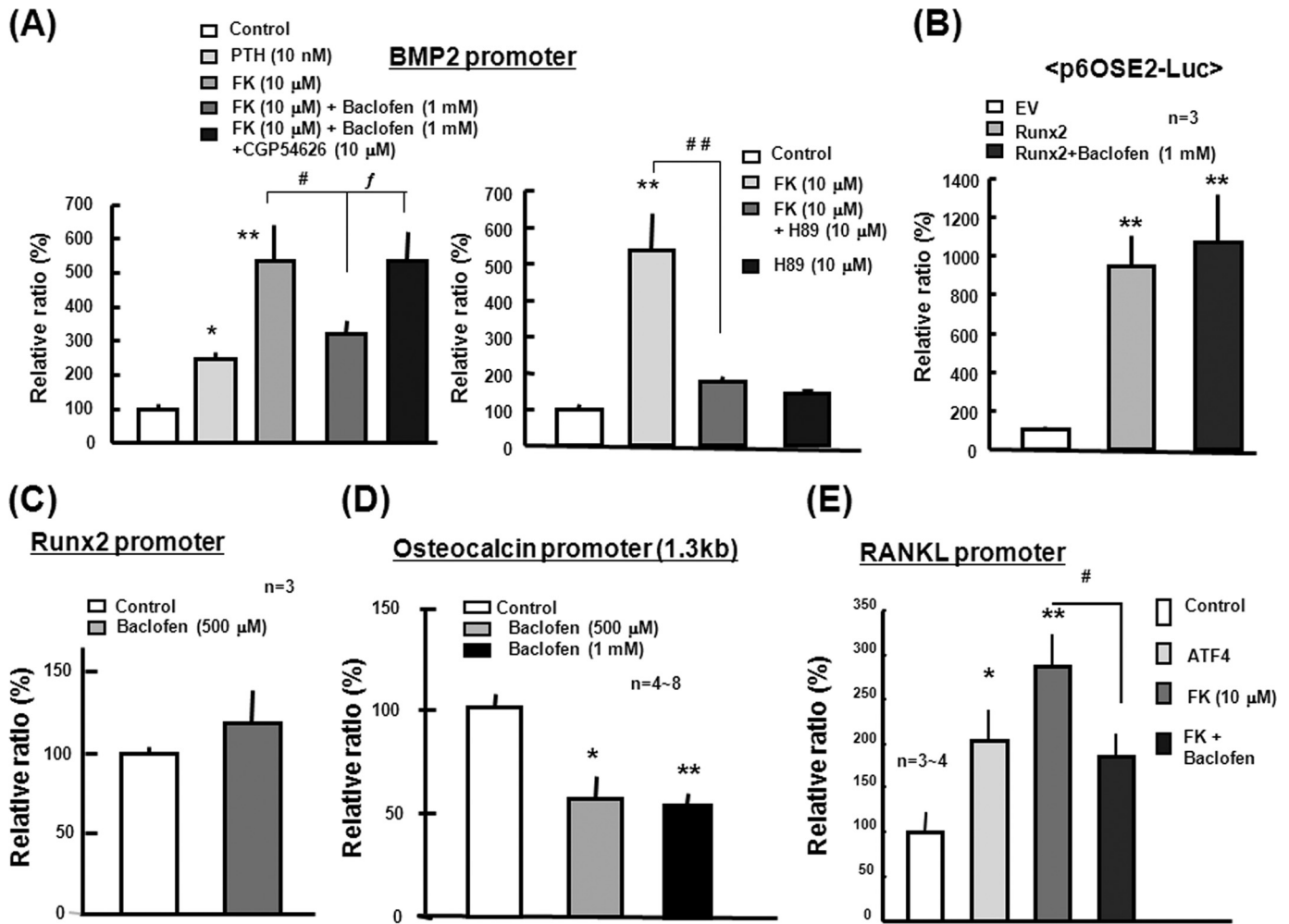


FIGURE 3. Luciferase reporter activity in MC3T3-E1 cells. MC3T3-E1 cells were transfected with a luciferase reporter plasmid containing BMP2 promoter (A), six tandem copies of the Runx2 binding site OSE2 (B), Runx2 promoter (C), osteocalcin promoter (D), or RANKL promoter (E) in either the presence or absence of Runx2 expression vector for 1 h, followed by further culture for an additional 24 h and subsequent incubation with different test drugs for 8–24 h. The values are the means \pm S.E. from three to eight different experiments. **, $p < 0.01$, significantly different from each control value obtained in the absence of any added test drugs or Runx2 expression vector; #, $p < 0.05$; ##, $p < 0.01$, significantly different from the value obtained in the presence of forskolin (FK) alone. *f*, $p < 0.05$, significantly different from the value obtained in the presence of both forskolin and baclofen.

$\alpha 6$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ subunits of GABA_A receptor, $\rho 1$, $\rho 2$, and $\rho 3$ subunits of GABA_CR, GAT3 isoform, or vesicular GAT (Fig. 1A). In mouse osteoblasts cultured for 7 days, moreover, immunoreactive GABA_BR1a, GABA_BR1b and GABA_BR2 subunits were all detected at the respectively corresponding molecular weight sizes on immunoblotting analysis (Fig. 1B).

GABA_BR Signaling in Mouse Osteoblasts—An attempt was next made to determine whether GABA_BR is functional for the formation of cAMP in mouse calvarial osteoblasts. The cells were exposed for 30 min to 10 μ M forskolin in either the presence or absence of the GABA_BR agonist baclofen at 1 mM. The addition of forskolin markedly increased the endogenous level of cAMP in a manner sensitive to the prevention by baclofen, whereas the prevention by baclofen was significantly blocked by the GABA_BR antagonist saclofen at 1 mM (Fig. 2A). In cultured osteoblasts exposed to baclofen for 28 consecutive days, significant decreases were seen in ALP activity (Fig. 2B) and Ca²⁺ content (Fig. 2C) as osteoblastic maturation indices. Baclofen significantly decreased mRNA expression of BMP2, osteocalcin, and osterix in cells cultured for 28 days but not in those for

7 days, without significantly affecting mRNA expression of type I collagen, osteopontin, or Runx2 irrespective of the culture periods examined (Fig. 2D). Therefore, GABA_BR would be functionally expressed by murine calvarial osteoblasts to negatively regulate cellular differentiation after tonic activation.

Gene Transactivation—Osteoblastic MC3T3-E1 cells were transfected with luciferase reporter plasmid linked to BMP2 promoter, followed by culture for an additional 8 h in either the presence or absence of test drugs. Forskolin at 10 μ M was more effective in significantly increasing luciferase activity than parathyroid hormone at 10 nM, whereas further addition of 1 mM baclofen significantly prevented the increase by forskolin in a manner sensitive to the GABA_BR antagonist CGP54626 (Fig. 3A, left panel). The addition of the protein kinase A inhibitor H89 also prevented the increase by forskolin without affecting luciferase activity alone (Fig. 3A, right panel). MC3T3-E1 cells were then transfected with osteoblast-specific element (OSE2) reporter plasmid containing six tandem copies of Runx2-binding element in either the presence or absence of Runx2 expression vector, followed by exposure to 1 mM baclofen for 8 h.

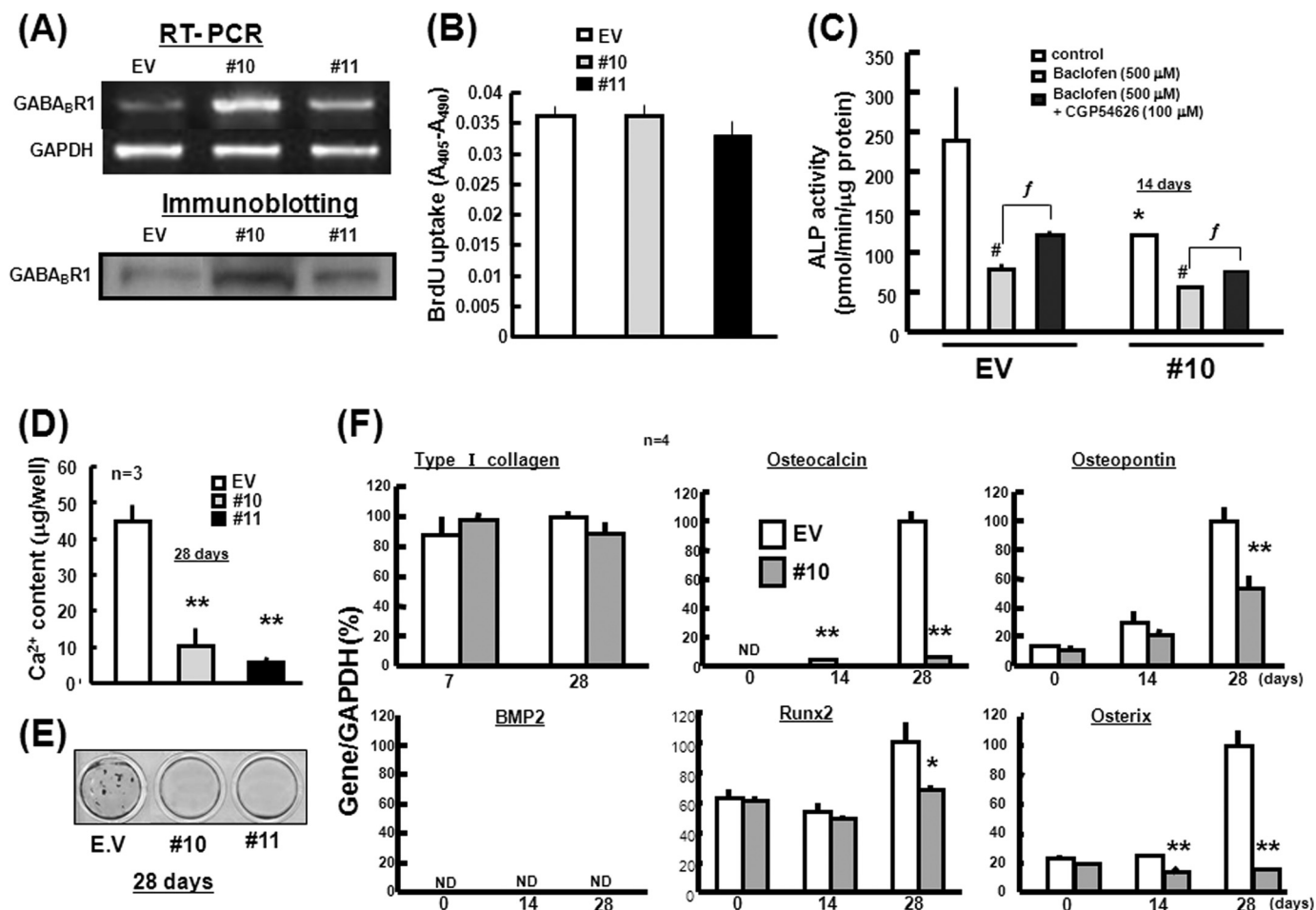


FIGURE 4. Establishment of GABA_BR1 stable transfectants. *A*, MC3T3-E1 cells were stably transfected with GABA_BR1 expression vector or EV, followed by determination of expression levels of mRNA (upper panel) and corresponding protein (lower panel) for GABA_BR1. *B*, stable transfectant clones were cultured for 24 h in the absence of ascorbic acid and β-glycerophosphate, followed by determination of BrdU incorporation. *C*, stable transfectant clones were cultured for 14 days in either the presence or the absence of baclofen, followed by determination of ALP activity. GABA_BR1 antagonist CGP54626 was also added as needed at 10 μM. *D* and *E*, stable transfectant clones were cultured for 28 days in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate, followed by determination of Ca²⁺ accumulation (*D*) and von Kossa staining (*E*). *F*, total RNA was extracted from stable transfectant clones, followed by determination of mRNA expression. The values are the means ± S.E. in three to four different experiments. *, *p* < 0.05; **, *p* < 0.01, significantly different from each control value obtained in cells transfected with EV alone. #, *p* < 0.05, significantly different from the value in cells not exposed to any test drugs. *f*, *p* < 0.05, significantly different from the value obtained in cells exposed to baclofen alone. ND, not detectable.

Introduction of Runx2 significantly increased luciferase activity, whereas baclofen did not significantly affect the luciferase activity elevated by Runx2 introduction (Fig. 3B). MC3T3-E1 cells were further transfected with luciferase reporter plasmid linked to Runx2 promoter, followed by exposure to baclofen for 24 h. However, baclofen failed to significantly affect luciferase activity (Fig. 3C). MC3T3-E1 cells were next transfected with osteocalcin promoter reporter plasmid, followed by exposure to baclofen at concentrations of 0.5–1 mM. Baclofen was effective in significantly decreasing osteocalcin promoter activity at concentrations of 0.5–1 mM (Fig. 3D). Transfection with activating transcription factor-4 expression vector led to a significant increase in luciferase activity in MC3T3-E1 cells with RANKL promoter, whereas forskolin more than doubled luciferase activity in a manner sensitive to the prevention by baclofen in cells with RANKL promoter (Fig. 3E). Accordingly, activation of GABA_BR would disturb cellular differentiation through a mechanism relevant to the interference with transactivation of BMP2, but not of Runx2, in murine osteoblasts.

Overexpression of GABA_BR1 Subunit—To further analyze the role of GABA_BR in osteoblastogenesis, osteoblastic MC3T3-E1 cells were stably transfected with GABA_BR1 subunit expression vector or EV. Several transfectant clones were established with mRNA (Fig. 4A, upper panel) and corresponding protein (Fig. 4A, lower panel) for GABA_BR1 subunit. However, stable GABA_BR1 transfection failed to significantly affect BrdU incorporation into osteoblastic cells before differentiation (Fig. 4B). To next investigate whether GABA_BR1 subunit affects cellular differentiation in osteoblasts, stable transfectants were cultured with differentiation inducers such as ascorbic acid and β-glycerophosphate for determination of mRNA expression of cellular differentiation markers, in addition to ALP activity, Ca²⁺ accumulation, and von Kossa staining. In stable GABA_BR1 transfectants cultured for 14 days, a significant decrease was seen in ALP activity, whereas baclofen similarly inhibited ALP activity in a manner sensitive to CGP54626 in both GABA_BR1 and EV transfectants (Fig. 4C). Stable GABA_BR1 transfection led to significant reductions of Ca²⁺

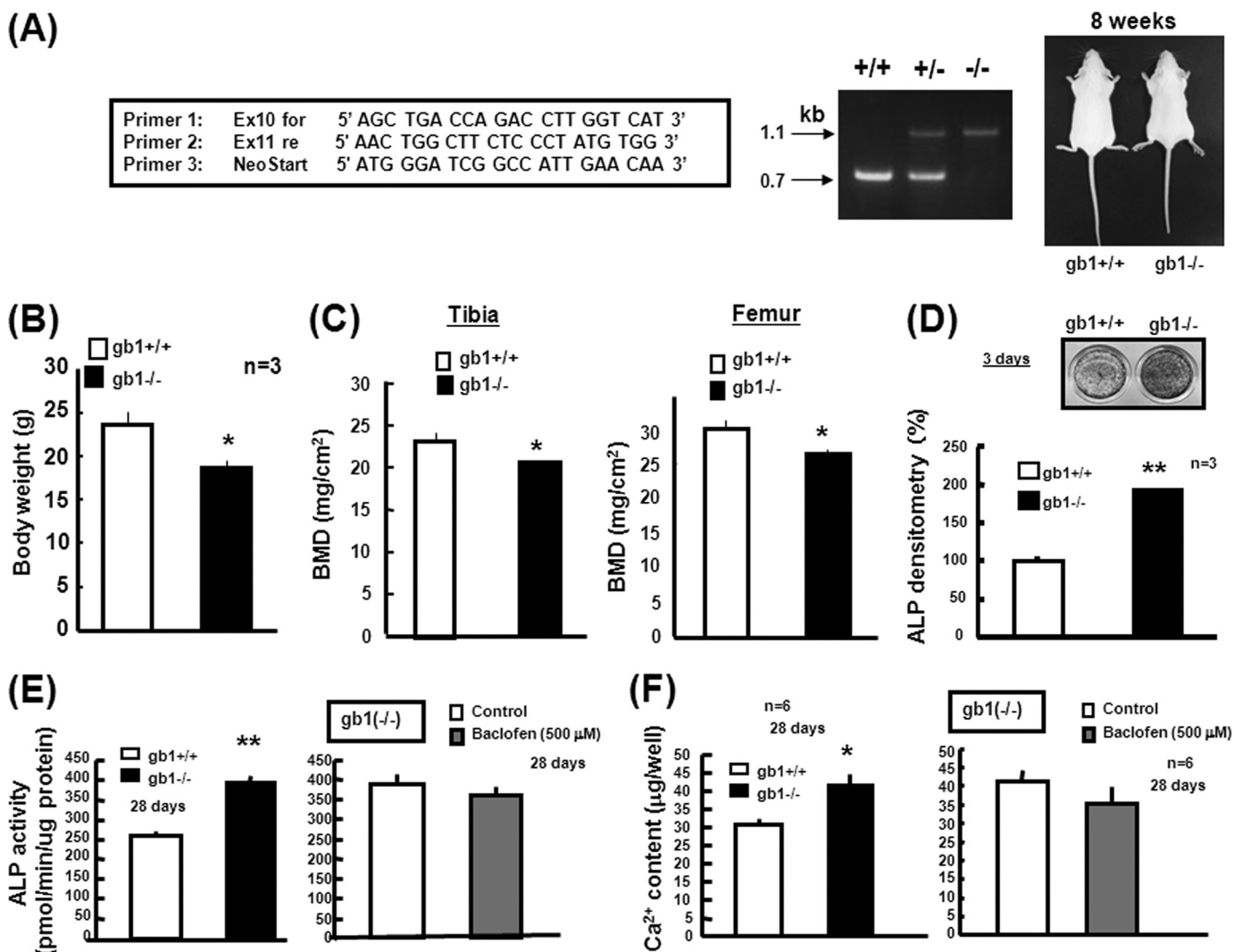


FIGURE 5. Phenotypes in GABA_BR1-null mice. *A*, both primers 1 and 2 were used for wild-type allele, whereas both primers 2 and 3 were used for knock-out ($gb1^{-/-}$) allele. Genomic DNA was extracted from the tails of neonatal mice, followed by PCR genotyping using two sets of primers. Primer 1 and 2 and primers 3 and 2 yielded 0.7- and 1.1-kb fragments, respectively. *B* and *C*, body weight (*B*) and bone mineral density (*C*) were individually determined in mice at 8 weeks old. *D*, bone marrow stromal cells were isolated from adult mice at 8 weeks old, followed by culture with osteoblastic inducers for 3 days and subsequent staining with ALP. *E* and *F*, calvarial osteoblasts were cultured for 28 days, followed by determination of ALP activity (*E*) and Ca²⁺ accumulation (*F*). Cells isolated from GABA_BR1-null mice were also cultured in either the presence or absence of 0.5 mM baclofen for 28 days. The values are the means \pm S.E. in three to six separate experiments. *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in WT mice ($gb1^{+/+}$).

accumulation (Fig. 4D) and of von Kossa staining for nodules (Fig. 4E) in cells cultured for 28 days.

In contrast to mouse calvarial osteoblasts, however, no marked expression was seen with BMP2 mRNA in MC3T3-E1 transfectants cultured for a period up to 28 days (Fig. 4F). In stable EV transfectants, mRNA expression was increased in proportion to increasing days in culture for osteocalcin, osteopontin, osterix, and Runx2, but not for type I collagen. However, stable GABA_BR1 transfection resulted in significantly decreased expression of mRNA for osteocalcin, osteopontin, osterix, and Runx2 in cells cultured for 28 days. These results suggest that GABA_BR is functionally expressed by osteoblastic MC3T3-E1 cells to negatively regulate cellular differentiation without affecting cellular proliferation through a mechanism similar to that in primary mouse calvarial osteoblasts.

GABA_BR1-null Mice—For further evaluation of the possible negative modulation by GABA_BR of osteoblastogenesis, an

attempt was made to determine whether the absence of GABA_BR1 subunit indeed affects a variety of phenotypes in skeleton. Genotyping by PCR using different primers clearly revealed the complete absence of GABA_BR1 subunit mRNA from particular embryos delivered after the mating of mice with genetically heterologous genes (Fig. 5A). General body size was apparently smaller in GABA_BR1-null ($gb1^{-/-}$) mice than in WT ($gb1^{+/+}$) mice at 8 weeks old (Fig. 5A). Body weight was significantly lower in GABA_BR1-null mice than in WT mice at 8 weeks old (Fig. 5B), along with significantly lower bone mineral density in GABA_BR1-null mice than in WT mice for both tibia (Fig. 5C, left panel) and femur (Fig. 5C, right panel). In osteoblasts differentiated from bone marrow stromal cells prepared from GABA_BR1-null mice, moreover, ALP staining intensity was significantly higher than those from WT mice on 3 days in culture (Fig. 5D). In calvarial osteoblasts devoid of GABA_BR1 subunit, a significant increase was again seen in ALP

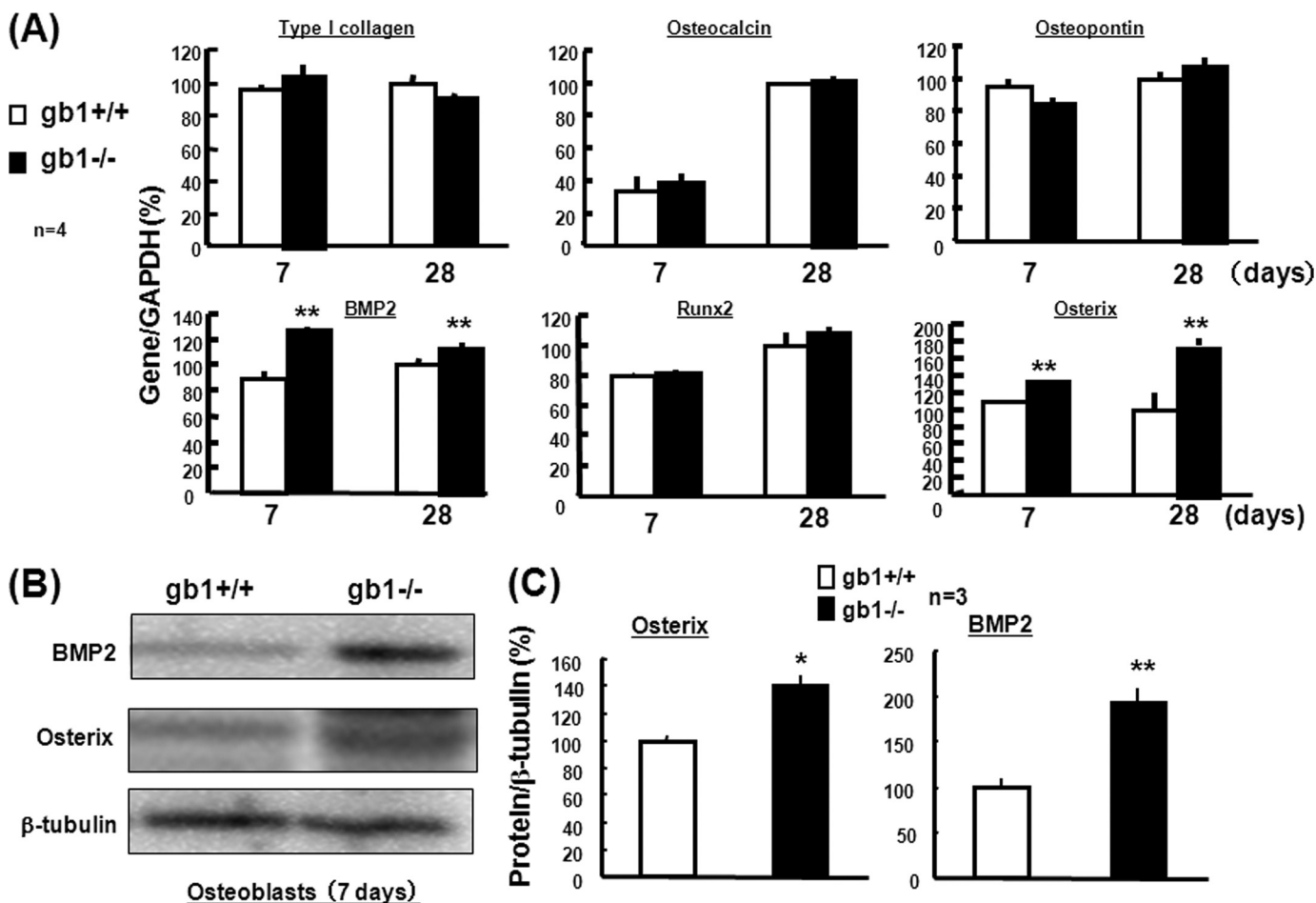


FIGURE 6. Osteoblastic gene expression in GABA_BR1-null mice. *A*, calvarial osteoblasts were cultured for 28 days, followed by determination of mRNA expression of different osteoblastic markers. The cells were cultured for 7 days, followed by determination of protein expression of osterix and BMP2. *B* and *C*, typical pictures are shown in *B*, whereas in *C*, quantitative data are shown for the corresponding protein levels. The values are the means \pm S.E. in three to four separate experiments. *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in WT mice (*gb1*^{+/+}).

activity, with baclofen being ineffective at 0.5 mM, when determined on 28 days in culture (Fig. 5E). Similarly, GABA_BR1 subunit defect led to a significant increase in Ca²⁺ accumulation and a lack of the effect of baclofen in calvarial osteoblasts cultured for 28 days (Fig. 5F). Thus, GABA_BR1 subunit knock-out would positively regulate osteoblastic differentiation *in vitro* along with reduced BMP in tibia and femur during skeletogenesis in mice *in vivo*.

Osteoblastic Marker Expression in GABA_BR1-null Mice—In calvarial osteoblasts from GABA_BR1-null (*gb1*^{-/-}) mice, significant increases were seen in mRNA expression of both BMP2 and osterix compared with those from WT (*gb1*^{+/+}) mice when cultured for 7–28 days (Fig. 6A). However, no significant differences were seen in mRNA expression of type I collagen, osteocalcin, osteopontin, and Runx2 between cultured calvarial osteoblasts prepared from GABA_BR1-null and WT mice, irrespective of the culture periods from 7 to 28 days. On Western blotting analysis, a marked increase was seen in protein levels of both BMP2 and osterix in calvarial osteoblasts from GABA_BR1-null mice when cultured for 7 days (Fig. 6B). Repetition and quantification of these experiments clearly revealed a significant increase in protein levels of BMP2 and osterix in GABA_BR1-null mouse osteoblasts (Fig. 6C).

Osteoclastogenesis in GABA_BR1-null Mice—To clarify the reason for the paradoxical data between the promotion of osteoblastic differentiation *in vitro* and the reduction of bone mineral density *in vivo*, an attempt was next made to determine whether osteoclastic activity is involved in bone phenotypes found in GABA_BR1-null mice. In WT mouse osteoclasts cultured for 5 days, mRNA expression was not seen for either GABA_BR1 or GABA_BR2 subunit (Fig. 7A). In cultured osteoclasts prepared from GABA_BR1-null mice, no marked change was found in the shape or the number of cells stained with TRAP compared with those from WT mice (Fig. 7B). Indeed, the number of TRAP-positive MNCs was not significantly different between cultured osteoclasts prepared from GABA_BR1-null and WT mice (Fig. 7C).

In tibial sections from 1-day-old GABA_BR1-null mice, by contrast, the intensity of TRAP staining was apparently higher than those from WT mice (Fig. 7D). Therefore, we next conducted co-culture of calvarial osteoblasts and osteoclasts prepared from GABA_BR1-null and WT mice for subsequent determination of the number of TRAP-positive MNCs as a marker of osteoclastic differentiation. Calvarial osteoblasts prepared from GABA_BR1-null and WT mice were cultured for 1 day, followed by lamination of osteoclasts over the osteoblastic layer

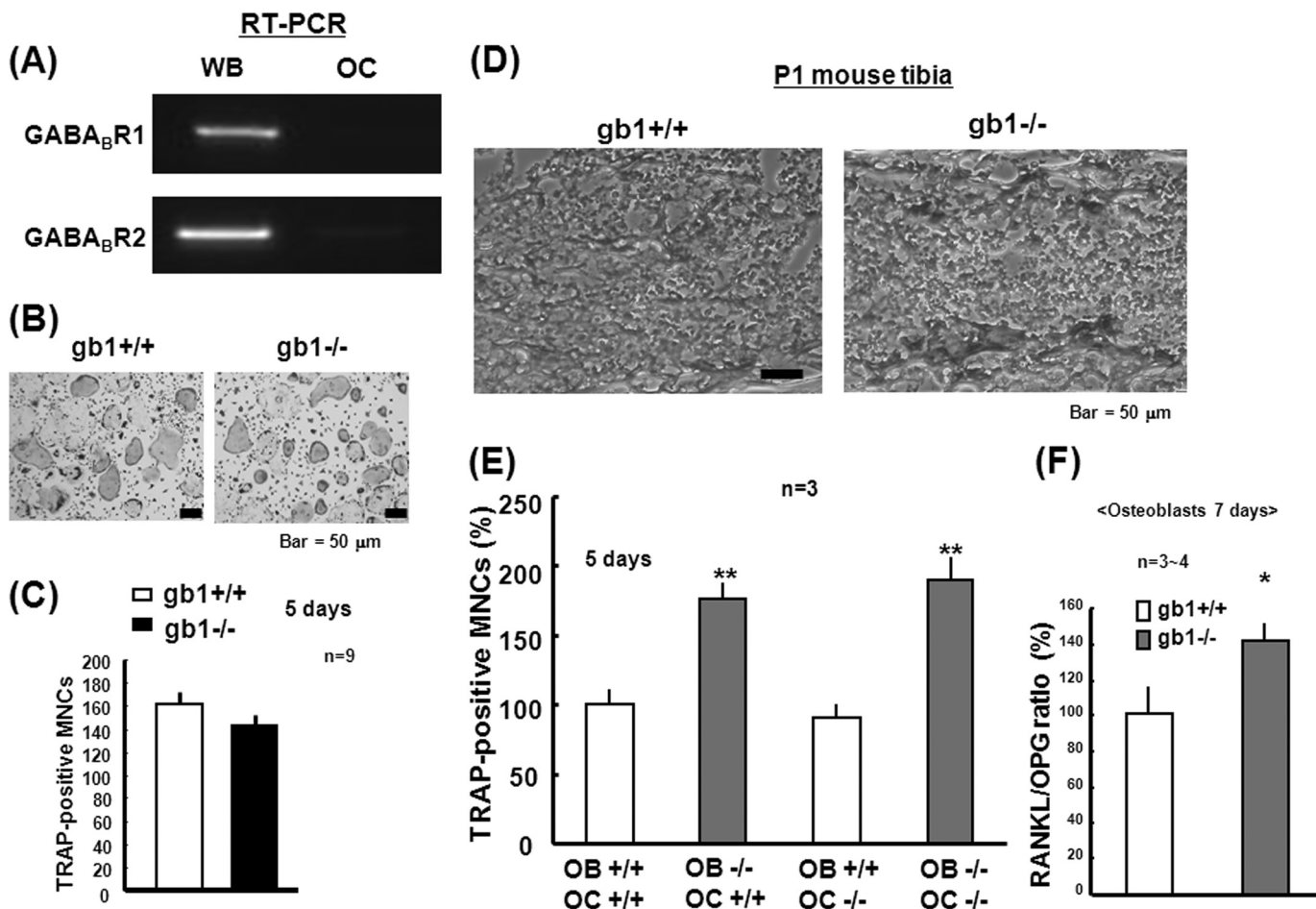


FIGURE 7. Osteoclastic differentiation in GABA_BR1-null mice. *A*, osteoclasts were cultured for 5 days, followed by extraction of total RNA for determination of mRNA expression of both GABA_BR1 and GABA_BR2 subunits. Osteoclasts cultured for 5 days were stained for TRAP, followed by counting of the number of TRAP-positive MNCs. *B* and *C*, typical pictures are shown in *B*, whereas in *C*, quantification data are presented for the number of TRAP-positive MNCs. *D*, tibias were isolated from 1-day-old mice, followed by dissection of frozen sections for subsequent TRAP staining. *E*, osteoclasts were cultured with osteoblasts under different combination conditions as described in the figure, followed by determination of the number of TRAP-positive MNCs. *F*, calvarial osteoblasts were cultured for 7 days, followed by determination of RANKL and OPG mRNA expression and subsequent calculation of the RANKL/OPG ratio. The values are the means ± S.E. in three to nine separate experiments. *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in WT mice. *WB*, whole brain; *OC*, osteoclast; *OB*, osteoblast.

and subsequent further culture in the absence of added RANKL for an additional 5 days. In osteoclasts plated on osteoblasts obtained from GABA_BR1-null mice, a significant increase was invariably seen in the number of TRAP-positive MNCs compared with osteoclasts cultured on osteoblasts from WT mice (Fig. 7E). In osteoblasts prepared from GABA_BR1-null mice, a significant increase was found in the ratio of RANKL over its decoy receptor OPG compared with those from WT mice within the culture for 7 days (Fig. 7F). Accordingly, impairment of GABA_BR function would promote osteoclastic differentiation through a mechanism relevant to up-regulation of RANKL expression after positive regulation of osteoblastogenesis in bone.

DISCUSSION

The essential importance of the present findings is that functional GABA_BR was predominantly expressed by osteoblasts rather than osteoclasts to negatively regulate cellular maturation through a mechanism related to down-regulation of BMP2, but not Runx2, expression at the level of gene transactivation without affecting proliferation *in vitro*. In osteoclasts

co-cultured with osteoblasts from GABA_BR1-deficient mice, moreover, cellular differentiation was markedly promoted along with the increased RANKL/OPG ratio in osteoblasts irrespective of the osteoclastic origin. To our knowledge, this is the first direct demonstration of negative regulation of osteoclastogenesis by functional GABA_BR predominantly expressed in osteoblasts through mechanisms relevant to down-regulation of both BMP2 and RANKL expression in bone. Although several previous studies including ours have demonstrated that GABA_BR subtype is expressed in different peripheral organs, no direct evidence for a pivotal role of the GABA_BR in the mechanisms underlying the cellular differentiation and maturation in osteoblasts is available in the literature to date. In bone, activation of GABA_BR would lead to the inhibition of cAMP formation required for transactivation of BMP2 and RANKL genes in osteoblasts and subsequent suppression of both osteoblastogenesis mediated by BMP2 and osteoclastogenesis mediated by RANKL, respectively (Fig. 8). Several independent lines of evidence indicate possible functional expression of the GABAergic signaling system in some non-neuronal

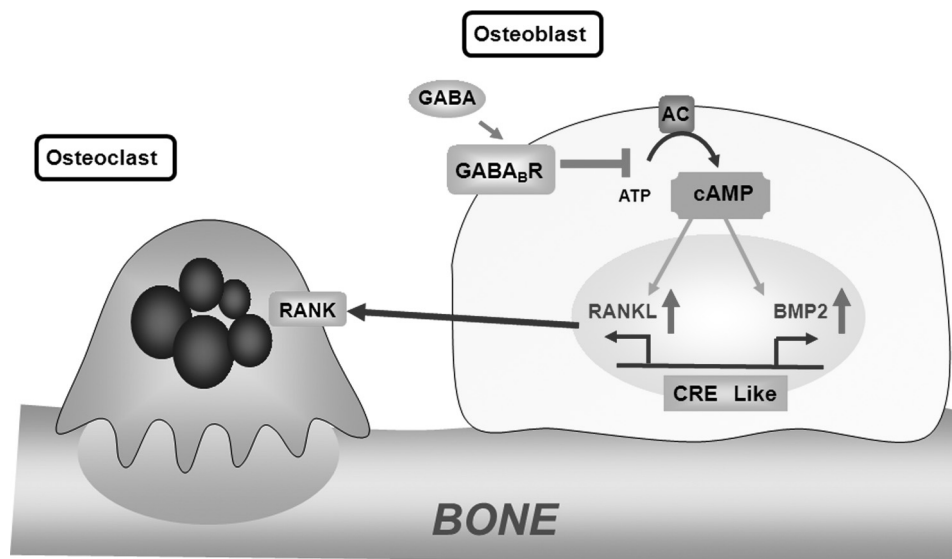


FIGURE 8. **Proposed model of GABAergic signaling in osteoblasts.** In bone, GABA may play a pivotal role in mechanisms underlying the negative regulation of osteoblastogenesis and osteoclastogenesis through the down-regulation of BMP2 and RANKL expression essential for each cellular differentiation and maturation after activation of GABA_BR predominantly expressed at osteoblastic cellular surfaces.

and peripheral organs. For example, GABA is highly concentrated in rat pancreatic islets (23) and fallopian tube (24) together with the enzyme glutamic acid decarboxylase responsible for biosynthesis. An RT-PCR analysis shows the expression of both GABA_BR1 splice variants in rat peripheral organs, in addition to brain, as seen in the present study. These include heart, spleen, lung, liver, small intestine, large intestine, kidney, stomach, adrenal, testis, ovary, and urinary bladder (25). The exact intracellular signal flows after activation of GABA_BR expressed at cell surface in osteoblasts, however, remain to be elucidated in future studies.

Mesenchymal stem cells have a self-renewal capacity and a multi-potentiality to differentiate into osteoblast, chondrocyte, adipocyte, and myocyte lineages, whereas BMP2 is shown to strongly promote differentiation of mesenchymal stem cells into osteoblasts and chondrocytes through up-regulation of the master regulator of osteoblastogenesis Runx2 by the Smads pathway (26, 27). The failure on Runx2 expression thus gives rise to an idea that BMP2 signaling could lead to promotion of osteoblastogenesis through a mechanism not related to up-regulation of Runx2 expression in osteoblasts. For instance, BMP2 is shown to induce several regulatory genes including *Msx2*, *Dlx3*, *Dlx2*, *Dlx5*, and *osterix* in osteoblasts (28, 29). Among these downstream genes, *osterix* (also referred to as *Sp7*) is a member of the Sp1 transcription factor family and plays a pivotal role in bone formation during skeletogenesis (13). Marked up-regulation is seen with ALP activity and osteocalcin expression in C2C12 cells and C3H10T1/2 cells with overexpression of *osterix* (13, 30), whereas *osterix* stimulates calcification in murine primary osteoblasts (29). Because Runx2 overexpression induces up-regulation of *osterix* expression as well (31), *osterix* should also function as a downstream signal of Runx2 in osteoblasts. In mesenchymal stem cells derived from Runx2-deficient mice, however, exposure to BMP2 results in *osterix* up-regulation along with increased ALP activity (12, 32). Overexpression of Smad1 and Smad4 up-regulates *osterix* expres-

sion, furthermore, whereas Smad6 markedly suppresses BMP2-induced *osterix* expression in osteoblasts prepared from Runx2-null mice (29). It is thus likely that *osterix* is a regulatory factor other than Runx2 for osteoblastic differentiation and maturation through a dual mechanism relevant to the regulation dependent on and independent of Runx2 expression.

The reason why activation of GABA_BR drastically decreased mRNA expression of several transcription factors essential for osteoblastogenesis, such as BMP2 and *osterix*, in addition to decreases in ALP activity, Ca²⁺ accumulation, and RANKL expression, is not clarified so far. On *in silico* data base search, we have found the presence of cAMP responsive element (CRE)-like sequences at the upstream of BMP2 gene. The present data from promoter analysis thus argue in favor of an idea that activation of GABA_BR would lead to inhibition of the transactivation of BMP2 gene through the prevention of cAMP formation required for the phosphorylation mediated by the protein kinase A pathway of a variety of target genes including CRE-binding protein, without affecting Runx2 expression, in osteoblasts. In addition to BMP2 expression, indeed, RANKL expression is shown to be regulated through the cAMP/protein kinase A pathway in osteoblastic cells (33). Phosphorylation of CRE-binding protein as well as its binding to CRE, nevertheless, should be at least demonstrated before drawing any conclusions on signal flows from the inhibition of BMP2 expression to suppressed osteoblastogenesis after activation of GABA_BR expressed by osteoblasts.

Further analysis on *osterix* promoter undoubtedly gives us a clue to elucidate molecular mechanisms underlying differential signal inputs from BMP2 and Runx2 toward the promotion of osteoblastogenesis. The absence of both GABA_BR1 and GABA_BR2 subunits from matured osteoclasts is highly suggestive of the view that the functional GABA_BR predominates in osteoblasts to negatively regulate bone remodeling as well as skeletogenesis in bone. Negative regulation of osteoblastogenesis would lead to subsequent disturbance of osteoclastogenesis

through down-regulation of the expression of RANKL absolutely required for osteoclastic differentiation and maturation. The present findings from *in vitro* co-culture experiments indeed give support for this proposal. The paradoxical data on osteocalcin mRNA expression profiles between osteoblasts exposed to baclofen and osteoblasts from GABA_BR1-null mice could be accounted for by taking into consideration the regulation of osteocalcin expression by activating transcription factor-4 (34), whose leucine zipper domain could interact with the C-terminal coiled-coil domain of GABA_BR1 subunit (35, 36). The complete absence of GABA_BR1 subunit would thus ignite compensatory mechanisms, other than the GABA_BR signaling pathway, required for osteocalcin expression in osteoblasts during postnatal skeletogenesis in GABA_BR1-null mice. The possibility that baclofen could be less effective than expected in inhibiting mRNA expression of osteocalcin, BMP2, and osterix because of the relatively low basal levels of endogenous cAMP in cultured osteoblasts, furthermore, is not ruled out.

It thus appears that functional GABA_BR is predominantly expressed by osteoblasts rather than osteoclasts during bone remodeling as well as skeletogenesis. Accordingly, GABAergic signaling machineries could be a novel target for the discovery and development of an innovative drug useful for the treatment and therapy of a variety of bone diseases relevant to abnormal bone remodeling in human beings.

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