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Positive Regulation by γ-Aminobutyric Acid B Receptor Subunit-1 of Chondrogenesis through Acceleration of Nuclear Translocation of Activating Transcription Factor-4*[S]

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Background: $GABA_BR$ is functionally expressed in a variety of peripheral organs outside the central nervous system.
Results: Mice defective in $GABA_BR$ subunit-1 ($GABA_BR1$) show delayed calcification.
Conclusion: $GABA_BR1$ molecule alone positively regulates chondrogenesis through accelerating nuclear translocation of activating transcription factor-4.
Significance: $GABA_BR1$ is a target candidate for the drug therapy of bone diseases relevant to endochondral ossification impairments.

A view that signaling machineries for the neurotransmitter γ-aminobutyric acid (GABA) are functionally expressed by cells outside the central nervous system is now prevailing. In this study, we attempted to demonstrate functional expression of GABAergic signaling molecules by chondrocytes. In cultured murine costal chondrocytes, mRNA was constitutively expressed for metabotropic $GABA_B$ receptor subunit-1 ($GABA_BR1$), but not for $GABA_BR2$. Immunohistochemical analysis revealed the predominant expression of $GABA_BR1$ by prehypertrophic to hypertrophic chondrocytes in tibial sections of newborn mice. The $GABA_BR$ agonist baclofen failed to significantly affect chondrocytic differentiation determined by Alcian blue staining and alkaline phosphatase activity in cultured chondrocytes, whereas newborn mice knocked out of $GABA_BR1$ (KO) showed a decreased body size and delayed calcification in hyoid bone and forelimb and hindlimb digits. Delayed calcification was also seen in cultured metatarsals from KO mice with a marked reduction of Indian hedgehog gene (*Ihh*) expression. Introduction of $GABA_BR1$ led to synergistic promotion of the transcriptional activity of activating transcription factor-4 (ATF4) essential for normal chondrogenesis, in addition to facilitating ATF4-dependent *Ihh* promoter activation. Although immunoreactive ATF4 was negligibly detected in the nucleus of chondrocytes from KO mice, ATF4 expression was again seen in the nucleus and cytoplasm after the retroviral introduction of $GABA_BR1$ into cultured chondrocytes from KO mice. In nuclear extracts of KO chondrocytes, a marked decrease was seen in ATF4 DNA binding. These results suggest that $GABA_BR1$ positively regulates chondrogenesis through a mechanism relevant to the acceleration of nuclear translocation of ATF4 for *Ihh* expression in chondrocytes.

γ-Aminobutyric acid (GABA) is one of the most abundant amino acid neurotransmitters in the mammalian central nervous system (CNS) with three different signal receptor subtypes, including $GABA_A$ receptor ($GABA_AR$),[3] $GABA_B$ receptor ($GABA_BR$), and $GABA_C$ receptor ($GABA_CR$) (1). The ionotropic $GABA_AR$ and $GABA_CR$ subtypes are responsible for the rapid component of inhibitory postsynaptic potentials through activation of ion channels permeable to chloride ions. The $GABA_AR$ is a heteromeric protein complex composed of five isoforms of a number of different subunits, whereas the $GABA_CR$ is derived from the assembly between various isoforms of the rho subunit (2). By contrast, the metabotropic $GABA_BR$ subtype belongs to a superfamily of the seven-transmembrane domain receptors coupled to adenylyl cyclase through trimeric G proteins to inhibit intracellular cAMP formation, in addition to inhibiting voltage-sensitive Ca^{2+} channels and activating voltage-sensitive K^+ channels, respectively (3). The $GABA_BR$ is orchestrated by a heterodimer comprising members of $GABA_BR1$ and $GABA_BR2$ subunits. Any $GABA_BR1$ subunits are unable to activate K^+ channels alone (4), whereas the 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 (6, 7). For example, both GABA (8) and L-glutamic acid decarboxylase (GAD) essential for GABA synthesis (9) are highly condensed in β-cells of Langerhans islets in pancreas. GABA is released

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[3] The abbreviations used are: $GABA_AR$, $GABA_BR$, $GABA_CR$, GABA types A, B, and C receptor, respectively; ALP, alkaline phosphatase; ATF4, activating transcription factor-4; Col2a1, α1(II)Collagen; Col10a1, α1(X)Collagen; GAD, L-glutamic acid decarboxylase; GAT, γ-aminobutyric acid transporter; Ihh, Indian hedgehog; PFA, paraformaldehyde; RANKL, receptor activator of nuclear factor-κB ligand; OSE1, osteocalcin specific element1.

from β-cells to exert an inhibitory paracrine action on glucagon secretion from neighboring α-cells through activating GABA$_A$R (10), as well as a suppressive autocrine effect on insulin secretion through activation of GABA$_B$R (11), in pancreas. In our previous study, functional GABA$_B$R is expressed to inhibit the formation of cAMP in a manner dependent on the pertussis toxin-sensitive G_i protein in cultured rat calvarial osteoblasts toward negative regulation of osteoblastogenesis (12). In fact, both GABA$_B$R1 and GABA$_B$R2 subunits are predominantly expressed by bone-forming osteoblasts rather than bone-resorbing osteoclasts to negatively regulate osteoblastic differentiation for maturation after down-regulation of bone morphogenetic protein-2 expression toward the inhibition of the synthesis of receptor activator of nuclear factor-κB ligand, which is absolutely required for the bone resorption by osteoclasts in mouse bone (13).

Endochondral ossification is an essential process for skeletal development in the fetal and neonatal periods, in addition to subsequent bone growth (14). Mesenchymal precursor cells differentiate into skeletal elements by forming a cartilaginous model during embryogenesis, which in turn induces bone formation in the vertebral column and long bone (15). Through this endochondral ossification process, the cartilaginous rudiment, which is a tightly regulated area of both differentiation and maturation of chondrocytes, undergoes developmental growth toward maturation. Within the cartilaginous rudiment, chondrocytes differentiate, progressing through the resting, proliferating, hypertrophic, and calcifying stages, which leads to mineralization of the cartilage matrix around the central region of the rudiment in the area of hypertrophic chondrocytes. Shortly after the mineralization process takes place, most hypertrophic chondrocytes undergo sustained apoptosis. Upon apoptotic death of chondrocytes after mineralization, osteoblasts, osteoclasts and capillaries begin to invade the cartilage matrix to produce a new bone, leading to the growth of endochondral bones. Although GABAergic signaling machineries could regulate the endochondral bone formation through directly regulating chondrocytic differentiation in addition to modulating osteoblastogenesis, little attention has been paid to the possible expression of GABAergic signaling machineries in chondrocytes to date.

In this study, we have attempted to demonstrate the functional expression of particular GABAergic signaling machineries by chondrocytes to clarify the physiological and pathological significance of GABAergic signals in chondrocyte differentiation and maturation during endochondral bone formation using *in vitro* and *in vivo* experimental techniques.

EXPERIMENTAL PROCEDURES

Materials—The pMX vector and PLAT-E cells (16) (17) were generous gift from Dr. Kitamura (Tokyo University, Tokyo, Japan). ATDC5 cells were purchased from RIKEN Cell Bank. α-Minimal essential medium, Dulbecco's modified Eagle's medium (DMEM) and DMEM and Ham's F-12 medium (DMEM/F12) were obtained from Invitrogen. Baclofen was purchased from TOCRIS. r*Taq* polymerase was obtained from Takara Bio. Dual luciferase assay system was purchased from Promega. M-MLV reverse transcriptase and Lipofectamine 2000 were supplied by Invitrogen. Anti-GABA$_B$R1 antibody, anti-ATF4 antibody, anti-rabbit IgG, and anti-mouse IgG were purchased from Santa Cruz Biotechnology. ISOGEN was obtained from WAKO. Both ECL™ detection reagent and protein G-Sepharose were obtained from Amersham Biosciences. LightShift Chemiluminescent EMSA kit was obtained from Thermo Fisher Scientific. Other chemicals used were all of the highest purity commercially available.

Culture of Primary Chondrocytes and ATDC5 Cells—The protocol used here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University (AP-101806). The BALB/c GABA$_B$R1-KO mice were kindly provided from Dr. Kaupmann (Novartis International AG, Basel, Switzerland). Both WT and KO mice were obtained from heterozygous breeding in our university animal facility at room temperature, under a 12-h light/dark cycle with light-on at 8:45 a.m. with access to food and water *ad libitum*. Genotyping was performed using specific primers as described previously (18). Mouse primary chondrocytes were prepared from thoracic cages of pups at postnatal 3–5 days old by the sequential enzymatic digestion method as described previously (19). In brief, thoracic cages were dissected and subsequently incubated with 0.3% collagenase for 90 min to remove soft tissues. The thoracic cages were further digested with 0.3% collagenase for 6 h. Finally, cells were collected in DMEM containing 10% fetal bovine serum (FBS) and antibiotics, followed by plating at a density of 1×10^4 cells/cm^2 for culturing under 5% CO_2. A differentiation inducer mixture containing 50 μg/ml ascorbic acid, 1 mM pyruvate, and 1 mM cysteine was used for the culture of primary chondrocytes. Prechondrocytic ATDC5 cells were plated at a density of 1×10^4 cells/cm^2 in DMEM/F12 containing 5% FBS. For induction of cellular differentiation, culture medium was replaced with DMEM/F12 containing 10 μg/ml transferrin, 30 nM sodium selenite, and 10 μg/ml bovine insulin.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis—Cultured cells were washed with ice-cold phosphate-buffered saline (PBS) twice, followed by extraction of total RNA according to the standard procedure. The individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, PCR buffer, dNTPs, the relevant sense and antisense primers (supplemental Table S1) and r*Taq* DNA polymerase. Quantitative analysis was done at the cycle number with high linearity between mRNA expression and cDNA production using primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as described previously (20).

Immunohistochemical Analysis—For immunohistochemical analysis, mouse tibia was fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose. The tibia was then dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections were fixed with 4% paraformaldehyde (PFA), followed by treatment with 0.3% H_2O_2 in methanol. After blocking with bovine serum albumin (BSA), sections were incubated with primary antibodies and subsequently with biotinylated secondary antibodies. Finally, visualization was performed using 0.05% diaminobenzidine and 0.03% hydrogen peroxide.

Determination of ALP Activity and Alcian Blue Staining—Determination of ALP activity and Alcian blue staining were done as described previously (21). In brief, chondrocytes were solubilized with 0.1% Triton X-100 followed by determination of the ALP activity in lysates using *p*-nitrophenol phosphate as a substrate. For Alcian blue staining, cells were fixed with 4% PFA and stained with 1% Alcian blue 8GX solution. Staining intensity was finally quantified using ImageJ.

Skeletal Preparation—Pups at 1 day old were put into 95% ethanol for 24 h for fixation, followed by staining for 24 h with Alcian blue solution consisting of 0.015% Alcian blue 8GX, 20% acetic acid, and 70% ethanol. After rinsing with 95% ethanol twice, whole bodies were digested with 2% KOH at room temperature until skeleton became clearly visible. The specimens were then stained with alizarin red solution consisting of 0.025% alizarin red and 1% KOH for 24 h. Finally, specimens were kept in 100% glycerol and observed under microscope.

Electron Microscopic Analysis—Mineralization of the cartilage matrix was analyzed by electron microscopy according to the procedures described previously (22). In brief, tibias of embryonic mice at 15.5 fetal days were prefixed in 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 24 h, followed by fixation with 2% OsO_4 for 3 h and subsequent dehydration for embedding in epoxy resin. Sections were then double stained with uranyl acetate and Reynolds' lead citrate for the examination in JEM2000EX at 100 kV.

Embryonic Metatarsal Rudiment Organ Culture—Metatarsal organ culture was conducted after the isolation from embryonic mice at 15.5 fetal days before vascularization as described previously (23). The area of the whole metatarsal was calculated as the total area, and the area of the middle mineralized part was determined by alizarin red staining as the mineralization area.

In Situ Hybridization Analysis—*In situ* hybridization was performed as described previously (19). In brief, metatarsals were dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections mounted on slide glasses were fixed with 4% PFA and successively treated with 0.2 M HCl and 10 μg/ml proteinase K. Sections were then subjected to the acetylation in 0.1 M triethanolamine, 0.25% acetic anhydride. After prehybridization, sections were covered with digoxigenin-labeled cRNA probes at 65 °C for 16 h. Sections were then washed and treated with 4 μg/ml RNase A. Sections were further incubated with anti-digoxigenin-AP Fab fragments for 16 h. After washing, sections were treated with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate for different periods.

Luciferase Assay—Reporter vectors were co-transfected with a SV40-*Renilla* luciferase construct into ATDC5 or COS-7 cells using Lipofectamine 2000 Plus reagent. Two days after transfection, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer according to the manufacturer's protocol. Transfection efficiency was normalized by determining the activity of *Renilla* luciferase.

Electrophoretic Mobility Shift Assay (EMSA)—Nucleotide sequences of the *Ihh* promoter were used as double-stranded oligonucleotide probers for detecting ATF4 DNA binding; OSE1-like sequence was 5′-GATCTGGCCCTGGCAACATTCCCTTTG-3′, and the point-mutated OSE1-like sequence was the ΔOSE1-like sequence, 5′-GATCTGGCCCCGGAAACATTCCCTTTG-3′. Annealed double-stranded oligonucleotides were labeled with biotin for the use as probes in EMSA with nuclear extracts of primary cultured chondrocytes according to the manufacturer's procedure.

Immunoprecipitation Assay—Primary chondrocytes transfected with Myc-ATF4 were solubilized in lysis buffer containing 1% Nonidet P-40, followed by incubation with the anti-myc antibody for 3 h at 4 °C and subsequent immunoprecipitation with protein G-Sepharose. Immunoprecipitates were washed five times with lysis buffer and boiled in SDS- sample buffer. Samples were then separated by SDS-PAGE, followed by transfer to membranes and subsequent immunoblotting assay.

Retroviral Transfection—The pMX-based retroviral vector for GABA$_B$R1 expression was generated by insertion of a mouse GABA$_B$R1 cDNA fragment into pMX vector. The pMX-GABA$_B$R1 vector was transfected into PLAT-E cells using the calcium carbonate method. Virus supernatant was collected 48 h after transfection, and then chondrocytes were infected with this virus supernatant for 48 h in the presence of 4 μg/ml Polybrene. Cells were then selected by 5 μg/ml blasticidin for 3 days before using for experiments.

Immunoblotting Analysis—Cultured cells were solubilized in lysis buffer containing 1% Nonidet P-40. Samples were then subjected to SDS-PAGE, followed by transfer to nitrocellulose membranes and subsequent immunoblotting assay. Quantification was performed by densitometry using ImageJ.

Immunocytochemical Analysis—For immunocytochemical analysis, cells were plated on a chamber slide coated with poly-L-lysine. Cultured cells were fixed with 4% PFA in PBS, followed by washing and subsequent treatment with BSA in PBS containing 0.1% Triton X-100. Cells were then incubated with a primary antibody diluted with PBS containing BSA and 0.3% Triton X-100. Finally, cells were reacted with the corresponding secondary antibody, and subsequently chamber slides were mounted with the FluorSave reagent.

Data Analysis—Results are all expressed as the mean ± S.E., and the statistical significance was determined by the two-tailed and unpaired Students' *t* test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

RESULTS

Expression of GABA$_B$R1 Subunit by Chondrocytes—We first examined the expression profile of a variety of chondrocyte differentiation markers in mouse costal chondrocytes cultured with a differentiation inducer mixture for different periods. A drastic increase was seen in mRNA expression of all chondrocytic marker genes examined in proportion to culture periods up to 21 days. These include α1(II)Collagen (*Col2a1*), α1(X)Collagen (*Col10a1*), and osteopontin (*Opn*) (supplemental Fig. S1).

However, mRNA expression was not seen for GABA$_A$$\alpha$1–6, β1–3, and γ1–3 subunits of GABA$_A$R (Fig. 1*A*), and ρ1–3 subunits of GABA$_C$R (Fig. 1*C*) in chondrocytes cultured for 7 and 21 days, whereas mRNA was constitutively detected only for GABA$_B$R1 subunit, but not for GABA$_B$R2 subunit, in chondrocytes cultured for 7–21 days (Fig. 1*B*). Although mRNA was

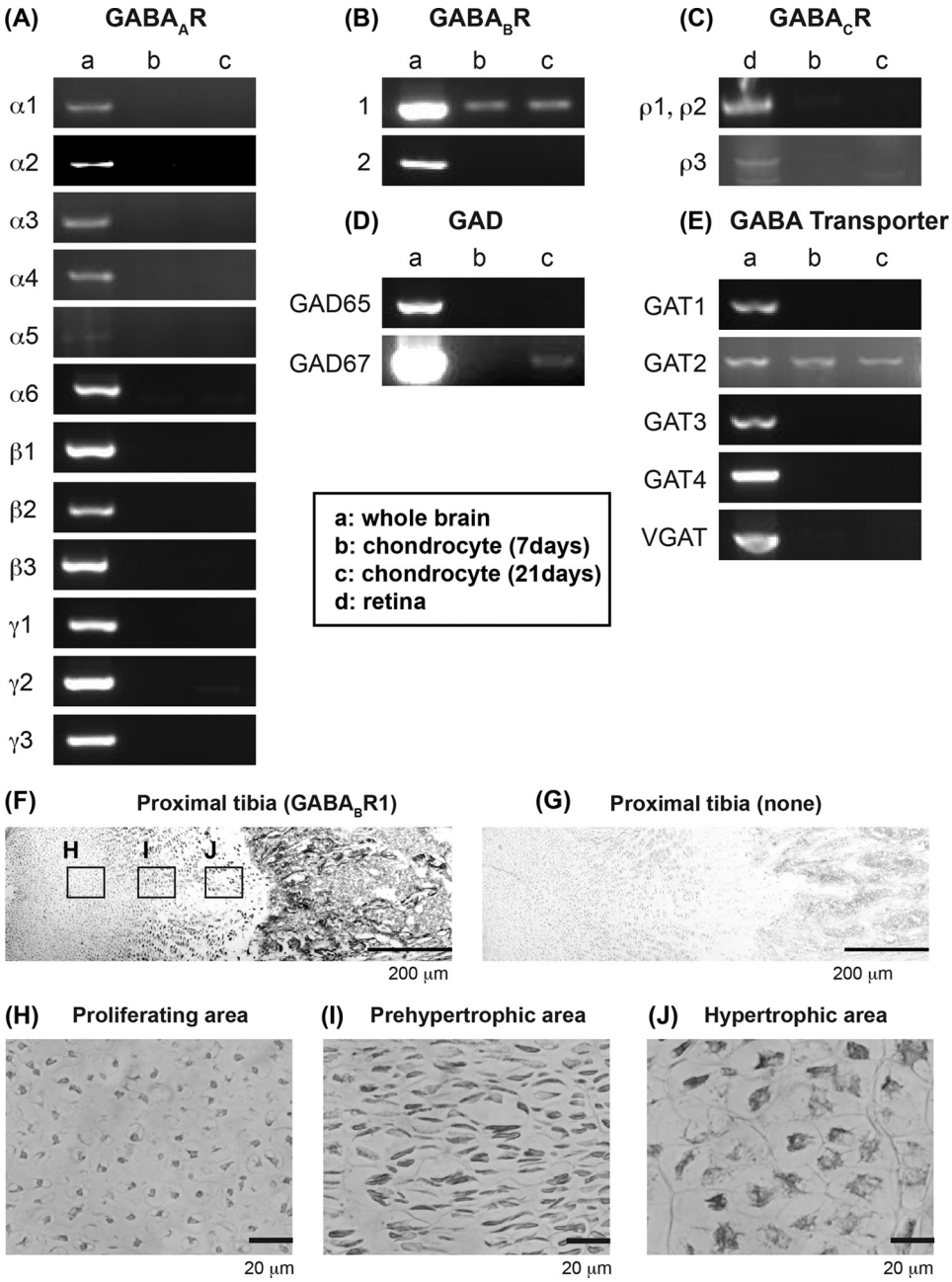

FIGURE 1. **Expression profiles of GABAergic signaling molecules in chondrocytes.** *A–E*, total RNA was extracted from mouse costal chondrocytes cultured for 7–21 days, followed by RT-PCR using specific primers for GABA$_A$R (*A*), GABA$_B$R (*B*), GABA$_C$R (*C*), GAD (*D*), and GABA transporters (*E*). Mouse whole brain and retina were used as a positive control. *F* and *G*, tibia was isolated from 1 day-old mice, followed by sectioning for subsequent immunohistochemical analysis using the anti-GABA$_B$R1 antibody (*F*) or the secondary antibody only (*G*). *H–J*, high magnification images are shown in proliferating (*H*), prehypertrophic (*I*), and hypertrophic chondrocytic layers (*J*) marked by *black rectangular boxes* in *F*.

only detected for GAD67 in chondrocytes cultured for 21 days (Fig. 1*D*), mRNA expression was constitutively detected for GABA transporter 2 (GAT2) in cultured chondrocytes (Fig. 1*E*). No detectable mRNA expression was seen for GAD65, GAT1, GAT3, GAT4, and vesicular GABA transporter in murine costal chondrocytes cultured for 7–21 days.

Immunohistochemical analysis revealed that GABA$_B$R1 subunit protein was detected predominantly in areas enriched of prehypertrophic to hypertrophic chondrocytes, in addition to osteoblasts attached on cancellous bone, in tibial sections from postnatal 1-day-old mice (Fig. 1*F*), whereas no marked immunoreactivity was detected in sections not treated with the primary antibody against GABA$_B$R1 subunit (Fig. 1*G*).

Skeletal Phenotypes in GABA$_B$R1 Knock-out (KO) Mice—An attempt was next made to determine whether the GABA$_B$R agonist baclofen affects chondrocytic differentiation. Primary chondrocytes were strongly stained with Alcian blue after the culture for 21 days (supplemental Fig. S2*A*, *upper*), whereas sustained exposure to baclofen failed to significantly affect the intensity of Alcian blue staining at concentrations from 10 to 500 μM (supplemental Fig. S2*A*, *lower*). Furthermore, sustained exposure to baclofen did not affect ALP activity at concentra-

tions from 10 to 500 μM in chondrocytes cultured for 21 days (supplemental Fig. S2*B*). Accordingly, the aforementioned results clearly revealed the absence of canonical functional GABA$_B$R composed of both GABA$_B$R1 and GABA$_B$R2 subunits from chondrocytes, in contrast to osteoblasts expressing both subunits (13).

To further evaluate properties of GABA$_B$R1 subunit expressed alone by chondrocytes, skeletal phenotypes were analyzed in newborn mice genetically knocked out of GABA$_B$R1 subunit at the age of 1 day after birth by using Alcian blue/alizarin red double staining of skeletons. Alcian blue staining has been used for the detection of extracellular matrix such as aggrecan, which is composed of chondroitin sulfate and keratan sulfate chains, whereas alizarin red staining has been used to detect mineralization of extracellular matrix. General appearance was apparently smaller in KO mice than in wild-type (WT) mice (Fig. 2*A*), whereas KO mice exhibited small body skeleton stained with both dyes (Fig. 2*B*), with a reduced size of skull (Fig. 2*C*), compared with WT mice. Lengths of femur (Fig. 2*D*) and tibia (Fig. 2*E*) were significantly shorter in KO mice than in WT mice, whereas delayed calcification was seen in hyoid bone (Fig. 2*F*) and forelimb (Fig. 2*G*) and hindlimb (Fig. 2*H*) digits in terms of the lower intensity of alizarin red staining compared with those in WT mice. Furthermore, mineralization of the cartilage matrix was analyzed in WT and KO hypertrophic cells by electron microscopy. Both the number and size of matrix granules were much less in KO chondrocytes than in WT chondrocytes (Fig. 2*I*, *arrows*).

Calcification and Ihh Expression in Cultured Metatarsals and Chondrocytes from KO Mice—Metatarsals were isolated before vascularization from embryonic mice at 15.5 days after gestation for subsequent *ex vivo* culture for 5 consecutive days. Although no marked difference was seen in the total areas of metatarsals from WT and KO mice (Fig. 3 *A* and *B*), the calcified areas were significantly decreased in metatarsals from KO mice compared with those from WT mice as revealed by alizarin red staining (Fig. 3*C*). *In situ* hybridization analysis revealed that markedly decreased expression was seen for *Ihh* expressed in prehypertrophic to hypertrophic chondrocytic layers (Fig. 3*F*), but not for *Col2a1* expressed in proliferating chondrocytic layer (Fig. 3*D*) and *Col10a1* expressed in prehypertrophic to hypertrophic layers (Fig. 3*E*), among cellular markers predominantly expressed by chondrocytes at different differentiation stages in cultured metatarsals from KO mice than in WT mice. Repetition for quantification clearly showed a significant decrease in the expression area of *Ihh* in cultured metatarsals from KO mice (Fig. 3*G*). Costal chondrocytes were next cultured for 14 days, followed by determination of Alcian blue staining, ALP activity, and *Ihh* expression. A significant decrease was invariably seen in Alcian blue staining intensity (Fig. 3*H*), ALP activity (Fig. 3*I*), and *Ihh* expression (Fig. 3*J*), in chondrocytes from KO mice.

Because chondrocytes are also known to express RANKL, we next examined the endogenous levels of *RANKL* in cultured chondrocytes from KO and WT mice. However, no significant difference was seen in *RANKL* expression in WT and KO chondrocytes (supplemental Fig. S3*A*). Similarly, transfection with GABA$_B$R1 expression vector failed to significantly affect *RANKL* expression in prechondrocytic ATDC5 cells (supplemental Fig. S3*B*).

Promotion by GABA$_B$R1 of Transcriptional Activity of ATF4 in Chondrocytes—Although ATF4 positively regulates chondrocyte differentiation by activating *Ihh* expression (24), GABA$_B$R1 subunit protein is shown to physically interact with ATF4 protein (25). We thus performed immunoprecipitation analysis for determination of the possible physical interaction between GABA$_B$R1 subunit and ATF4 protein using chondrocytes transiently transfected with Myc-ATF4. In immunoprecipitates with the myc-antibody from costal chondrocytes, immunoreactive GABA$_B$R1 subunit was clearly detected in a manner similar to that in COS-7 cells with overexpression of both GABA$_B$R1 and Myc-ATF4 (Fig. 4*A*). We thus examined whether GABA$_B$R1 regulates *Ihh* expression through interacting with ATF4 in chondrocytes. Chondrocytic ATDC5 cells were transiently transfected with expression vectors of ATF4, GABA$_B$R1, and GABA$_B$R2, followed by determination of the luciferase activity after introduction of different reporter plasmids as an index of the promoter activity. The introduction of ATF4 alone significantly increased the luciferase activity of a reporter vector containing six tandem copies of ATF4 binding site (6XOSE1) (Fig. 4*B*) and *Ihh* promoter fragment (Fig. 4*C*). Although GABA$_B$R1 alone did not significantly affect the luciferase activity of both reporter plasmids, co-introduction of GABA$_B$R1 significantly accelerated the ATF4-induced increase in luciferase activity of 6XOSE1 and *Ihh* promoter plasmids. By contrast, further introduction of GABA$_B$R2 subunit significantly inhibited the GABA$_B$R1-induced increases in luciferase activity in both 6XOSE1 and *Ihh* promoter reporters. A GABA$_B$R1 expression vector was next introduced into ATDC5 cells, followed by determination of Alcian blue staining and ALP activity as indices of chondral bone formation. The introduction of GABA$_B$R1 alone induced significant increases in both Alcian blue staining intensity (Fig. 4*D*) and ALP activity (Fig. 4*E*) in ATDC5 cells cultured for 10 days. Although osteoblasts are known to express both Ihh and ATF4, no significant differences were seen in both *Ihh* and *ATF4* expression in osteoblasts from WT and KO mice (supplemental Fig. S4, *A* and *B*).

Nuclear Translocation of ATF4 in Chondrocytes—We next investigated whether GABA$_B$R1 affects nuclear translocation of the transcription factor ATF4 in chondrocytes. Costal chondrocytes were prepared from KO and WT mice, followed by culture for 7 days and subsequent fractionation of both nuclear and cytoplasmic fractions for determination of ATF4 expression on immunoblotting. Although ATF4 was found in both nuclear and cytoplasmic fractions of chondrocytes from WT mice, ATF4 levels were greatly lower in nuclear fractions of chondrocytes from KO mice than in those from WT mice (Fig. 5*A*). By contrast, cytoplasmic ATF4 expression was not markedly affected in chondrocytes irrespective of the deficiency of GABA$_B$R1. Retroviral infection with a GABA$_B$R1 expression vector led to markedly increased levels of ATF4 in nuclear, but not cytoplasmic, fractions of chondrocytes from KO mice. Immunoreactive ATF4 was highly localized in the cytoplasm rather than the nucleus in chondrocytes from KO mice, whereas ATF4 expression was seen in both the nucleus and

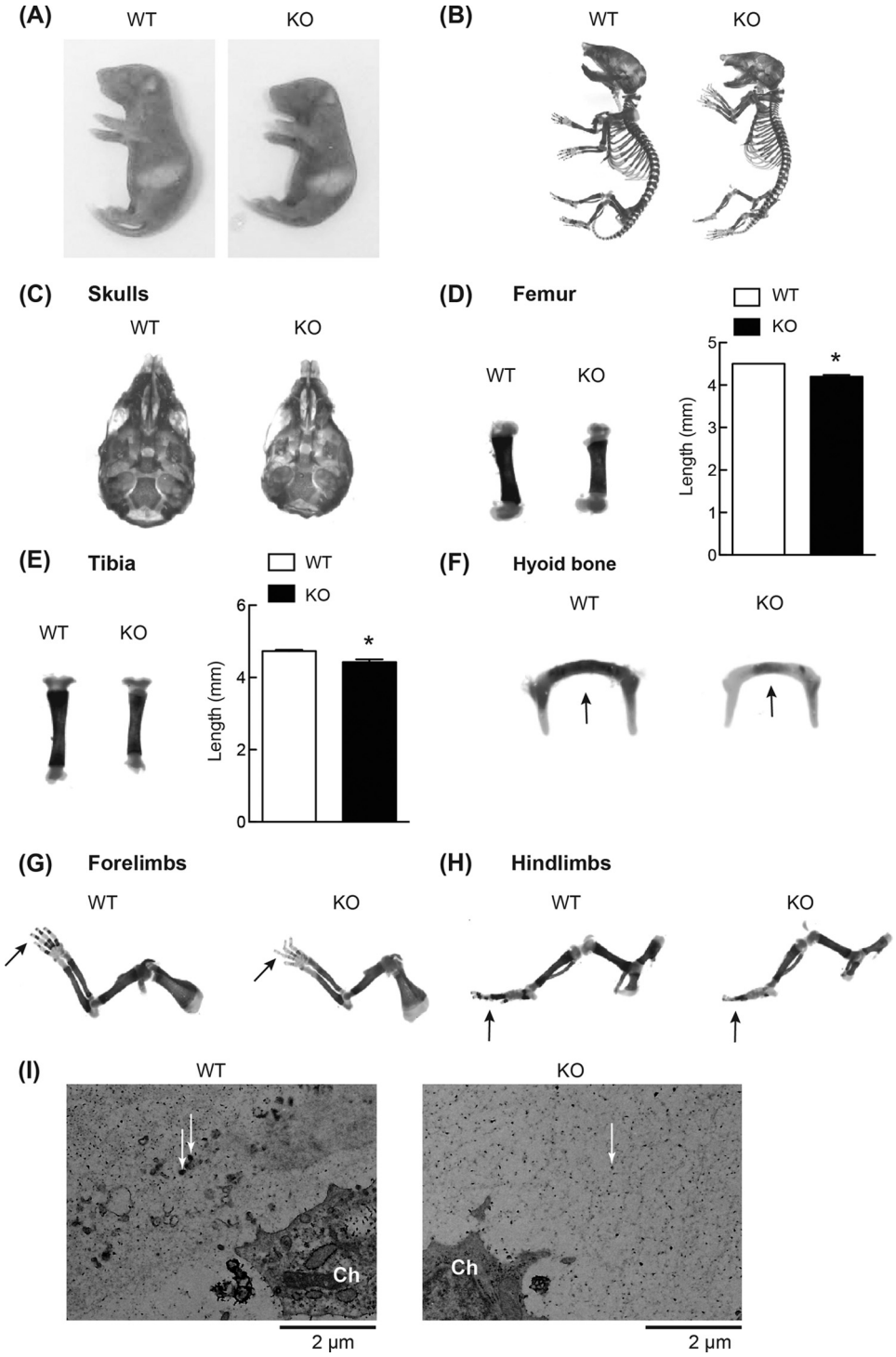

FIGURE 2. **Skeletal phenotypes in KO mice.** *A*, growth appearance of 1-day-old WT and KO mice. *B*– h, Alcian blue/alizarin red double staining of whole skeleton (*B*), skull (*C*), femur (*D*), tibia (*E*), hyoid bone (*F*), forelimb (*G*), and hindlimb (*H*) in WT and KO mice. Lengths of femur and tibia were also quantitatively measured. *. $p < 0.05$, significantly different from each control value obtained in WT mice. *Arrows* indicate the portion of delayed calcification with alizarin red staining. *I*, electron microscopy on hypertrophic cells in tibia of embryonic mice. The matrix consists of matrix granules (*arrows*) and extremely thin collagenous fibrils. Magnification, ×5500. *Ch*, chondrocyte.

cytoplasm in GABA$_B$R1-null chondrocytes with the retroviral infection of GABA$_B$R1 expression vector (Fig. 5*B*).

We thus examined ATF4 DNA binding to the *Ihh* promoter fragment in nuclear extracts of chondrocytes from WT and KO mice. Although ATF4 DNA binding was detected in nuclear extracts of chondrocytes from both WT and KO mice, ATF4 DNA binding was clearly lower in KO chondrocytes than in those from WT mice (Fig. 5*C*). The addition of the anti-ATF4 antibody resulted in a marked decrease in ATF4 DNA binding to the OSE1-like probe, whereas no marked DNA binding was detected at the position of the probe-protein complex to the ΔOSE1-like probe with a point mutation (Fig. 5*D*).

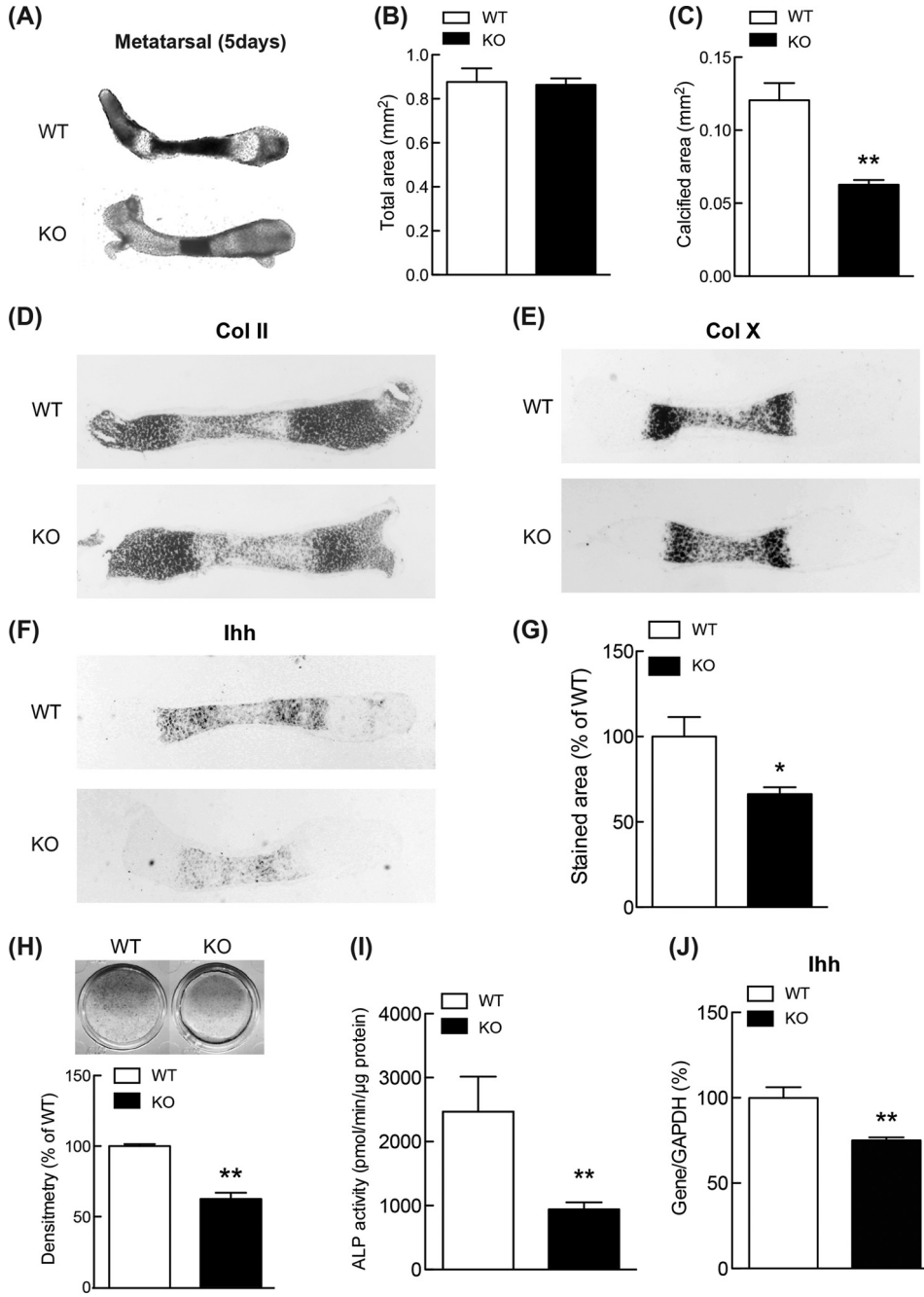

FIGURE 3. **Analysis of cultured metatarsal and chondrocytes from KO mice.** *A*, metatarsals were isolated from WT and KO mice, followed by culture for 5 days and subsequent quantitative measurement of the total (*B*) and mineralization (*C*) areas. *D–F*, metatarsals of WT and KO mice were cultured for 5 days, followed by determination of the expression pattern of the chondrocyte-specific marker genes, *Col2a1* (*D*), *Col10a1* (*E*), and *Ihh* (*F*) on *in situ* hybridization analysis. *G*, *Ihh* expression was quantified in cultured metatarsals. *H–J*, costal chondrocytes were isolated from WT and KO mice, followed by culture for 14 days and subsequent determination of Alcian blue staining intensity (*H*), ALP activity (*I*), and *Ihh* expression (*J*). *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in WT mice.

DISCUSSION

The essential importance of the present findings is that GABA$_B$R1 subunit was constitutively expressed by chondrocytes to positively regulate transactivation of *Ihh* expression toward promotion of cellular differentiation and maturation. In this study, mRNA was not detected for the heterodimeric partner GABA$_B$R2 subunit essential for the assembly to functional GABA$_B$R in cultured murine chondrocytes. The functional GABA$_B$R is believed to be orchestrated by a heterodimeric assembly between members of GABA$_B$R1 and GABA$_B$R2 subunits, neither of which is fully functional when expressed individually (4). In hippocampal slices from mice defective in GABA$_B$R2 subunit, however, the GABA$_B$R agonist baclofen still induces atypical inhibition of K^+ channels, in place of the typical activation, as electrophysiological responses in a manner sensitive to a GABA$_B$R antagonist (26), suggesting that GABA$_B$R1 subunit alone plays an additional and atypical role in the central GABAergic synaptic neurotransmission even in the

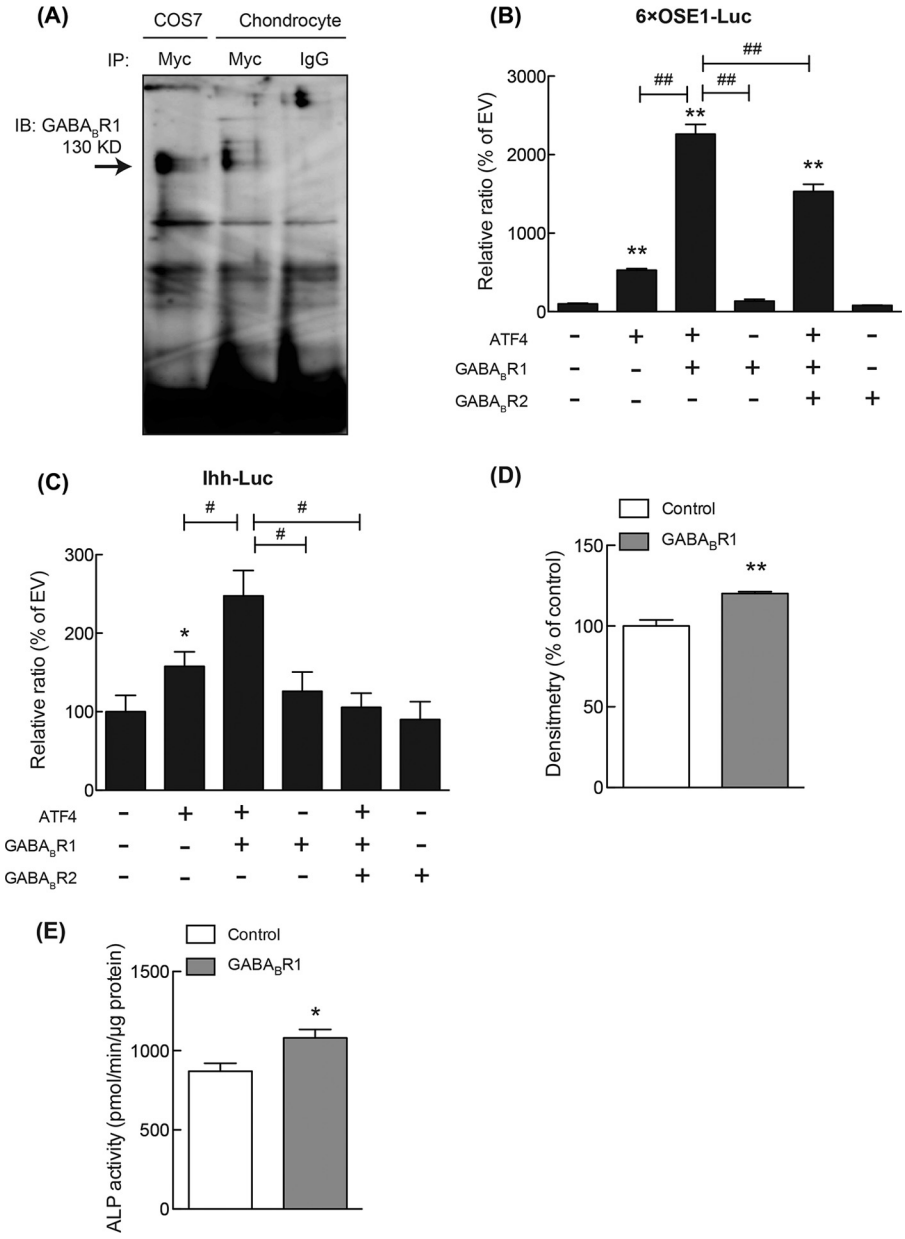

FIGURE 4. **Effects of GABA$_B$R1 introduction.** *A*, costal chondrocytes were transfected with Myc-ATF4, followed by further culture for an additional 3 days and subsequent immunoprecipitation analysis on GABA$_B$R1 and ATF4. COS-7 cells were also transfected with both GABA$_B$R1 and ATF4 to serve as a positive control. *B*, COS-7 cells were transfected with the reporter plasmid 6XOSE1 in either presence or absence of expression vectors of ATF4, GABA$_B$R1, and GABA$_B$R2, followed by further culture for an additional 1 day and subsequent determination of luciferase activity. *C*, ATDC5 cells were transfected with a reporter plasmid containing *Ihh* promoter in either the presence or absence of expression vectors of ATF4, GABA$_B$R1, and GABA$_B$R2, followed by further culture for an additional 1 day and subsequent determination of luciferase activity. *, $p < 0.05$; **, $p < 0.01$, significantly different from the value obtained in cells transfected with empty vectors (*EV*). #, $p < 0.05$; ##, $p < 0.01$, significantly different from the value obtained in the presence of both ATF4 and GABA$_B$R1 vectors. *D* and *E*, ATDC5 cells were transiently transfected with EV or GABA$_B$R1 expression vector, followed by further culture for an additional 10 days and subsequent determination of Alcian blue staining (*D*) and ALP activity determination (*E*). *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in cell with empty vectors (*EV*).

absence of the dimerization partner, GABA$_B$R2 subunit, required for orchestration of functional metabotropic receptors linked to a Gi protein. In our previous study, similarly, the GABA$_B$R1 subunit alone is constitutively expressed by adipocytes to positively regulate leptin expression at the transcriptional level through a mechanism entirely irrelevant to the typical role as a functional GABA$_B$R assembly partner (20). The present inhibition of the GABA$_B$R1-induced increases in luciferase activity in both 6XOSE1 and *Ihh* promoter reporters by co-introduction of GABA$_B$R2 subunit essential for orchestration of functional GABA$_B$R is favorable for the complete irrelevance of the promoted chondrogenesis by GABA$_B$R1 subunit to properties as a functional GABA$_B$R composed of both subunits in chondrocytes. In osteoblasts, by contrast, both GABA$_B$R1 and GABA$_B$R2 subunits are similarly expressed to negatively regulate osteoblastogenesis as a functional GABA$_B$R through a mechanism relevant to the inhibition of cAMP formation (13). Accordingly, one possible speculation is that GABA$_B$R1 subunit would be endowed to play a dual role as a dimerization partner of the canonical functional GABA$_B$R and

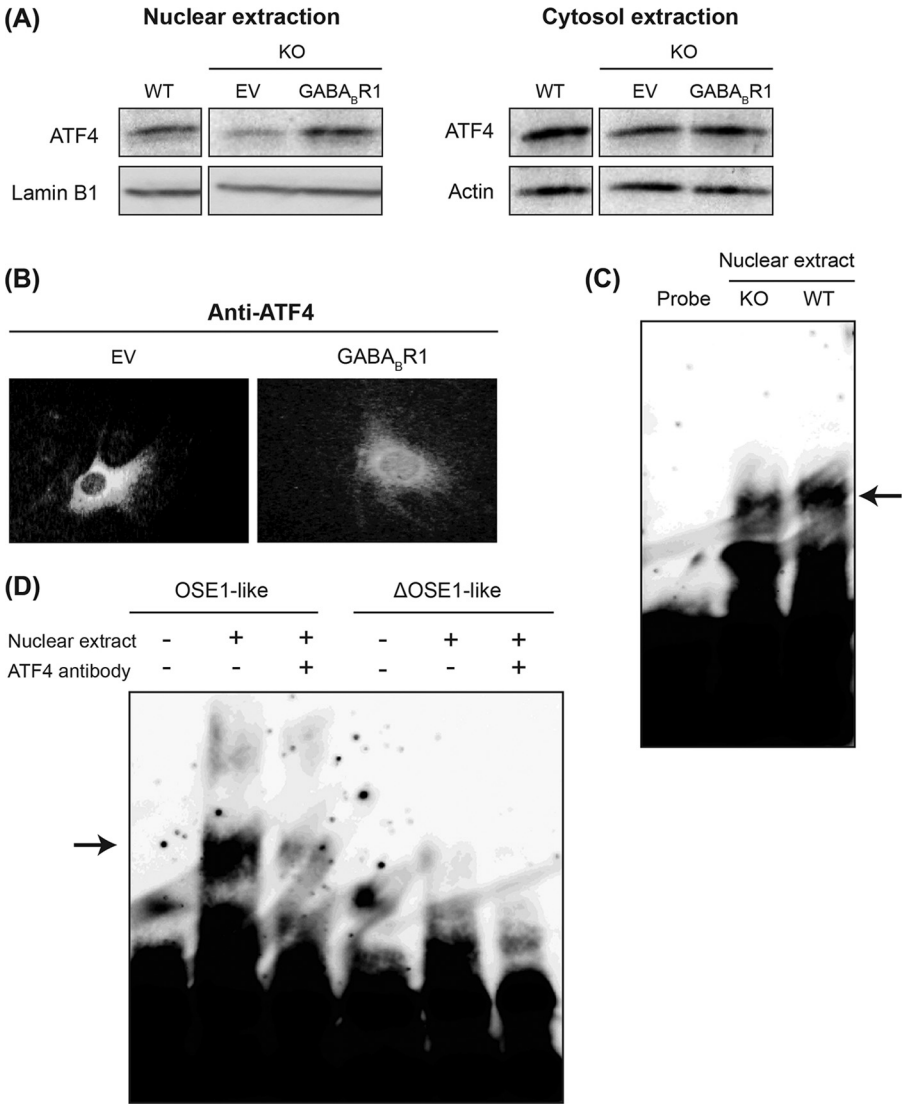

FIGURE 5. **Nuclear ATF4 expression in chondrocytes.** *A*, costal chondrocytes were prepared from WT and KO mice, followed by culture for 7 days and subsequent fractionation of nuclear and cytoplasmic fractions for determination of ATF4 levels on immunoblotting. Cells were also transfected with retroviral vectors of EV and GABA$_B$R1 as needed. *EV*, empty vectors. *B*, costal chondrocytes were prepared from KO mice, followed by culture for 3 days and subsequent retroviral infection with GABA$_B$R1 expression vector for determination of ATF4 expression on immunocytochemistry. *C*, nuclear extracts were prepared from WT and KO chondrocytes, followed by determination of DNA binding on EMSA using biotin-labeled oligonucleotide probes containing ATF4 binding site (OSE1-like; TGGCAACA) or point mutated ATF4 binding site (ΔOSE1-like; CGGAAACA) in the *Ihh* promoter. *D*, nuclear extracts were prepared from WT chondrocytes, followed by EMSA using OSE1-like or ΔOSE1-like oligonucleotide probe in either the presence or absence of the anti-ATF4 antibody. The *arrows* indicate the position of the probe-protein complex.

a modulator of gene transactivation by ATF4 in a manner dependent on the concurrent expression of GABA$_B$R2 subunit in a particular situation. To our knowledge, this is the first direct demonstration of constitutive and functional expression of GABA$_B$R1 subunit protein alone by cells outside the CNS with capability of positively regulating chondrocytic cellular maturation required for endochondral ossification during postnatal skeletogenesis.

The fact that the present phenotypes are quite similar to those seen in mice defective in ATF4 (24) argues in favor of an idea that GABA$_B$R1 subunit would play a role in chondrogenesis through up-regulation of *Ihh* expression after the interaction with the nuclear transcription factor ATF4 in a manner apart from the role as a partner of the heteromeric orchestration of functional GABA$_B$R in chondrocytes. Abnormal endochondral bone formation and mineralization are shown in mice conditionally deleted of *Ihh* from *Col2a1*-expressing cells (27, 28), which is a quite similar phenotype, such as delayed calcification in forelimbs and hyoid bones, found with GABA$_B$R1-KO mice in this study. From this point of view, it should be emphasized that immunoreactive ATF4 was highly localized in the cytoplasm of chondrocytes from KO mice on immunoblotting and immunocytochemistry in this study. The findings that ATF4 expression was seen in both the nucleus and cytoplasm in GABA$_B$R1-null chondrocytes transfected with the GABA$_B$R1 expression vector altogether give rise to a proposal that the GABA$_B$R1 subunit is endowed to accelerate the translocation of cytoplasmic ATF4 into the nucleus to promote gene transactivation for *Ihh* expression in chondrocytes. The present promoter reporter analysis undoubtedly gives support to this proposal.

In fact, the GABA$_B$R1 subunit is shown to interact physically with ATF4 through the basic leucine zipper domain of ATF4 and the coiled-coil domain in the C terminus of GABA$_B$R1 subunit to modulate the expression of target genes in the CNS (25, 29). Although ATF4 is a transcription factor expressed predominantly by osteoblasts to play a role as a regulator of a variety of functions linked to the maintenance of bone mass (30, 31), this transcription factor also regulates chondrocytic proliferation and differentiation through up-regulation of *Ihh* expression during endochondral bone ossification (24). Moreover, *Ihh* promoter activity and expression are shown to be under the control by a cell-specific member of the Runt family of transcription factors, runt-related transcription factor-2 (Runx2) (32), which is responsible for cellular differentiation processes in osteoblasts and chondrocytes, besides ATF4. In our preliminary experiments, however, a physical interaction was not confirmed between GABA$_B$R1 and Runx2 on the yeast two-hybrid system and promoter reporter analysis (data not shown). Taken together, it is highly conceivable that GABA$_B$R1 subunit alone would positively regulate chondrocytic differentiation and maturation through up-regulation of *Ihh* expression during skeletal development after the acceleration of nuclear translocation of ATF4 toward promotion of the transactivation of particular target genes such as *Ihh*.

Nevertheless, GABA$_B$R1 subunit is shown to interact functionally with members other than GABA$_B$R2 subunit of the G protein-coupled receptor superfamily with seven transmembrane domains. For example, co-expression of GABA$_B$R1a with the metabotropic glutamate receptor isoform GluR4 leads to the expression of GABA$_B$R1a subunit at the cell surface, without forming a heterodimer between GABA$_B$R1a and metabotropic glutamate receptor 4, nor coupling to adenylyl cyclase and K^+ channels (33). A view that GABA$_B$R2 subunit is required for the surface trafficking of GABA$_B$R1 subunit responsible for the recognition of the agonist GABA and for coupling to G protein is indeed prevailing (26). By taking into consideration such unique properties of GABA$_B$R in the CNS, therefore, the possibility that GABA$_B$R1 subunit would regulate chondrogenesis during skeletal development through a mechanism associated with a hitherto unidentified functional interaction with particular members of G protein-coupled receptor superfamily expressed by chondrocytes is not ruled out so far.

It thus appears that GABA$_B$R1 subunit alone is constitutively expressed by chondrocytes to positively regulate chondrogenesis during skeletal development after acceleration of nuclear translocation of ATF4 toward up-regulation of *Ihh* expression in a manner irrelevant to the role as a dimeric partner for the canonical functional GABA$_B$R composed of both GABA$_B$R1 and GABA$_B$R2 subunits. Accordingly, GABA$_B$R1 subunit could be a potential candidate for the discovery and development of a drug useful for the treatment and therapy of a variety of cartilage and bone diseases relevant to abnormal endochondral ossification in human beings.

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