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Recql4 haploinsufficiency in mice leads to defects in osteoblast progenitors: Implications for low bone mass phenotype ☆

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Abstract

The cellular and molecular mechanisms that underlie skeletal abnormalities in defective Recql4-related syndromes are poorly understood. Our objective in this study was to explore the function of Recql4 in osteoblast biology both in vitro and in vivo. Immunohistochemistry on adult mouse bone showed Recql4 protein localization in active osteoblasts around growth plate, but not in fully differentiated osteocytes. Consistent with this finding, Recql4 gene expression was high in proliferating mouse osteoblastic MC3T3.E1 cells and decreased as cells progressively lost their proliferation activity during differentiation. Recql4 overexpression in osteoblastic cells exhibited higher proliferation activity, while its depletion impeded cell growth. In addition, bone marrow stromal cells from male Recql4+/− mice had fewer progenitor cells, including osteoprogenitors, indicated by reduced total fibroblast colony forming units (CFU-f) and alkaline phosphatase-positive CFU-f colonies concomitant with reduced bone mass. These findings provide evidence that Recql4 functions as a regulatory protein during osteoprogenitor proliferation, a critical cellular event during skeleton development.

Keywords: Recql4 overexpression; Osteoprogenitors; Proliferation; Differentiation; Recql4+/− mice; Bone mass

RecQ DNA helicases are known to be involved in the maintenance of genomic integrity at the cellular level (for reviews, see [1,2]). While yeast and bacteria possess a single RecQ helicase, there are five apparent orthologs in humans. Mutations inactivating the human Recql4 helicase occur in some cases of Rothmund–Thomson syndrome (RTS) [2]. Recql4 has high expression in actively proliferating tissues and its expression level is cell cycle regulated, with peak expression during the S-phase [3]. Recql4 was found to have a DNA-dependent ATPase function and participate in the repair of DNA double-strand breaks by homologous recombination [4]. The direct connection of Recql4 with DNA process is emphasized in recent studies showing that Recql4 is indispensable for initiation of DNA replication and cell proliferation [5–7].

RTS (OMIM 268400) with mutated Recql4 is characterized by chromosome fragility, predisposition to osteosarcoma and skeletal abnormalities [8–12]. Distinct Recql4 mutations also occur in RAPADILINO (OMIM 266280) and Baller–Gerold syndromes (OMIM 218600), where they are associated with skeletal malformations but not cancer predisposition [10,11].

Three Recql4 knockout mouse models have been generated to recapitulate the clinical symptoms of human Recql4 diseases [5,6,13]. The Recql4 knockout mouse construct with extensive Recql4 disruption was embryonic lethal and accompanied by defective cell growth [5]. Mice with Recql4 exon 13 deletion had a 5% survival rate by 2 weeks of age. Surviving mice were growth-retarded and exhibited bone defects and impeded cell division [6]. Mice with

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deletion of Recql4 helicase domain exhibited skeletal abnormalities and high frequency of aneuploidy in bone marrow stromal cells [13]. Findings obtained on different systems and by different approaches corroborate our understanding of skeletal phenotypes of Recql4 mutant alleles. However, the precise function of Recql4 in bone biology remains largely unknown.

In this report, we show that Recql4 protein was abundantly expressed in active osteoblasts around growth plate, but not in the fully differentiated osteocytes in adult murine skeleton. Recql4 gene expression was decreased as mouse osteoblastic MC3T3.E1 cells gradually lost their proliferation ability during differentiation. Recql4 overexpression in mouse osteoblastic cells resulted in increased proliferation, while its depletion impeded cell growth. Recql4 haploinsufficiency in mice decreased bone marrow stromal total fibroblastic colony forming units (CFU-f) and alkaline phosphatase-positive CFU-f colonies. The implications of these findings on low bone mass phenotype in Recql4-deficient mice are described.

Materials and methods

Immunohistochemistry. Immunohistochemistry for Recql4 protein expression was conducted in tibiae of 4-week-old C57/B6 mice ($n = 3$). Tibiae were fixed in 10% zinc formalin for 48 h, decalcified in 10% EDTA, and rapidly processed for paraffin wax embedding, using an automated tissue processor (ThermoShandon, Cheshire, UK). Sagittal sections of the proximal and middle tibia, 5 μm thin, were used to detect Recql4. The primary antibody, goat polyclonal anti-mouse Recql4 antibody (sc-16927, diluted 1:100, Santa Cruz Biotech, Santa Cruz, CA), was applied to each section and incubated at 4 °C overnight. The secondary biotinylated antibody and the streptavidin–horseradish peroxidase (HRP) conjugate complex from Vectastain ABC kit (Vector Laboratories, Burlingame, CA) were applied in a humidified chamber for 30 and 60 min, respectively. After washing in PBS, the chromagen, diaminobenzidine, was applied for 5 min, followed by counterstaining with methyl green. Negative controls included substituting the primary antisera with preimmune sera from the same species and omitting the primary antibody.

Cell culture. Mouse calvarial osteoprogenitor MC3T3.E1 clone 4 (American Type Culture Collection, ATCC, Manassas, VA) was cultured in α-MEM (Gibco-BRL, Grand Island, NY). Media were supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (Gibco-BRL). To induce osteoblast differentiation, MC3T3.E1 cells were seeded at a density of 1.8×10^5 cells/well in 6-well plates and induced to differentiate by enriching media with 50 μg/ml L-ascorbic acid and 10 mM β-glycerol phosphate. The culture medium was changed every 2 days. RNA and protein samples were collected on day 1, 5, 10, and 16.

Western analysis. Protein concentrations of whole cell lysates were determined spectrophotometrically using the BCA Protein Assay kit (Pierce Chemical, Rockford, IL). Ten microgram of protein extract was separated by 10% SDS–PAGE and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were probed overnight at 4 °C with mouse monoclonal anti-PCNA (ab29-100, Abcam, Cambridge, MA) at 1:1000 dilution and mouse monoclonal anti-actin (MAB1501R, Chemicon, Temecula, CA) at 1:500 dilution. Blots were further incubated for 1 h at 20 °C in the presence of a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at a dilution of 1:2000. The immune complexes were detected using ECL chemiluminescence substrate (Amersham Pharmacia Biotech). Actin was used as loading control.

Reverse transcription-polymerase chain reaction. Total RNA was prepared for RT-PCR using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized by Superscript System for RT-PCR (Invitrogen, Grand Island, NY). PCR was performed by RedTaq ReadyMix PCR Mix with $MgCl_2$ kit (Sigma, St. Louis, MO) in an Eppendorf Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Primers, corresponding annealing temperature, and fragment length of PCR product size are listed in Table 1. PCR products were run in 1% agarose gel. Real-time PCR primers and probes for Recql4 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Foster City, CA). Gene expression was evaluated as the ratio of target gene to GAPDH expression. The analyses were performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden). All experiments were repeated in triplicate.

Overexpression of Recql4 in MC3T3.E1 cells. MC3T3.E1 cells were seeded at a density of 2×10^5 cells/well in 6-well plate. The expression vector pCMV Tag-Recql4 (Recql4) and empty vector pCMV Tag-2A(Mock) [6] were transiently transfected into MC3T3.E1 cells using Lipofectamine Plus reagent (Invitrogen, Grand Island, NY) following the manufacturer's protocol. Recql4 overexpression was confirmed by Western analysis.

Silencing of Recql4 with siRNA in MC3T3.E1 cells. SiRNAs against Recql4 (Recql4-F, 5′AAATAGACTGCGTATTCTGCGCCTGTCTC-3′; Recql4-R, 5′AACGCAGAATACGCAGTCTATCCTGTCTC-3′) or a null control were cloned into the p*Silencer*™ 2.1-U6 hygro vector (Ambion, TX). Transfection in MC3T3.E1 cells was carried out using Lipofectamine Plus reagent as described above. Silencing of Recql4-specific siRNA was confirmed by RT-PCR.

BrdUrd proliferation assay. Bromo-deoxyuridine (BrdUrd) was used to label cycling MC3T3.E1cells in S-phase using standard protocol. Briefly, cells were treated with 10 μM BrdUrd for 1 h before harvest and then washed twice with phosphate-buffered saline (PBS), pH 7.4, and fixed with ice-cold methanol at 20 °C for 5 min. Chamber slides were blocked in PBS containing 10% goat serum and incubated at room temperature for 2 h with mouse monoclonal anti-BrdUrd antibody (Bu20a; diluted at 1:200, DAKO, Carpinteria, CA). Secondary antibody incubation and color development were done, using the protocol provided in the Vectastain ABC kit (Vector Laboratory). Slides were counterstained with methyl green. The percentage of BrdUrd-positive cells was calculated by dividing the number of BrdUrd-positive cells by total cell number, counted in 6–8 randomly selected fields for 3 individual wells.

Recql4 genetically modified animals. Indiana University School of Dentistry Animal Care and Use Committee approved all animal protocols. Age-matched 3 month male Recql4+/− and Recql4+/+ littermates were used for in vivo studies, or at age of 2 months for in vitro studies. Mice were genotyped by Taconic (Germantown, NY) as described previously by Dr. Masumi Abe's group at the National Institute of Radiological Sciences, Chiba, Japan [6]. Mice were housed under standard conditions of 12:12-h light/dark cycle, and given water and food ad

Table 1
Primers for PCR

Gene	Forward primer	Reverse primer	Annealing temp. (°C)	PCR product (bp)
Recql4	CCTGACATCCAGTCAGAAGT	CTGTGTTTCCTCACCAGAGT	57	666
Runx2	CCGGGAATGATGAGAACTAC	AGCTTCTGTCTGTGCCTTCT	60	230
Osteocalcin	CAAGTCCCACACAGCAGCTT	AAAGCCGAGCTGCCAGAGTT	60	370
GAPDH	TGACCACAGTCCATGCCATC	GACGGACACATTGGGGTAG	58	203

libitum (Purina Rodent Lab Chow 5001, containing 0.95% Ca, 0.67% P, and vitamin D3 4500 IU/kg). Mice were euthanized by CO_2 inhalation; thoracic transection assured death.

Dual energy X-ray absorptiometry analysis. Dual energy X-ray absorptiometry (DEXA) analysis on right femur bone mass was carried out under anesthesia as described previously [14]. Bone mineral content (BMC, g/cm), bone area (BA, cm^2), and bone mineral density (BMD, g/cm^2) were determined on 6–8 mice from each genotype.

Progenitor assay. To study the total number of progenitors of bone marrow stromal cells (BMSCs), including osteoprogenitors in vitro, we performed colony forming unit fibroblast (CFU-f) assay followed by alkaline phosphatase staining. Marrow cells were harvested from tibia of 5–6 mice of 2-month-old Recql4+/+ and Recql4+/− mice by flushing diaphyseal marrow through a 21-G needle. Cells were plated at a density of 4×10^6 cells/well on 6-well plates. Medium was first changed at 4 days and then every 2 days for 11 days supplemented with 50 μg/ml L-ascorbic acid and 10 mM β-glycerol phosphate. Cells were fixed and stained for alkaline phosphatase (ALP) according to the manufacturer's instructions (Sigma, St. Louis, MO). The number of fibroblastic colony forming units (CFU-f) and alkaline phosphatase-positive CFU-f (ALP + CFU-f) containing >50 cells was determined by microscopic examination.

Statistical analyses. Data were analyzed using JMP Statistical Discovery Software 4.1 (SAS Institute, Cary, NC). Data are expressed as means ± standard deviation (SD). Statistical analyses were performed with Student's *t* test. A *P* value <0.05 denoted the presence of a statistically significant difference.

Results

Recql4 protein was expressed in active osteoblasts and bone lining cells, but not in osteocytes

In an initial investigation of Recql4 protein expression pattern in adult murine skeleton, we performed immunohistochemistry on tibia sections of 4-week-old C57/B6 mice (Fig. 1). Intensive brown staining for Recql4 protein was observed in growth plate chondrocytes and osteoblasts lining the trabecular surface in tibial proximal metaphysis region, a region of active bone formation (Fig. 1A). Recql4 protein was also present in bone lining cells of endocortical surface, but not in the fully differentiated osteocytes in cortical bone (Fig. 1B).

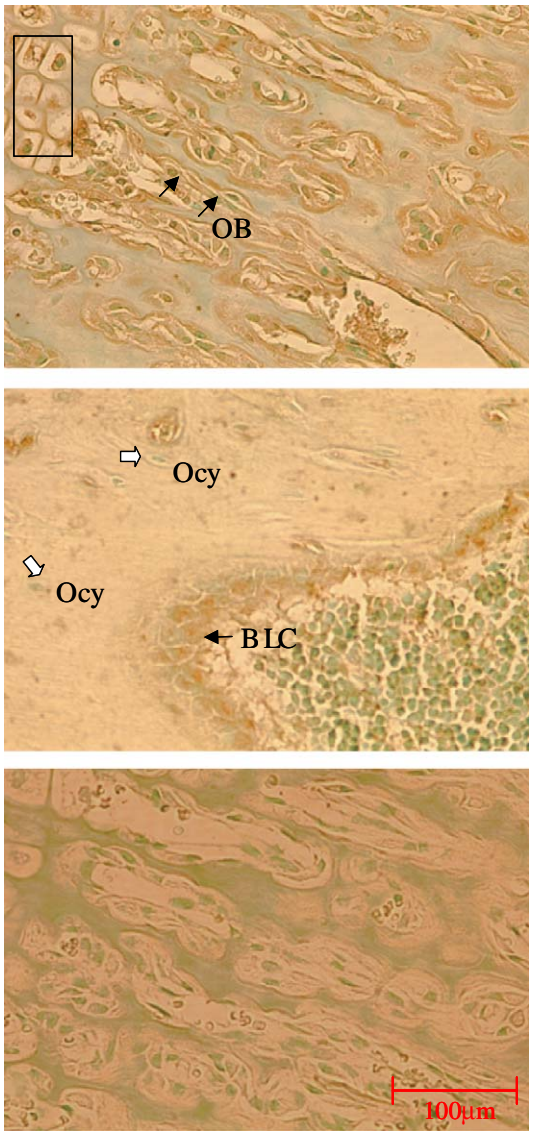

Fig. 1. Recql4 expression in tibia. Decalcified and wax embedded sections from tibia were stained by immunohistochemistry to detect Recql4 protein. (A) In trabecular primary spongiosa, osteoblasts lining trabeculae surface (arrow) and chondrocytes (boxed region) exhibited intensive brown staining for Recql4 expression. (B) Endocortical bone lining cells (arrow) revealed intense brown staining for Recql4 expression, but not in osteocytes (open arrow). (C) Negative control: absence of primary antibody. Secondary antibody alone yielded no staining of the tissue (note the prevalence of blue nuclei). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Recql4 gene expression was downregulated during osteoblast differentiation in vitro

Since Recql4 was detected in osteoblasts, we further investigated the expression pattern of Recql4 during osteoblast differentiation in vitro. Mouse osteoblastic MC3T3.E1 cells were induced to osteoblast differentiation in the presence of osteogenic media. Runx2 gene, an indicator of early commitment to osteoblast differentiation, constantly expressed during osteoblast differentiation; while osteocalcin, a marker of late stage osteoblast differentiation, increased over time (Fig. 2A). Cell proliferation marker, PCNA, was downregulated after day 10 (Fig. 2A). Maximal expression of Recql4 mRNA occurred at day 1 and then declined about 3-fold between day 5 and day 16 (Figs. 2A and B).

Overexpression of Recql4 induces higher proliferation activity and depletion of Recql4 impedes proliferation in MC3T3.E1 cells

We have shown that expression of Recql4 protein/gene was correlated with cell proliferation activity (Figs. 1 and 2). To investigate if Recql4 contributes to the regulation of osteoprogenitor proliferation, we examined the cell proliferation activity of MC3T3.E1 cells with Recql4 overexpression by measuring BrdUrd incorporation. Transfection of

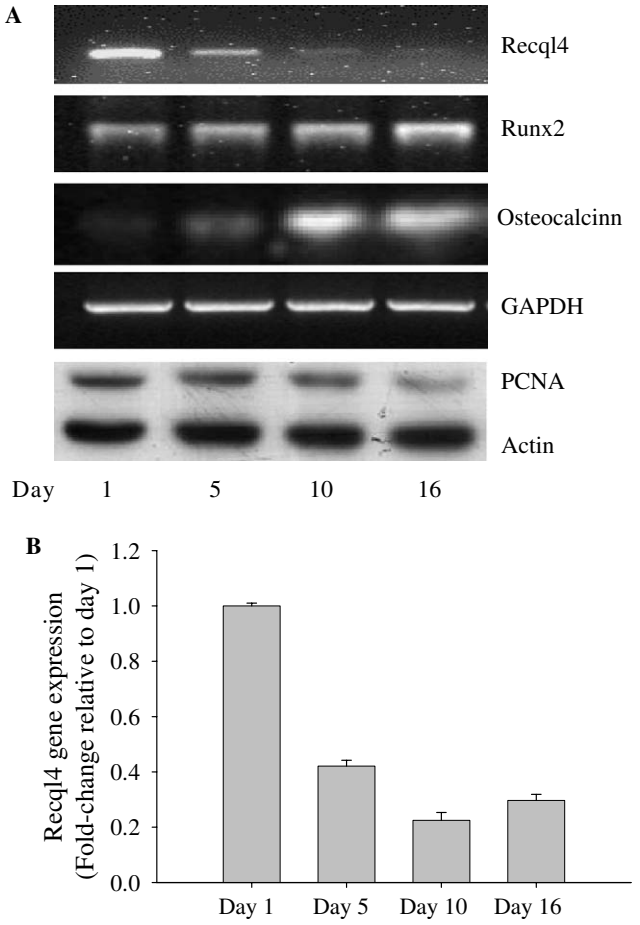

Fig. 2. Downregulation of Recql4 gene expression during osteoblast differentiation in MC3T3.E1 cells. (A) RT-PCR was used to determine Recql4, and osteoblastic specific genes (Runx2, and osteocalcin) mRNA expression levels in MC3T3.E1 cells induced to differentiate as described in Materials and methods. GAPDH was used as a control to verify that equal amounts of mRNA were used in the reactions. Western blotting was used to detect PCNA protein levels as a marker of cell proliferation activity. Actin was used as a control to verify equal protein loading. (B) Real-time PCR was used to confirm Recql4 gene expression. Data were normalized to GAPDH expression levels and indicated as means ± standard deviation ($n = 3$).

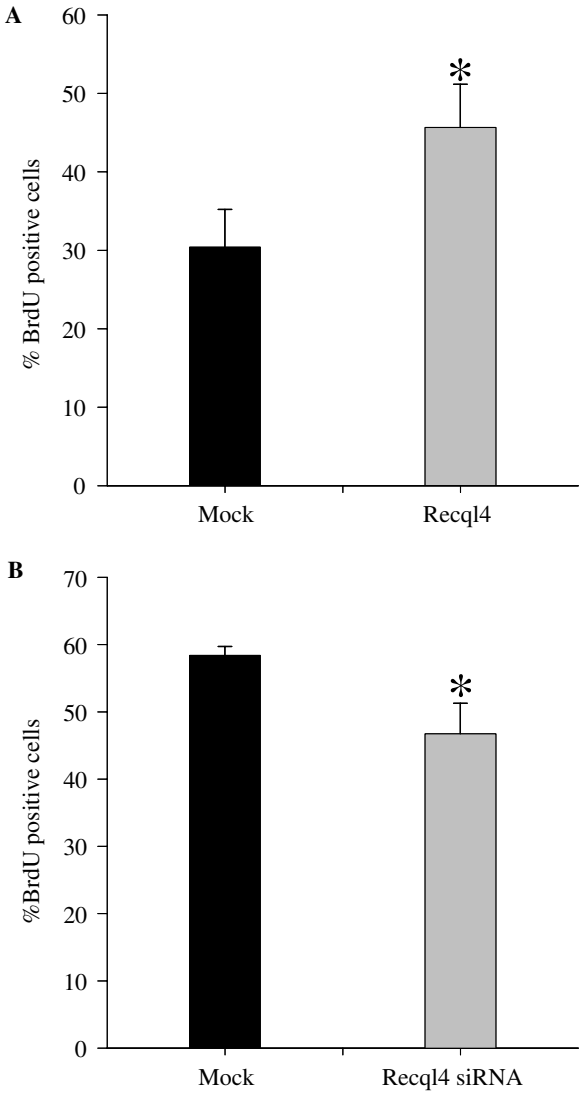

Fig. 3. Overexpression of Recql4 induces higher proliferation activity and depletion of Recql4 obstructs growth in MC3T3.E1 cells. Cell proliferation assay was performed by measuring percentage of BrdUrd-positive cells as described in Materials and methods. (A) Recql4 overexpression (Recql4) in MC3T3.E1 cells significantly increased the percentage of BrdUrd-positive cells compared to cells transfected with empty vector (mock). (B) Recql4 silencing by siRNA targeting Recql4 in MC3T3.E1 cells decreased the percentage of BrdUrd-positive cells compared to cells transfected with scrambled siRNA expression vector (mock). Bar graphs represent the mean ± standard deviation ($n = 3$), $^{*}P < 0.05$ as compared to mock culture.

MC3T3.E1 cells with Recql4 expression vector increased the percentage of BrdUrd-positive cells (S-phase cells) by 50% during 14 h serum stimulation compared to MC3T3.E1 cells transfected with control empty vector (Fig. 3A). In contrast, Recql4 silencing by siRNA targeting Recql4 decreases the proliferation of MC3T3.E1 cells in culture when compared to cells transfected with an empty siRNA expression vector (Fig. 3B). These results indicate that overexpression of Recql4 increases cell growth, while its silencing obstructs proliferation in mouse osteoblastic cells.

Recql4+/− mice exhibited reduced bone mass with concomitant defect in stromal progenitors

The regulatory role of Recql4 in osteoprogenitor proliferation suggests that a defect in osteoblastic lineage may be responsible for the skeletal hypoplasia reported in Recql4−/− mice [6]. Recql4−/− embryos in our study died before day 10 in uteri, we used Recql4+/− mice as a surrogate model to study how defective Recql4 affects osteoblast lineage cells. We first showed that Recql4+/− mice had detectable reduced bone mass: BMC (85.7%) and BMD (87.8%) in their right femora compared to Recql4+/+ mice by DEXA analysis (Fig. 4).

We then performed in vitro analysis of osteoprogenitor to determine if osteoblast defect contributes to reduced bone mass in Recql4+/− mice by using CFU-f assay followed by alkaline staining [15]. CFU-f assay demonstrated that Recql4+/− bone marrow has reduced progenitors

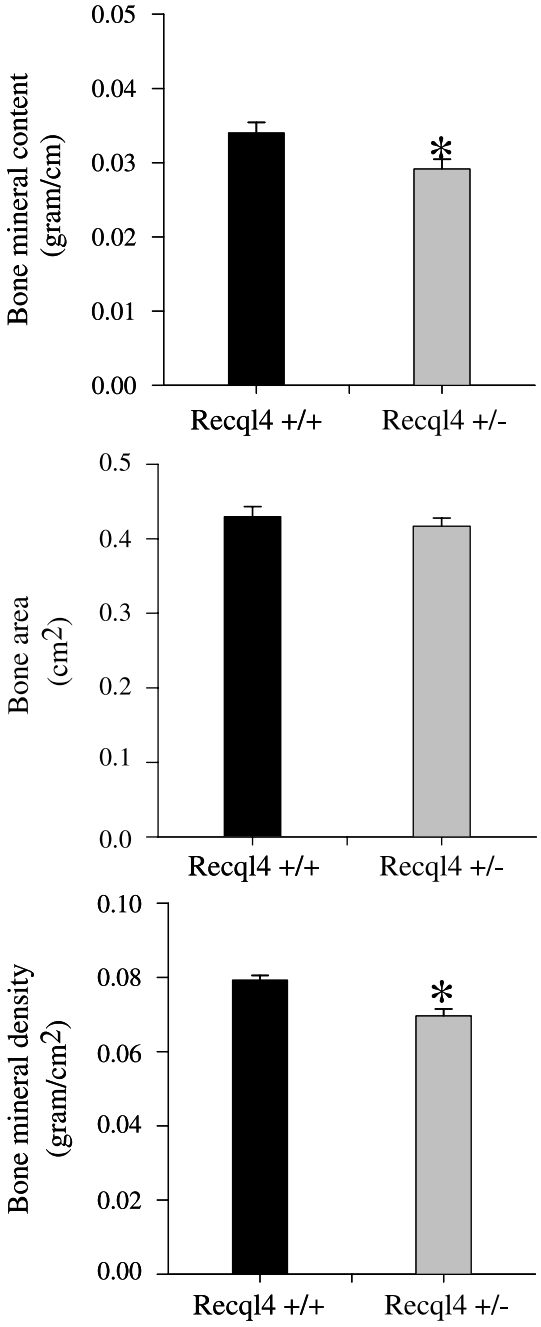

Fig. 4. Effects of Recql4 haploinsufficiency on bone mass in mice. The effects of single copy gene loss of Recql4 on bone mass were investigated by examining bone mass of the right femora by DEXA analyses. Significantly lower BMC (85.7%) and BMD (87.8%) were observed in Recql4+/− mice at 3 months of age compared to Recql4+/+ mice. Data are expressed as means ± standard error of mean. $^{*}P < 0.05$ compared to wild type littermates ($n = 6$ animals per genotype).

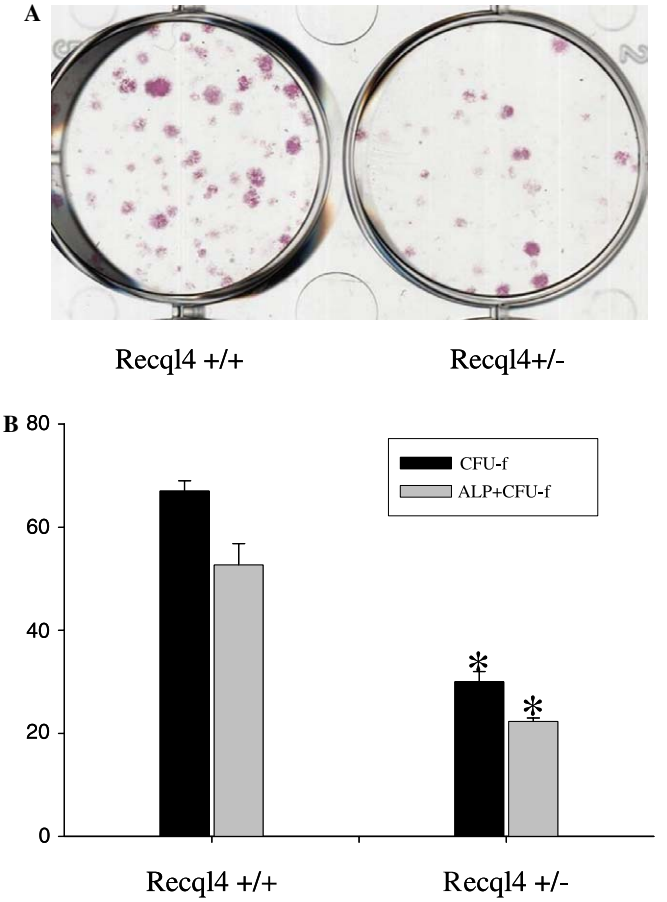

Fig. 5. Effects of Recql4 haploinsufficiency on primary bone marrow stromal cell culture (BMSCs). (A) BMSCs from Recql4+/− and Recql4+/+ mice were seeded in 6-well plates for CFU-f assay followed by alkaline phosphatase staining, as marker for osteogenic differentiation. Images shown are CFU-f cultures from Recql4+/− and Recql4+/+ mice after staining for alkaline phosphatase. (B) The total number of CFU-f colonies (ALP positive and negative colonies) and ALP + CFU-f colonies from primary bone marrow stromal cultures derived from 2-month-old Recql4+/− mice is <60% compared to cells derived from Recql4+/+ mice. Data are expressed as means ± standard deviation. $^{*}P < 0.05$ compared to wild type littermates.

(CFU-f) by <40% accompanied by fewer ALP + CFU-f/osteoprogenitors (Fig. 5).

Discussion

Report to date from both human and mouse Recql4 mutant alleles suggests a key role of Recql4 in the early development, including skeletal system [5,6,9–13,16,17]. One mechanism by which defective Recql4 may exert its effects on skeletal development is by affecting the proliferation of osteoblasts. Recql4 has been implicated as a proliferation-related protein in some tissues [3,5–7,18]. However, no study has been done so far to examine the function of Recql4 in osteoblast proliferation and differentiation.

In the present study, we have demonstrated that Recql4 protein was abundantly expressed in osteoblasts on trabecular tibiae bone surface around growth plate, but not in the fully differentiated osteocytes. Consistent with the published results, we also found intense staining for Recql4 protein in chondrocytes [10]. Expression of Recql4 was also observed in endocortical bone lining cells, which are considered as static cells and without active proliferation activity [19]. In these cells Recql4 expression may still be sustained as these cells can be readily induced into proliferation stage in response to several inductive signals in vivo,

such as mechanical stress and hormone regulation among others [19,20].

The in vivo Recql4 protein expression patterns in bone sections parallel the in vitro gene expression profile in mouse osteoblastic MC3T3.E1 cells showing downregulation during osteoblast differentiation, a process of losing proliferation activity progressively. It has been reported previously that intentional depletion of Recql4 in mouse embryo fibroblasts leads to defective cell proliferation, suggesting an indispensable role in normal cell growth [6,7]. These observations are further supported by our data showing that overexpression of Recql4 in osteoblastic cells increases cell growth, while its depletion impedes cell proliferation. Thus, taken together, our findings suggest that Recql4 acts as a regulatory protein during osteoprogenitor proliferation and its defects may lead to proliferation failure.

The Recql4 knockout mouse model was reported to have 5% survival rate by 2-week-old [6], but Recql4−/− embryos in our study died before day 10. We used Recql4+/− mice as a surrogate model to study how defective Recql4 affects osteoblast lineage cells. We first confirmed that Recql4+/− mice exhibited skeletal defects with reduced bone mass, but somewhat to a lesser degree compared to the skeletal hypoplasias reported in Recql4−/− mice [6]. The size of osteoblast populations during skeletal development contribute largely to the attainment of peak bone mass [21–24]. In the present study, reduced osteoprogenitor frequency may be the cellular causes for low bone mass phenotype detected in Recql4+/− mice compared to wild type littermates. To determine if osteoprogenitors are affected in Recql4+/− mice and therefore contributes to reduced bone mass, equal numbers of nucleated BMSCs from 2-month-old Recql4+/− and Recql4+/+ mice were cultured. The number of ALP + CFU-f colonies, a measure of osteoprogenitors, and number of CFU-f colonies, a measure of total mesenchymal precursors including stem cells and committed progenitors [15], were significantly lower. However, we did not detect significant differences in cell proliferation activity in the primary BMSC cultures between two genotypes (data not shown). This may be because highly proliferative embryonic stem cells/progenitors in the early developmental stages require strict mechanism to maintain chromosome integrity to guarantee cell viability [25,26]. Stem cells or early progenitor cells in early development stages may weaken their proliferation and/or viability due to Recql4 haploinsufficiency resulting in decreased cell number.

In conclusion, our data indicate that Recql4 functions in the regulation of osteoprogenitor proliferation, which may be one of the cellular mechanisms that underlie skeletal anomalies common to defective Recql4 syndromes. Bone is a dynamic tissue and undergoes constant remodeling by way of bone formation and resorption by the activity of osteoblasts and osteoclasts. Defects in osteoblasts or osteoclasts with decreased rate of their production from progenitors and/or proliferation failure may lead to skeletal malformation. However, the effects of Recql4 haploinsufficiency in mice on osteoclast functions need to be determined.

Acknowledgments

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