Akt1 in Chondrocytes Controls Cartilage Calcification during Skeletal Growth and Osteophyte Formation in Osteoarthritis

INTRODUCTION
Endochondral ossification plays crucial roles in skeletal growth and osteoarthritis progression. Since the phosphoinositide-dependent serine-threonine protein kinase Akt is known to be a pivotal signaling molecule for several factors regulating cartilage metabolism, this study examined the possible involvement of Akt in the endochondral ossification process under physiological and pathological conditions.

METHODS
Expressions of the Akt isoforms, chondrocyte differentiation and calcification markers, and inorganic pyrophosphate (PPi)-related factors were assessed by real-time RT-PCR or Western blotting in primary cartilaginous chondrocytes from neonatal mice or mouse chondrogenic ATDC5 cells. To know the in vivo role of Akt1, we compared the skeletal phenotypes between homozygous Akt1-deficient (Akt1−/−) mice and the wild-type littermates by radiological and histological analyses, including HE, Safranin-O, and von Kossa stainings, BrdU labeling, and immunostainings of type X collagen (COL10) and vascular endothelial growth factor (VEGF). An experimental osteoarthritis model was created surgically by inducing instability in the medial joint of 9-week-old mice, and osteoarthritis severity was quantified 8 weeks after the surgery using our histopathology assessment system. For the functional analyses, we established stable lines of ATDC5 cells with retroviral overexpression of constitutively active Akt1 (ca-Akt1) or small interfering RNA of Akt1 (si-Akt1). Cell proliferation was assessed by CCK-8 assay. The chondrocyte differentiation was determined by Alcian blue staining and COL10 mRNA level under the stimulation of insulin. The cartilage calcification was assessed by Alizarin red and von Kossa stainings, and VEGF expression under the stimulation of insulin and phosphate. Promoter activity of COL10, ANK or NPP1 was assessed by luciferase assay by the transfection of ca-Akt1, dominant negative mutant of Akt1 (dn-Akt1), or the control EV in ATDC5 cells with a reporter construct containing a respective promoter fragment.

RESULTS
Among the Akt isoforms (Akt1, 2 & 3) Akt1 was most highly expressed in primary cartilaginous, and both phosphorylated and unphosphorylated Akt proteins were considerably decreased in the Akt1−/− chondrocytes, indicating a major role of Akt1. The Akt1−/− mice exhibited dwarfism with shorter limbs and trunks than the wild-type littermates (Figure 1A). In the Akt1−/−/+ growth plate, BrdU-positive proliferative and COL10-positive hypertrophic zones were normal; however, cartilage calcification at the bottom of the von Kossa staining was significantly suppressed (Figure 1B). Under the osteoarthritis induction in knee joints of the two genotypes, cartilage calcification, and suppressed by that of si-Akt1 (Figure 2A, middle and right columns, *p<0.05 vs. +/+). The present in vivo and in vitro evidence revealed that Akt1 promotes cartilage calcification by inhibiting PPi accumulation in chondrocytes, and thereby controls endochondral ossification in the skeletal growth and in the osteophyte formation of osteoarthritis. Interestingly, neither the proliferation nor hypertrophic differentiation of chondrocytes was affected by the gain- or loss-of-function of Akt1. Although it seems difficult to target this molecule directly in order to yield novel therapeutics for skeletal disorders like growth retardation and osteoarthritis because of its ubiquitous expression and diverse functions, further understanding of the molecular network related to the Akt1 / PPi axis will greatly help us to unravel the complex mechanism that modulates the endochondral ossification process under physiological and pathological conditions.

DISCUSSION
The proliferation nor hypertrophic differentiation of chondrocytes was affected by the gain- or loss-of-function of Akt1. Although it seems difficult to target this molecule directly in order to yield novel therapeutics for skeletal disorders like growth retardation and osteoarthritis because of its ubiquitous expression and diverse functions, further understanding of the molecular network related to the Akt1 / PPi axis will greatly help us to unravel the complex mechanism that modulates the endochondral ossification process under physiological and pathological conditions.

Figure 1. (A) Radiographic images of the whole body of wild-type (+/+) and Akt1−/− littermates at 8 weeks of age. The graph shows the time course of the naso-anal lengths of the littermates after birth. (B) Histology of the growth plates in proximal tibia of neonatal mice. Graphs show the percentage of width of the calcified osteophyte area. Graphs show the quantifications of osteoarthritis progression by our histopathology assessment system. Means (bars) ± SEM (error bars) for 7-13 mice.

Figure 2. (A) Effects of gain- and loss-of-functions of Akt1 on cartilage calcification in cultures. Alizarin red and von Kossa stainings, and mRNA levels of VEGF, ANK and NPP1 in in vivo cultures of costal cartilages from wild-type (+/+) and Akt1−/− littermates (left column), and stable lines of ATDC5 cells retrovirally transfected with ca-Akt1 (middle column) and si-Akt1 (right column) or the respective control vectors (EV and si-GFP). (B) Luciferase activities by the transfection of ca-Akt1, dn-Akt1, or the control EV in ATDC5 cells with a luciferase reporter gene construct containing a fragment of the promoter regions of ANK or NPP1 gene shown above. Means (bars) ± SEM (error bars) for 3 cultures. *p<0.05 vs. +/+.