KRÜPPEL-LIKE FACTOR 5 CAUSES CARTILAGE DEGRADATION THROUGH TRANSACTIVATION OF MATRIX METALLOPROTEINASE 9

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Although degradation of cartilage matrix has been suggested to be a rate-limiting step for endochondral ossification during skeletal development, little is known about the transcriptional regulation. This study investigated the involvement of Krüppel-like factor 5 (KLF5), an Sp/KLF family member, in the skeletal development. KLF5 was expressed in chondrocytes and osteoblasts, but not in osteoclasts. The heterozygous deficient (KLF5+/-) mice exhibited skeletal growth retardation in the perinatal period. Although chondrocyte proliferation and differentiation were normal, cartilage matrix degradation was impaired in KLF5+/- mice, causing delay in replacement of cartilage with bone at the primary ossification center in the embryonic limbs and elongation of hypertrophic chondrocyte layer in the neonatal growth plates. Microarray analyses identified matrix metalloproteinase 9 (MMP9) as a transcriptional target since it was strongly up-regulated by adenoviral transfection of KLF5 in chondrogenic cell line OUMS27. The KLF5 overexpression caused gelatin degradation by stimulating promoter activity of MMP9 without affecting chondrocyte differentiation or vascular endothelial growth factor expression in the culture of chondrogenic cells; however, in osteoclast precursors, it affected neither MMP9 expression nor osteoclastic differentiation. KLF5 dysfunction by the genetic hetero-deficiency or the RNA interference was confirmed to cause reduction of MMP9 expression in cultured chondrogenic cells. MMP9 expression was decreased in the limbs of KLF5+/- embryos, which was correlated with suppression of matrix degradation, calcification, and vascularization. We conclude that KLF5 causes cartilage matrix degradation through transcriptional induction of MMP9, providing the first evidence that transcriptional regulation of a proteinase contributes to endochondral ossification and skeletal development.

Endochondral ossification is an essential process for skeletal development and growth (1). During the process, chondrocytes undergo proliferation and hypertrophic differentiation. The hypertrophic chondrocytes then secrete a specialized extracellular matrix rich in type X collagen (COL10) which is replaced by bone matrix. The ossification begins with chondrocyte apoptosis, cartilage matrix degradation, calcification, vascular invasion from perichondrium and bone marrow, and deposition of bone matrix by osteoblasts (2). Among these individual steps, previous studies have indicated that degradation of cartilage matrix is particularly crucial (3-6). This step requires proteolytic breakdown by a variety of proteinases, among which members of the matrix metalloproteinase (MMP) family are of special interest due to their ability to cleave collagens and aggrecan, the two principal matrix components of cartilage (7,8). However, little is known about transcriptional regulation of MMPs in the endochondral ossification process.

Members of the Krüppel-like factor (KLF) family are important transcription factors that regulate development, cellular differentiation and growth, and pathogenesis of atherosclerosis and tumor development, by controlling the expression...
of a large number of genes with GC/GT-rich promoters (9). There are currently 17 known members of the mammalian KLF family (10), each of which has individually important biological functions (11). Among the members, KLF5 (IKLF, BTEB2) was identified as a positive regulator of SMemb, a marker gene for activated smooth muscle cells in vascular disease (12). KLF5 shows temporal changes in expression during embryogenesis, with diverse functions in cell differentiation and embryonic development (13,14). Although KLF5 homozygous knockout (KLF5−/−) mice die in utero before embryonic day (E)8.5, the heterozygous knockout (KLF5+/−) mice are apparently normal and fertile (15). Further analyses of these mice revealed that KLF5 mediates cardiovascular remodeling since the mice exhibited attenuated cardiac hypertrophy and fibrosis, as well as much less granulation formation in response to vascular injury (15). The neonatal KLF5+/− mice also exhibited a marked deficiency in white adipose tissue development, suggesting a contribution to adipogenesis (16).

The KLF family shares similar zinc finger structures with the Sp family, some members of which are known to be essential for skeletal development and growth. For example, Sp3 and Sp7 (osterix) are required for skeletal ossification (17) and osteoblast differentiation (18), respectively. The present study initially detected KLF5 expression in cells of bone and cartilage. To learn the role of KLF5, we analyzed the skeleton of KLF5+/− mice, and found that the KLF5 insufficiency caused impaired degradation of cartilage matrix in the perinatal period. We further investigated the underlying molecular mechanisms.

**Experimental Procedures**

*Mice*- Generation of KLF5+/− mice was described previously (15). All mice were maintained in the C57BL/6 background with a standard diet. In each experiment, male mice of KLF5+/− and the wild-type littermates were compared. All experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

*Cell cultures*- Mouse osteoblastic cell line MC3T3-E1, mouse chondrogenic cell line ATDC5, and mouse monocyte-macrophagic cell line RAW264.7 were purchased from RIKEN. Human chondrogenic cell line OUMS27 was purchased from Health Science Research Resources Bank.

For isolation of primary osteoblasts, calvariae of neonatal wild-type mice were digested for 10 min at 37°C in an enzyme solution containing 0.1% collagenase and 0.2% dispase 5 times, and cells isolated by the last four digestions were combined. Primary chondrocytes were prepared from ventral rib cages (excluding the sternum) of E18.5 wild-type mouse embryos as previously described (19). For mature osteoclasts, macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages (M-BMMφ) which are known to be osteoclast precursors were isolated from bone marrow of 6-8 week-old mice as previously described (20), and cultured in the presence of M-CSF (30 ng/mL) and soluble receptor activator of nuclear factor κB ligand (RANKL; 100 ng/mL) for 3 d. Primary osteoblasts and MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM) containing 10% FBS. Primary chondrocytes, ATDC5, OUMS27, and Raw264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. For proliferation assay of primary chondrocytes, cell number was counted using the Cell Counting Kit-8 (Dojindo).

**Histological analyses**- The whole skeletons of WT and KLF5+/− littermate embryos (E16.5) were fixed in 99.5% ethanol, transferred to acetone, and stained in a solution containing Alizarin red S and Alcian blue 8GX (Sigma). Tibial limbs were fixed in 4% paraformaldehyde/PBS, embedded in paraffin, sectioned in 5-µm slices, and stained with hematoxylin and eosin (HE), toluidine blue (TB), Safranin-O, and 5% silver nitrate (von Kossa), according to the standard procedures. TUNEL staining was performed using an Apoptosis in situ Detection Kit (Wako), according to the manufacturer’s instruction. TRAP-positive cells were stained at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate in N,N-dimethyl formamide as the substrate. For immunohistochemistry, rabbit anti-mouse antibodies against KLF5 (1:100; KM1785) (15), type II collagen (COL2) (1:1000; LSL), COL10 (1:1000; LSL), and osteopontin...
(1:1000; LSL), a goat anti-mouse antibody against MMP9 (1:150; R&D), a mouse anti-mouse antibody against the aggrecan DIPEN neoepitope (1:100; ab3777, abcam), and a rat anti-mouse antibody against CD34 clone MEC14.7 (1:50; Hycult Biotech) were used. For BrdU labeling, animals were injected intraperitoneally with BrdU (25 μg/g body weight; Sigma) 2 h prior to sacrifice, and the sections were stained with a BrdU staining Kit (ZYMED) according to the manufacturer’s instruction.

**Viral transfections and osteoclastogenesis assay—**
For adenovirus infection of KLF5 to OUMS27 cells, the construction of adenovirus KLF5 expression vector was described previously (21). Adenovirus expressing KLF5 or the control empty vector (EV) was transduced to the cells at 40 or 100 multiplicities of infection (MOI). At 72h after transfection, the cells were harvested and used for subsequent assays. For retrovirus infection of KLF5 to M-BMMΦ, the construction of retrovirus KLF5 expression vector was described previously (16). M-BMMΦ were infected with KLF5 or control retroviral particles, and further cultured with M-CSF (30 ng/ml) and soluble RANKL (100 ng/ml) for 4-5 additional days, then the number of cells positively stained for TRAP and containing more than three nuclei were counted as osteoclasts.

**RNA interference—** Small interfering RNA (siRNA) oligonucleotides were constructed by DHARMACON. They were transfected to OUMS27 cells in concentrations of 200 nM, according to the manufacturer’s instruction, and cultured for 72 h for subsequent assays. siRNA probe sequences are described in the Supplemental Information online.

**Real-time RT-PCR, Western blotting, and gelatin zymography—** For RT-PCR, total RNAs were reverse-transcribed with MultiScribe reverse transcriptase (ABI). Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (ABI) using QuantiTect SYBR Green PCR Master Mix (Qiagen), according to the manufacturer’s instructions. Sequence information is described in the Supplemental Information online. For Western blot analysis, primary antibody to KLF5 (1:1000; KM1785) (15), MMP9 (1:1000; R&D), or β-actin (1:2000; Sigma) was used. For gelatin zymography, 10 μg of cell lysates was loaded to a zymogram acrylamide gel. The gel was electrophoresed and incubated with developing buffer for 40 h using a Zymography Electrophoresis kit (Cell Garage).

**Microarray analysis—** Target genes of KLF5 were identified by comparing mRNA expression in OUMS27 cells with adenoviral introduction of KLF5 or EV using two systems of microarray analyses: APHS-016 (for human extracellular matrix and adhesion molecules; Supplemental Table 1) and APHS-024 (for human angiogenesis molecules; Supplemental Table 2) in an RT2 Profiler PCR Array System (Super Array Bioscience). For MMP19, 20, ADAMTS 4, 5, 9 and 15, which are not included in the array systems, real-time RT-PCR analysis was separately performed. Sequence information is described in the Supplemental Information online.

**Luciferase assay—** The human MMP9 promoter regions from −1,250 bp relative to the transcriptional start site were cloned into the pGL4-Basic vector (Promega). The luciferase assay was performed with a dual-luciferase-reporter assay system (Promega) using GloMax™ 96 Microplate Luminometer (Promega).

**Radiological analyses—** Plain radiographs were taken using a soft X-ray apparatus (CMB-2, SOFTEX), and bone mineral density (BMD) was measured by dual energy X-ray absorptiometry using a bone mineral analyzer (PIXImus, Lunar Corp).

**Bone fracture experiment—** A transverse osteotomy was created at the midshaft of the right tibia using a bone saw, and was internally stabilized with an intramedullary nail using the inner pin of a spinal needle of 22- or 23-gauge diameter depending on the size of the cavity, as we reported previously (22,23). For histological analyses, specimens of the harvested tibias were fixed with 4% paraformaldehyde, decalcified with EDTA, dehydrated with ethanol, embedded in paraffin, and cut into 5 μm sections. The sections were stained with HE or toluidine blue.

**Arthritis experiment—** Arthritis was induced by the modified method of Terato et al. (24,25). Briefly, mice were injected intraperitoneally with 10 mg of an anti-COL2 monoclonal antibody (Immuno-Biological Laboratory) on day 0. On days 2 and 7, 50 μg of lipopolysaccharide (LPS;
100 μL of 500 μg/mL solution in saline) was injected intraperitoneally followed by an intermittent LPS injection every 3 d to the end of the experiment. As a control, 2.5 and 0.1 mL of saline was injected similarly to the antibody and LPS, respectively. The clinical severity of arthritis was graded on a 0–3 scale as follows: 0, normal; 1, swelling of ankle or wrist, or limited to digits; 2, swelling of the entire paw; 3, maximal swelling. Each limb was graded by a single blinded observer, allowing a maximum arthritis score of 12 for each animal.

Statistical analysis- Means of groups were compared by ANOVA and significance of differences was determined by post-hoc testing using Bonferroni’s method.

RESULTS

KLF5 expression in bone and cartilage. To know the involvement of KLF5 in skeletal metabolism, we initially examined the expression of KLF5 mRNA in cells of bone and cartilage by RT-PCR analysis (Fig. 1A, Supplemental Fig. 1A). It was expressed in primary osteoblasts and chondrocytes derived from mouse neonatal calvaria and costal cartilage, respectively, as well as in osteoblastic cell line MC3T3E1 and chondrogenic cell lines ATDC5 and OUMS27. Meanwhile, the expression was hardly detected in osteoclasts formed from the precursor M-BMMΦ (20) or in monocyte-macrophagic cell line RAW264.7. Immunohistochemical analysis of tibial limbs of mouse embryos and neonates showed extensive expression of KLF5 in cells of all layers of cartilage and perichondrium, as well as in osteoblasts on primary spongiosa (Fig. 1B). The expression in chondrocytes of limb cartilage was visible as early as E13.5 (Supplemental Fig. 1B).

Impaired cartilage degradation and remodeling in KLF5+/- limbs. To learn the physiological function of KLF5 in vivo, we investigated the skeletal phenotype of KLF5+/- mice, because KLF5−/− mice died before E8.5 (15). Although KLF5+/- embryos (E16.5) showed normal skeletal patterning without abnormality in major organ development, they were smaller in size compared to wild-type littermates (Figs. 2A & 2B). The femoral and tibial limbs of KLF5+/- embryos were 10-15% shorter than those of the wild-type littermates. In the KLF5+/- limb shaft, formation of bone and bone marrow tissues around the primary ossification center was delayed (Fig. 2B), and cartilage matrix like COL2 or COL10 remained undegraded in the shaft (Fig. 2C). Accordingly, TUNEL-positive apoptotic chondrocytes and tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts / chondroclasts were decreased in the KLF5+/- limb. In the growth plate of neonates, although there was no difference in proliferative and prehypertrophic layers between the genotypes, the hypertrophic layer was elongated in KLF5+/- mice (Figs. 2D & 2E). The Safranin-O-positive proteoglycan matrix remained in the hypertrophic layer, while the von Kossa-positive calcification layer was reduced in the KLF5+/- growth plate. These results indicate that the KLF5 insufficiency causes impairment of cartilage degradation and calcification in the perinatal period.

In an earlier period prior to the occurrence of ossification in the limb shaft of wild-type mice (E15.5), the limbs were filled with chondrocytes with comparable production of proteoglycan, COL10, and osteopontin in KLF5+/- and the wild-type littermates, indicating that KLF5 insufficiency did not affect chondrocyte differentiation in early stages up to hypertrophic differentiation (Supplemental Fig. 2A). Chondrocyte proliferation determined by BrdU uptake was also similar between the genotypes (Supplemental Fig. 2B).

After birth, skeletal growth of KLF5+/- mice caught up with that of wild-type, and they became comparable at 4 weeks of age (Supplemental Figs. 3A & 3B). KLF5 did not affect bone remodeling after birth either, since bone density of KLF5+/- long bones was normal (Supplemental Figs. 3C & 3D).

MMP9 as a transcriptional target of KLF5 in chondrocytes. To know the molecular mechanism whereby KLF5 contributes to cartilage degradation, we searched for the transcriptional targets by comparing mRNA levels in human chondrogenic cell line OUMS27 adenovirally transfected with KLF5 and the empty vector using microarray and real-time RT-PCR analyses (Table 1). Among molecules related to matrix degradation, MMP9 expression was most strongly
up-regulated by the KLF5 overexpression.

MMP9 mRNA level was confirmed to be increased in a dose-dependent manner of adenoviral KLF5 overexpression in OUMS27 cells (Fig. 3A). In addition, gelatin zymography revealed an increase of gelatin degradation by KLF5, which was compatible with MMP9 activity, but not with MMP2 activity, indicating that KLF5 exhibited proteinase activity via the induction of MMP9. Contrarily, osteopontin mRNA level was not altered by the overexpression, suggesting that chondrocyte differentiation at later stages was not affected by KLF5 (Fig. 3B). MMP13 was moderately decreased while vascular endothelial growth factor A (VEGFA) was little regulated by KLF5 in OUMS27 cells, both of which were consistent with the results of microarray analyses (Table 1, Supplemental Table 1 & 2). To examine the transcriptional regulation, a luciferase-reporter gene construct of the MMP9 5'-end flanking region was transfected into OUMS27, ATDC5 and human hepatocytic Huh7 cells. The transcriptional activity determined by the luciferase-reporter assay was enhanced by co-transfection with KLF5 in all cells, demonstrating the transcriptional induction of MMP9 by KLF5 (Fig. 3C).

The expression patterns of KLF5 and MMP-9 during chondrocyte differentiation were confirmed to be similar in cultured OUMS-27 cells. (Supplemental Fig. 1A). In addition, the time course analyses by immunostainings of embryonic limbs showed that KLF5 expression was seen early from E13.5 while MMP9 expression could be detected at E15.5 and enhanced at E16.5 (Supplemental Fig. 1B). Since the embryonic stage when the suppression of cartilage degradation in the KLF5+/− limb was initiated around E16.5 (Fig. 2, Supplemental Fig. 2), these time courses support the hypothesis that MMP9 induction by KLF5 may lead to the cartilage degradation during endochondral ossification in skeletal development.

Since MMP9 is known to be strongly expressed in osteoclastic cells (26) and to play an important role in skeletal remodeling (3), we next investigated the effects of KLF5 on the culture of osteoclast-precursor M-BMMΦ by retroviral overexpression of KLF5. Neither the osteoclastogenesis nor the MMP9 mRNA level was affected by the overexpression (Figs. 3D & 3E). In the monocyte-macrophagic Raw264.7 cell culture as well, the adenoviral overexpression of KLF5 did not affect endogenous MMP9 mRNA level (Supplemental Fig. 4A), while it increased the activity of the exogenously transfected luciferase-reporter construct containing the MMP9 promoter above (Supplemental Fig. 4B). These indicate that KLF5 does not have an important physiological function in osteoclastic cells.

To further examine the effect of loss-of-function of KLF5 in chondrocytes, we cultured primary costal chondrocytes derived from KLF5+/− mice, and confirmed the KLF5 mRNA level was decreased to about half that of wild-type chondrocytes (Fig. 4A). Among the molecules related to terminal differentiation of chondrocytes, cartilage degradation and remodeling, only MMP9, but not osteopontin, MMP13 or VEGFA, was significantly suppressed by the KLF5 haploinsufficiency. Proliferative ability was comparable between cultured chondrocytes from KLF5+/− and the wild-type littermates (Fig. 4B). Gene silencing of KLF5 by RNA interference also caused the reduction of MMP9 mRNA expression in OUMS27 cells (Fig. 4C).

Finally, an immunohistochemical staining confirmed that MMP9 expression seen in the cartilage layer and the perichondrium of wild-type limb was scarcely detected in KLF5+/− (Fig. 4D). In the cartilage layer, the decreased MMP9 expression was correlated with the suppression of COL2 degradation and chondrocyte calcification, determined by immunohistochemical and von Kossa stainings, respectively. In the perichondrium, the MMP decrease was correlated with the suppression of DIPEN which is a neoepitope at the aggrecan cleavage site generated by MMPs, and with that of CD34 which is an endothelial antigen representing blood vessels, determined by respective immunostainings. Quantitative analyses actually revealed significant decreases in the width of the von Kossa-positive calcified layer and the number of CD34-positive blood vessels in the KLF5+/− limb (Fig. 4E).

**DISCUSSION**

The present in vivo analyses revealed that KLF5 haploinsufficiency caused impairment of cartilage matrix degradation and the subsequent
remodeling to bone tissue, without affecting chondrocyte proliferation or differentiation. Microarray and cell culture analyses demonstrated that KLF5 contributes to the cartilage degradation through transcriptional induction of MMP9. MMP9 is known to be a potent proteinase that degrades denatured collagens and activates other MMPs and cytokines (4,8,27). In fact, the homozygous deficient mice (MMP9−/− mice) are reported to exhibit skeletal abnormality similar to KLF5+/− mice; elongation of hypertrophic layer, impaired vascularization, and delayed formation of bone and bone marrow cavity in the limbs (3), indicating a role of the KLF5-MMP9 axis during skeletal development.

It is, however, of note that the defect of KLF5 is physiologically more critical than that of MMP9, since KLF5−/− mice die in utero before E8.5 while MMP9−/− mice grow normally after birth and MMP9+/− mice show no abnormality from embryos (3). This might be because KLF5 regulates molecules other than MMP9, since the present microarray analyses revealed up-regulation of several molecules like α-E-catenin (CTNNA1), a disintegrin and metalloproteinase with thrombospondin-like repeat 4 (ADAMTS4), interleukin-1 (IL-1), and MMP14 by the KLF5 overexpression in chondrogenic cells (Table 1, Supplemental Table 1 & 2). α-E-catenin, a prototypic member of the α-catenin family and a component of the cadherin-catenin complex (28), is known to be required to sustain adhesion between cells during mammalian morphogenetic events (29). Although it is mainly expressed in epithelial tissues and the loss-of-function mutation causes human squamous cell carcinoma of the skin (30), the involvement in matrix degradation or angiogenesis remains unknown. Meanwhile, ADAMTS4 is a principal proteinase for aggrecan (31), a major cartilage matrix component that is degraded before collagenases cleave collagens in the hypertrophic layer (32). Hence, KLF5 might possibly lead to aggrecan degradation through induction of ADAMTS4, which is followed by matrix degradation by MMP9 (5). IL-1, a representative proinflammatory cytokine, is also known to be a potent stimulator of MMPs, ADAMTSs, and other catabolic cytokines (32), so that IL-1 and MMP9 induced by KLF5 might initiate the subsequent catabolic changes of the cartilage. MMP14, although the induction by KLF5 was not strong (Table 1), is also a key proteinase in growth plate resorption, since the MMP-14-deficient mice exhibited dwarfism due to impaired endochondral ossification and angiogenesis, similarly to the KLF5−/− mice (33,34). KLF5 may therefore be a crucial transcription factor that controls the molecular network for cartilage matrix degradation during endochondral ossification.

Another difference in the effects of insufficiency of KLF5 and MMP9 is their function in osteoclasts or chondroclasts. MMP9−/− mice showed abnormal bone remodeling after birth with impaired osteoclast recruitment while KLF5+/− mice showed normal bone remodeling. Expression of MMP9 by osteoclastic cells may physiologically be important for skeletal development, since transplantation of wild-type bone marrow cells including osteoclast progenitors rescues the skeletal phenotype (3). In fact, MMP9 is abundantly expressed (26) while KLF5 was barely detected in osteoclastic cells (Fig. 1). The finding that the KLF5 overexpression in osteoclast precursors failed to alter endogenous MMP9 expression and osteoclastic differentiation (Figs. 3D & 3E, Supplemental Fig. 4) supports the importance of MMP9 expression in osteoclastic cells. The present study, however, demonstrated that other than in osteoclasts, MMP9 was expressed in chondrocytes and perichondrium during skeletal development, and was dramatically decreased by the KLF5 haploinsufficiency (Fig. 4D). This decrease was correlated with the suppression of COL2 degradation and an aggrecan cleavage neoepitope DIPEN. In addition, the induced MMP9 in chondrocytes by the KLF5 overexpression exerted a potent enzyme activity by gelatin zymography (Fig. 3A), as previously reported (35). These indicate a significant role of cell-autonomous action of MMP9 in chondrocytes in the process of cartilage degradation.

For endochondral ossification of hypertrophic chondrocytes, chondrocyte apoptosis, cartilage matrix degradation, and vascularization are tightly coupled (2,5); however, which of these steps is rate-limiting remains unclear. A recent study on knockout mice of an anti-apoptotic protein galectin 3 has shown that acceleration of chondrocyte apoptosis was not associated with
endochondral ossification (36), suggesting that chondrocyte apoptosis might be dispensable for the process. Several reports have indicated the matrix degradation and vascularization as the crucial steps (3,5,37-39), and, in fact, the present in vivo analyses showed that suppressions of MMP9 expression and cartilage matrix degradation by KLF5 insufficiency led to impairment of skeletal development accompanied by decreased vascularization (Fig. 4D). In vitro cultures, however, showed a principal angiogenic factor VEGFA (37-40) was little influenced by gain- or loss-of-function of KLF5 in chondrocytes (Fig. 3B & Fig. 4A), nor were other angiogenic factors VEGF, VEGFD (41), HGF (42), FGF1, or FGF2 (43,44) in the microarray analyses (Supplemental Table 2). Hence, KLF5 is likely to regulate vascularization indirectly as a secondary effect of MMP9 secretion and matrix degradation in the cartilage layer and perichondrium, although the details need to be further investigated. In fact, cartilage explants from MMP9−/− mice in culture are reported to show a delayed angiogenesis (3). A previous report on MMP13−/− and MMP9−/− mice also showed that the cartilage matrix degradation was decreased in parallel with the pace that vasculature recruitment maintains with the slower rate of endochondral ossification (5). This evidence suggests that the matrix degradation may create a permissive environment for blood vessels to invade or make angiogenic factors accessible, leading to a hypothesis that cartilage degradation is a rate-limiting step for endochondral ossification of hypertrophic chondrocytes.

The skeletal abnormality of KLF5+/− mice was limited to the perinatal period and disappeared as the animals grew up after birth under physiological conditions. This may be due to compensatory mechanisms for endochondral ossification such as an increase of proteinases other than MMP9. In fact, proteinases such as MMP13 tended to be regulated oppositely to MMP9 by the KLF5 overexpression (Fig. 3B, Table 1). Since MMPs are known to play roles under various pathological conditions including wound healing, arthritis, and tumor development (45-47), we examined the effects of KLF5 insufficiency on bone fracture and arthritis by making the models in KLF5+/- mice at 8 weeks of age (Supplemental Figs. 5A & 5B). In results, there was no difference in fracture healing or arthritis development between KLF5+/- and the wild-type littermates. KLF5 may therefore be indispensable for skeletal development only in the perinatal period, but be dispensable after birth under physiological and pathological conditions. Another possible compensatory mechanism is bone formation by osteoblasts, in spite of the expression of KLF5 in the cells (Fig. 1). This osteoblastic compensation may be sufficient to make up for the KLF5 dysfunction in chondrocytes after a substantial number of osteoblasts have appeared after birth, while insufficient in the perinatal period when chondrocytes play central roles in endochondral ossification. Generation and evaluation of conditional knockout mice will clarify the tissue specific roles of KLF5. In addition, further understanding of the molecular network related to the KLF5-MMP9 axis will greatly help us to unravel the complex mechanism modulating endochondral ossification.

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FOOTNOTES

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The abbreviations used are : ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like repeat; COL2, type II collagen; COL10, type X collagen; KLF, Krüppel-like factor; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; M-CSF, macrophage-colony stimulation factor; M-BMMΦ, M-CSF dependent-bone marrow macrophages; MMP, matrix metalloproteinase; TRAP, tartrate-resistant acid phosphate

FIGURE LEGENDS

Fig. 1. Expression of KLF5 in cells of bone and cartilage. (A) mRNA expression of KLF5 and GAPDH as the loading control, determined by RT-PCR in mouse primary cells (upper lane); neonatal calvarial
osteoblasts, mature osteoclasts formed from M-BMMΦ with M-CSF and RANKL stimulation, neonatal costal chondrocytes, and cell lines (lower lane); osteoblastic cell line MC3T3E1, monocyte-macrophagic cell line Raw264.7, chondrogenic cell lines ATDC5 and OUMS27. (B) Localization of KLF5 determined by immunohistostaining by an antibody to KLF5 or the control non-immune serum in tibial limbs of mouse embryo (E15.5) and neonate (1 d). Inset boxes in the left panels indicate the regions of the respective right panels. Scale bars, 100 μm and 50 μm in left and right panels, respectively.

**Fig. 2.** Skeletal phenotypes of KLF5+/- mice in the perinatal period. (A) Skeletal staining with Alizarin red and Alcian blue and (B) HE staining of the tibial limbs of wild-type (+/+) and KLF5+/- embryos (E16.5). Scale bars, 1 mm and 200 μm, respectively. (C) HE, COL2 and COL10 immunostainings, TUNEL and TRAP stainings of the tibial limbs of wild-type and KLF5+/- embryos (E16.5). Inset boxes in the left COL2 panels indicate the regions of the respective right COL2 panels. Blue, red, and green bars indicate proliferative (P), hypertrophic (H) layers, and bone area (B), respectively. Scale bars, 50 μm. (D) HE, Safranin-O, and von Kossa stainings of the growth plates in proximal tibias of wild-type and KLF5+/- neonates (1 d). Scale bars, 50 μm. (E) Relative lengths of hypertrophic layer (left) and calcified layer (right) in KLF5+/- growth plate compared to those in wild-type (1 d). Data are expressed as means (bars) ± SEM (error bars) for 4 mice/group. *P<0.05, **P<0.01 vs. wild-type.

**Fig. 3.** Induction of MMP9 by KLF5 overexpression. (A) MMP9 and KLF5 mRNA levels determined by real-time RT-PCR analysis after 3 d culture of human chondrogenic OUMS27 cells adenovirally transfected with empty vector (EV) or KLF5 (KL) at 40 or 100 MOI (graphs). Gelatinase activity was determined by gelatin zymography. The right lane (M) shows markers using recombinant proteins of pro-MMP9, pro-MMP2 and MMP2. KLF5 and Actin protein levels were determined by Western blotting. (B) Osteopontin, MMP13, and VEGFA mRNA levels determined by real-time RT-PCR analysis in 3 d culture of OUMS27 cells adenovirally transfected with 100 MOI of EV or KLF5. (C) MMP9 promoter activity determined by luciferase assay in OUMS27, ATDC5, and Huh7 cells co-transfected with a reporter construct containing 1,250 bp MMP9 5'-end flanking region and plasmid vector of EV or KLF5. Data are expressed as relative values compared to EV. (D) TRAP staining of the osteoclast-precursor M-BMMΦ that were retrovirally transfected with EV or KLF5, and cultured with M-CSF and RANKL for 4-5 d. Scale bars, 100 μm. The graph shows the number of TRAP-positive multinucleated osteoclasts. (E) mRNA levels of KLF5 and MMP9 determined by real-time RT-PCR analysis in the M-BMMΦ cultures. Data are expressed as means (bars) ± SEM (error bars). *P<0.05, **P<0.01 vs. EV.

**Fig. 4.** Suppression of MMP9 by KLF5 insufficiency. (A) KLF5, MMP9, osteopontin, MMP13 and VEGFA mRNA levels determined by real-time RT-PCR analysis in 3 d culture of primary costal chondrocytes isolated from wild-type (+/+) and KLF5+/- littermates. Data are expressed as means (bars) ± SEM (error bars) for 4 mice/group. *P<0.05 vs. wild-type. (B) Time course of the number of the primary costal chondrocytes above during 5 d of culture. Data are expressed as means (symbols) ± SEM (error bars) of the ratios of day 0 for 3 wells/group. (C) MMP9 and KLF5 mRNA levels determined by real-time RT-PCR analysis after 3 d culture of OUMS27 cells transfected with siSEAP (secreted form of the human placental alkaline phosphatase; control) or siKLF5 oligonucleotides (graphs). Data are expressed as means (bars) ± SEM (error bars) for 3 wells/group. *P<0.05 vs. siSEAP. MMP9, KLF5, and actin protein levels were determined by Western blotting. (D) MMP9, COL2, DIPEN and CD34 immunostainings, and von Kossa staining of the tibial limbs of E15.5 wild-type and KLF5+/- embryos. Blue and red bars indicate proliferative (P) and hypertrophic (H) layers, respectively. Scale bars, 50 μm. (E) The percent width of calcified layer to the entire hypertrophic layer determined by the von Kossa staining (top) and the number of blood vessels around the hypertrophic layer determined by the CD34 immunostaining (bottom) in the growth plates of E15.5 wild-type and KLF5+/- embryos. Data are expressed as means (bars) ± SEM (error bars) for 3 mice/group. *P<0.05 vs. wild-type.
Table 1
Changes in expression of genes related to matrix degradation by KLF5 overexpression

| Gene symbol | GenBank accession no. | Fold increase |
|-------------|-----------------------|---------------|
| MMP1        | NM_002421             | 3.1           |
| MMP2        | NM_004530             | 1.6           |
| MMP3        | NM_002422             | -1.3          |
| MMP7        | NM_002423             | -1.4          |
| MMP8        | NM_002424             | 2.1           |
| MMP9        | NM_004994             | 2143.9        |
| MMP10       | NM_002425             | -1.1          |
| MMP11       | NM_005940             | -1.8          |
| MMP12       | NM_002426             | 1.4           |
| MMP13       | NM_002427             | -2.1          |
| MMP14       | NM_004995             | 2.6           |
| MMP15       | NM_002428             | 3.9           |
| MMP16       | NM_005941             | -1.6          |
| MMP19       | NM_002429             | 1.6           |
| MMP20       | NM_004771             | ND            |
| ADAMTS1     | NM_006988             | -2.5          |
| ADAMTS4     | NM_005099             | 46.6          |
| ADAMTS5     | NM_007038             | -1.4          |
| ADAMTS8     | NM_007037             | 8.4           |
| ADAMTS9     | NM_182920             | 1.1           |
| ADAMTS13    | NM_139028             | 1.6           |
| ADAMTS15    | NM_139055             | 3.1           |
| TIMP1       | NM_003254             | 3.0           |
| TIMP2       | NM_003255             | 3.8           |
| TIMP3       | NM_000362             | 7.7           |

mRNA levels in OUMS27 cells with adenoviral introduction of KLF5 and the control empty vector were compared by RT2 profiler PCR array (APHS-016; SuperArray Bioscience). For MMP19, 20, ADAMTS 4, 5, 9 and 15, real-time RT-PCR was performed using primer sets shown in the Supplemental Information online. ND; not detected.
**Figure 1**

### A

| Primary cells | Osteoblasts | Osteoclasts | Chondrocytes |
|---------------|-------------|-------------|--------------|
| RT            | +           | -           | +            |
| KLF5          | -           | -           | +            |
| GAPDH         | -           | -           | -            |

### B

| Cell lines | MC3T3E1 | Raw264.7 | ATDC5 | OUMS27 |
|------------|---------|----------|-------|--------|
| RT         | +       | -        | +     | -      |
| KLF5       | -       | -        | +     | -      |
| GAPDH      | -       | -        | -     | -      |

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![Image of Figure 1 A and B](http://www.jbc.org/Downloaded from)
Figure 2
Figure 3
Figure 4
Krüppel-like factor 5 causes cartilage degradation through transactivation of matrix metalloproteinase 9
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