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Perlecan modulates VEGF signaling and is essential for vascularization in endochondral bone formation

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Abstract

Perlecan (Hspg2) is a heparan sulfate proteoglycan expressed in basement membranes and cartilage. Perlecan deficiency (Hspg2$^{-/-}$) in mice and humans causes lethal chondrodysplasia, which indicates that perlecan is essential for cartilage development. However, the function of perlecan in endochondral ossification is not clear. Here, we report the critical role of perlecan in VEGF signaling and angiogenesis in growth plate formation. The Hspg2$^{-/-}$ growth plate was significantly wider but shorter due to severely impaired endochondral bone formation. Hypertrophic chondrocytes were differentiated in Hspg2$^{-/-}$ growth plates; however, removal of the hypertrophic matrix and calcified cartilage was inhibited. Although the expression of MMP-13, CTGF, and VEGFA was significantly upregulated in Hspg2$^{-/-}$ growth plates, vascular invasion into the hypertrophic zone was impaired, which resulted in an almost complete lack of bone marrow and trabecular bone. We demonstrated that cartilage perlecan promoted activation of VEGF/VEGFR by binding to the VEGFR of endothelial cells. Expression of the perlecan transgene specific to the cartilage of Hspg2$^{-/-}$ mice rescued their perinatal lethality and growth plate abnormalities, and vascularization into the growth plate was restored, indicating that perlecan in the growth plate, not in endothelial cells, is critical in this process. These results suggest that perlecan in cartilage is required for activating VEGFR signaling of endothelial cells for vascular invasion and for osteoblast migration into the growth plate. Thus, perlecan in cartilage plays a critical role in endochondral bone formation by promoting angiogenesis essential for cartilage matrix remodeling and subsequent endochondral bone formation.

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Keywords

Perlecan; Endochondral bone formation; Growth plate; Vascular invasion; VEGF signaling

1. Introduction

Most bones, such as the long bones, are formed by endochondral ossification, in which cartilage during growth is first formed as a template and then replaced by bone (Karsenty, 2003; Kronenberg, 2003). Endochondral ossification is initiated by the condensation of mesenchymal cells, which differentiate into chondrocytes. The cells surrounding the mesenchyme condensation differentiate into the perichondrium. Proliferating chondrocytes produce a large number of matrix molecules, such as collagen II and aggrecan, to expand the cartilage template, cease proliferation at the prehypertrophic zone in the middle of the growth plate, and further differentiate into collagen X-expressing hypertrophic chondrocytes. The matrix surrounding mature hypertrophic chondrocytes is mineralized and replaced with osteoblasts. Although cartilage is a neovascular tissue, factors such as vascular endothelial growth factor (VEGF) produced by hypertrophic chondrocytes induce vascular invasion into the perichondrium and cartilage near the terminal region of the cartilage template, which is required for cartilage matrix remodeling and osteoblast migration from the perichondrium for ossification and bone marrow formation (Zelzer et al., 2004). This indicates that endochondral bone formation is a process highly coordinated between chondrogenesis and osteogenesis.

Perlecan plays critical roles in normal development, tissue functions, and diseases (DeCarlo and Whitelock, 2006; Knox and Whitelock, 2006; Olsen, 1999; Zoeller et al., 2009). Perlecan is a proteoglycan present in all basement membranes and other tissues, such as cartilage, plays important roles in development and tissue functions, and is associated with various diseases (DeCarlo and Whitelock, 2006; Knox and Whitelock, 2006; Morita et al., 2005; Olsen, 1999; Zoeller et al., 2009). Perlecan consists of a large elongated core protein with a complex modular structure and is usually substituted with several heparan sulfate and/or chondroitin sulfate chains (Noonan et al., 1991). Perlecan binds basement membrane components, such as laminins and collagen IV, providing scaffolding for cells in many tissues and creating a barrier to the passage of molecules in the kidneys (Hopf et al., 1999; Morita et al., 2005). Perlecan also binds other extracellular proteins, such as fibronectin and fibulin. Perlecan modulates cell proliferation and differentiation through interaction with cell surface receptors such as integrins and with growth factors such as FGFs (Aviezer et al., 1994; Brown et al., 1997). Many biological functions of perlecan have been reported, such as aiding supramolecular organization of basement membranes and cell-matrix interactions (Brown et al., 1997; Hopf et al., 1999; Kvist et al., 2006), storage and release of various cytokines (Ghiselli et al., 2001; Govindraj et al., 2006; Klein et al., 1995; Smith et al., 2007; Whitelock et al., 1996), control of extracellular proteolysis and macromolecular filtration (Mongiat et al., 2003; Morita et al., 2005), and angiogenesis (Aviezer et al., 1994; Jiang and Couchman, 2003; Segev et al., 2004; Zhou et al., 2004).

Studies in knockout mice and mutations in the perlecan gene (HSPG2) in humans revealed that perlecan is essential for cartilage development (Arikawa-Hirasawa et al., 1999; Arikawa-Hirasawa et al., 2001a; Arikawa-Hirasawa et al., 2001b; Costell et al., 1999). Perlecan knockout (Hspg2$^{-/-}$) mice develop severe skeletal dysplasia characterized by shortened bones and craniofacial abnormalities and die shortly after birth of respiratory failure due to the cartilage defects of the rib cage (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). Proliferation of chondrocytes is reduced in Hspg2$^{-/-}$ mice (Arikawa-Hirasawa et al., 1999). The cartilage matrix of the knockout mice contains disorganized collagen fibrils and glycosaminoglycans, which suggests that perlecan plays an important role in the cartilage matrix structure (Kvist et al., 2006). A human disorder, dyssegmental dysplasia, Silverman-Handmaker type (DDSH), was identified as a functional null mutation of perlecan and causes skeletal abnormalities similar to those of the knockout mice (Arikawa-Hirasawa et al., 2001a; Arikawa-Hirasawa et al., 2001b). In addition, subtle functional mutations of perlecan cause Schwartz-Jampel Syndrome (SJS), a rare autosomal recessive osteochondrodysplasia associated with myotonia (Arikawa-Hirasawa et al., 2002; Nicole et al., 2000; Rodgers et al., 2007). Patients with SJS survive and show much milder skeletal dysplasia compared to those with DDSH. We also showed that perlecan is critical for maintaining fast muscle mass and fiber composition by regulating myostatin signaling using Hspg2$^{-/-}$ mice perinatal lethality rescued (Hspg2$^{-/-}$-Tg) mice by expressing recombinant perlecan specifically in the cartilage of the perlecan-null (Hspg2$^{-/-}$) genetic background (Xu et al., 2010), where perlecan is expressed in cartilage but absent in muscle, endothelial basement membranes, and other tissues.

Although perlecan plays a critical role in growth plate development, the function of perlecan in endochondral ossification is not clear. Here we analyzed the growth plates of Hspg2$^{-/-}$ and Hspg2$^{-/-}$-Tg mice and demonstrated that perlecan in cartilage, not in endothelial basement membranes, is required for vascular invasion and cartilage matrix remodeling that is essential for the formation of the trabecular bone and bone marrow.

2. Results

2.1. Endochondral ossification is inhibited in the growth plate of Hspg2$^{-/-}$ mice

Perlecan is essential for cartilage development, as perlecan deficiency in mice displays perinatal lethal chondrodysplasia (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). In humans, null mutations of perlecan cause severe chondrodysplasia and DDSH, similar to Hspg2$^{-/-}$ mice. The humeri of E16.5 Hspg2$^{-/-}$ mice were shorter and wider than those of wild-type mice (Fig. 1A). Quantification analyses revealed that the humeri of Hspg2$^{-/-}$ mice were significantly shorter (70% of wild-type, Fig. 1B) and wider (180% of wild-type, Fig. 1C) compared to those of wild-type mice. The humeri of Hspg2$^{-/-}$ mice contained reduced levels of glycosaminoglycan, as shown with Safranin-O staining (Fig. 1D, red) and as described previously (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). The striking abnormality of the growth plate of mutant mice is the almost complete lack of bone marrow cavities and trabecular bone (Fig. 1). In the E16.5 growth plates of wild-type mice, von Kossa staining (brown) showed that bone collar and trabecular bone were formed, and calcification of the matrix surrounding mature hypertrophic chondrocytes, as well as of the matrix surrounding the periosteum (bone collar) and trabecular bone, was observed (Fig. 1D). In contrast, in Hspg2$^{-/-}$ mice, thin calcified layers were observed along the bottom border of the cartilage and separated two cartilage regions, which were located close together in the almost complete absence of bone marrow.

2.2. Defect of perlecan in chondrocytes inhibits vascular invasion into the hypertrophic chondrocyte

The inhibition of the formation of bone marrow and trabecular bone suggests a defect in angiogenesis in the hypertrophic zone in Hspg2$^{-/-}$ growth plates. Therefore, we examined vascular invasion into the hypertrophic zone. Immunostaining of CD31 (PCAM-1), a marker of endothelial cells, showed that endothelial cells invaded cartilage from surrounding tissues, including the perichondrium and bone marrow, in wild-type mice (Fig. 2A). The chondro-osseous region was arranged perpendicularly to the long axis of the bone (Fig. 2A) in wild-type mice. In Hspg2$^{-/-}$ mice, bone marrow was almost completely absent, and CD31-positive endothelial cells (black) were observed in the perichondrium/periosteum and surrounding tissues (Fig. 2A). In addition, the proximal and distal cartilage areas were separated by thin calcified layers in the humeri of the Hspg2$^{-/-}$ mice (Fig. 1A, B, and 2A). These results suggest that vascular invasion into the hypertrophic zone from the surrounding tissues was severely inhibited in the absence of perlecan. TRAP-positive osteoclasts with multiple nuclei, which are differentiated from hematopoietic stem cells, migrated into the cartilage-bone interface and trabecular bone through the vasculature in wild-type mice (Fig. 2B). TRAP-positive osteoclasts (dark red) were present, but their numbers were reduced in the bone collar and surrounding region of the Hspg2$^{-/-}$ growth plates (Fig. 2B). These results further indicate that vascular invasion into the growth plate is impaired in Hspg2$^{-/-}$ mice.

2.3. VEGFA expression is increased in chondrocytes of Hspg2$^{-/-}$ mice

Vascular invasion is a crucial step in removing the cartilage matrix for endochondral ossification. VEGFA plays an important role in vascular invasion for endochondral ossification (Maes et al., 2002; Maes et al., 2004; Zelzer et al., 2004; Zelzer et al., 2002). Therefore, we examined the VEGFA protein expression level in chondrocytes in HSPG2$^{-/-}$ mice with immunostaining and confirmed with Western blot (Fig. 3). In Hspg2$^{-/-}$ mice, the expression levels of VEGFA proteins in hypertrophic chondrocytes were substantially increased compared with the control mice (Fig. 3A). Consistent with this immunostaining result, Western blotting revealed that VEGFA protein levels in the Hspg2$^{-/-}$ growth plate were increased compared to those in the wild-type growth plate (Fig. 3B).

2.4.VEGF$_{164}$ expression is increased in chondrocytes of Hspg2$^{-/-}$ mice

VEGFA consists of three splice variants, VEGF$_{120}$, VEGF$_{164}$, and VEGF$_{188}$ (Ruhrberg et al., 2002). Although VEGF$_{120}$, which does not have a heparan sulfate binding site in the C-terminal region, is important for vascular invasion in the epiphysis of the growth plate to form the secondary ossification center (Maes et al., 2004), VEGF$_{164}$, which contains one of the two heparan sulfate binding sites, is critical for vascular invasion to form trabecular bone in the growth plate (Zelzer et al., 2004). We examined the mRNA expression levels of these isoforms of VEGFA in the growth plate chondrocytes in Hspg2$^{-/-}$ mice. Although two VEGF mRNA isoforms were expressed in chondrocytes prepared from the growth plates of wild-type mice, the expression levels of VEGF$_{164}$ were more dominant than those of VEGF$_{120}$ (Fig. 4A). In Hspg2$^{-/-}$ mice, these VEGF mRNA were also expressed, but their expression levels were found to be increased further than those in wild-type mice by using semi-quantitative RT-PCR analysis (Fig. 4A). Real-time PCR analysis of the expression levels of these three isoforms showed that, although these isoforms of VEGF mRNA were significantly increased in chondrocytes of Hspg2$^{-/-}$ mice compared to those of wild-type mice, the VEGF$_{164}$ mRNA levels were most profoundly increased in the absence of perlecan (Fig. 4B). These results indicate that the VEGFA expression level was not a major cause of the defect in vascular invasion into the growth plate cartilage of Hspg2$^{-/-}$ mice.

2.5. Expression of CTGF, Chm-1, and MMPs in the hypertrophic chondrocytes of Hspg2$^{-/-}$ mice

In addition to VEGFA, connective tissue growth factor (CTGF) plays an important role in vascular invasion in endochondral ossification. The absence of CTGF impairs vascular invasion (Ivkovic et al., 2003), while the absence of chondromodulin-1 (Ch-1) in vivo does not impair vascular invasion (Brandau et al., 2002). We found that the CTGF and Ch-1 mRNA levels in Hspg2$^{-/-}$ mice were significantly increased compared with those of the wild-type mice (Fig. 5A, B). Matrix metalloproteinases (MMPs) are also important for vascular invasion, as MMPs degrade most components of the extracellular matrix (ECM) that allow promotion of sprouting and migration of endothelial cells (Noel et al., 2004; Sottile, 2004). We found that the MMP13 mRNA levels were significantly increased in the growth plates of Hspg2$^{-/-}$ mice compared with those of wild-type mice (Fig. 5C). MMP-9 is also expressed in the hypertrophic chondrocytes of wild-type mice and Hspg2$^{-/-}$ mice (data not shown)(Gustafsson et al., 2003). These results indicate that the inhibition of vascular invasion in the growth plates of Hspg2$^{-/-}$ mice was not due to the reduced expression levels of CTGF and MMP.

2.6. Removal of hypertrophic matrix is inhibited in the absence of perlecan

We examined cartilage calcification and osteopontin (OPN) expression in E18.5 Hspg2$^{-/-}$ growth plates. Type X collagen expression was observed in Hspg2$^{-/-}$ mice (data not shown), as reported previously, although its expression levels are decreased in Hspg2$^{-/-}$ mice compared with those in wild-type mice (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). At E18.5, formation of hydroxyapatite nodules (calcospherites) was observed in the hypertrophic zone of Hspg2$^{-/-}$ mice, which is one of the characteristics of the defects in perlecan-deficient cartilage in mice and humans (Arikawa-Hirasawa et al., 1999)(Fig. 6A). In addition, although the calcified matrix was observed only in the last few layers of hypertrophic chondrocytes in wild-type mice, the calcified matrix was found in multiple layers in the hypertrophic zone in perlecan-deficient mice, suggesting impaired remodeling of the calcified cartilage matrix. The expression of OPN mRNA, a marker of terminally differentiated mature chondrocytes, was significantly increased in chondrocytes in Hspg2$^{-/-}$ mice compared with those in wild-type mice (Fig. 6B). In wild-type growth plates, perlecan (red) was expressed and surrounded hypertrophic chondrocytes, while perlecan was absent in Hspg2$^{-/-}$ mice (Fig. 6C) (Xu et al., 2010).

Immunohistochemical analyses revealed that the OPN protein (green) was expressed at the end layer of hypertrophic chondrocytes in wild-type mice. However, in Hspg2$^{-/-}$ growth plates, OPN protein expression was observed in multiple layers of hypertrophic chondrocytes (Fig. 6C). These results suggest that remodeling of the hypertrophic matrix was inhibited in Hspg2$^{-/-}$ mice.

2.7.Inhibition of vascular invasion is due to the defect of perlecan in chondrocytes, but not in endothelial cells

Perlecan is expressed not only in chondrocytes but also in all basement membranes, including vessel walls. Therefore, the inhibition of vascular invasion in the hypertrophic zone in the absence of perlecan could have been due to the absence of perlecan in the basement membranes of endothelial cells. To exclude this possibility, mice in which perlecan was expressed specifically in chondrocytes were created by introducing the transgene (Per-Tg) under the control of a cartilage-specific Col2a1 promoter and enhancer in the Hspg2$^{-/-}$ genetic background (Hspg2$^{-/-}$-Tg mice) (Xu et al., 2010). In the Hspg2$^{-/-}$-Tg mice, perlecan is expressed in cartilage but absent in the basement membranes of blood vessels, muscle, and other tissues surrounding cartilage (Xu et al., 2010). The bone sizes of the HSPG2$^{-/-}$-Tg mice were similar to those of wild-type mice (Fig. 7A). Histological analysis showed that the columnar structure of the growth plate can be restored in Hspg2$^{-/-}$-Tg mice (Fig. 7B). Type II collagen, type X collagen, and osteopontin expression of Hspg2$^{-/-}$-Tg mice were similar to those of wild-type mice.

Vascular invasion into the chondro-osseous region observed in the Hspg2$^{-/-}$-Tg mice was also similar to that of wild-type mice (Fig. 7D). Since Hspg2$^{-/-}$-Tg mice expressed perlecan in cartridge but not surrounding tissues, these results indicate that the inhibition of vascular invasion in Hspg2$^{-/-}$mice is due to the absence of perlecan in cartilage but not to its absence in endothelial cells.

2.8. Perlecan promotes VEGF-induced VEGFR2 activation in endothelial cells

$VEGF_{164}$ is expressed in hypertrophic chondrocytes and is critical for inducing vascular invasion into the hypertrophic zone (Zelzer et al., 2004). As $VEGF_{164}$ contains a heparan sulfate binding site, perlecan may bind to $VEGF_{164}$, which promotes VEGF signaling of endothelial cells for angiogenesis. We tested the binding of purified perlecan from cartilage to $VEGF_{164}$ in a solid phase binding assay using perlecan-coated dishes with different amounts of growth factors or growth factor receptors (Fig. 8A). Perlecan bound to FGF2 and to FGF receptors 2 and 3, as reported (Knox et al., 2002; Knox and Whitelock, 2006; Patel et al., 2007; Smith et al., 2007). Perlecan bound to VEGF receptor 2 (VFGFR2), in agreement with recent findings (Goyal et al., 2011). However, perlecan did not bind to $VEGF_{164}$. To test whether perlecan promotes $VEGF_{164}$-mediated VEGFR2 activation, primary endothelial cells from wild-type mouse skin were incubated with $VEGF_{164}$ in the presence of various amounts of perlecan. After being incubated for 5 min at 37 °C, the cells were lysed, and VEGFR2 was immunoprecipitated with anti-VEGFR2 antibody. The proteins were analyzed with Western blotting using anti-pVEGFR2 (Tyr951) and anti-VEGFR2 antibodies as described in the Materials and Methods section. We found that perlecan promoted VEGFR2 phosphorylation (Fig. 8B). The cartilage perlecan-promoted activation of VEGF/VEGFR2 is consistent with the VEGFR2 activation by perlecan from endothelial cells (Goyal et al., 2011; Zoeller et al., 2009).

3. Discussion

Endochondral bone formation occurs through highly coordinated biological processes, including chondrocyte hypertrophy, deposition and remodeling of the cartilage matrix, vascular invasion, apoptosis, osteoblast replacement, and subsequent trabecular bone formation. In this study, we demonstrated that perlecan present in cartilage, but not in capillary basement membranes, is essential for cartilage matrix remodeling, vascular invasion, and the formation of bone marrow and trabecular bone.

Perlecan had been suggested to play crucial roles not only in vasculogenesis but also in the maturation and maintenance of differentiated tissues, including cartilage (Handler et al., 1997). In growth plates of $Hspg2^{-/-}$ mice, the matrix structure is disorganized, and glycosaminoglycans are reduced (Fig. 1A) (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). Biochemical studies in vitro confirmed that perlecan is required for cartilage collagen fibril formation (Kvist et al., 2006). In $Hspg2^{-/-}$ mice, growth plate cartilage is wider, and chondrocytes are located more sparsely in the cartilage matrix than in wild-type mice (Fig. 1). These phenotypes are different from those of cartilage-deficient (cmd/cmd) mice, which are caused by the absence of functional aggrecan, a major chondroitin sulfate proteoglycan in cartilage (Watanabe et al., 1994). These cmd/cmd mice die perinatally; the width of the cartilage of the long bone is narrow, and chondrocytes are rather densely packed in the matrix (Watanabe and Yamada, 2002). In the absence of perlecan, chondrocytes were able to differentiate into mature hypertrophic chondrocytes, which express VEGF (Figs. 3 and 4), MMP13 (Fig. 5C), and osteopontin (Fig. 6B,C). Removal of the hypertrophic matrix and its calcified regions is essential for endochondral bone formation. In wild-type growth plates, only a few layers containing osteopontin and calcification surround mature hypertrophic chondrocytes in the chondroosseous boundary (Fig. 6). However, in $Hspg2^{-/-}$ growth plates, multiple layers were accumulated near the end of the hypertrophic zone, indicating that hypertrophic cartilage removal was inhibited in the absence of perlecan (Fig. 6). Matrix metalloproteinases, such as MMP-9 and MMP-13, are involved in degradation of the hypertrophic matrix (Engsig et al., 2000; Inada et al., 2004; Stickens et al., 2004; Vu et al., 1998). However, we found that MMP-13 expression was increased in $Hspg2^{-/-}$ growth plates compared to those of wild-type mice. MMP-9 is also expressed in the growth plates of $Hspg2^{-/-}$ mice. Since perlecan interacts with MMPs and is most abundantly expressed in the hypertrophic zone compared with other chondrocyte zones, perlecan may play a role in the activation of MMPs for cartilage remodeling.

Although matrix components are expressed in the cartilage of Hspg2$^{-/-}$ growth plates, the fibrillar formation and density are especially reduced in the hypertrophic zone (Gustafsson et al., 2003). In addition, the columnar structure of hypertrophic chondrocytes is disorganized, and the hypertrophic matrix is often disrupted in Hspg2$^{-/-}$ mice, especially during later stages such as birth (Arikawa-Hirasawa et al., 2002; Arikawa-Hirasawa et al., 1999; Costell et al., 1999). These observations suggest that perlecan provides the strength and rigidity of the hypertrophic matrix structure by interacting with matrix molecules for proper growth plate development. In Hspg2$^{-/-}$ growth plates, the ossified periosteum is formed but apparently curved into the hypertrophic zone, in contrast to the longitudinal growth seen in bone collars of wild-type mice (Fig. 1). Because the formation of bone marrow and the trabecular bone was severely inhibited, the bone collar structure separated two adjacent cartilage molds within the humerus close together (Fig. 1). The abnormal alignment of the bone collar seen in Hspg2$^{-/-}$ growth plates is likely due in part to the less rigid hypertrophic matrix structure. In addition to MMP-9 and MMP-13, other molecules, such as CTGF (CCN2) and Ch-1, are implicated in matrix remodeling and vascular invasion in the growth plates (Ivkovic et al., 2003). In Hspg2$^{-/-}$ growth plates, the expression levels of CTGF and Ch-1 were significantly increased (Fig. 5). VEGFA plays an important role in angiogenesis for endochondral ossification. Administration of an inhibitor of VEGFA activity in mice reduced vascular invasion into the hypertrophic zone and inhibited endochondral bone formation (Gerber et al., 1999). Conditional VEGFA knockout in mice specific in chondrocytes using Col2a1Cre displayed an expansion of the hypertrophic zone, delayed vascular invasion, and impaired endochondral ossification (Zelzer et al., 2004). Forced expression of Runx2 in hypertrophic chondrocytes using the Col10a1 promoter reduced VEGFA expression and resulted in impaired cartilage matrix remodeling and an almost complete lack of bone marrow due to the inhibition of vascular invasion into hypertrophic cartilage (Hattori et al., 2010). In the Hspg2$^{-/-}$ growth plates, the VEGF protein levels were increased. VEGF$_{120}$ and VEGF$_{164}$ mRNA were expressed in the growth plates of wild-type mice. The mRNA expression levels for VEGF$_{120}$ and VEGF$_{164}$ were increased in Hspg2$^{-/-}$ growth plates, with the highest level for VEGF$_{164}$ (Fig. 4B). In the zebrafish, perlecan regulates angiogenic blood vessel formation, and perlecan knockdown results in an abnormal increase and relocation of the VEGFA proteins (Zoeller et al., 2009). In wild-type mice, perlecan is expressed not only in cartilage but also in blood vessel basement membranes. We therefore examined whether cartilage or endothelial perlecan is important for normal vascular invasion into the hypertrophic zone and endochondral bone formation by expressing the perlecan (Hspg2) transgene (Tg) specifically in the cartilage of HSPG2$^{-/-}$ mice (Xu et al., 2010). The mutant mice (Hspg2$^{-/-}$-Tg) expressed perlecan in cartilage but not in surrounding tissues (Fig. 7C)(Xu et al., 2010). Hspg2$^{-/-}$-Tg mice survived and showed normal cartilage development and endochondral bone formation (Fig. 7A, B, D). These results suggest that perlecan is critical in cartilage but not in endothelial cell basement membranes for vascular invasion into the hypertrophic zone. Since vascularization in other tissues occurred without perlecan, the mechanism of angiogenesis must be unique in endochondral ossification processes.

Perlecan purified from bovine cartilage enhanced the activation of VEGF$_{164}$-VEGFR2 signaling in endothelial cells (Fig. 8). This activation is facilitated via direct binding of perlecan to VEGFR2 (Fig. 8A). Perlecan from endothelial cell culture and recombinant endorepellin, the C-terminal part of perlecan, binds to VEGFR1 and 2 (Goyal et al., 2011). Perlecan promotes angiogenesis, while its fragment acts as an anti-angiogenic factor by disrupting the actin assembly of endothelial cells through interaction with $\alpha 2\beta 1$ integrin (Bix et al., 2004). Endorepellin attenuated VEGFA-mediated activation of VEGFR2 in endothelial cells, and this attenuation is required for α2β1 integrin (Goyal et al., 2011). Cartilage perlecan did not bind to $VEGF_{164}$, while endothelial cell perlecan binds to the heparin-binding site containing VEGFA (Goyal et al., 2011). This difference may be because cartilage perlecan is substituted with not only heparan sulfate chains but also chondroitin sulfate chains, which may inhibit perlecan interactions with $VEGF_{164}$.

Studies by Takimoto et al., (Takimoto et al., 2009), with overexpression of VEGFA in cartilage in transgenic mice and in chick embryonic forelimbs, revealed the perichondrium prevents vascular invasion into cartilage from highly vascularized surrounding tissues at early stages, but at later stages, perichondrial angiogenesis occurs and is followed by vascular invasion into the hypertrophic zone. This process is required for heparin-binding VEGF isoforms. In $Hspg2^{-/-}$ mice, heparin-binding $VEGF_{164}$ was excessively expressed in the hypertrophic zone, and vasculature in the perichondrium and bony collar was observed, but osteoclasts were reduced, and vascular invasion was inhibited (Fig. 2A). Since perlecan is not expressed in the perichondrium (data not shown) (Melrose et al., 2004; Smith et al., 2010), other molecules, such as Nrp1 and Nrp2, which are expressed in the vasculature of surrounding tissues (Takimoto et al., 2009), enhance VEGFR2 signaling via binding to VEGFA as a receptor (Herve et al., 2008; Staton et al., 2007).

In summary, we demonstrated that perlecan in cartilage is essential for vascular invasion from the perichondrium into the hypertrophic zone. We showed that cartilage perlecan enhances VEGF/VEGFR signaling of endothelial cells in culture. In $Hspg2^{-/-}$ growth plates, hypertrophic chondrocytes express molecules such as MMP-13, OPN, CTGF, and VEGFA, which are important for cartilage matrix remodeling and for vascular invasion. However, without perlecan in cartilage, the osteopontin-expressing hypertrophic chondrocyte layers and calcified areas expand, and formation of bone marrow and the trabecular bone is inhibited. The defect in vascular invasion results in the inhibition of cartilage remodeling and replacement of hypertrophic chondrocytes with osteoblasts, which leads to severe defects in endochondral bone formation of $Hspg2^{-/-}$ mice.

4. Experimental procedures

4.1. Mice

Perlecan knockout ($Hspg2^{-/-}$) mice were generated as described previously (Arikawa-Hirasawa et al., 1999). About half of the $Hspg2^{-/-}$ mice died around embryonic day (E) 10 of hemorrhage due to defective myocardium basement membranes (Arikawa-Hirasawa et al., 1999; Costell et al., 1999; Xu et al., 2010). Perinatal lethality-rescued perlecan knockout mice ($Hspg2^{-/-}$-Tg, $Hspg2^{-/-}$; Col2a1-$Hspg2^{Tg/-}$) were created by expressing a transgene for perlecan under the control of the Col2a1 promoter and enhancer specifically to cartilage (Col2a1-PerTg) in $Hspg2^{-/-}$ mice (Xu et al., 2010). The animal protocol approved by the NIDCR ACU Committee was used for maintaining and handling mice, and all mice were housed in a mouse facility affiliated with the American Association for the Accreditation of Laboratory Animal Care.

4.2. Skeletal histology

Paraffin sections (5 μm) of mouse embryos were deparaffinized using xylene, rehydrated through an alcohol gradient series to water, and then used for histological and immunohistochemical analysis. Paraformaldehyde (4%) was used for fixation in histology and immunohistochemistry. Double staining for Safranin-O and von Kossa staining was performed as described (Aszodi et al., 2003). Immunostaining was performed using a Histostain-SP kit (Zymed) according to the manufacturer's instructions. The following antibodies were used for immunohistochemical studies: a rabbit polyclonal antibody for perlecan from Dr. T. Sasaki (University of Erlangen-Nuremberg, Erlangen, Germany), a monoclonal antibody for osteopontin (R&D Systems), a monoclonal antibody for VEGF (R&D Systems), a monoclonal antibody for type II collagen (Hybridoma Bank, University of Iowa), and a type X collagen chain from Greg Lunstrum (Shriners Hospital for Children Research Center, Portland). Immunostaining for CD31 was performed using a monoclonal antibody for CD31 (Pharmingen) as described previously (Colnot et al., 2005). For the immunofluorescence study, fluorescein isothiocyanate (FITC)-conjugated or Alexa-488-conjugated (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Tartrate-resistant acid phosphatase (TRAP) was stained as described previously (Ishijima et al., 2001; Ishijima et al., 2007).

4.3.Skeletal analysis

Bones and cartilage of newborn mice were stained with Alizarin red and Alcian blue as described previously (Arikawa-Hirasawa et al., 1999). The bone length and width were histologically measured, and the relative length and width of the humeri in the wild-type mice were set at 100%.

4.4. RT-PCR analysis

Total RNA was extracted from growth plate cartilage of the distal end of the femora and the proximal end of the tibiae of E16.5 or E18.5 embryos using TRIzol (Invitrogen). For reverse transcription, 2 μg of total RNA were used to generate cDNA, which was used as a template for PCRs with gene-specific primers. cDNA was amplified with an initial denaturation at 95 °C for 3 min, and then at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 25 cycles. A final elongation step was conducted at 72 °C for 5 min, and then the cDNA was separated on agarose gels. Real-time PCR analysis was performed using a TaqMan Real-Time PCR detection system (ABI7000, Applied Biosystems). TaqMan Universal Master Mix and TaqMan Gene Expression Assays Hs99999901_s1 and Hs01078483_g1 (Applied Biosystems) were used according to the manufacturer's protocol, with a final reaction volume of 25 μl. Sequences for VEGF-120, -164, -188, CTGF, Ch-1, MMP13, and OPN specific PCR primers are available from the authors upon request.

4.5. Western blotting

Growth plate cartilage of the distal end of the femora and the proximal end of the tibiae of E16 mice was dissected from the right side of the knee joint and then lysed. The lysates were run on a 10% SDS-PAGE. A monoclonal antibody for VEGF (C-1, Santa Cruz) was used for Western blot analysis.

For the phosphorylation assay of VEGFR2, primary endothelial cells were prepared from the wild-type mouse skin at postnatal day 4 by immunopanning using anti-ICAM2 antibody (BD Biosciences), as described previously (Kataoka et al., 2003). The endothelial cells were cultured in DMEM: F12=1:1 (Invitrogen) containing 100 μM nonessential amino acid (Invitrogen), 20% fetal calf serum (FCS; Hyclone), 100 μg/ml heparin (Sigma-Aldrich), 100 units penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 50 μg/ml endothelial cell growth supplement (ECGS; BD Biosciences). For starvation of the phosphorylation assay, FCS, heparin, and ECGS were eliminated from the medium, and endothelial cells were incubated overnight. $VEGF_{164}$ and various amounts of perlecan were mixed and preincubated at room temperature for 1 hour. After preincubation, the mixture of $VEGF_{164}$ and perlecan was added to the starved endothelial cell culture, and the cells were incubated for 5 min at 37 °C. The cells were lysed with the lysis buffer (1% Triton-X100, 1.5 mM EDTA, 1 mM Na_3PO_4, 25 mM NaF, and 1 mM Na_3VO_4 in Tris-buffered saline) for 5 min on ice. The cell lysate was centrifuged at 15,000 rpm for 30 min at 4 °C and separated from the cell pellet. Fifty μg of the cell lysate was incubated with anti-VEGFR2 antibody (Cell

Signaling) in the binding/washing buffer (0.1% Triton-X100, 1.5 mM EDTA, 1 mM Na_3PO_4, 25 mM NaF, and 1 mM Na_3VO_4 in Tris-buffered saline) for 1 hour at 4 °C. Then, protein-G Sepharose beads (Invitrogen) were added to the reaction mixture and incubated for 1 hour at 4 °C. After incubation, the beads were washed with the binding/washing buffer. Proteins bound to the beads were eluted with the LDS-sample buffer (Invitrogen) with 10 μM DTT. The proteins were detected with Western blotting using anti-pVEGFR2 (Tyr951) and anti-VEGFR2 antibodies (Cell Signaling).

4.6. Binding assays

A solid-phase binding assay was performed using purified perlecan from bovine cartilage (Govindraj et al., 2002). Two hundred and fifty ng of perlecan was coated onto 96-well plates at 4 °C overnight. The wells were blocked with the blocking buffer (3% bovine serum albumin: Sigma-Aldrich) at room temperature for 2 hours. After blocking, various amounts of FGF-basic (PeproTech), $VEGF_{164}$ (R&D Systems), and recombinant fusion proteins of extracellular domains of FGFR2, FGFR3, and VEGFR2 with the human IgG Fc portion (R&D Systems) in the blocking buffer were added and incubated at room temperature for 1 hr. The proteins bound to perlecan were detected with anti-FGF-basic (Millipore), anti-VEGF (Santa Cruz Biotech.), biotinylated anti-human IgG (Jackson ImmunoResearch Inc.), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo) and streptavidin (Sigma-Aldrich). After incubation with appropriate antibodies, 3,3',5,5'-tetramethyl-benzidine solution (Sigma-Aldrich) was added to the wells and incubated for 10 min at room temperature. After 0.5 N H_2SO_4 was added to stop the colorimetric reaction by HRP, the optical density at 450 nm was measured using a microplate reader (Safire, Tecan Ltd.).

4.7.Statistical analysis

Group means were compared with analysis of variance, and the significance of differences was determined by using an unpaired t-test. P values less than 0.05 were considered significant.

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Highlight

1. The perlecan knockout mice showed severely impaired endochondral bone formation.
2. In Hspg2−/− growth plates, formation of the trabecular bone was almost completely defective.
3. VEGFA and MMPs expression was upregulated in Hspg2−/− growth plates.
4. Vascular invasion into the hypertrophic zone was inhibited.
5. Cartilage perlecan promoted activation of VEGF/VEGFR by binding to VEGFR of endothelial cells

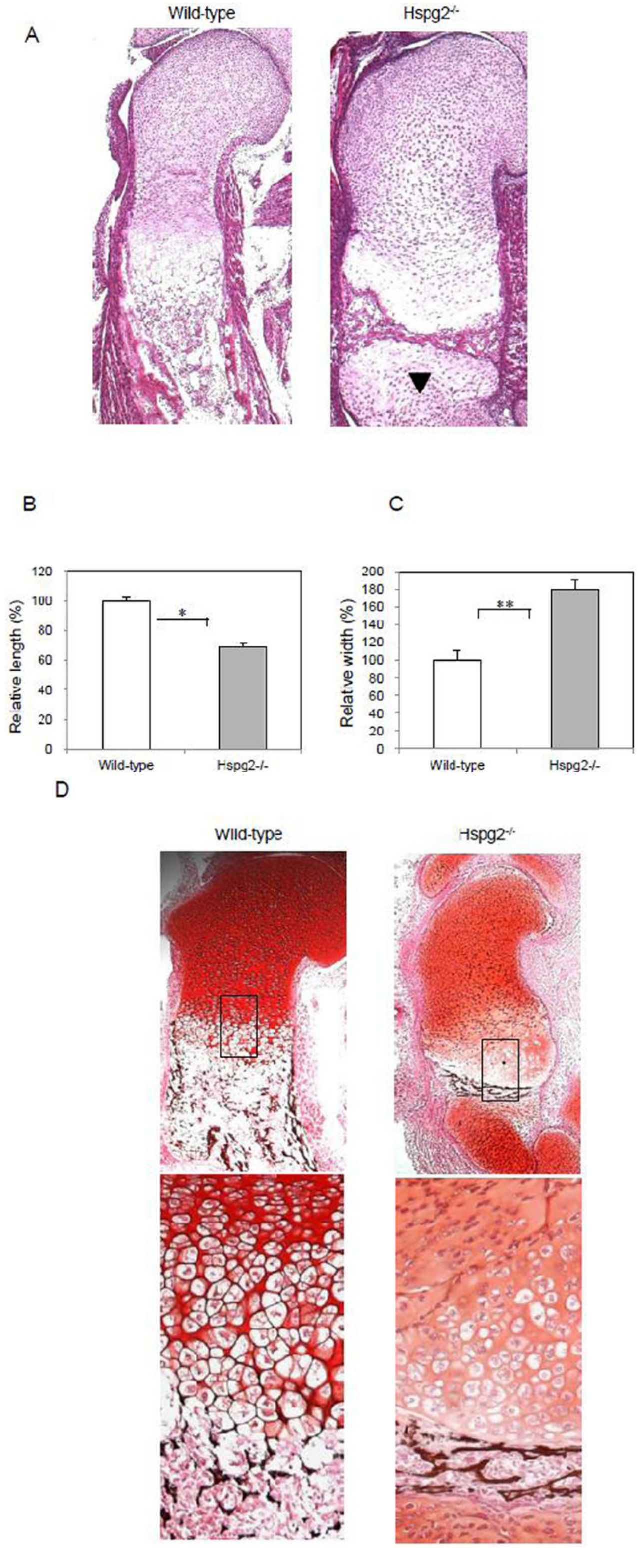


Fig. 1. Endochondral ossification was impaired in the growth plate of Hspg2$^{-/-}$ mice

(A) H-E staining of the proximal end of the humerus in E16.5 wild-type and Hspg2$^{-/-}$ mice. The long bones of Hspg2$^{-/-}$ mice were shorter than those of wild-type mice, as the distal end of the growth plate (arrowhead) was observed in Hspg2$^{-/-}$ mice but not in wild-type mice. **(B)** Comparison of the humeral length of Hspg2$^{-/-}$ mice with that of wild-type mice. The relative length of the humerus in wild-type mice was set at 100%. * indicates $p<0.05$. **(C)** Comparison of the humeral width of Hspg2$^{-/-}$ mice with that of wild-type. The relative width of the humerus in wild-type mice was set at 100%. ** indicates $p<0.01$. **(D)** Double staining of Safranin-O (red) and von Kossa (brown) staining of the proximal end of the humerus at E16.5 in wild-type and Hspg2$^{-/-}$ mice. Reduced glycosaminoglycan levels in the growth plate of Hspg2$^{-/-}$ mice were observed and compared with those of wild-type mice. In the growth plates of wild-type mice, the terminal hypertrophic zone was replaced with trabecular bone, and the perichondrium formed bone collars by membranous ossification. The bone collar aligns parallel to the longitudinal axis of the limb. In Hspg2$^{-/-}$ mice, levels of the calcified matrix in the terminal cartilage template were increased, and levels in the trabecular bone were reduced, likely causing the bone collar to migrate into the longitudinal axis of the limb. Boxed areas are enlarged and shown below.

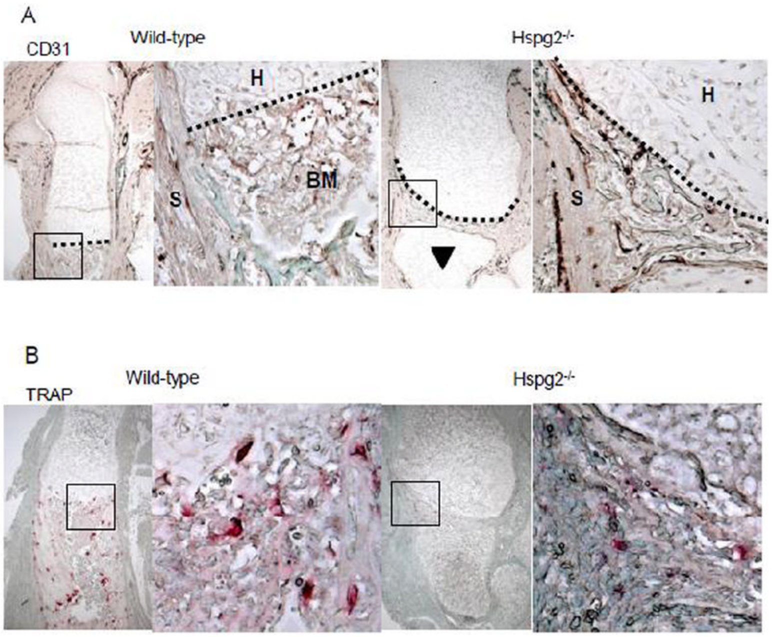


Fig. 2. Vascular invasion into the hypertrophic zone is inhibited in Hspg2$^{-/-}$ mice

(A) Immunostaining of CD31 (PCAM-1) (black) shows that endothelial cells invaded the chondro-osseous boundary and bone marrow from surrounding tissues in wild-type mice. Dotted line, chondro-osseous boundary; bone marrow (BM); hypertrophic zone (H); surrounding tissues (S). The dotted line in the growth plates of Hspg2$^{-/-}$ mice indicates the boundary between the hypertrophic zone and the abnormally curved perichondrium layer and bone collar surrounding the terminal hypertrophic zone. **(B)** Osteoclast differentiation was inhibited in the growth plates of Hspg2$^{-/-}$ mice. Multinucleated TRAP-positive osteoclasts (dark red) were observed in the chondro-osseous boundary as well as bone marrow in wild-type mice. In Hspg2$^{-/-}$ mice, TRAP-positive osteoclast numbers are lower in the boundary, and none of the TRAP-positive cells are multinucleated.

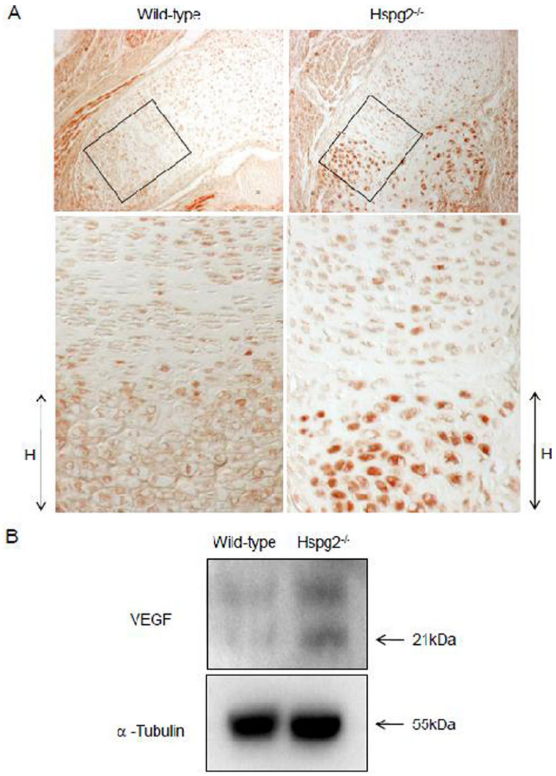


Fig. 3. Increased VEGF expression by chondrocytes in Hspg2$^{-/-}$ mice

(A) Immunostaining of VEGFA. Expression of VEGFA in prehypertrophic and hypertrophic chondrocytes was increased in Hspg2$^{-/-}$ mice compared to that in wild-type mice. Hypertrophic zone (H). **(B)** Western blot of VEGFA protein. The expression level of the VEGFA proteins was increased further in the chondrocytes of Hspg2$^{-/-}$ mice compared to that in wild-type mice.

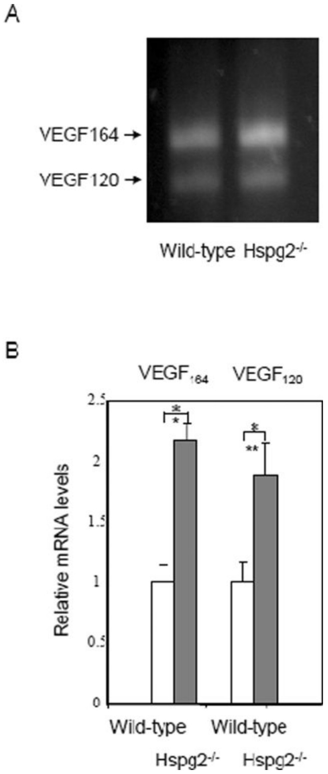


Fig. 4. VEGF$_{164}$ expression is increased in chondrocytes of Hspg2$^{-/-}$ mice

(A) Semiquantitative RT-PCR of VEGF isoforms. VEGF$_{120}$ and VEGF$_{164}$ mRNA were expressed in chondrocytes prepared from the growth plates of wild-type mice. Expression levels of VEGF$_{164}$ mRNA were most prominent in wild-type mice. In Hspg2$^{-/-}$ mice, these VEGF mRNA were also expressed, with the highest level for VEGF$_{164}$. (B) Real-time RT-PCR analysis of the expression levels of VEGF mRNA in chondrocytes in wild-type and Hspg2$^{-/-}$ mice. VEGF$_{120}$ and VEGF$_{164}$ mRNA were increased in the chondrocytes of Hspg2$^{-/-}$ mice. The increase in the levels of VEGF$_{164}$ mRNA was largest in the absence of perlecan. The relative expression levels of each isoform in wild-type mice were set as 1. * indicates $p<0.05$.

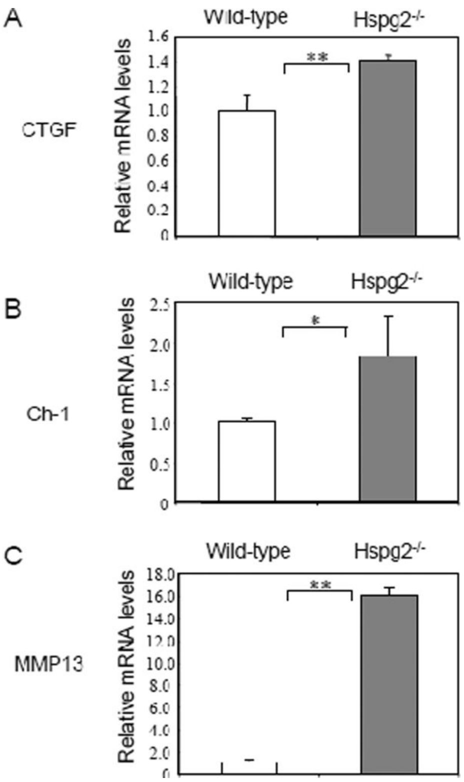


Fig. 5. Expression of molecules involved in vascular invasion

(A, B) Quantitative RT-PCR. Expression of mRNA for the connective tissue growth factor (CTGF) and chondromodulin-1 (Ch-1), which are known to be involved in vascular invasion in endochondral ossification, were significantly increased in chondrocytes from Hspg2$^{-/-}$ mice compared to those in wild-type mice**.** * and ** indicate p<0.05 and <0.01, respectively. **(C)** Expression of MMP13, which is expressed by hypertrophic chondrocytes, was strongly increased in the growth plates of Hspg2$^{-/-}$ mice. ** indicates p<0.01.

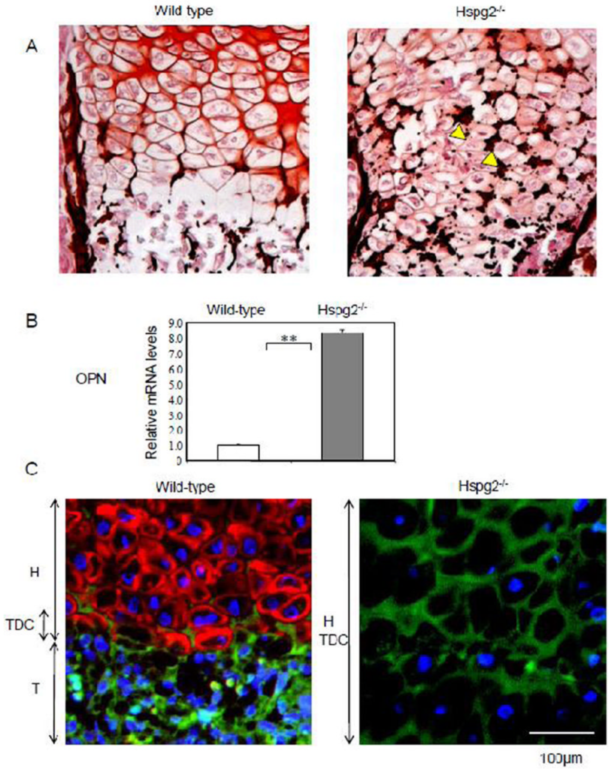
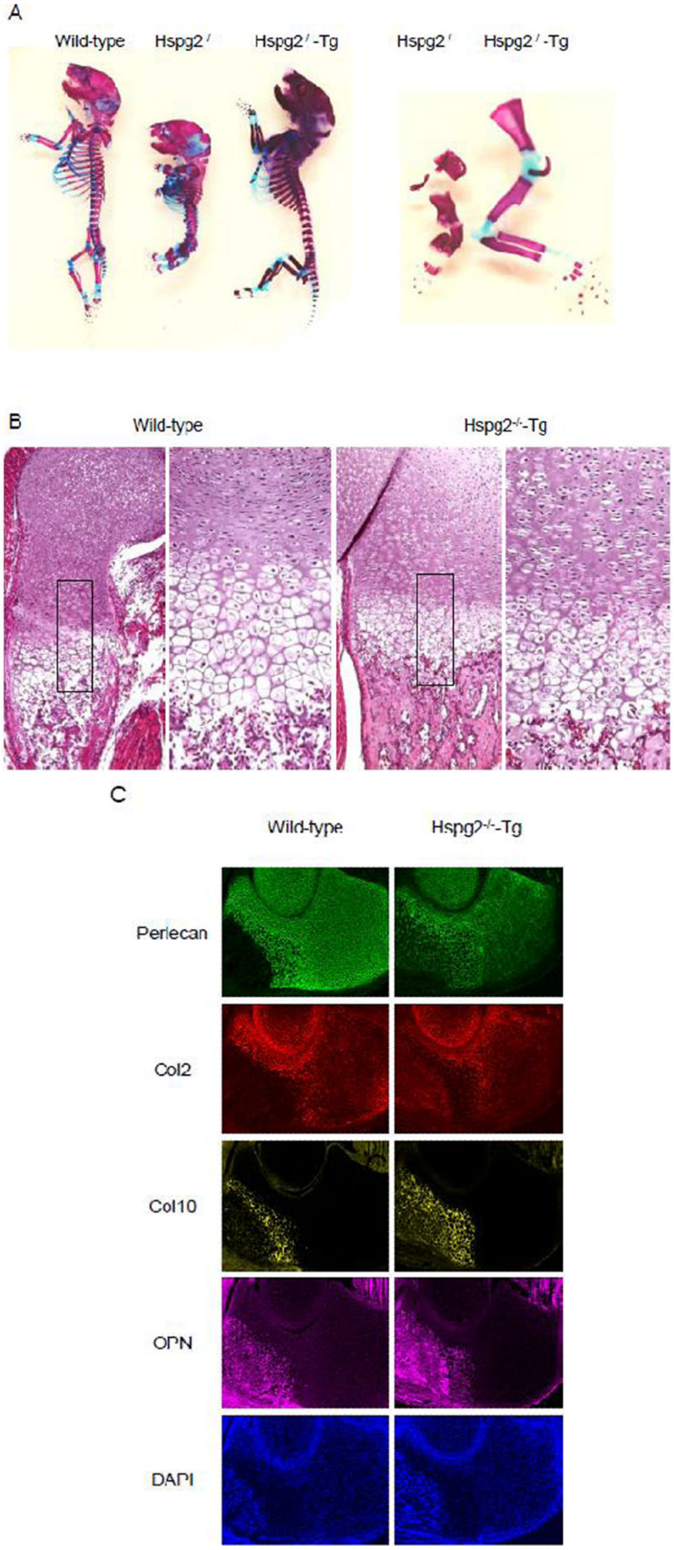


Fig. 6. Accumulation of mature population of hypertrophic chondrocytes in the growth plates of Hspg2$^{-/-}$ mice

(A) Double staining of Safranin-O (red) and von Kossa (brown) staining at the proximal end of the humerus in E18.5 wild-type and Hspg2$^{-/-}$ mice. In E18.5 Hspg2$^{-/-}$ mice, the matrix surrounding multiple layers of hypertrophic chondrocytes is calcified, whereas in the wild-type growth plate, the matrix surrounding only a few hypertrophic layers at the end of cartilage is calcified. Arrowheads indicate the formation of hydroxyapatite nodules (calcospherites) in the hypertrophic zone of Hspg2$^{-/-}$ mice. **(B)** Real-time RT-PCR analysis. Osteopontin OPN mRNA expression was significantly increased in the chondrocytes of E18.5 Hspg2$^{-/-}$ mice compared with the expression in wild-type mice. ** indicates $p<0.01$. **(C)** Double immunostaining for OPN (green) and perlecan (red) of the proximal end of the humerus in E18.5 wild-type and Hspg2$^{-/-}$ mice. OPN-expressing mature hypertrophic chondrocytes accumulated in the growth plates of Hspg2$^{-/-}$ mice. Only a few hypertrophic cell layers expressed OPN in the terminally differentiated chondrocyte area (TDC) of the wild-type mice. Perlecan was expressed in the area of hypertrophic chondrocytes (H) and TDC, but not in the trabecular bone area (T) of the growth plate of wild-type mice. Perlecan was completely absent in all of these areas in Hspg2$^{-/-}$ mice. Scales show 100 μm in length.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DAPI

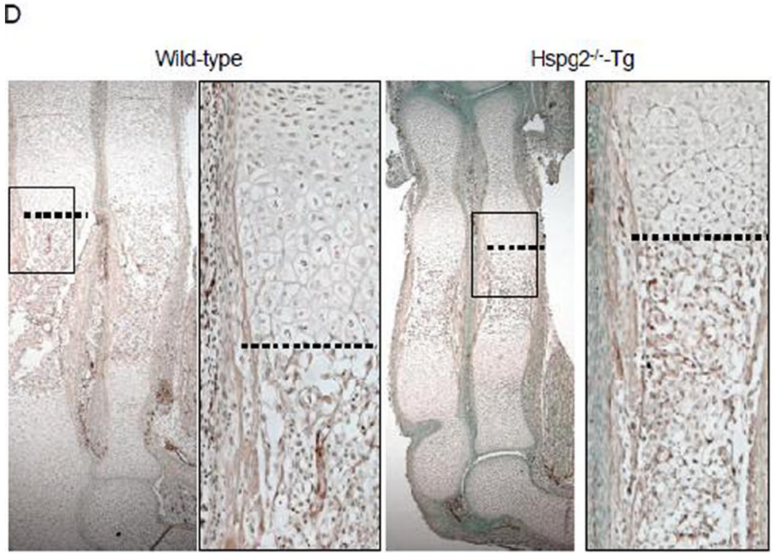

Fig. 7. Restore skeletal abnormalities of Hspg2$^{-/-}$ mice to normal by mating with transgenic mice (Col2a1-PerTg)

(A) Skeletal abnormalities of Hspg2$^{-/-}$ mice were rescued by creating Hspg2$^{-/-}$ mice containing the Hspg2 transgene (Col2a1-PerTg) under the control of a chondrocyte-specific Col2a1 promoter and enhancer (Hspg2$^{-/-}$-Tg). The left three panels show skeletal preparations of E18.5 whole embryos (wild-type, Hspg2$^{-/-}$, and Hspg2$^{-/-}$-Tg); the right two panels show hind limbs from E18.5 Hspg2$^{-/-}$ and Hspg2$^{-/-}$-Tg mice. Cartilage was stained with Alcian blue, and bone was stained with Alizarin red S. **(B)** Histological sections of the growth plate of E18.5 hind limbs of wild-type mice stained with H-E. Columnar structure of the growth plate was restored in Hspg2$^{-/-}$-Tg mice. **(C)** Immunostaining of extracellular matrix proteins in E18.5 growth plates from wild-type and Hspg2$^{-/-}$-Tg mice. Perlecan was expressed in the growth plates of Hspg2$^{-/-}$-Tg mice. Expression of type II collagen (Col2), type X collagen (Col10), and osteopontin (OPN) was similar for the wild-type and Hspg2$^{-/-}$-Tg mice. **(D)** Immunostaining of CD31 in the growth plate of E18.5 wild-type and Hspg2$^{-/-}$-Tg mice. The vascular invasion of the chondro-osseous boundary and bone marrow observed in Hspg2$^{-/-}$-Tg mice was similar to that in wild-type mice.

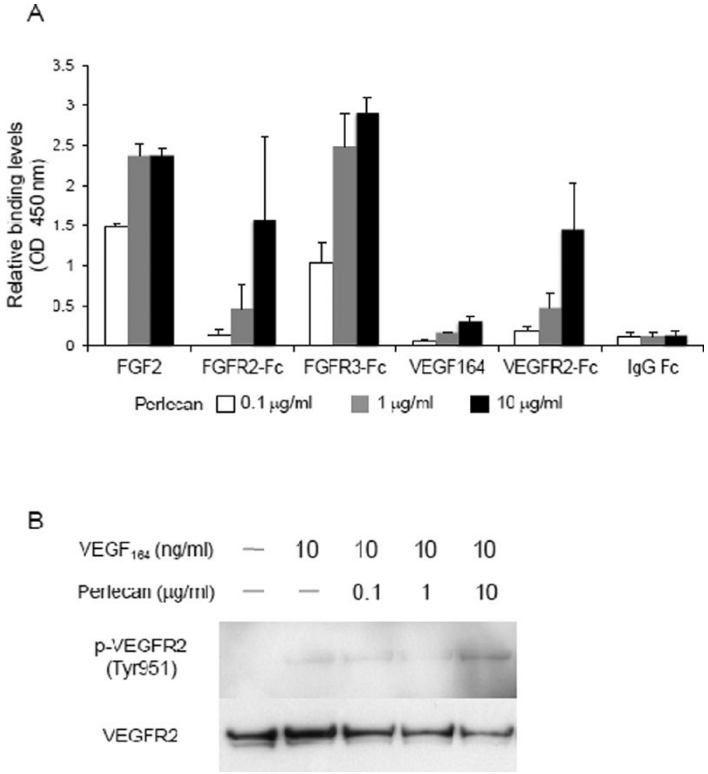

Fig. 8. Perlecan binds to VEGFR2 and promotes VEGF-induced VEGFR2 activation in endothelial cells

Binding of cartilage perlecan to FGF2 and $VEGF_{164}$ and their receptors at various concentrations in solid phase assays. Perlecan bound to FGF2 and FGFR2. Perlecan did not bind to $VEGF_{164}$ but bound to its receptor, VEGFR2. (B) Western blots of VEGFR2 and phosphorylated VEGFR2 of endothelial cells treated with $VEGF_{164}$ and perlecan. Perlecan promoted the phosphorylation levels of VEGFR2 in the presence of VEGFA.