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IGF-1R Signaling in Chondrocytes Modulates Growth Plate Development by Interacting With the PTHrP/Ihh Pathway

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Abstract

Systemic derangements and perinatal death of generalized insulin-like growth factor 1 (IGF-1) and IGF-1 receptor (IGF-1R) knockout mice preclude definitive assessment of IGF-1R actions in growth-plate (GP) chondrocytes. We generated cartilage-specific *Igf1r* knockout (*CartIgf1r^{-/-}*) mice to investigate local control of chondrocyte differentiation in the GP by this receptor. These mice died shortly after birth and showed disorganized chondrocyte columns, delayed ossification and vascular invasion, decreased cell proliferation, increased apoptosis, and increased expression of parathyroid hormone-related protein (*Pthrp*) RNA and protein in their GPs. The increased *Pthrp* expression in the knockout GPs likely was due to an increase in gene transcription, as determined by the increased activity of a *LacZ* reporter that was inserted downstream of the endogenous PTHrP promoter and bred into the knockout mice. To circumvent the early death of *CartIgf1r^{-/-}* mice and investigate the role of IGF-1R during postnatal growth, we made tamoxifen (Tam)–inducible, cartilage-specific *Igf1r* knockout (*TamCartIgf1r^{-/-}*) mice. At 2 weeks of age and 7 to 8 days after Tam injection, the *TamCartIgf1r^{-/-}* mice showed growth retardation with a disorganized GP, reduced chondrocyte proliferation, decreased type 2 collagen and Indian Hedgehog (Ihh) expression, but increased expression of PTHrP. Consistent with in vivo observations, in vitro knockout of the *Igf1r* gene by adenoviral expression of *Cre* recombinase suppressed cell proliferation, promoted apoptosis, and increased *Pthrp* expression. Our data indicate that the IGF-1R in chondrocytes controls cell growth, survival, and differentiation in embryonic and postnatal GPs in part by suppression of *Pthrp* expression.

Keywords

IGF-1 RECEPTOR; CHONDROCYTE; GROWTH PLATE DEVELOPMENT; IHH; PTHRP

Introduction

Skeletal development in vertebrates begins in early embryos and continues postnatally until peak bone mass is reached in the adulthood.⁽¹⁾ Endochondral bone formation is the major process controlling longitudinal bone growth. This process is initiated by the condensation of mesenchymal progenitor cells, followed by the differentiation of chondrocytes to form the cartilage anlagen. Chondrocytes in the latter structure proliferate to increase the number of cells and produce the extracellular matrix, together increasing the size of the cartilage template that becomes the growth plate (GP). Following the stage of cell proliferation, GP chondrocytes (GPCs) mature, hypertrophy, and begin to deposit mineral in the surrounding matrix. They secrete vascular endothelial growth factors (VEGFs) to facilitate the invasion of blood vessels and produce other factors to support the differentiation of incoming osteoblast and osteoclast progenitors. Finally, terminally differentiated chondrocytes undergo apoptosis and are replaced by bone cells.^(2–4) GPs continue to support longitudinal bone growth through cycles of the preceding steps until they are closed naturally in adulthood or abnormally under pathologic conditions.⁽⁵⁾ Numerous endocrine, autocrine, and paracrine factors coordinate to control the steps of GP development.

The parathyroid hormone–related protein/Indian Hedgehog (PTHrP/Ihh) feedback loop is a well-established autocrine/paracrine pathway that controls the pace of chondrocyte differentiation.^(6,7) PTHrP is synthesized by perichondrial and reserve cells in the embryonic skeleton and diffuses into the proliferation zone to activate the type 1 PTH/PTHrP receptor (PTH/PTHrP-1R) in proliferating chondrocytes to sustain their proliferation and delay their maturation.^(8,9) When proliferating chondrocytes mature, they increase the production of Ihh, which acts on its receptor Patched (ptch) in the neighboring cells via unknown mechanisms to increase the production of PTHrP, thus slowing down cell differentiation to prevent early closure of the GP.^(7,10) During postnatal growth, PTHrP is expressed in maturing/prehypertrophic chondrocytes, which also produce PTHrP, supporting a local autocrine/paracrine interaction.⁽¹¹⁾

In the vertebrates, insulin-like growth factor 1 (IGF-1) is a major growth-promoting signal for skeletal development.^(12,13) In global *Igf1* knockout embryos, most of which died shortly after birth,⁽¹⁴⁾ IGF-1 deficiency led to markedly reduced chondrocyte proliferation, increased apoptosis, and delayed maturation and differentiation of chondrocytes, resulting in a severely shortened and undermineralized skeleton.⁽¹⁵⁾ Global *Igf1r* knockout also resulted in perinatal death of the mice, and these mice showed even more severe growth retardation and skeletal defects than the global *Igf1* knockout mice, suggesting that both IGF-1 and IGF-2 acting via the IGF-1R are involved in regulating skeletal development in embryos. Because *Igf1* and *Igf1r* double-knockout mice are comparable in phenocopy to the growth and skeletal defects in *Igf1r* knockout mice, it is proposed that the IGF-1R mediates all activities induced by this growth factor.⁽¹⁶⁾ Because chondrocytes, osteoblasts, osteoclasts, and many other cells in the bone express IGF-1R and IGF-1, it is difficult to specify the action of IGF-1R in chondrocytes and exclude the impact of other systemic derangements on the skeletons in the global knockout animals. Furthermore, their early death precludes studies of IGF-1R actions in GPs during postnatal growth. We report herein the making of *Cart^{Cre}Igf1r^{-/-}* mice in which the *Igf1r* gene was ablated specifically in chondrocytes by *Cre-lox* recombination. We also made tamoxifen (Tam)-inducible *TamCart^{Cre}Igf1r^{-/-}* mice that permit cartilage-specific knockout of the *Igf1r* gene at specific time points during postnatal development. We use these animal models and in vitro chondrocyte cultures to define the action of IGF-1R signaling on chondrocyte differentiation and its interaction with the PTHrP/Ihh feedback mechanism.

Materials and Methods

Generation of cartilage-specific *Igf1r* knockout mice (with or without *Pthrp-LacZ* reporter)

The *CartIgf1r*^{-/-} mice were made by breeding *floxed-Igf1r* (*Igf1r*^{flox/flox}) mice that carry *loxP* sequences flanking exon 3 of the gene⁽¹⁷⁾ with transgenic mice (*CreCart*) expressing Cre recombinase under control of a type 2 collagen [α_1 (II)] promoter⁽¹⁸⁾ (Jackson Laboratories, Bar Harbor, ME, USA). Skeletons from 14.5- to 18.5-day postconception (dpc) *CartIgf1r*^{-/-} embryos and newborn pups and their wild-type littermates were analyzed.

To determine the effect of *Igf1r* knockout on the transcriptional activity of the *Pthrp* gene in GPs, we generated *CartPthrpIgf1r*^{-/-} mice by crossing *CartIgf1r*^{-/-} mice with *Pthrp-LacZ* mice (gift from Dr Arthur E Broadus) expressing a *LacZ* reporter that was inserted downstream of the endogenous *Pthrp* gene promoter.^(19,20) This *Pthrp-LacZ* knock-in mouse model provides a 5- to 10-fold increase in sensitivity over conventional in situ hybridization and immunohistochemistry that measure mRNA and protein levels, respectively. The triple transgenic *CartPthrpIgf1r*^{-/-} mice carry one copy of the *Pthrp-LacZ* knock-in allele and the *Col(II)-Cre* transgene and homozygous *floxed-Igf1r* alleles. Their littermates, carrying the same gene alleles, except the *Cre* transgene, were used as controls.

The inducible *TamCartIgf1r*^{flox/flox} mice were generated by breeding *Igf1r*^{flox/flox} mice with mice expressing a *CreER*^{Cart} transgene, which encodes a fusion protein of the Cre recombinase and a mutated estrogen-responsive element to confer sensitivity to Tam,^(21,22) and is regulated by the α_1 (II) promoter for cartilage-specific expression.⁽²²⁾ The resulting *TamCartIgf1r*^{flox/flox} mice were viable and fertile in the absence of Tam. To produce *Igf1r* gene knockout in *TamCartIgf1r*^{-/-} mice during postnatal growth, *TamCartIgf1r*^{flox/flox} mice were injected with four doses of Tam (0.2 mg/mouse) at 2-day intervals beginning on day 5 after birth. Bones from the *TamCartIgf1r*^{-/-} mice and their control littermates (*Igf1r*^{flox/flox}) injected with Tam were harvested on day 14 for analysis. All animal studies were approved by the Animal Use Committee of the San Francisco Veterans Affairs Medical Center, where the animals were raised and studied.

Genotyping and determination of tissue-specific deletion of the *Igf1r* gene

Genomic DNA was extracted from tail snips and other tissues (GP cartilage, heart, liver, kidney, brain, intestine, spleen, lung, and bone) of the mice using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) analyses of the DNA were performed to detect *Cre* and *floxed-Igf1r* alleles using corresponding primer sets with a standard condition (1 minute at 94°C, 1 minute at 61°C, and 1 minute at 72°C for 30 cycles). PCR was performed with a mixture of three primers (two forward primers, 5'-CTT CCC AGC TTG CTA CTC TAG G-3' and 5'-TGA GAC GTA GCG AGA TTG CTG TA-3', and a reverse primer, 5'-CAG GCTTGC AAT GAG ACA TGG G-3') to detect 320- and 120-bp products from the excised and nonexcised gene alleles, respectively,⁽²³⁾ or with a forward primer, 5'-AAT TGA ATT ATG GCC CAC AC-3', and a reverse primer, 5'-AAT TCC GCC GAT ACT GAC-3', to identify a 192-bp *LacZ* coding region.⁽¹⁹⁾ The *cre* transgene was detected by PCR using the primers 5'-GCA AAA CAG GCT CTA GCG TTC G-3' (forward) and 5'-CTG TTT CAC TAT CCZ GGT TAC GG-3' (reverse) to amplify a 560-bp DNA product.

Skeletal preparations for whole-mount staining and histology

For whole-mount skeletal staining, 13.5 to 18.5 dpc *CartIgf1r*^{-/-} embryos and 1-day-old neonates were eviscerated, fixed in 99% ethanol overnight, and stained with alizarin red for calcified tissue and alcian blue for cartilage, as described previously.^(24,25) To assess β -galactosidase (β -gal) activity in cartilage, skinned E18.5 embryos were fixed in 4%

paraformaldehyde (PFA) in PBS for 4 hours at 4°C and incubated in 1 mg/mL of X-gal buffer at 32°C for 24 hours. After postfixation in 4% PFA overnight, tibias were dissected, embedded in paraffin, sectioned (8 µm), and counterstained with nuclear fast red (Poly Scientific; R&D Biosystems, Minneapolis, MN, USA). To determine changes in chondrocyte proliferation rate in GPs, 5-bromo-2-deoxyuridine (BrdU; 50 µg/g of body weight), which is incorporated into genomic DNA in the S phase of the cell cycle, was given to pregnant mice (18.5 dpc) by intraperitoneal injections 2 hours prior euthanization. For standard histology, tibias and/or the lumbar spines (L₁–L₅) from *CartIgf1r*^{-/-}, *TamCartIgf1r*^{-/-}, and corresponding control littermates were fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated in ethanol and xylene, embedded in paraffin, and sectioned (5 µm thickness). Bone sections were stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry with antisera against proliferating cell nuclear antigen (PCNA; Invitrogen, Carlsbad, CA, USA), BrdU (Invitrogen), α₁(II) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Ihh (Santa Cruz Biotechnology, Inc.), PTHrP (Santa Cruz Biotechnology, Inc.), CD31 (PECAM-1; BD Pharmingen, Franklin Lakes, NJ, USA), and active caspase-3 (Abcam, Cambridge, MA, USA).⁽¹⁵⁾ Apoptotic cells in the sections were detected with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Billerica, MA, USA). For histologic studies, we analyzed three bone sections from each mouse, and at least three mice per group were examined.

Cultures and viral infection of GPCs

Epiphyseal GPs from 2- to 4-day-old *floxed-Igf1r* mice were obtained, and chondrocytes were released by enzymatic digestion and cultured as described previously.⁽²⁶⁾ To knock out *Igf1r* expression in vitro, confluent GPCs (4 to 5 days after plating) were infected with adenoviruses carrying a *Cre* recombinase cDNA (Ad-Cre) at 1, 2, 4, and 8 plaque-forming units (pfu)/cell for 72 hours. Mock infection with PBS or viruses expressing empty vector (Ad-DNR) were performed as controls.⁽²⁶⁾ Proliferation of infected GPCs was determined after cells were labeled with BrdU for 4 hours, and the BrdU signals were detected by a staining kit (Invitrogen). Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon).

Quantitative real-time PCR

Total RNA was extracted from epiphyseal GPs and cultured GPCs and reverse transcribed into cDNA, as described previously.⁽²⁶⁾ Expression of *Igf1r*, *Ihh*, *Patch*, *Pthrp*, and *osteopontin* (*Opn*) was determined by quantitative real-time PCR using specific primers and probes as described in previous reports.^(15,25)

Statistical analysis

Results were presented as mean±SD and compared using the unpaired Student's *t* test. Significance was assigned for *p*<.05. Samples of at least three animals were analyzed for each experimental and control group.

Results

The role of IGF-1R signaling in GPCs in embryonic bone development

We confirmed the specificity of gene excision in the *CartIgf1r*^{-/-} mice by PCR analyses of genomic DNA from different tissues with primers flanking exon 3 of the *Igf1r* gene. As shown in Fig. 1A, gene excision (Δ -*Igf1r*) occurred only in GP cartilage from the *CartIgf1r*^{-/-} mice but not in other tissues from the same animals or in GP cartilage from the *CartIgf1r*^{-/-} (wild-type) mice. In the GPs of *CartIgf1r*^{-/-} (wild-type) mice, IGF-1R

immunoreactivity, indicated by brown 3,3'-diaminobenzidine (DAB) staining, was detected in resting, proliferating, and prehypertrophic or maturing chondrocytes and, to a lesser extent, in hypertrophic cells (Fig. 1B). The expression of IGF-1R protein was profoundly reduced in all cell layers in the knockout GPs (Fig. 1B).

Most of the *CartIgf1r*^{-/-} mice died shortly after birth and had blunted growth. The body weights of newborn knockout mice (1.28±0.13 g, *n*=10) were approximately 90% (*p*<.05) of those of their wild-type littermates (1.42±0.15 g, *n*=17). Whole-mount alcian blue and alizarin red staining showed normal skeletal patterning in *CartIgf1r*^{-/-} embryos and neonates (Fig. 2A), but their skeletons clearly were smaller than those of their wild-type counterparts (Fig. 2A). Mineralization in calvaria, rib cages, and long bones in the extremities of the knockout mice also was clearly reduced, beginning as early as 14.5 dpc and remaining evident in 18.5-dpc embryos and neonates (Fig. 2A).

In tibias of *CartIgf1r*^{-/-} mice, the arrangement of proliferating chondrocytes was disorganized compared with the orderly cell columns in wild-type GPs (Fig. 2B, Tibia). In the knockout GPs, the proliferation zones (PZs) also were expanded (1198±112 µm in the wild-type mice versus 1425±135 µm in the knockout mice, *p*<.05, *n*=4 mice), but their hypertrophic zones (HZs) were shortened (wild-type mice: 607±79 µm; knockout mice: 489±38 µm, *p*<.05, *n*=4 mice; Fig. 2B). Such morphologic changes were in the same direction but did not achieve statistical significance in the distal femur (PZ length: 278.53±9.99 µm in wild-type mice versus 313.66±26.97 µm in knockout mice, *p*=.051; HZ length: 176.67±17.62 µm in wild-type mice versus 153.67±21.5 µm in knockout mice, *p*=.13). In the spinal columns of knockout mice, the formation of ossification centers was delayed and most marrow components were absent compared with wild-type littermates (Fig. 2B, Spine). The preceding morphologic changes in long bones and spine support a delay in the progression of GPC differentiation. There also was a clear reduction in vascular elements in the spinal columns from the knockout mice (Fig. 2B, Spine). Immunohistochemistry with antisera against PECAM-1 (CD31), an endothelial cell marker, showed only trace amount of signal localized to perichondrial areas in the knockout spinal columns (Fig. 2C, *arrowheads*), as opposed to the abundant immunoreactivity across the ossification centers of wild-type *CartIgf1r*^{+/+} mice (Fig. 2C, *arrowheads*), indicating delayed vascular invasion.

To test whether *Igf1r* knockout affects the proliferation and survival of chondrocytes in the GP, we performed PCNA and TUNEL staining, respectively (Fig. 3). The number of PCNA⁺ cells was significantly decreased in the PZ of knockout GPs compared with that of wild-type GPs (16.4%±4.2% in wild-type mice versus 12.3%±3.5% in knockout mice, *p*<.05, *n*=4 to 5 mice; Fig. 3A, PZ). In supporting the hypoproliferation phenotype, BrdU incorporation in the tibial GP of 18.5-dpc knockout embryos was decreased significantly in both resting and proliferating zones (Fig. 3A, and data not shown) when compared with the wild-type embryos. In contrast, the number of apoptotic cells was increased significantly in the resting (Fig. 3B, RZ) and proliferating (Fig. 3B, PZ) zones of the knockout versus control GPs and unchanged in the hypertrophic zone (Fig. 3B, HZ). Increased immunohistochemical staining of active caspase-3 further supports the increased apoptosis in the resting, proliferating, and hypertrophic zones of the knockout GP versus wild-type controls noted by TUNEL analysis (Fig. 3C).

To examine whether deletion of *Igf1r* affects chondrocyte terminal differentiation and the PTHrP-Ihh signaling pathway, we measured mRNA levels of *Opn*, *Ihh*, *Patched*, and *Pthrp* in the GPs by quantitative real-time PCR (qPCR; Fig. 4A). mRNA levels for *Opn* were decreased by approximately 60% in the GPs of *CartIgf1r*^{-/-} mice, indicating a delay in cell differentiation. *Igf1r* knockout had no effect on the expression of *Ihh* and modestly

decreased the expression of *Patched* by 9%. Interestingly, *Igf1r* knockout significantly increased mRNA levels of *Pthrp* by more than 35%. To further examine the transcriptional regulation of the *Pthrp* gene by *Igf1r* knockout, we crossed the *CartIgf1r^{-/-}* mice with the *Pthrp-LacZ* reporter mouse. In the resulting E18.5 *CartPthrpIgf1r^{-/-}* embryos, β -galactosidase (β -Gal) activity, stained in blue, was clearly increased in the cartilage of the long bones and ribcages (Fig. 4B, panels 1 and 2, KO) when compared with the controls without deletion of the *Igf1r* gene (Fig. 4B, panel 2, Cont). High-power views of bone sections from the proximal tibias of control mice (Fig. 4B, panels 3 and 5) showed that the β -Gal activity was localized mainly to the RZ chondrocytes adjacent to the perichondrium. In the corresponding region of the knockout GP, β -Gal activity was profoundly increased (Fig. 4B, panels 4 and 6 versus panels 3 and 5).

The role of IGF-1 signaling in postnatal bone development

Although the gene knockout was targeted specifically to chondrocytes, most (>95%) of the *CartIgf1r^{-/-}* mice still died shortly after birth, precluding studies on GP development during postnatal growth. To overcome this deficiency, we generated *TamCartIgf1r^{flox/flox}* mice in which the *flox*-*Igf1r* gene could be excised in chondrocytes by a Tam-induced translocation of the Cre-ER fusion protein into the nuclei of the cells.⁽²²⁾ We compared the skeletal phenotypes of the Tam-injected *TamCartIgf1r^{flox/flox}* (*TamCartIgf1r^{-/-}*) mice with those of controls (Cont, *Igf1r^{flox/flox}*), which carried the *flox*-*Igf1r* alleles without the *Cre-ER* transgene and also were injected with the same amount of Tam.

The *TamCartIgf1r^{-/-}* mice showed significant growth retardation (Fig. 5A). At 2 weeks of age and 7 to 8 days after induction of gene excision by Tam, their body weights (3.59 ± 0.13 g) were about 70% ($p < .01$) of their control littermates (5.14 ± 0.28 g). Ablation of *Igf1r* gene was confirmed by a decrease (by >90%) in RNA expression by qPCR (data not shown) and marked reduction in IGF-1R immunoreactivity in the knockout GP (Fig. 5B) compared with control mice. In the GP of *TamCartIgf1r^{-/-}* mice, the HZ was significantly shortened, whereas the PZ was somewhat disorganized (Fig. 5C).

In the knockout GPs, the number of PCNA-positive cells was decreased by more than 85% in the PZ (Fig. 5D), as was the expression of Col(II) protein (Fig. 5E). In the control GPs, the expression of *Ihh* protein was mainly localized to prehypertrophic or maturing chondrocytes but was absent in the lower HZ (Fig. 5F). In the knockout GPs, *Ihh* expression, however, was detected throughout the region below the PZ, although the level of expression was substantially reduced compared with controls. Similarly, the expression of PTHrP was abnormally broadened in all cell layers across the GPs in the knockout mice compared with the pattern of expression, which was restricted to the maturing and prehypertrophic chondrocytes, in control mice (Fig. 5G). These data indicate that IGF-1R signaling modulates the expression of *Ihh* and PTHrP to mediate cell proliferation and survival while controlling the pace of cell differentiation and matrix production in GPs during postnatal growth.

Studies of chondrocyte proliferation, survival, and differentiation in vitro

To further confirm the action of *Igf1r* on GPC differentiation, we examined the effects of acute *Igf1r* gene knockout on growth, survival, and gene expression in cultured chondrocytes. We blocked *Igf1r* expression by infecting chondrocytes cultured from epiphyseal GPs of *Igf1r^{flox/flox}* mice with adenoviruses expressing Cre recombinase (Ad-Cre) or blank vector (DNR).⁽²⁵⁾ Western blotting of protein lysates from the infected cells showed that IGF-1R protein levels were dose-dependently decreased with the titers of Ad-Cre virus compared with mock or Ad-DNR controls (Fig. 6A). Real-time PCR analyses also

showed a profound reduction (by >80%) in *Igf1r* RNA levels in Ad-Cre-infected cultures versus mock or Ad-DNR controls (Fig. 6A).

Cell proliferation, determined by BrdU incorporation, was decreased by about 50% in Ad-Cre- versus Ad-DNR-infected cultures (Fig. 6B). On the other hand, the number of apoptotic cells assessed by TUNEL staining (Fig. 6C) was increased by 50% in cultures infected with Ad-Cre. Furthermore, OPN expression was decreased by 50% in the Ad-Cre-infected cells (Fig. 6D), further suggesting a delay in the progression of chondrocyte differentiation. These data are consistent with our in vivo observations in *Igf1r* KO mice and support the idea that IGF-1R signaling in GPCs is essential for cell growth and differentiation. The expression of *Pthrp* RNA (Fig. 6D) was increased in the Ad-Cre-infected cultures, as it was in vivo in the *Igf1r* KO mice, further supporting an interaction between IGF-1R signaling and the *Ihh*/PTHrP pathway.

Discussion

In this study, we generated chondrocyte-specific *Igf1r* knockout mice to investigate the direct actions of IGF-1R in chondrocytes during embryonic and postnatal skeletal development. Our data confirm a critical role for this receptor in sustaining proliferation, survival, and differentiation of the chondrocyte and its interaction with the PTHrP/*Ihh* signaling pathway. Deleting *Igf1r* in chondrocytes in *CartIgf1r^{-/-}* and *TamCartIgf1r^{-/-}* mice led to their hypoproliferation and increased apoptosis. The actions of IGF-1R on chondrocytes appear to be direct because acute knockout of *Igf1r* in cultured chondrocytes produced similar growth and apoptotic effects. The IGF-1R in chondrocytes is also critically involved in cell differentiation because its deletion delays cell maturation and hypertrophy, decreases the expression of $\alpha_1(\text{II})$, and blocks vascular invasion into the cartilage. These dysregulated activities together caused disorganized growth plates and a shortened skeleton.^(27,28) The morphologic changes—expanded PZ and shortened HZ—in the GP of knockout mice further indicate unsynchronized cell proliferation and differentiation. These morphologic changes are more evident in the tibial GP but are less profound in the femoral GP, suggesting a site-dependent effect of *Igf1r* knockout.

The hypoproliferation phenotype of *Igf1r* knockout mice was clearly demonstrated by the reduced PCNA expression and decreased BrdU incorporation in the tibial GP. The latter observation indicates a disproportionately prolonged S phase of cell cycle in knockout GPCs. Interestingly, another chondrocyte-specific *Igf1r* KO mouse model made by Long and colleagues did not show a reduction in BrdU labeling in the femoral GP in comparison with Wt controls.⁽²⁹⁾ The authors suggested that the unchanged BrdU⁺ cell fraction might be due to the proportional lengthening of all phases of the cell cycle in chondrocytes in the PZ, resulting in an overall decrease in proliferation without a significant change in the ratio of BrdU⁺ cells over the total number of cells.⁽²⁹⁾ This is different from our observations with the tibial GP in our model. It is possible that these discordant results of BrdU labeling are due in part to a site-specific effect of the gene knockout because we assessed the BrdU labeling in the tibial GP in this study, whereas Long and colleagues examined the femoral GP. This site-dependent effect is further supported by less change in the morphology of the femoral GP than of the tibial GP in our knockout mice and in the model of Long and colleagues, which showed relatively normal cell organization in the femoral GP of the knockout mice.⁽²⁹⁾

In addition to hypoproliferation and increased apoptosis of chondrocytes, *CartIgf1r^{-/-}* mice had a significant delay in chondrocyte terminal differentiation. The latter change likely caused the accumulation of cells in the PZ, resulting in its disorganization and expansion in

the knockout mice, similar to that seen in the global *Igf1* knockout mouse.⁽¹⁴⁾ The delayed terminal differentiation also was reflected by a shortened HZ and a reduced bone length.

The similarity in the skeletal phenotypes between the *Cart⁺Igf1^{-/-}* and global *Igf1* knockout mice indicates that IGF-1 is likely the main ligand acting on the receptor in the chondrocytes, although a role for IGF-2 is not excluded by our data. The degree of skeletal defect in the *Cart⁺Igf1^{-/-}* mice, however, is less profound than that in the generalized *Igf1* knockout mice, suggesting that the function of IGF-1R in other relevant cells, such as osteoblasts,⁽²³⁾ osteoclasts,⁽³⁰⁾ and osteocytes,⁽³¹⁾ also may contribute to the GP development. Alternatively, the IGF1-1R may be involved in earlier chondrogenic events that take place before the activation of the α_1 (II) promoter that we used to drive the expression of the *Cre* transgene in our knockout mice.

Ihh and PTHrP constitute a feedback loop to control the pace of chondrocyte proliferation and maturation. Interestingly, ablation of *Igf1r* increased the expression of PTHrP protein and RNA, and this was likely due to an increase in gene transcription based on the study using a β -Gal reporter system. The increased PTHrP expression might have contributed to the delayed cell differentiation and mineralization and the reduced α_1 (II) expression in the GPs in our knockout mouse models⁽³²⁾ because their phenotypes are similar to those of transgenic mice overexpressing PTHrP.⁽³³⁾ However, the latter mice have increased proliferation and decreased apoptosis. It appears that *Igf1r* knockout prevents the increased growth and survival of chondrocytes otherwise expected with increased PTHrP expression, suggesting that the increased expression of PTHrP is an effort to compensate for the reduced proliferation in the *Igf1r* knockout. It remains unclear how the *Igf1r* knockout affects PTHrP expression in GPCs. It has been proposed that Ihh is a critical modulator that promotes PTHrP expression in the GP. We, however, observed a decrease in the expression of Ihh protein in the prehypertrophic chondrocytes that are immediately adjacent to the PTHrP-expressing cells in the GP of 2-week-old *TamCart⁺Igf1^{-/-}* mice, supporting an Ihh-independent regulation of PTHrP expression by *Igf1r* knockout.⁽²⁹⁾ This notion is further supported by the observation that the expression of *Pthrp* was upregulated even in cell layers of the GP that do not express this gene normally.

It is worth pointing out that we did not dissect out subregions of GPs in preparing RNA for the qPCR analyses. Because we normalized gene expression to the expression of ribosomal protein *L19* gene, inclusion of RNA from the disproportionately expanded PZ, which does not express *Pthrp*, might have underestimated the impact of *Igf1r* knockout on the *Pthrp* expression assessed by qPCR. This diluting effect may explain in part the discrepancy between the modest change (approximately 35%) in *Pthrp* RNA levels assessed by qPCR, as shown in Fig. 4A, compared with the more robust *Pthrp* reporter activity determined by the β -Gal staining in Fig. 4B. Furthermore, the potentially longer half-life of the *LacZ* transcript compared with that of *Pthrp* mRNA also may contribute to the enhanced sensitivity in detecting the transcription activity of the *Pthrp* gene. Nevertheless, these observations, together with study of cultured chondrocytes, support the impact of *Igf1r* knockout on PTHrP expression in GPCs.

Our studies also demonstrate an important role for the IGF-1R in the induction of angiogenesis and vascular invasion in the GPs. In our mouse models, *Igf1r* knockout delays development of the vasculature and overall formation of the ossification center in the spinal column. These data are consistent with previous studies demonstrating that IGF-1R signaling is required to mediate the hypoxia-inducible factor 1(HIF-1)-induced angiogenesis and expression vascular endothelial growth factor (VEGF).^(34,35)

Our *TamCarIgf1r^{-/-}* mice are the first to allow assessment of IGF-1R actions in cartilage development during postnatal growth studies. Our initial characterization of the mice demonstrates that the IGF-1R modulates functions in postnatal GPs, as it does in embryonic GPs. This inducible knockout model will be invaluable in future studies on the role of IGF-1R signaling in the development of cartilage diseases such as osteoarthritis and endochondral repair of bone fractures.

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Fig. 1. Ablation of the *Igf1r* gene in *CartIgf1r^{-/-}* mice. (A) PCR analyses of genomic DNA extracted from different tissues as specified from the *CartIgf1r^{-/-}* (KO) and *CartIgf1r^{+/+}* (WT) littermates with primer sets for the *Cre* transgene, *floxed-Igf1r* allele, and sequences after gene excision (Δ -IGF-1R) as described in “Materials and Methods.” (B) Immunohistochemistry for the expression of IGF-1R in tibial GPs of knockout and wild-type neonates (P₀, same day of birth). Signals were detected by brown DAB stain, and bone sections were counterstained with hematoxylin. RZ=resting zone; PZ=proliferation zone; HZ=hypertrophic zone. Bar=100 μ m.

Fig. 2. Skeletal development in *CartIgf1r*^{-/-} (KO) and *CartIgf1r*^{+/+} (WT) mice. (A) Whole-mount alizarin red and alcian blue staining of skeletons from 14.5- and 18.5-dpc wild-type and knockout embryos and P₀ neonates. Bar=1 cm. (B) Hematoxylin and eosin staining of tibias and spinal columns from P₀ wild-type and knockout mice. Arrowheads indicate capillaries in the bone marrow of wild-type spine. RZ=resting zone; PZ=proliferation zone; HZ=hypertrophic zone. Bar=200 μm. (C) Immunohistochemistry for the expression of CD31, an endothelial cell marker, in the spine of P₀ wild-type and knockout mice. Signals are indicated by brown DAB stain, and sections were counterstained with hematoxylin. Arrowheads indicate CD31⁺ capillaries. Bar=50 μm.

Fig. 3.

Effects of *Igflr* knockout on proliferation and apoptosis of GPCs in *CartIgf1r*^{-/-} (KO) and *CartIgf1r*^{+/+} (WT) mice. (A) Cell proliferation in tibial GPs of P₀ wild-type and knockout mice was assessed by immunocytochemistry with antiserum against PCNA and BrdU. Sections were counterstained with hematoxylin. Bar graphs show numbers of PCNA⁺ or BrdU⁺ cells as a percent of total cell number in the PZ. Bar=100 μ m. (B) Apoptotic cells in the P₀ wild-type and knockout GPs were detected by TUNEL staining, as described in "Materials and Methods." Bar graphs show numbers of TUNEL⁺ cells as a percent of total cell number in each zone specified. Results are expressed as means $\pm$ SD. (C) Cell apoptosis in tibial GPs of P₀ wild-type and knockout mice was further assessed by immunocytochemistry with antiserum against active caspase-3 (brown). RZ=resting zone; PZ=proliferating zone; HZ=hypertrophic zone in both B and C. Bar=50 μ m.

Fig. 4. Effects of *Igf1r* knockout on the expression of critical components of the PTHrP/Ihh feedback mechanism in E18.5 *CartIgf1r^{-/-}* (KO) and *CartIgf1r^{+/+}* (Cont) embryos. (A) mRNA levels from the tibia-femur joints from control (*solid bars*) and knockout (*open bars*) mice were quantified by qPCR for the expression of *Ihh*, *Patched*, *Pthrp*, and *Opn* genes. RNA expression was presented as percent expression of *L19*, a housekeeping gene, and expressed as means±SD. ^a*p*<.05 KO versus Cont. (B) β-Gal staining of *CartPthrpIgf1r^{-/-}* (KO) and control (Cont) embryos. (Panel 1) Whole-mount staining of *CartPthrpIgf1r^{-/-}* embryos. (Panel 2) Staining of β-Gal activity in femurs from knockout and control embryos. (Panels 3 and 4) Sections of tibia from control and knockout embryos. (Panels 5 and 6) High magnification for panels 3 and 4. Scale bar for panels 3 to 6 =100 μm.

Fig. 5. Postnatal growth and GP development in *TamCartIgf1r^{-/-}* (KO) and *TamIgf1r^{lox/lox}* (Cont) mice. Postnatal deletion of IGF-1R in chondrocytes was induced by injecting *TamCartIgf1r^{lox/lox}* mice with four doses of Tam (0.2 mg/mouse) at 2-day intervals starting at 5 days. For controls (*TamIgf1r^{-lox/lox}*), *Igf1r^{lox/lox}* mice, which did not express the *Cre* transgene, were injected with Tam as described earlier. Mice were studied on day 14 (3 days after the last injection). (A) Photographs of the 14-day-old control and knockout littermates. (B) Immunohistochemistry of tibial GPs of control and knockout mice for the expression of IGF-1R was performed as described earlier. (C) H&E staining of tibial GPs of control and knockout mice. PZ= proliferating zone; HZ=hypertrophic zone. (D–G) Immunohistochemistry of tibial GPs of control and knockout mice for the expression of PCNA (D), α_1 (II) (E), Ihh (F), and PTHrP (G). Signals were indicated by brown DAB stain. Sections for immunohistochemistry were counterstained with hematoxylin. Bar=100 μ m.

Fig. 6.

Effect of *Igflr* KO on proliferation, apoptosis, and gene expression in cultured GPCs. GPCs from 2-to4-day-old *Igflr^{flx/flx}* mice infected with Ad-Cre (1 to 8 pfu/cell) or Ad-DNR (8 pfu/cell) viruses or mock solution. (A) Protein and RNA expression of IGF-1R in cultured cells was determined by Western blotting and qPCR, respectively, 72 hours after infection. mRNA levels were normalized to the level of *L19* as a loading control and presented as percent of the levels in mock-infected cultures (control). (B) Proliferation of GPCs was determined 72 hours after infection. The cells were labeled with 5-bromo-2-deoxyuridine (BrdU) for 4 hours, and the BrdU signals were detected by DAB stain (arrows). Bar graphs show numbers of BrdU⁺ cells as PERCENT of total cell number in the cultures. (C) Apoptosis was assessed by TUNEL staining, and signals were detected by DAB-stain (arrows). Bar graphs show numbers of TUNEL⁺ cells as percent of total cell number in the cultures. (D) RNA expression of *Opn* and *Pthrp* in cultured cells was determined 72 hours after infection by qPCR. All results are expressed as mean $\pm$ SD. ^a $p < .05$ versus control cultures.