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Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes

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Chondrocyte proliferation and differentiation requires their attachment to the collagen type II–rich matrix of developing bone. This interaction is mediated by integrins and their cytoplasmic effectors, such as the integrin-linked kinase (ILK). To elucidate the molecular mechanisms whereby integrins control these processes, we have specifically inactivated the ILK gene in growth plate chondrocytes using the Cre-lox methodology. Mice carrying an ILK allele flanked by loxP sites (ILK-fl) were crossed to transgenic mice expressing the Cre recombinase under the control of the collagen type II promoter. Inactivation of both copies of the ILK-fl allele lead to a chondrodysplasia characterized by a disorganized growth plate and to dwarfism. Expression of chondrocyte differentiation markers such as collagen type II, collagen type X, Indian hedgehog and the PTH-PTHrP receptor was normal in ILK-deficient growth plates. In contrast, chondrocyte proliferation, assessed by BrdU or proliferating cell nuclear antigen labeling, was markedly reduced in the mutant growth plates. Cell-based assays showed that integrin-mediated adhesion of primary cultures of chondrocytes from mutant animals to collagen type II was impaired. ILK inactivation in chondrocytes resulted in reduced cyclin D1 expression, and this most likely explains the defect in chondrocyte proliferation observed when ILK is inactivated in growth plate cells.

Introduction

Endochondral bone formation is a multistep process in which the conversion of a cartilaginous model to bone leads to longitudinal growth. This process starts with mesenchymal cells that condense and differentiate into two types of cells: chondrocytes that form cartilage elements and express specific molecular markers, such as α1(II) collagen (collagen type II), and perichondrial cells that surround the cartilage model. Starting from the center of the cartilage elements, chondrocytes undergo several steps of maturation from proliferating chondrocytes, forming characteristic orderly columns, to nonproliferating, hypertrophic cells (Shum and Nuckolls, 2002). Hypertrophic chondrocytes express the specific marker α1(X) collagen (collagen type X) (Linsenmayer et al., 1991). Hypertrophy of these chondrocytes is associated with apoptosis, matrix calcification, and vascular invasion, after which the calcified cartilage will be removed and replaced by trabecular bone and bone marrow. This sequential process of chondrocyte proliferation, hypertrophy, and replacement by osteoblasts responsible for longitudinal bone growth is retained at the extremities of long bones and organized into the specialized structure known as the growth plate (Poole, 1991). Although multiple extracellular signaling molecules have been implicated in the control of chondrocyte proliferation and maturation (Karsenty and Wagner, 2002), our understanding of the intracellular signaling pathways involved in these processes remains incomplete.

Chondrocyte proliferation and differentiation requires an integrin-mediated interaction with the collagen type II–rich

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matrix of developing bone. After engagement with extracellular matrix components, integrin receptors signal via multiple downstream effectors (Hynes, 2002), including integrin-linked kinase (ILK)* (Hannigan et al., 1996; Wu and Dedhar, 2001). The kinase activity of ILK is stimulated in a phosphatidylinositol 3,4,5-trisphosphate–dependent manner (Delcommenne et al., 1998) after engagement of integrins and growth factor receptors (Wu and Dedhar, 2001). Once activated, ILK can phosphorylate its downstream effectors, which include protein kinase B (PKB)/Akt and glycogen-synthase kinase (GSK)-3 (Delcommenne et al., 1998). Ultimately, the ILK signaling cascade affects gene expression and cell proliferation and survival. Overexpression of ILK potentiates c-jun–dependent activating protein-1 transcriptional activity (Troussard et al., 1999) and can also lead to translocation of β-catenin to the nucleus, which results in activation of lymphocyte enhancer factor-1–dependent transcription (Novak et al., 1998). Constitutive ILK expression, or activation, has been shown to stimulate cyclin D1 expression (Radeva et al., 1997; D'Amico et al., 2000; Persad et al., 2001b). In addition, unregulated ILK expression also promotes anchorage-independent growth, fibronectin matrix assembly, and tumorigenesis (Hannigan et al., 1996; Radeva et al., 1997; Wu et al., 1998). Together, these results show that ILK is a key effector of downstream integrin signaling and an important regulator of gene expression and cellular activity.

Inactivation of the ILK gene in genetic model systems such as *Drosophila* and *Caenorhabditis elegans* resulted in defects similar to loss of integrin function (Zervas et al., 2001; Mackinnon et al., 2002). To understand the role of ILK signaling in the adhesion, proliferation, differentiation, and function of cells from the growth plate and therefore in the control of skeletal growth, we have specifically inactivated the ILK gene in growth plate chondrocytes using the Cre-lox methodology. Mice carrying an ILK allele flanked by loxP sites (ILK-fl) were crossed to transgenic mice expressing the Cre recombinase under the control of the collagen type II promoter/enhancer.

We show that inactivation of both copies of the ILK-fl allele lead to reduced chondrocyte proliferation and adhesion and caused dwarfism. Cyclin D1 expression was markedly reduced in ILK-null chondrocytes. Our results demonstrate that ILK-mediated signaling is essential for chondrocyte proliferation and growth plate structure and function.

Results

ILK expression in growth plate chondrocytes

We first examined ILK expression in chondrocytes using immunohistochemistry. The ILK protein was abundantly expressed in embryonic chondrocytes at embryonic day (E)14.5 and E16.5 (Fig. 1, A and B). Expression was maintained

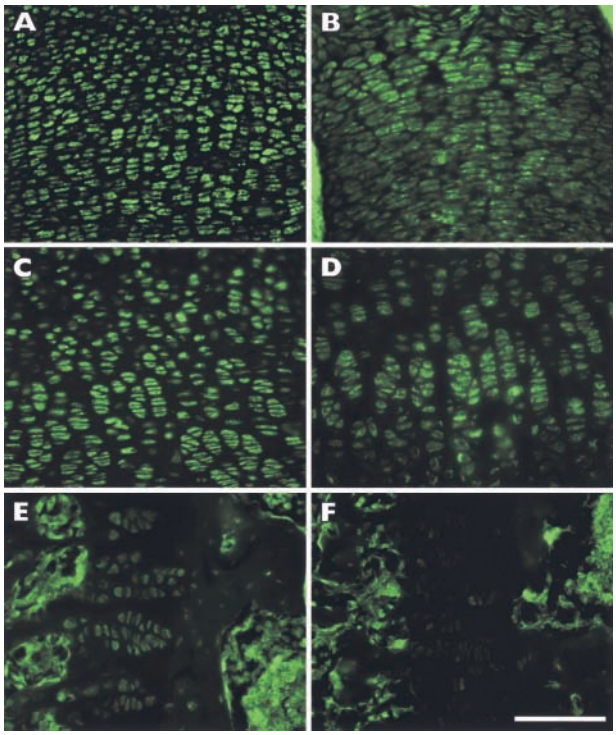

Figure 1. **ILK protein expression pattern in chondrocytes.** Indirect immunofluorescence staining of chondrocytes with anti-ILK antibody. ILK protein was detected in proliferating and prehypertrophic chondrocytes at E14.5 (A), E16.5 (B), and postnatal days 4 (C), 21 (D), and 90 (3 mo, E). Background staining of chondrocytes was negligible, but bone tissue showed background autofluorescence (F). Bar, 100 μm.

in proliferating and prehypertrophic chondrocytes of the growth plate from birth to adulthood (Fig. 1, C–E). ILK was not expressed in hypertrophic chondrocytes at all ages examined (Fig. 1, D and E; unpublished data). Although adjacent bone tissue generated background autofluorescence, no signal was detected in chondrocytes under control staining conditions (Fig. 1 F; unpublished data). This specific ILK expression pattern suggested a role for ILK-mediated signaling in growth plate development and function. We studied this putative role by specifically inactivating the ILK gene in proliferating, collagen type II–expressing chondrocytes using Cre-mediated excision of a loxP-flanked (floxed) ILK locus.

Characterization of the Col2-Cre transgenic mice

To document the ability of the collagen type II gene promoter driving Cre recombinase transgene (Col2-Cre) to induce specific recombination in chondrocytes, the Col2-Cre mice were bred with the targeted gene trap strain, ROSA26 reporter (R26R) (Soriano, 1999). Cre-mediated recombination in R26R mice allows LacZ expression, which can readily be monitored by LacZ staining. This was performed in Col2-Cre;R26R transgenic newborn pups. LacZ staining could be detected in the growth plate of all bones and in costochondral chondrocytes (Fig. 2, middle). Analysis of sections from bone tissue revealed LacZ staining in proliferating, collagen type II–expressing chondrocytes but not in differentiated hypertrophic cells (Fig. 2, right). Immu-

*Abbreviations used in this paper: Col2-Cre, collagen type II gene promoter driving Cre recombinase transgene; CREB, cyclic AMP response element binding protein; DMEM, Dulbecco's Minimal Essential Medium; E, embryonic day; GSK, glycogen synthase kinase; Ihh, Indian hedgehog; ILK, integrin-linked kinase; ILK-fl, ILK allele flanked by loxP sites; PCNA, proliferating cell nuclear antigen; PKB, protein kinase B; PTHR1, PTH-PTHrP receptor.

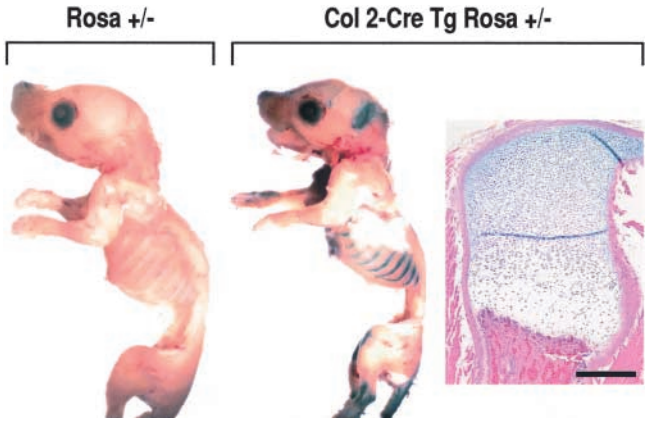

Figure 2. **Chondrocyte-specific Cre-mediated recombination at the ROSA26 reporter locus.** The Col2-Cre transgenic strain (Col2-Cre Tg) was crossed to the ROSA26 reporter (Rosa +/−) strain. Cre-mediated recombination allowed expression of the β-Gal gene, which was monitored using LacZ staining in newborns (left) and tissue sections (right). Bar, 500 μm.

nostaining with anti-Cre antibodies also confirmed specific expression of the Col2-Cre transgene in proliferating chondrocytes in the growth plate of transgenic animals (Fig. 3, B and C). No staining was detected in any other tissue or in R26R control mice (Fig. 2; unpublished data). Together, these data indicate that the Col2-Cre transgene was specifically expressed in chondrocytes at levels allowing recombination at the ROSA26 locus.

Excision at the ILK locus

LoxP sites were inserted downstream from exons 4 and 12 at the ILK locus through homologous recombination in embryonic stem cells (Fig. 3 A). This strategy should delete all the kinase domain of ILK upon Cre-mediated excision and create a null allele. By mating floxed ILK mice with Col2-Cre transgenic mice, mice heterozygous for the recombined floxed ILK allele were generated (genotype: ILK$^{fl/+}$,Cre). These mice had no phenotype (Fig. 4 A) and were fertile. They were backcrossed to the homozygous floxed ILK mice to inactivate both ILK alleles by Cre-mediated excision (genotype: ILK$^{fl/fl}$,Cre). The ability of Cre to recombine the floxed ILK allele was examined by PCR analysis of tail genomic DNA, which contains a sizable proportion of chondrocytes. The 230-bp amplimer corresponding to the excised floxed ILK allele was detectable in both ILK$^{fl/+}$,Cre and ILK$^{fl/fl}$,Cre mice, indicating that Cre was capable of recombining the loxP sites within the ILK gene (Fig. 3 D).

The ILK protein was expressed at high levels in the proliferating chondrocytes of the growth plate from wild-type (Fig. 1) and ILK$^{fl/+}$,Cre mice (Fig. 3 E). Inactivation of both copies of the floxed ILK allele caused a significant reduction of the expression of the ILK protein in proliferating chondrocytes (Fig. 3 F). Since the efficiency of the Cre-mediated excision varied between mutant animals of the ILK$^{fl/fl}$,Cre genotype, the expression levels of the ILK protein varied accordingly (unpublished data). The efficiency of the recombination induced by Cre was estimated to be

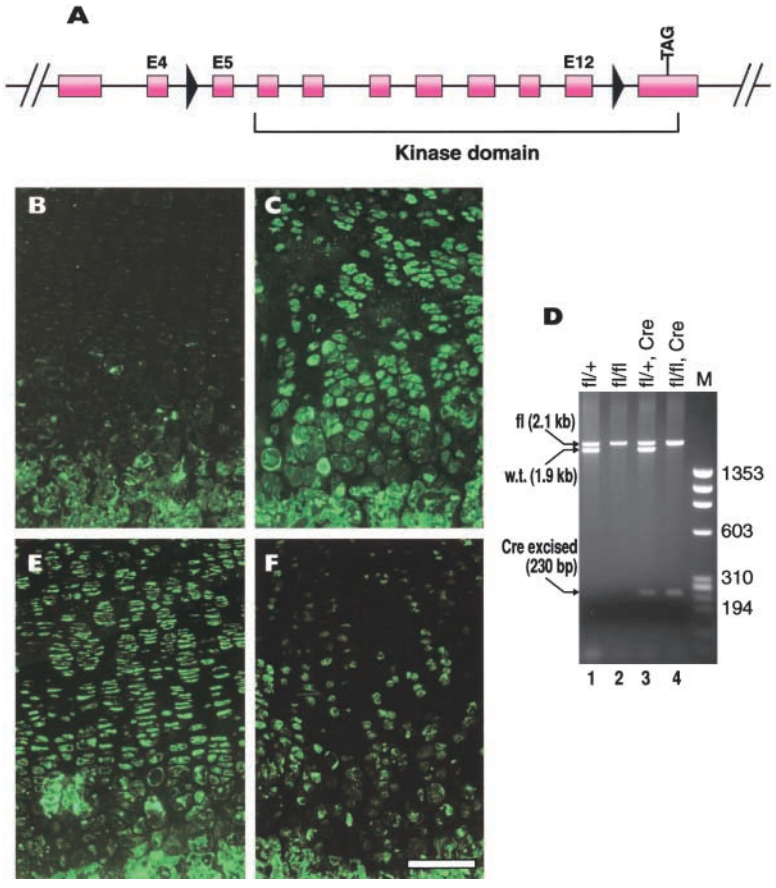

Figure 3. **Targeted inactivation of ILK in chondrocytes.** (A) Structure of the floxed ILK allele. Excision by the Cre recombinase deletes the kinase domain. (black triangles), loxP sites; E, exon; TAG, translational stop codon. (B and C) Cre expression in wild-type (B, ILK$^{fl/+}$) and transgenic (C, ILK$^{fl/fl}$,Cre) littermates. Indirect immunofluorescence staining of growth plate from neonatal mouse femur with anti-Cre antibody. (D) Cre-mediated excision of the floxed ILK allele in chondrocytes. DNA was prepared from tail clippings and analyzed by PCR with appropriate primers. The wild-type (w.t.,1.9 kb), floxed (fl, 2.1 kb) and Cre excised (230 bp) bands are indicated. Genotypes of a representative litter are indicated above lanes 1–4. M, DNA size markers. (E and F) ILK protein expression. Indirect immunofluorescence staining of growth plate from neonatal mouse femur. Note strong ILK signal in proliferating wild-type chondrocytes (E) that express type II collagen and reduced ILK expression in ILK$^{fl/fl}$,Cre chondrocytes (F). Bar, 100 μm.

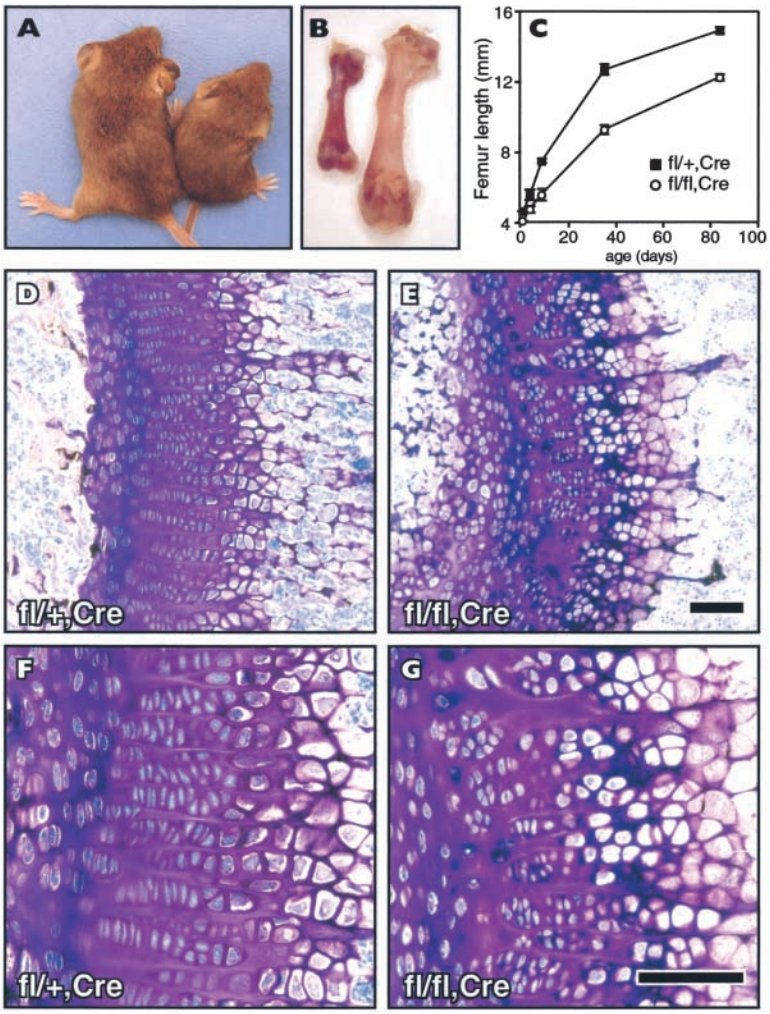

Figure 4. **Reduced growth and abnormal growth plate structure after chondrocyte-specific inactivation of ILK.** (A) Phenotypic appearance of ILK$^{fl/+}$,Cre (wild-type, left) and ILK$^{fl/fl}$,Cre (mutant, right) littermates at 18 d. (B and C) Reduced femur length after chondrocyte-specific inactivation of ILK (B). Femurs were smaller at all ages examined, but the divergence in size was more evident in older animals (C). (D–G) Toluidine blue staining of distal femoral growth plate from control 18-d-old mouse (D and F) and corresponding sample from mutant littermate (ILK$^{fl/fl}$,Cre, E and G). Note the perturbed columnar organization of the chondrocytes in the mutant sample. The disorganized arrangement of the chondrocytes at the secondary ossification center is also evident on the low power micrographs. Bars, 100 μm.

superior to 50%, closer to 75%. All ILK$^{fl/fl}$,Cre mice showed the same phenotype.

Dwarfism and chondrodysplasia in ILK$^{fl/fl}$,Cre mice

Newborn ILK$^{fl/fl}$,Cre mice were born with the expected Mendelian ratio (unpublished data). Postnatal death occurred sporadically, mostly due to cannibalism. Newborn ILK$^{fl/fl}$,Cre mutant mice were smaller than control littermates, with a typical shortening of the forelimbs and the hindlimbs (unpublished data). This smaller size was more evident as the mice aged (Fig. 4 A). Mutant limbs were obviously shorter but not deformed when compared with control littermates (Fig. 4 B). Other organs were not affected. The long bones of ILK$^{fl/fl}$,Cre mice were shorter than controls at birth and at all ages examined. The divergence in size was more evident in older animals (Fig. 4 C), confirming the dwarfism phenotype.

At the histological level, ILK$^{fl/fl}$,Cre mice showed moderate chondrodysplasia. When the characteristic zones of chondrocyte differentiation throughout the growth plate (resting, proliferating, prehypertrophic, and hypertrophic) were compared, the most remarkable effect was seen in the proliferating zone; the other zones appeared to be less affected. Low and high power micrographs of the toluidine blue-stained growth plate from 18-d-old animals is shown in Fig. 4. The proliferating zone of wild-type and ILK$^{fl/+}$,Cre mice was normally organized in the characteristic orderly columns (Fig. 4, D and F). However, the proliferating zone of ILK$^{fl/fl}$,Cre animals showed poor columnar organization (Fig. 4, E and G). Also, the shape of the mutant proliferating chondrocytes appeared to be highly abnormal. Instead of the normal typical flat shape (Fig. 4, D and F), mutant proliferating chondrocytes were rounded (Fig. 4, E and G; also Fig. 3, C and F).

The disorganized columnar organization of proliferating and prehypertrophic chondrocytes in ILK$^{fl/fl}$,Cre mutant animals led to a more poorly structured hypertrophic zone. This appeared more evident at the secondary site of ossification (Fig. 4 E). The development and morphology of the secondary ossification center will be examined in subsequent studies. The size of the hypertrophic zone and the morphology of the hypertrophic chondrocytes from ILK$^{fl/fl}$,Cre mutant mice appeared normal at the primary spongiosa (Fig. 4, E and G), however, suggesting that chondrocyte differentiation itself was not affected by the engineered ILK mutation.

Expression of chondrocyte differentiation markers

To confirm that chondrocyte differentiation was normal in the absence of ILK, we analyzed the expression of chondrocyte differentiation markers using in situ hybridization. The

expression of collagen type II, a marker of immature, proliferating chondrocytes, and collagen type X, a marker of hypertrophic chondrocytes, was first monitored in embryonic long bones. At E16.5, chondrocytes from control ($ILK^{fl/+}$,Cre) and mutant ($ILK^{fl/fl}$,Cre) mice expressed α1 (II) and α1(X) collagen (Fig. 5, A–D). The size of the collagen type II–expressing, proliferating, and prehypertrophic chondrocyte zone was similar between the two genotypes (Fig. 5, A and B). There was also no major change in the size of the hypertrophic chondrocyte zone expressing collagen type X (Fig. 5, C and D). The expression of collagen type II and collagen type X was also confirmed by immunochemistry on postnatal days 4 and 18 (unpublished data).

The signaling molecule Indian hedgehog (Ihh) regulates key events of skeletal morphogenesis, including chondrocyte proliferation and maturation (St-Jacques et al., 1999). Ihh expression is restricted to the prehypertrophic chondrocytes in late embryogenesis (Bitgood and McMahon, 1995; St-Jacques et al., 1999). This wild-type pattern of expression was observed in $ILK^{fl/+}$,Cre embryos (Fig. 5 E) and was not affected in $ILK^{fl/fl}$,Cre mice (Fig. 5 F). Prehypertrophic chondrocytes also express the PTH-PTHrP receptor (PTHR1) (Lanske et al., 1996). We observed no differences in the PTHR1 expression pattern in either $ILK^{fl/+}$,Cre (Fig. 5 G) or $ILK^{fl/fl}$,Cre (Fig. 5 H) E16.5 embryos. Since the normal spatial and temporal pattern of expression of chondrocyte differentiation markers was maintained in $ILK^{fl/fl}$,Cre embryos, we conclude that the mutation did not affect chondrocyte differentiation.

Normal apoptosis in $ILK^{fl/fl}$,Cre growth plates

The normal differentiation pattern of chondrocytes from $ILK^{fl/fl}$,Cre embryos suggested that the observed dwarfism phenotype could be due to decreased chondrocyte proliferation and/or increased apoptotic rates. We examined the effect of ILK inactivation on expression levels of caspase-cleaved cytokeratin, a marker of apoptosis. In the growth plate, apoptosis is restricted to regions of terminal differentiation, the prehypertrophic and hypertrophic zones. There was no difference in the staining for caspase-cleaved cytokeratin in the prehypertrophic and hypertrophic zones between mutant and control animals (Fig. 6, A and B). The proliferative zone of the growth plate from animals of all genotypes did not stain for the apoptosis marker (Fig. 6, A and B). This data indicates that chondrocyte apoptosis is not influenced by ILK inactivation.

Reduced proliferation of $ILK^{fl/fl}$,Cre chondrocytes

We assessed chondrocyte proliferation by quantifying BrdU and proliferating cell nuclear antigen (PCNA) labeling in the growth plate of femurs from 4-d-old animals. There was a significant reduction in the percentage of PCNA- (Fig. 6 G) and BrdU- (Fig. 6 H) positive cells in the proliferating zone of the distal femoral growth plate of $ILK^{fl/fl}$,Cre mice compared with the same region in $ILK^{fl/+}$,Cre control mice. Cyclin D1 is an important regulator of the cell cycle, since it promotes the transduction of the G1 phase to the S phase (Inoshita et al., 1999). The expression of cyclin D1 has also been shown to be regulated by ILK (Radeva et al., 1997; D'Amico et al., 2000; Persad et al., 2000), suggesting that the reduced proliferation may be a consequence of reduced cyclin D1 expression in the ILK mutant chondrocytes. In $ILK^{fl/+}$,Cre control mice, cyclin D1 was abundantly expressed in the proliferating zone and showed a perinuclear and cytosolic localization (Fig. 6 C; corresponding DAPI stain in E). However, in $ILK^{fl/fl}$,Cre mutant animals, levels of cyclin D1 protein were greatly reduced in the proliferat-

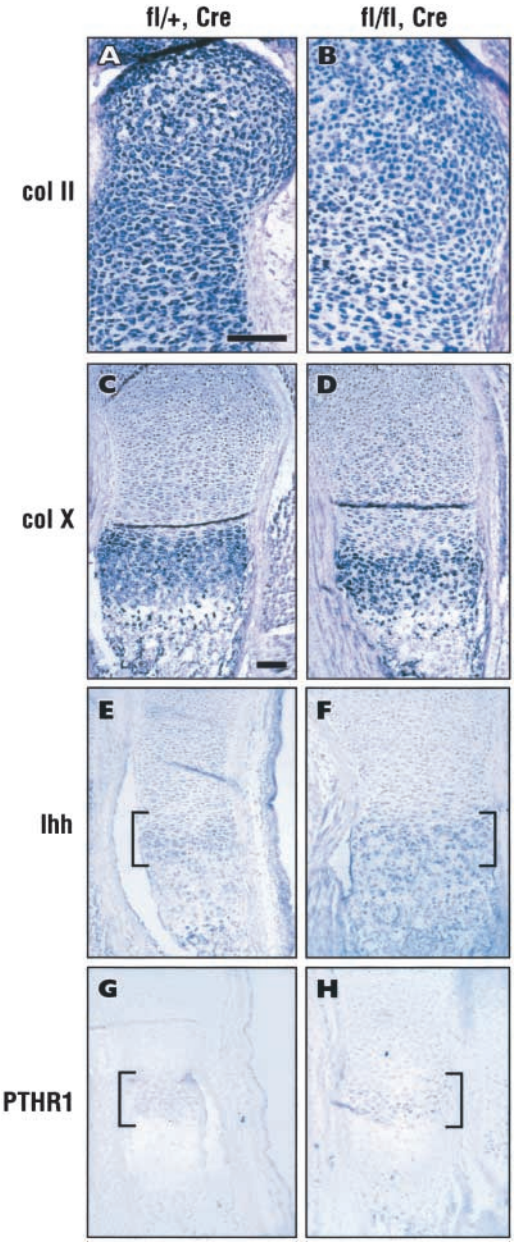

Figure 5. **Normal differentiation of ILK-deficient chondrocytes.** In situ hybridization of growth plate from control (fl/+, Cre, A, C, E, and G) or mutant (fl/fl, Cre, B, D, F, and H) E16.5 mouse femur. (A and B) Collagen type II (col II), antisense probe. (C and D) Collagen type X (col X), antisense probe. (E and F) Ihh, antisense probe. (G and H) PTHR1, antisense probe. The zone of expression is bracketed in E–H. Note that these markers of differentiation exhibit the same pattern of expression in control and mutant littermates. Background staining with sense probes was undetectable (not depicted). Bars, 100 μm.

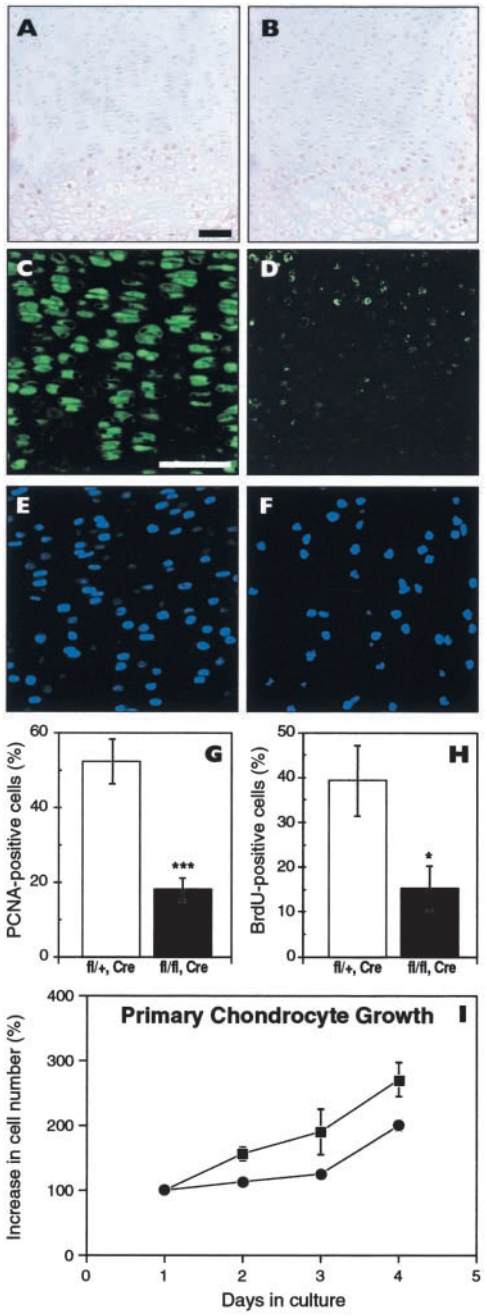

Figure 6. **Normal apoptosis and reduced expression of proliferation markers in mutant (fl/fl, Cre) mice.** (A and B) Immunostaining of caspase-cleaved cytokeratin 18. A comparable signal can be observed in control (A) and mutant fl/fl,Cre (B) mice. (C and D) Indirect immunofluorescence staining with anti–cyclin D1 antibody of growth plate from neonatal mouse femur. (C) Control mouse (fl/+, Cre). (D) Mutant littermate (fl/fl, Cre). E and F show the DAPI signal for the fields shown in C and D, respectively. (G and H) Percentage of PCNA- (G) or BrdU- (H) positive cells in the growth plate of neonatal control and mutant mouse femurs. Mean ± SEM of three sections from two control and two mutant mice are shown. *$P < 0.05$; ***$P < 0.001$. (I) Growth curves of control (fl/+,Cre; ■) and mutant (fl/fl,Cre; ●) primary chondrocytes. Cells were seeded at low density and viable cells (trypan blue exclusion) were counted daily with a hemocytometer. Results are shown as the percentage of increase relative to number of cells seeded.

ing zone (Fig. 6 D). DAPI staining confirmed the presence of cyclin D1–negative chondrocytes in the proliferating zone of the ILK-deficient growth plate (Fig. 6 F). There was no positive staining for cyclin D1 in hypertrophic chondrocytes from growth plates of either genotype, although some speckled background signal could be observed in some sections (unpublished data).

We also measured reduced proliferation rates in primary cultures of chondrocytes from $ILK^{fl/fl}$,Cre mutant mice when compared with wild-type or $ILK^{fl/+}$,Cre genotypes (Fig. 6 I). Together, these data demonstrate that inactivating both ILK alleles significantly inhibited chondrocyte proliferation.

Impaired adhesion of $ILK^{fl/fl}$,Cre chondrocytes to collagen type II

Primary cultures of costochondral chondrocytes were derived to further characterize the effects of the inactivation of ILK in these cells. Initially, adhesion to extracellular matrix components was measured. Chondrocytes of all genotypes adhered poorly to the BSA-coated negative control plates (Fig. 7). Wild-type chondrocytes (fl/fl and fl/+) adhered strongly to collagen type II–coated dishes, and this characteristic was not affected by the expression of the Cre transgene in heterozygous floxed chondrocytes (Fig. 7, fl/+,Cre). Primary chondrocytes isolated from $ILK^{fl/fl}$,Cre mice (fl/fl,Cre), however, were severely hampered in their capacity to attach to a collagen type II substratum (Fig. 7). This was not due to reduced expression of β_1-integrins (unpublished data). Adhesion of $ILK^{fl/fl}$,Cre chondrocytes to fibronectin was also reduced (unpublished data). Thus, perturbing signaling downstream from integrins impaired chondrocyte adhesion to extracellular matrix molecules.

Discussion

To investigate the importance of signaling downstream from integrins in growth plate chondrocytes, we have specifically

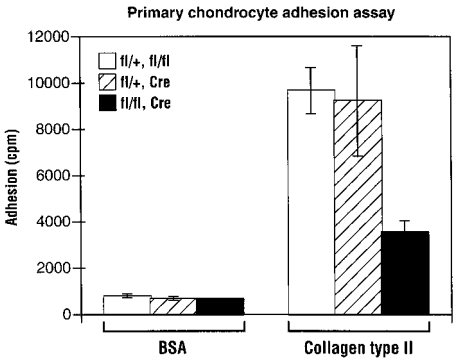

Figure 7. **Reduced adhesion of ILK-deficient chondrocytes.** Chondrocytes were harvested from the ribs of newborn mice and cultured for 2 d before labeling with ^{35}S-methionine and performing the adhesion assay. Adhesion is quantified by counting the radioactivity of labeled, adherent cells (in cpm). Background adhesion was measured using BSA-coated plates. The chondrocytes from mutant mice (fl/fl, Cre; black bars), which do not express ILK, are impaired in their capacity to bind a collagen type II matrix.

inactivated both alleles of ILK in collagen type II–expressing chondrocytes using the Cre-lox technique. Our results showed that ILK is highly expressed in proliferating and prehypertrophic chondrocytes. Specific ablation of ILK in a proportion of those cells in the growth plate led to dwarfism and chondrodysplasia. Inactivation of both ILK alleles in chondrocytes reduced adhesion and proliferation of the cells without major effects on differentiation or apoptosis. There was a marked reduction in cyclin D1 protein expression. Overall, our data demonstrate the importance of ILK-mediated signaling for chondrocyte proliferation and for maintaining normal growth plate architecture and function.

Several groups have engineered transgenic strains that specifically express the Cre recombinase in type II collagen–expressing chondrocytes (Ovchinnikov et al., 2000; Long et al., 2001b; Schipani et al., 2001). The Col2-Cre strain that we engineered achieved high levels of expression of the Cre recombinase that led to efficient recombination at the R26R locus. We observed variable efficiency of the recombination event at the ILK locus. Nonetheless, the dwarfism and chondrodysplasia phenotype of $ILK^{fl/fl}$,Cre mice was 100% penetrant. This suggests that inhibiting ILK-mediated signal transduction in even a subpopulation of proliferating chondrocytes is severely deleterious for growth plate structure and function.

The $ILK^{fl/fl}$,Cre animals exhibited dwarfism with shortening of the limbs. The most striking effect of ILK inactivation was seen in the proliferating zone, with a reduced height and abnormally round-shaped, disorganized cells. Femur length of neonatal $ILK^{fl/fl}$,Cre animals was smaller than wild-type and $ILK^{fl/+}$,Cre controls. With increasing age, the femur length of $ILK^{fl/fl}$,Cre mice further negatively diverged from controls. Thus, postnatal growth is severely affected in mutants.

We observed normal spatial and temporal expression of several differentiation markers of the chondrocyte phenotype, both during development (Fig. 5) and postnatally (unpublished data). In particular, Ihh expression was normal, demonstrating that the reduced proliferation phenotype observed in $ILK^{fl/fl}$,Cre chondrocytes is not secondary to a perturbation of the Ihh signaling pathway. Ectopic expression of collagen type X was not seen in mutant growth plates. Furthermore, the hypertrophic region was not enlarged. These findings indicate that the size reduction of the proliferative zone was not caused by a higher differentiating rate toward the hypertrophic chondrocyte fate. Similarly, we found no major changes in apoptosis of developing chondrocytes. Therefore, shortening of the proliferative zone cannot be explained by an increase in apoptotic rate.

The reduced height of the proliferative zone and the dwarfism phenotype appeared mainly due to a significant decrease in proliferation of collagen type II–expressing chondrocytes as demonstrated by the dramatic reduction in the expression of the proliferation markers BrdU, PCNA, and cyclin D1. The significant down-regulation of cyclin D1 expression in the ILK mutant chondrocytes suggests an essential role of ILK in the regulation of the expression of cyclin D1 in chondrocytes. These data support previous findings implicating ILK as a regulator of cyclin D1 expression (Radeva et al., 1997; D'Amico et al., 2000; Persad et al., 2001b). The expression of cyclin D1 by ILK has been shown to be regulated at the transcriptional level and may involve the activation of several transcription factors regulated by the ILK signaling pathway. These transcription factors include activating protein-1, Tcf(LEF)/β-catenin, and cyclic AMP response element binding protein (CREB), all of which can bind to the cyclin D1 promoter and regulate transcription from the cyclin D1 promoter. The precise signaling pathways activated by ILK in the regulation of cyclin D1 expression may vary according to the cell type. Thus, although ILK has been shown to regulate cyclin D1 expression in a GSK-3–dependent manner (D'Amico et al., 2000; Persad et al., 2001b), the phosphorylation of GSK-3 β on serine 9, the kinase inactivation site, does not seem to be altered in the ILK mutant chondrocytes (unpublished data). It is possible that in chondrocytes ILK regulates cyclin D1 expression in a PKB/Akt-dependent but GSK-3–independent manner. Cyclin D1 transcription is strongly regulated by binding of CREB or activating transcription factor 2 to the cAMP response element within its promoter (Watanabe et al., 1996; Beier et al., 1999; D'Amico et al., 2000), and CREB has been shown to be a target of the PKB/Akt kinase activity (Du and Montminy, 1998). It is likely that the control of cyclin D1 transcription by ILK in chondrocytes involves CREB and/or activating transcription factor 2, since these transcriptional regulators have been shown to be important for cyclin D1 transcription in chondrocytes (Beier et al., 1999) and for chondrocyte proliferation (Long et al., 2001a). PKB/Akt can also regulate cyclin D1 transcription by inactivation of the negative regulator of cyclin D1 expression, the FoxO forkhead transcription factors (Schmidt et al., 2002). The precise mechanism of the ILK-mediated regulation of cyclin D1 expression in chondrocytes remains to be elucidated.

In cultured cells, ILK has been shown previously to be a major regulator of the phosphorylation of PKB/Akt on serine 473 (Delcommenne et al., 1998; Persad et al., 2000, 2001a; Di-Poi et al., 2002). We have also used the floxed-ILK mice described here to isolate macrophages from these mice and knock-down ILK expression in these cells by expression of Cre recombinase using adenoviral infection (Kaartinen and Nagy, 2001). In these cells, we not only observe a dramatic and specific inhibition of PKB/Akt serine 473 phosphorylation and down-regulation of cyclin D1 expression but also inhibition of GSK-3 phosphorylation on serine-9 (Troussard et al., 2003), further supporting cell-type specific regulation of GSK-3 phosphorylation by the ILK-PKB/Akt pathway.

Recent data have shown that ILK can interact with several proteins involved in coupling integrins to the actin cytoskeleton and localization of ILK to focal adhesion plaques (Wu and Dedhar, 2001). The interaction of ILK with CH-ILKBP and affixin regulates early stages of cell spreading. Furthermore, ILK can interact with paxillin, and this interaction may be responsible for the localization of ILK to focal adhesion plaques (Wu and Dedhar, 2001). Thus, in addition to its signaling properties as a kinase in the regulation of PKB/Akt, GSK-3, and cyclin D1, ILK also functions as an adaptor protein in coupling integrins to the actin cytoskeleton. ILK regulates cell adhesion and spreading via its interactions with the integrin cytoplasmic domains and focal ad-

hesion plaque components mentioned above, and recent genetic data from *Drosophila* and *C. elegans* (Zervas et al., 2001; Mackinnon et al., 2002) provide strong support for an essential role for ILK in the regulation of cell adhesion and spreading. Our findings reported here that the ILK mutant chondrocytes are defective in cell attachment and spreading on type II collagen and fibronectin provide further support for an essential role of ILK in the regulation of cell attachment and spreading on extracellular matrix. The disorganization of the chondrocytes in the $ILK^{fl/fl}$,Cre mice may be a reflection of these altered cell attachment and spreading properties.

It could be argued that the inhibition of cyclin D1 expression resulting from the loss of ILK expression is a consequence of decreased attachment of the chondrocytes to the extracellular matrix. However, this is unlikely to be the case since we have shown recently that inhibition of integrin function results in the inhibition of cell proliferation in an ILK- and a PKB/Akt-dependent manner irrespective of effects on cell adhesion (Cruet-Hennequart et al., 2003). In addition, ILK regulates PKB/Akt phosphorylation and cyclin D1 expression in an anchorage-independent manner in cancer cells (Radeva et al., 1997; Attwell et al., 2000; Persad et al., 2000, 2001b). ILK activity is also regulated by several growth factors and chemokines (Delcommenne et al., 1998; Wu and Dedhar, 2001; Friedrich et al., 2002) in addition to cell adhesion. The chondrodysplasia observed as a consequence of the down-regulation of ILK expression is therefore likely to be due to a combination of effects on cell attachment and spreading and altered proliferation secondary to impaired signal transduction.

While this manuscript was under revision, a similar study was published by Grashoff et al. (2003). Their findings confirm the chondrodysplasia and decreased chondrocyte proliferation and adhesion associated with ILK inactivation in growth plate chondrocytes. Grashoff et al. (2003) reported that all mice with chondrocyte-specific disruption of ILK died at birth due to respiratory distress, whereas the mutant $ILK^{fl/fl}$,Cre mice that we engineered survive to adulthood with dwarfism. The somewhat less severe mutation that we have engineered should allow to gain further insight into the ILK signaling cascade and into the impact of chondrocyte-specific ILK inactivation on the formation of the secondary ossification center and postnatal bone growth and structure.

Materials and methods

Engineering of Cre transgenic line and conditional ILK knockout

The Col2-Cre transgenic strain was engineered by subcloning the full-length cDNA of the bacterial Cre recombinase (Sauer and Henderson, 1990) between a 3-kb fragment of the α1(II) promoter and its 3-kb chondrocyte-specific enhancer region (Zhou et al., 1998). Transgenic founders were generated by pronuclear injection according to standard protocols (Hogan et al., 1994).

The full-length human ILK cDNA (Hannigan et al., 1996) was used as probe to isolate a 15.5-kb murine ILK genomic clone from a 129Sv strain DNA library (Stratagene). A nearly full-length 6.5-kb EagI/EcoRI fragment was subcloned into pBluescript (Stratagene) to create the targeting vector. From this starting vector, a 1,316-bp NsiI-SphI fragment encompassing from the end of exon 4 to the middle of exon 10 was subcloned into pBluescript and used to insert by inverse PCR a single 34-bp loxP site with a BamHI restriction site downstream from exon 4. The loxP-containing subfragment was then reinserted into the parental targeting vector. The previously described ploxPGK-neoNTRtkpAloxP cassette (He et al., 2001) was inserted by blunt end ligation into the single Eco47III site downstream of exon 12.

R1 embryonic stem cells (Nagy et al., 1993) were electroporated with the linearized targeting vector, and G418-resistant colonies were picked and screened for homologous recombination by Southern blotting on BamH1 digests. The probe used was an EagI-PstI 900-bp fragment from downstream of the region of homology. Targeted ES cells were then transfected with the Cre expression vector, pBS185 (Life Technologies), to excise the neo cassette. We confirmed the in vitro excision using Southern blotting of Bam H1 genomic digests with the Eag1-Pst1 probe. The resulting loxP-flanked (floxed) ILK locus contained one loxP site downstream from exon 4 and a second loxP site, in the same orientation, downstream from exon 12 (Fig. 3 A). The injection of the embryonic stem cells carrying the floxed ILK allele into C57BL/6 embryos at the blastocyst stage was performed using standard methodology (Hogan et al., 1994).

Chondrocyte-specific inactivation of the ILK gene was accomplished by mating mice containing the floxed ILK allele (genotype: $ILK^{fl/fl}$) to Col2-Cre transgenic mice to generate mice bearing Col2-Cre and a floxed allele in their germline (genotype: $ILK^{fl/+}$,Cre). These were backcrossed to homozygote floxed mice in the following cross: $ILK^{fl/+}$,Cre X $ILK^{fl/fl}$ to generate mice with both alleles inactivated in chondrocytes (genotype: $ILK^{fl/fl}$,Cre). Routine genotyping of tail DNA samples (Laird et al., 1991) used the following primer pairs: for the Cre transgene, 5′-CCTGGAAAATGCTTCTGTCCG-3′ and 5′-CAGGGTGTTATAACAATCCC-3′, yielding a 391-bp diagnostic fragment. For monitoring the ILK genotype, the primers used were: 5′-CCAGGTGGCAGAGGTAAGTA-3′ and 5′-CAAGGAATAAGGTGAGCTTCAGAA-3′. These primers yielded amplimers of 1.9-kb, 2.1-kb, and, 230-bp for the wild-type, floxed, and Cre-excised alleles, respectively (Fig. 3 D).

All mice were housed in a virus- and parasite-free barrier facility. They were exposed to a 12-h light–dark cycle and fed tap water and regular chow at libitum. All procedures involving animals were approved previously by the Institutional Animal Care and Use Committee.

Histology

Limbs were dissected, fixed overnight at 4°C in 4% PFA buffered in PBS, decalcified in 25% EDTA at 37°C with daily changes until soft (Takeda et al., 2001), dehydrated, embedded in paraffin, sectioned at 6 μm, and stained with toluidine blue.

Immunofluorescence staining

Sections from femurs of $ILK^{fl/+}$,Cre and $ILK^{fl/fl}$,Cre mice were dewaxed and rehydrated. To unmask antigens, we performed antigen retrieval using BD Retrievagen A (BD PharMingen) for 10 min at 94°C with a cool down period of 15 min. Sections were blocked using the MOM kit (Vector Laboratories) followed by incubation with first antibodies overnight at 4°C (dilution 1:50). Sections were incubated with fluorescein isothiocyanate-conjugated secondary antibodies for 1–2 h at room temperature (dilution 1:200) and mounted in Vectashield (with DAPI) mounting medium (Vector Laboratories). When necessary, the avidin-biotin amplification system was used according to the manufacturer's protocol (Vector Laboratories) to gain more signal.

Antibodies directed against the following antigens were used: ILK (Upstate Biotechnology Inc.), Cre-recombinase (Novagen), collagen type II (Research Diagnostics Inc.), collagen type X (a gift from Dr. Eunice Lee, Shriners Hospital for Children, Montreal, QC, Canada), caspase-cleaved cytokeratin 18 (M30 CytoDeath kit; Roche Molecular Biochemicals), cyclin D1 (Santa Cruz Biotechnology, Inc.), and β1-integrin (Santa Cruz Biotechnology, Inc.). Conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

In situ hybridization

Femurs from E16.5 embryos were dissected, fixed, and sectioned as described above. In situ hybridization to sections was performed as described by Wilkinson (1992) using digoxigenin-labeled riboprobes. Probes were labeled using the MAXIscript in vitro transcription kit (Ambion) and digoxigenin-UTP (Roche Molecular Biochemicals). Signal detection was with the DIG nucleic acid detection kit (Roche). The probes used were described before: Ihh (Bitgood and McMahon, 1995), a gift from Dr. Benoit St-Jacques (Shriners Hospital) and PTHR1, α1(II) collagen, and α1(X) collagen (Lee et al., 1996) provided by Dr. Henry Kronenberg (Harvard Medical School, Boston, MA).

Proliferation assay

Mice were injected with 50 μg/g body weight i.p. of BrdU (Sigma-Aldrich) 1 h before sacrifice. Bone sections were stained with the BrdU staining kit

(Zymed Laboratories). Additional sections were stained with the PCNA staining kit (Zymed Laboratories). Total and BrdU- or PCNA-positive chondrocytes were counted on two to four sections from 2 $ILK^{fl/+}$,Cre and 2 $ILK^{fl/fl}$,Cre mice. Statistical significance was assessed using Student's *t* test, and $P < 0.05$ was considered significant.

Primary cultures of chondrocytes

Primary cultures of chondrocytes from rib growth plates of newborn mice were prepared using the culture system developed by Lefebvre et al. (1994). Briefly, the rib cage and sternum were dissected from 1-d-old mice, rinsed in PBS, and incubated at 37°C for 2 h in 2 mg/ml of collagenase D in Dulbecco's Minimal Essential Medium (DMEM). Soft tissues were detached from the cartilage by repeated gentle up and down pipetting. The denser cartilage was allowed to sediment, and the soft tissues were aspirated; the cartilage was then further digested with collagenase D for 3 h. Undigested bony parts were discarded, and the remaining cell suspension was aspirated repeatedly with a Pasteur pipette, filtered through a 70-μm cell strainer (Falcon; Becton Dickinson), rinsed first in PBS then in serum-free DMEM, and counted. Chondrocytes were seeded in 6-well plates at a density of 700,000 cells per well in DMEM with antibiotics, ascorbic acid, and 10% FBS. Primary chondrocytes prepared under these conditions retain their differentiated phenotype for several days in culture (Lefebvre et al., 1994). Proliferation of the primary chondrocytes was assessed by plating cells at low density (5,000 cells/cm^2) in 24-well tissue culture plates and counting the number of viable cells (trypan blue exclusion) daily with a hemocytometer until confluence.

For the adhesion assay, chondrocytes were cultured for 2 d before labeling with ^{35}S-methionine (Seger et al., 2001) and performing the adhesion assay on collagen type II–coated 24-well plates (Loeser et al., 2000). Labeled cells were collected using 5 mM EDTA into serum-free medium, centrifuged, and resuspended at 10^6 cells/ml into serum-free medium. Cells in suspension (250 μl) were added to each coated well for 30 min at 37°C. After washing, the adhered cells were treated with 0.5 ml 1% Triton X-100 in PBS for 5 min. Adhesion was quantified by counting 0.4-ml aliquots from the adherent cells and expressed in cpm. Background adhesion was measured using BSA-coated plates.

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