A mutation in the gene encoding the α2 chain of the fibril-associated collagen IX, *COL9A2*, causes multiple epiphyseal dysplasia (EDM2)

Yasuteru Muragaki[1], Edwin C.M. Mariman[2], Sylvia E.C. van Beersum[2], Merja Perälä[3], Jan B.A. van Mourik[4], Matthew L. Warman[5], Bjorn R. Olsen[1] & Ben C.J. Hamel[2]

Multiple epiphyseal dysplasia, an autosomal dominant disease, is among the more common inherited osteochondrodysplasias. Symptoms range from stiffness and pain in large joints to frank osteoarthritis associated with short stature and stubby fingers[1–7]. Linkage analyses of multiple epiphyseal dysplasia families suggest at least three loci. One locus, *EDM1*, maps to chromosome 19 (ref. 4), and is caused by mutations in cartilage oligomeric matrix protein (COMP)[8]. Mutations in COMP have also been identified in patients with pseudoachondroplasia (PSACH)[8,9], consistent with previous analyses which suggested that EDM1 and PSACH could be allelic disorders[10]. A second locus, *EDM2*, maps to chromosome 1 in the vicinity of the *COL9A2* gene[6]. Finally, exclusion of *EDM1* and *EDM2* in other families suggests the existence of at least one additional locus[11]. We now show that affected members of a large kindred with multiple epiphyseal dysplasia linked to the *EDM2* locus are heterozygous for a splice site mutation within *COL9A2*, causing exon skipping during RNA splicing and an in-frame loss of 12 amino acid residues within the α2(IX) collagen chain. The results provide the first *in vivo* evidence for the role of collagen IX in human articular cartilage.

[1]*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA*
[2]*Department of Human Genetics, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands*
[3]*Department of Medical Biochemistry, University of Turku, 20520 Turku, Finland*
[4]*Department of Orthopaedic Surgery, St. Joseph Hospital, 5500 MB Veldhoven, The Netherlands*
[5]*Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, Ohio 441067, USA*

Correspondence should be addressed to B.R.O. or B.C.J.H.

Clinical features in part of the present family have been previously reported[12,13]. Affected individuals typically presented during childhood and adolescence with waddling gait and stiffness and/or pain in the knees. Few patients experienced involvement of other joints such as the elbow, wrist, or ankle. No one complained of hip or shoulder pain. Some patients were mildly short statured and/or had stubby hands. Several patients never sought medical advice because of the mildness of their complaints. There were no spine abnormalities. X-rays revealed flattened, irregular epiphyses (Fig. 1), varus/valgus deformity of the knees, and gradually appearing osteoarthritis with or without loose bodies. Since family members did not have complaints about their hips, X-rays of hips are limited to only a few cases. In these cases (including the patient whose knees are shown in Fig. 1) a variable phenotype is seen, ranging from almost normal for age to slight irregularity of epiphyseal surface and acetabulum.

Linkage analysis was performed with microsatellite markers from the *EDM1* (*D19S199, D19S212, D19S215, D19S222*) and *EDM2* (*D1S186* and *MYCL*) regions[4,6]. The *EDM1* locus was excluded (data not shown), whereas significant linkage was observed between the disorder and the *EDM2* locus. The maximal lod score z = 15.31 was obtained with the marker *MYCL* at θ = 0.016, based upon one recombination event occurring in an affected individual.

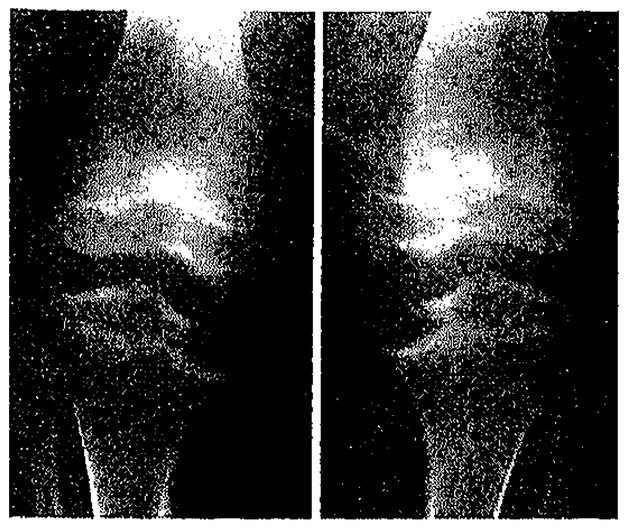

Fig. 1 X-rays of the right and left knee joints of an 11 years 9 months old affected boy in the Dutch family. Note irregular ossification and flattening of epiphyses.

To look for the causative mutation we first used the reverse transcription-polymerase chain reaction (RT-PCR). The RNA source was either total RNA from short term cultured chondrocytes (obtained during arthroscopic surgery) or Epstein-Barr virus (EBV) transformed lymphoblasts from an affected patient. An unaffected individual's lymphoblasts were used as control. Nested PCR reactions amplified overlapping cDNA fragments encoding the NC2, COL2, NC3, and COL3 domains and the carboxyl half of the signal peptide of the α2(IX) collagen chain. Fig. 2 shows a schematic representation of the collagen components in cartilage collagen fibrils. Fragments of cDNA encoding the NC1 and COL1 domains were amplified without nesting. Electrophoretic separation of the RT-PCR products of the COL3 domain (obtained with nested primers 7/9 and the more closely spaced primers, 7/13) showed migration as a single appropriately sized fragment in the control individual, whereas the affected individual had two fragments of equal intensity (Fig. 3*e*). The abnormally sized fragment was eluted from the gel and subjected to cycle sequence analysis. This revealed an in-frame deletion of 36 nucleotides when compared to the wild-type sequence (data not shown).

The sequence of the 36 nucleotide deletion corresponds to a single exon encoding the N-terminal region of the COL3 domain of the α2(IX) collagen polypeptide (Fig. 3*b*). Amplification of genomic DNA with PCR primers that represented exons flanking the deleted sequence showed identical sized genomic PCR products in both

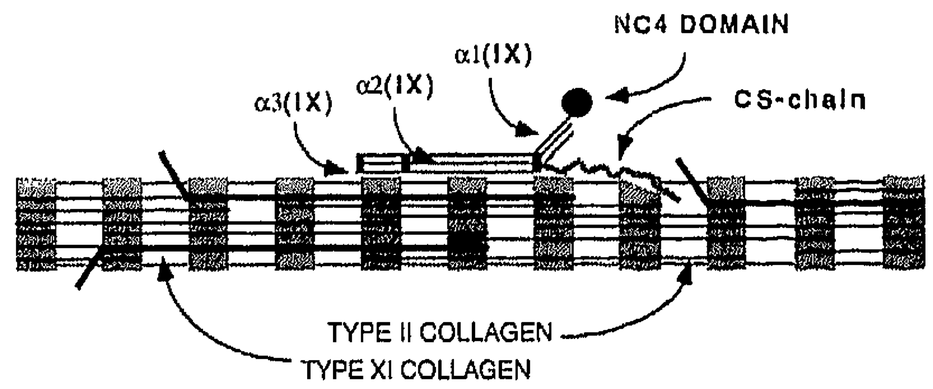

Fig. 2 The collagen components in cartilage collagen fibrils. The fibrillar collagens II and XI form the body of the fibril while the FACIT[21] molecule, collagen IX, is located on the fibril surface. Each of the three polypeptide subunits of collagen IX contains three triple-helical domains (straight lines) interrupted by non-triple-helical domains (bold vertical cross-bars). The α1(IX) chain contains a large N-terminal globular domain (NC4); a chondroitin sulfate (CS) chain is attached to the α2(IX) chain. A flexible kink in the CS-attachment region allows a portion of the molecule to project from the fibril surface into the perifibrillar space.

products revealed that the sequence of the splice donor site of intron 3, GTGAG, was converted to GCGAG in one allele of the affected patient (Fig. 3*a*). This change resulted in the loss of an *HphI* site in the genomic sequence of the affected allele; loss of this restriction site was used to test for the co-segregation of this change within the family (Fig. 4). All affected individuals, including the one affected patient who was recombinant at *MYCL*, were heterozygous for this change. The lod score for the mutation was $z = 17.55$ at $\theta = 0.0$.

The ability to amplify both mutant and wild-type alleles suggests that the mutation does not affect mRNA transcription or stability. Also, given the small size of the intron between exons 3 and 4 (about 150 nt), inclusion of this intron in the α2(IX) transcript as a result of the splice donor site mutation, should have been readily detected by our RT-PCR analysis (Fig. 3). We therefore conclude that the effect of the mutation in this pedigree is to cause an in-frame deletion in the COL3 domain of

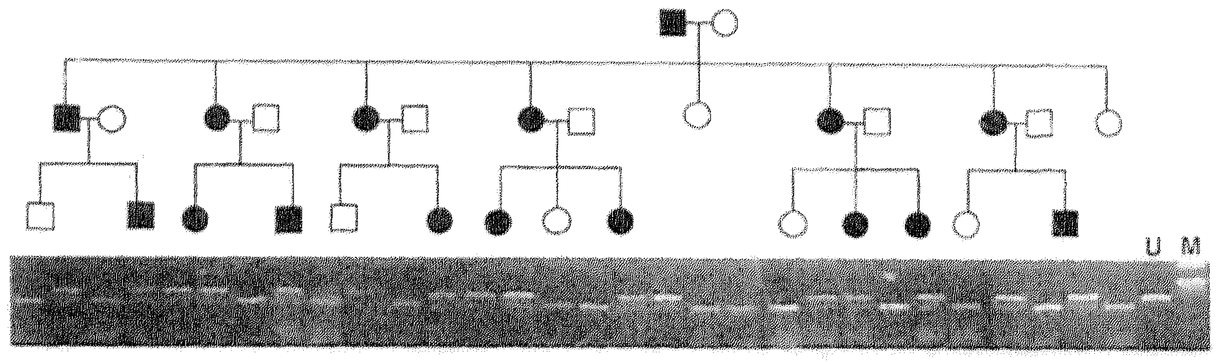

Fig. 4 Agarose gel electrophoresis of *COL9A2* genomic fragments, amplified by PCR with primers 16/13 (see Fig. 3) and digested with *HphI*, from a subset of the Dutch pedigree. Undigested DNA from an unaffected individual (lane U) and molecular weight markers (lane M) are shown on the right. All unaffected members of the family show cleavage of the PCR product to a smaller fragment, while all affected individuals show the presence of both uncleaved and cleaved products.

Our results support a role for collagen IX in contributing to the physical integrity of articular cartilage and/or participating in the process of cartilage matrix homeostasis. Joint pain and knee osteoarthritis were the most prominent features in the Dutch kindred. These complaints were also common in another large kindred linked to *EDM2* (ref. 6). In fact, in both families X-rays revealed flattened epiphyses particularly in the knees. Thus, mutations in *COL9A2* may have a particularly strong effect in the knee. Failure of normal joint function is a unifying feature in all forms of osteoarthritis, although the aetiologies by which this failure develops and progresses are heterogeneous[17,18]. Osteoarthritis linked to *EDM2* may be the consequence of altered physical integrity of cartilage, although joint shape abnormality cannot be precluded. That *COL9A2* contributes to the structural integrity of cartilage has already been suggested by its covalent cross-links to collagen II[19,20], its location on the surface of collagen II-containing fibrils, and the projection of its amino-terminal non-triple-helical domain into the perifibrillar space where it is likely to interact with other structural matrix elements[21,22]. It is also possible that a principal role of collagen IX may be in maintaining cartilage homeostasis. Rather than interacting with structural matrix molecules, the large amino-terminal domain of collagen IX might bind soluble growth factors, tissue inhibitors of matrix metalloproteases, or extracellular matrix proteins that would otherwise be particularly susceptible to degradation by matrix enzymes. Collagen IX may also, either directly or indirectly, interact with chondrocyte membrane receptors to provide the cells with information regarding the physical characteristics of their surrounding matrix.

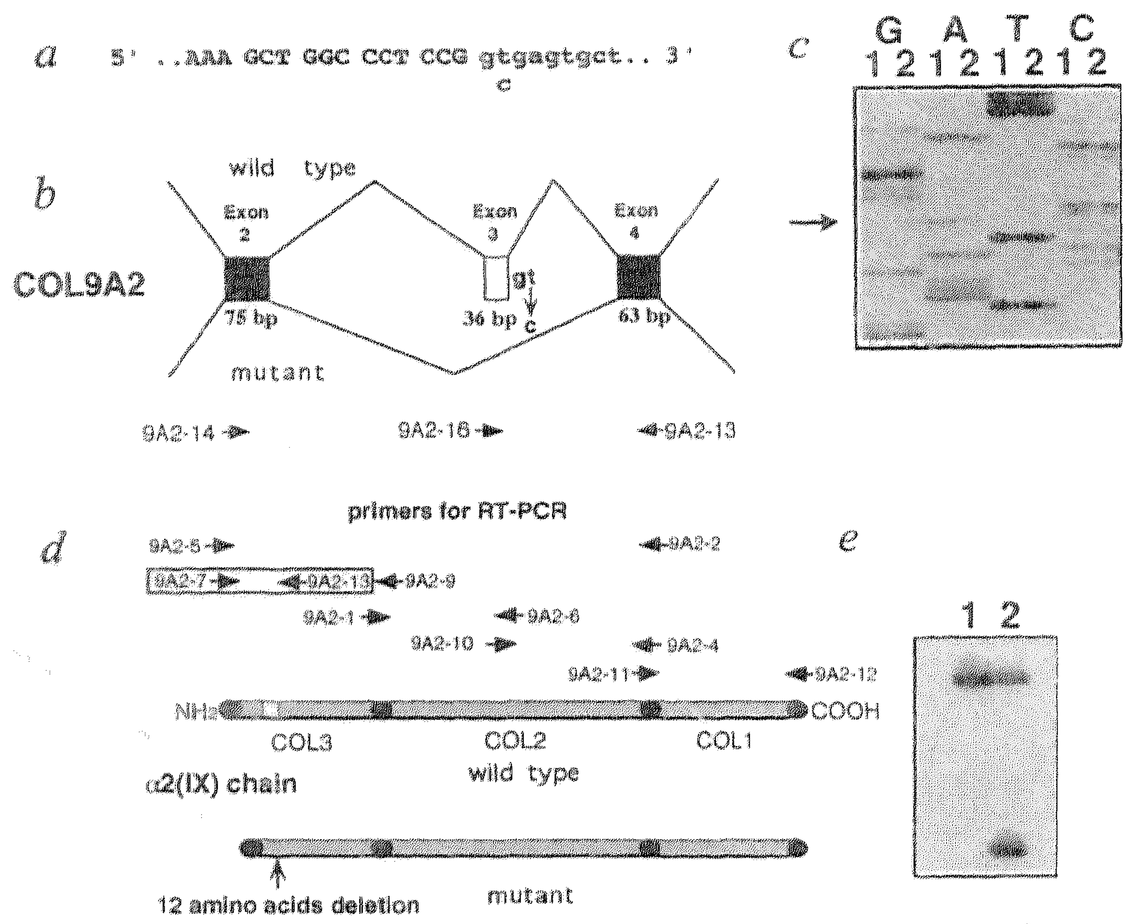

Fig. 3 *EDM2* splice site mutation and in-frame deletion in α2(IX) collagen caused by exon skipping. *a*, The nucleotide sequence of the 3' region of exon 3 (upper case letters) and the 5' end of intron 3 (lower case letters). The splice donor sequence gt is gc in the mutant. *b*, Exons 2, 3 and 4 splice patterns in wild-type and mutant *COL9A2*. *c*, The result of cycle sequencing of genomic DNA (antisense strand) from an unaffected individual (lanes 1) and an affected individual (lanes 2). The arrow indicates the point where the affected individual is heterozygous for A and G. Below the exons in (*b*), the primers used for amplification of genomic DNA by PCR are indicated. *d*, The location of the 12 amino acid residue deletion in the COL3 domain of the α2(IX) collagen chain caused by the mutation. (This deletion corresponds to exon 3 of the mouse *Col9a2* (ref. 27); the complete human exon structure has not yet been established.) Within the wild-type and mutant α2(IX) chain the triple-helical domains (COL1, COL2, and COL3) are indicated by rectangular areas; the non-triple-helical domains are indicated by the solid spheres. The 12 amino acid residue region in the COL3 domain deleted in the mutant is indicated by an open square. The position and direction of primers used for RT-PCR amplification of α2(IX) mRNA are shown. e, RT-PCR amplification of α2(IX) mRNA and acrylamide gel electrophoresis with RNA from an unaffected individual (lane 1) and an affected individual (lane 2). The primers 7/13 used for this amplification are boxed in (*d*).

affected and unaffected individuals (approximately 1.3 kb with primers 14/13, and about 180 bp with primers 16/13, data not shown). This suggests that the deletion detected at the level of the cDNA was not caused by a deletion of genomic DNA. Cycle sequencing of the genomic PCR

the α2(IX) collagen chain. Comparable mutations causing in-frame deletions in other cartilage collagen genes have little effect on polypeptide synthesis or collagen trimer assembly, however, they have profound effects on protein secretion and supramolecular assembly[14]. It is probable that the *COL9A2* mutation in this family causes a dominant negative effect upon collagen IX, at the level of either protein secretion or supramolecular assembly.

Our results confirm studies in mice that collagen IX does not appear to have a critical role during the process of skeletal element formation. Transgenic mice overexpressing a truncated α1(IX) chain had a mild chondrodysplasia and progressive osteoarthritis[15]. Mice homozygous for a null mutation in *Col9a1* exhibited normal skeletal morphogenesis and growth, but developed progressive osteoarthritis-like changes in articular cartilage[16]. Patients with multiple epiphyseal dysplasia linked to *EDM2* do not lack specific skeletal elements, nor do they have profound disturbances in skeletal element size or shape. The inconsistent feature of mild short stature and stubby fingers in some patients in our kindred may suggest a contribution of other modifying factors (epistatic, environmental, or stochastic) in the appearance of these traits. The process of endochondral ossification also appears to be minimally affected by the *COL9A2* mutation. Similar to patients with a disorder linked to *EDM1* (ref. 4), individuals with the *EDM2*-linked disorder can have mild reductions in height in comparison to their unaffected siblings[1]. This reduction may be due to altered physical integrity of the growth plate rather than to specific alterations in the cellular processes by which endochondral ossification progresses.

Methods

Genotyping and linkage analysis. Genomic DNA was prepared from venous blood[23]. Genotyping for each of the selected markers was performed by PCR amplification using 50 ng DNA and 30 ng of the appropriate primers (Genome DataBase, Isogen Bioscience, the Netherlands) in 15 μl amplification mixture (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.01% (w/v) gelatin, 200 μM of each dATP, dCTP, dTTP and 2.5 μM dCTP) with 0.06 U SuperTaq DNA polymerase (HT Biotechnology). During the 30 cycles of DNA amplification (1 min at 94 °C, 2 min at 55 °C and 1 min at 72 °C), 0.6 μCi α^{32}P-dCTP (10 mCi/ml, 3000 Ci/mmol) was included. Samples were analysed on 6.6% denaturing polyacrylamide gels. Allelic bands were visualized by overnight exposure of dried gels to Kodak X-OMAT S film. Linkage analyses were performed using the MLINK and ILINK options of the program package LINKAGE, version 5.10 (ref. 24). Full penetrance was assumed for the disorder, whereas the disease gene frequency was estimated at 0.0001. The allele frequencies of the genetic markers were obtained from the Genome Database.

Analysis of the *COL9A2* gene. RNA was extracted from EBV-transformed lymphoblasts and cultured chondrocytes by the acid guanidinium thiocyanate/phenol/chloroform method[25]. First strand cDNAs were synthesized with oligo(dT) primers using the Superscript Preamplification System (GIBCO BRL). PCR primers were designed to amplify the ~2-kb cDNA in four overlapping fragments. The second round PCR used nested primers. For amplification for the first round PCR, 35 cycles were performed at 94 °C for 0.5 min, 62 °C for 1 min, and 72 °C for 2 min, followed by an additional extension step at 72 °C for 10 min. The second round PCR was the same except that the annealing temperature was 58 °C. The sets of primers used were as follows (*sense and antisense*): 9A2–5, 5'–CTCCAGGTGGTAGTGCTCGCT–3' and 9A2–2, 5'–GGCTTCCCGCTTGGCACTCAC–3'; 9A2–11, 5'–GCCACTGACCAGCACATCGTG–3' and 9A2–12, 5'–TCAAGGCCCCTTGTAGGATCC–3'; 9A2–7, 5'–CTGGCGCAGATTAGAGGTCCA–3' and 9A2–9, 5'–ATGCCCCTTCACTCCCTGCAG–3'; 9A2–1, 5'–GCGGATTTCCTGTGTCCAACC–3' and 9A2–6, 5'–AGAGAATCCAGGAAGGCCCTG–3'; 9A2–10, 5'–CACCAGGGCCTAGCGGGTGTG–3' and 9A2–4, 5'–GGCGACCTCTGCCAGTTGCTC–3'. The primer pairs 5/2 and 11/12 were used for first round PCR. The product obtained with the pair 11/12 was analysed without further amplification. The product obtained with the pair 5/2 was amplified further by using primer pairs 7/9, 1/6, and 10/4 for second round PCR. All PCR reactions were done in a total volume of 50 μl containing 1 × PCR buffer, 200 μM dNTP, 0.5 μM (each) primer and 1U of *Taq* polymerase. The PCR products were analysed on 2% agarose gels. All products were the same size from affected and unaffected individuals except the product obtained with primer pair 7/9. This product migrated as a single band from unaffected individuals and as a double band from affected individuals. Dideoxy-nucleotide cycle sequencing (ampliCycle™, Perkin Elmer), showed a 36 nt deletion in the lower band. To better analyse this deletion, an additional anti-sense primer 9A2–13, 5'–CAATCCCGGGCTTCCCGTCTG–3' (closer than primer 9 to primer 7) was made and used for second round PCR with the 9A2–7 primer. PCR condition was 35 cycles at 94 °C for 0.5 min, 58 °C for 1 min, 72 °C for 2 min and additional 72 °C for 10 min at the end of cycles; 0.5 μl of [α-^{33}P] dCTP (10 mM, 2000 Ci/mmol) was added to the reaction. The product obtained with primers 7/13 was analysed on a 5% sequencing gel and subjected to cycle sequencing as well. For amplifying genomic DNA, two additional sense primers 9A2–14, 5'–CCTGGATCCGACGGCATCGAC–3' and 9A2–16, 5'–CAATGGGCCCCCTGGAAAAGC–3' were synthesized and the primer pairs 14/13 and 16/13 were used for PCR. The conditions were heating at 95 °C for 0.5 min, 64 °C for 1 min, and at 72 °C for 2 min, with a final extension at 72 °C for 10 min. These amplification products were also cycle sequenced. The products obtained with primers 16/13 from genomic DNA of 79 members of the family were digested with *Hph*I and analysed on 4% agarose gels. The primers were synthesized on the basis of both published[26] and unpublished sequences. The original GenBank/EMBL file (accession number M95610) has been updated to include the additional unpublished sequences.

Acknowledgments

We thank H.H. Ropers, H.G. Brunner and E. Vuorio for helpful discussions and comments. This work was supported by NIH grants AR36819 and AR36820 (to B.R.O.). Expert secretarial assistance was provided by Y. Pittel.

Received 28 July; accepted 20 September 1995.

1. Barrie, H., Carter, C. & Sutcliffe, J. Multiple epiphyseal dysplasia. *Br. Med. J.* **2**, 133–137 (1958).
2. Fairbank, T. Dysplasia epiphysialis multiplex. *Br. J. Surg.* **34**, 225–232 (1947).
3. Ribbing, S. Studien über hereditäre multiple Epiphysenstörungen. *Acta Radiol. Suppl.* **34**, 1–107 (1937).
4. Oehlmann, R. *et al.* Genetic linkage mapping of multiple epiphyseal dysplasia to the pericentromeric region of chromosome 19. *Am. J. Hum. Genet.* **54**, 3–10 (1994).
5. Briggs, M.D. *et al.* Genetic linkage of pseudoachondroplasia to markers in the pericentromeric region of chromosome 19. *Genomics* **18**, 656–660 (1993).
6. Briggs, M.D. *et al.* Genetic mapping of a locus for multiple epiphyseal dysplasia (EDM2) to a region of chromosome 1 containing a type IX collagen gene. *Am. J. Hum. Genet.* **55**, 678–684 (1994).
7. Rimoin, D.L. *et al.* A large family with features of pseudoachondroplasia and multiple epiphyseal dysplasia: exclusion of seven candidate gene loci that encode proteins of the cartilage extracellular matrix. *Am. J. Hum. Genet.* **93**, 236–242 (1994).
8. Briggs, M.D. *et al.* Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. *Nature Genet.* **10**, 330–336 (1995).
9. Hecht, J. *et al.* Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. *Nature Genet.* **10**, 325–329 (1995).
10. Stanescu, R. *et al.* Multiple epiphyseal dysplasia, Fairbank type: morphologic and biochemical study of cartilage. *Am. J. Med. Genet.* **45**, 501–507 (1993).
11. Deere, M. *et al.* Genetic heterogeneity in multiple epiphyseal dysplasia. *Am. J. Hum. Genet.* **56**, 698–704 (1995).
12. Versteijlen, R.J., Zwemmer, A., Lorié, C.A.M. & Schuur, K.H. Multiple epiphyseal dysplasia complicated by severe osteochondritis dissecans of the knee. Incidence in two families. *Skeletal Radiol.* **17**, 407–412 (1988).
13. van Mourik, J.B.A., Schaap, C. & Nolen A.J.G. Een Nederlandse familie met erfelijke gewrichtsklachten; multiple epifysaire dysplasie. *Ned Tijdschr. Geneeskd.* **137**, 32–36 (1993).
14. Byers, P.H. *Disorders of collagen biosynthesis and structure.The metabolic and molecular bases of inherited disease*, (eds Scriver, C.R. *et al.*) 4029–4078 (McGraw-Hill, New York, 1995).
15. Nakata, K. *et al.* Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing α1(IX) collagen chains with a central deletion. *Proc. Natl. Acad. Sci. USA* **90**, 2870–2874 (1993).
16. Fässler, R. *et al.* Mice lacking α1(IX) collagen develop noninflammatory degenerative joint disease. *Proc. Natl. Acad. Sci. USA* **91**, 5070–5074 (1994).
17. Howell, D.S., Treadwell, B.J., & Trippel, S.B. Etiopathogenesis of Osteoarthritis. *Osteoarthritis: Diagnosis and Medical/Surgical Management*, (eds Moskowitz, R.W. et al.) 233–252 (W.B. Saunders, Philadelphia, 1992).
18. Stanescu, V., Stanescu, R. & Maroteaux, P. Articular degeneration as a sequela of osteochondrodysplasias. *Clin. Rheum. Dis.* **11**, 239–270 (1985).
19. van der Rest, M. & Mayne, R. Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. *J. Biol. Chem.* **263**, 1615–1618 (1988).
20. Wu, J.J., Woods, P.E. & Eyre, D.R. Identification of cross-linking sites in bovine cartilage type IX collagen reveals an antiparallel type II-type IX molecular relationship and type IX to type II bonding. *J. Biol. Chem.* **267**, 23007–23014 (1992).
21. Shaw, L.M. & Olsen, B.R. Collagens in the FACIT group: diverse molecular bridges in extracellular matrices. *Trends Biochem. Sci.* **16**, 191–194 (1991).
22. Vaughan, L. *et al.* D-periodic distribution of collagen type IX along cartilage fibrils. *J. Cell Biol.* **106**, 991–997 (1988).
23. Miller, S.A., Dykes, D.D. & Polesky, H.F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* **16**, 1215 (1988).
24. Lathrop, G.M., Lalouel, J.M., Julier, C. & Ott, J. Multilocus linkage analysis in humans: detection of linkage and estimation of recombination. *Am. J. Hum. Genet.* **37**, 482–498 (1988).
25. Chomczynski, P. & Sacchi, K. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
26. Perälä, M. *et al.* Molecular cloning of the human α2(IX) collagen cDNA and assignment of the human COL9A2 gene to chromosome 1. *FEBS Lett.* **319**, 177–180 (1993).
27. Perälä, M. *et al.* The exon structure of the mouse α2(IX) collagen gene shows unexpected divergence from the chick gene. *J. Biol. Chem.* **269**, 5064–5071 (1994).