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UNRAVELING THE CONSEQUENCES OF COLLAGEN X MUTATIONS

Olena Jacenko[1,*] and Danny Chan[2,3]

[1]University of Pennsylvania School of Veterinary Medicine, Department of Animal Biology, Philadelphia, PA [2]University of Melbourne, Royal Children's Hospital, Department of Paediatrics, Parkville, Vic., Australia
[3]Current Address: Department of Biochemistry, University of Hong Kong, 5 Sassoon Road, Hong Kong

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Abstract

The characterization of *COL10A1* mutations in the human Schmid metaphyseal chondrodysplasia (SMCD), a heritable disorder affecting the endochondral skeleton, coupled with the analysis of *COL10A1* transgenic (Tg) mouse models, are providing insights into the biochemistry and function of collagen X. However, amidst these advances are controversies regarding the apparent phenotypic discrepancies between collagen X mutations in the mouse and human. This review brings together the most recent data on collagen X mutations, and addresses the possible biochemical basis underlying these defects, as well as the value of mutation analyses. The association of collagen X with endochondral ossification is presented in light of a previously unforeseen relationship between endochondral skeletogenesis and hematopoiesis.

Key Words: Chondrodysplasia, collagen X, collagen X mutations, transgenic mice.

*Address for correspondence
Olena Jacenko
University of Pennsylvania,
School of Veterinary Medicine,
Department of Animal Biology,
3800 Spruce Street,
Philadelphia, PA 19104-6046
Telephone Number: 215-573-9447
FAX Number: 215-898-9923
E-mail: jacenko@vet.upenn.edu

Collagen X and Endochondral Ossification

Collagen X, a 59 kDa homotrimer classified as a "short chain" non-fibrillar collagen (Jacenko *et al*., 1991), is distinct from other collagens because of its restricted pattern of expression in a subset of cartilage cells, the hypertrophic chondrocytes (Schmid and Linsenmayer, 1983; Kielty *et al*., 1985; Nerlich *et al*., 1992; Apte *et al*., 1993). Concomitant with and subsequent to chondrocyte hypertrophy, the morphogenetic events associated with endochondral ossification (EO) take place in this cartilage matrix. Specifically, as individual chondrocytes swell and undergo hypertrophy, the matrix changes from being avascular and non-calcified to one that is penetrable by blood vessels and capable of calcification; vascular invasion imports chondroclasts and stem cells for bone and marrow stroma, and trabecular bone forms on top of remaining hypertrophic cartilage spicules, while the marrow stroma establishes the appropriate conditions for hematopoiesis (Caplan, 1990). The transient expression of collagen X occurs at the onset of chondrocyte hypertrophy, and this protein represents the major biosynthetic product of these cells (Reginato *et al*., 1986). Collagen X synthesis is also reinitiated during fracture repair (Grant *et al*., 1987) and as a consequence of joint degeneration in osteoarthritis (Reichenberger *et al*., 1991). Despite its predominance in hypertrophic cartilage during fundamental events of EO under normal and pathologic conditions, the function of collagen X has eluded investigators since its discovery (Schmid and Conrad, 1982a, b; Gibson *et al*., 1982).

Proposed Structure and Function for Collagen X

Collagen X molecules are composed of three identical α1(X) chains encoded by a condensed gene (*COL10A1*) of three exons, one of which (exon 3) codes for the majority of the polypeptide chain including the entire triple helical domain (Fig. 1; LuValle *et al*., 1988; Thomas *et al*., 1991; Reichenberger *et al*., 1991; Elima *et al*., 1993). The α1(X) homotrimers consist of three distinct protein domains. Specifically, COL1 (amino acids 57-519 in humans) represents the short triple helical domain (in

List of Abbreviations

ECM	extracellular matrix
EO	endochondral ossification
KO	knock-out (null allele)
RHT	ruthenium hexamine trichloride
SMCD	Schmid metaphyseal chondrodysplasia
Tg	transgenic

comparison to fibrillar collagens) containing eight imperfections in the Gly-X-Y triplet repeat sequence. This triple helix is flanked by a small non-helical globular NC2 domain at the amino terminus (amino acids 19-56), and by a larger highly conserved non-helical carboxyl-terminal NC1 domain (amino acids 520-680).

Sequence comparisons of the NC1 domain of collagen X with the C-propeptides of fibrillar collagens reveal a conserved cluster of aromatic residues with a marked similarity in the hydrophilicity profile (Brass *et al*., 1992). Since the C-terminal propeptide domain of fibrillar collagens is involved in the initiation of intracellular α-chain selection, followed by trimer association and helix formation (Doege and Fessler, 1986), by analogy, the α1(X) NC1 domain is anticipated to play a crucial role in collagen X assembly. In contrast, the small non-helical N-terminal NC2 domain is less conserved among species. Unlike the case with fibrillar collagens, most studies suggest that the functional collagen X molecule is not processed except for the removal of the signal peptide (Thomas *et al*., 1991; Chan *et al*., 1995).

In vitro, the NC1 and NC2 domains are capable of spontaneous intermolecular interactions resulting in the formation of a hexagonal lattice (Kwan *et al*., 1991). *In vivo*, a similar lattice-like network (likely composed at least in part of collagen X) has been seen in the pericellular matrix of swine hypertrophic chondrocytes (Jacenko *et al*., 1991; LuValle *et al*., 1993b). Moreover, this network appears to be disrupted in mice with a dominant interference mutation for collagen X (see below). A number of different matrix configurations may involve the participation of collagen X, since it has also been detected near collagen II-XI-IX heterotypic fibrils (Poole and Pidoux, 1989; Chen *et al*., 1990; Schmid and Linsenmayer, 1990), as well as in mat-like aggregates (Schmid and Linsenmayer, 1990). Likewise, it cannot be overlooked that collagen VIII, a non-fibril-forming short chain collagen and a homolog of collagen X, has been demonstrated to form a similar lattice-like network in the Descemet's membrane (Sawada *et al*., 1990). Despite the spatio-temporal differences in expression between these two collagens, the similarity in amino acid sequence suggests a common function. Perhaps the short chain collagens act as stabilizers in the ECM by forming hexagonal networks.

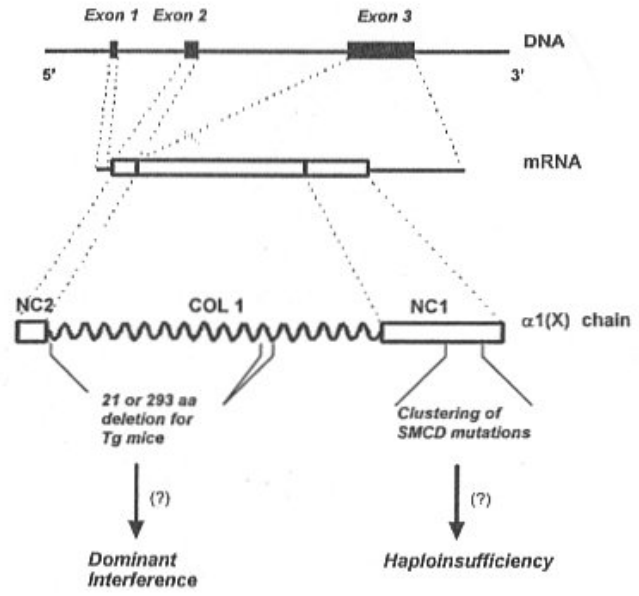

Figure 1. Diagram showing the organization of the collagen X gene, message, and protein, with potential disease mechanisms underlying mutations in Tg mice and in humans with SMCD indicated. The collagen X gene (top) contains three exons, which are represented by boxes and numbered starting from the 5' end of the gene. Dotted lines indicate correspondence between exons and mRNA (middle), and between mRNA and the translation product, an α1(X) chain (bottom). The globular regions of collagen X are marked NC1 and NC2, and the triple-helical region labeled COL1. Constructs encoding partially functional α1(X) chains with 21 or 293 amino acid deletions in the COL1 domain were generated for expression in Tg mice, and are postulated to act predominantly through a dominant interference mechanism. A clustering of mutations found in patients with SMCD is in the NC1 domain, and the disease likely results predominantly through haploinsufficiency.

Such lattices may in turn be stabilized by the sequestering of other molecules, thus establishing unique microenvironments.

Determining the precise supramolecular organization of collagen X and its interaction with other matrix components is a key to understanding its function. To date, the restricted expression pattern associated with EO has largely guided the thinking regarding the role of collagen X. Implicated functions include mineralization, matrix stability during degradation and remodeling, and vascular invasion. Many studies have focused on mineralization, where both *in vitro* (Bonen and Schmid, 1991) and *in vivo* (Reginato *et al*., 1993) analyses have demon-strated a direct relationship between calcium, collagen X production, and

cartilage hypertrophy. Collagen X binds calcium directly with high affinity (Kirsch and von der Mark, 1991) and was postulated to mediate the interaction with matrix vesicles (Kirsch *et al*., 1994, 1997a,b), which provide an essential nucleus for initial mineral crystal deposition and growth. However, conflicting studies have also reported a lack of interactions between matrix vesicles and collagen X (Poole and Pidoux, 1989; Inao and Conrad, 1993). Furthermore, studies by Caplan's group (Arias *et al*., 1991; Carrino *et al*., 1996) have proposed collagen X to be an inhibitor of mineral deposition. These investigators have localized collagen X to the inner, non-calcifiable membrane of the chicken eggshell and suggested that collagen X may provide an inhibitory boundary for biomineralization.

The temporo-spatial restriction of collagen X to hypertrophic cartilage implicates its involvement in the morphogenetic transition of EO. Accordingly, defects in collagen X biosynthesis and supramolecular organization were predicted to result in chondrodysplasias with primary growth plate involvement. Direct evidence for such associations between collagen X mutations and growth plate abnormalities came from studies of a murine transgenic (Tg) model (Jacenko *et al*., 1993a) and the identification of *COL10A1* mutations in a human chondrodysplasia (Wallis, 1993; Warman *et al*., 1993; Jacenko *et al*., 1994; summarized in Chan and Jacenko, 1998). Subsequently, mice with null alleles for collagen X were generated (Rosati *et al*., 1994; Kwan *et al*., 1997), and are contributing towards the elucidation of the role of this molecule. Lastly, previously unforeseen skeleto-hematopoietic relationships are being revealed through analysis of the existing murine models (Jacenko *et al*., 1996; Jacenko *et al*., manuscript in preparation #1; Healy *et al*., manuscript in preparation #2).

Skeleto-Hematopoietic Phenotype in Mice Transgenic for Collagen X

To gain insight into collagen X function in hypertrophic cartilage, transgenic (Tg) mice were generated expressing a defective collagen X variant (Jacenko *et al*., 1993a, b). Transgene constructs consisted of chicken α1(X) cDNA with in-frame deletions (encoding either 21 or 293 amino acids; Fig. 1) in regions encoding the central triple helical collagen X domain; transgene expression was driven by different lengths of the chicken collagen X promoter and regulatory elements (Jacenko *et al*., 1993a, b; LuValle *et al*., 1993a). Co-expression of truncated chicken with full-length mouse collagen X α-chains in an *in vitro* cell-free translation system yielded chick-mouse hybrid-trimers, supporting dominant interference as a possible mechanism for transgene action (Jacenko *et al*., manuscript in preparation #3).

Expression of transgene constructs in hypertrophic cartilage yielded similar skeleto-hematopoietic defects in 14 Tg mouse lines, representing all constructs. Murine phenotypes in each line ranged from a perinatal-lethal phenotype manifest as thoracolumbar kyphosis and wasting about week-3 after birth (in about 20-25% of Tg mice) (Fig. 2a), to variable dwarfism (in about 75-80% of Tg mice). The predominant dwarfism phenotype helped identify a human autosomal dominant disorder, Schmid Metaphyseal Chondrodysplasia (SMCD), resulting from collagen X mutations (see below).

Histomorphometry of perinatal-lethal and dwarfed Tg mice revealed growth plate compressions by week-3 after birth in hypertrophic zones of tissues arising by EO; the extent of hypertrophy and cell number were reduced, bony trabeculae were limited, osteopenia was prevalent, and mineral quality was altered (Jacenko *et al*., 1993a, b; Jacenko *et al*., 1996; Paschalis *et al*., 1996). Furthermore, electron microscopy detected a disruption of the hypertrophic chondrocyte pericellular matrix network (likely composed of collagen X, see above), which in control mice resembled a hexagonal lattice-like array. This network was observed when tissues were fixed in ruthenium hexamine trichloride (RHT), a cationic dye that preserves matrix integrity by precipitating proteoglycans (Hunziker *et al*., 1982, 1984; Farnum and Wilsman, 1989). In Tg mice, the hypertrophic chondrocyte pericellular matrix was dramatically reduced and did not maintain a network arrangement. In these mice, the entire growth plate appeared to be decompartmentalized; this was manifest by RHT-positive aggregates, likely proteoglycans, masking collagen fibrils in proliferative growth plate zones (Jacenko *et al*., manuscript in preparation #3).

Analysis of the subset of Tg mice with perinatal lethality revealed the most severe histological defects including greatest growth plate compressions, the least amount of bony trabeculae, and a depletion of the hematopoietic compartment in the marrow (Jacenko *et al*., manuscript in preparation #1). The latter was evidenced as a predominance of mature erythrocytes and a reduction of leukocytes, characteristic of marrow aplasia. Lymphopenia was confirmed by differential analysis of peripheral blood (Jacenko *et al*., 1996). Histology, immunohistochemistry, and flow cytometry of lymphatic organs revealed thymic reduction with a paucity of cortical immature T lymphocytes, and an overall decrease in apoptosis, consistent with the marrow's inability to replenish maturing cortical lymphocytes. Spleens were small and discolored, had poorly organized lymphatic nodules, and a reduced red pulp. Most important, splenic B cells were being depleted. Since B cells differentiate in the marrow prior to being released into the circulation, their depletion was again consistent with the marrow's inability to provide a nurturing environment for the development of these cells. Lastly, lymph

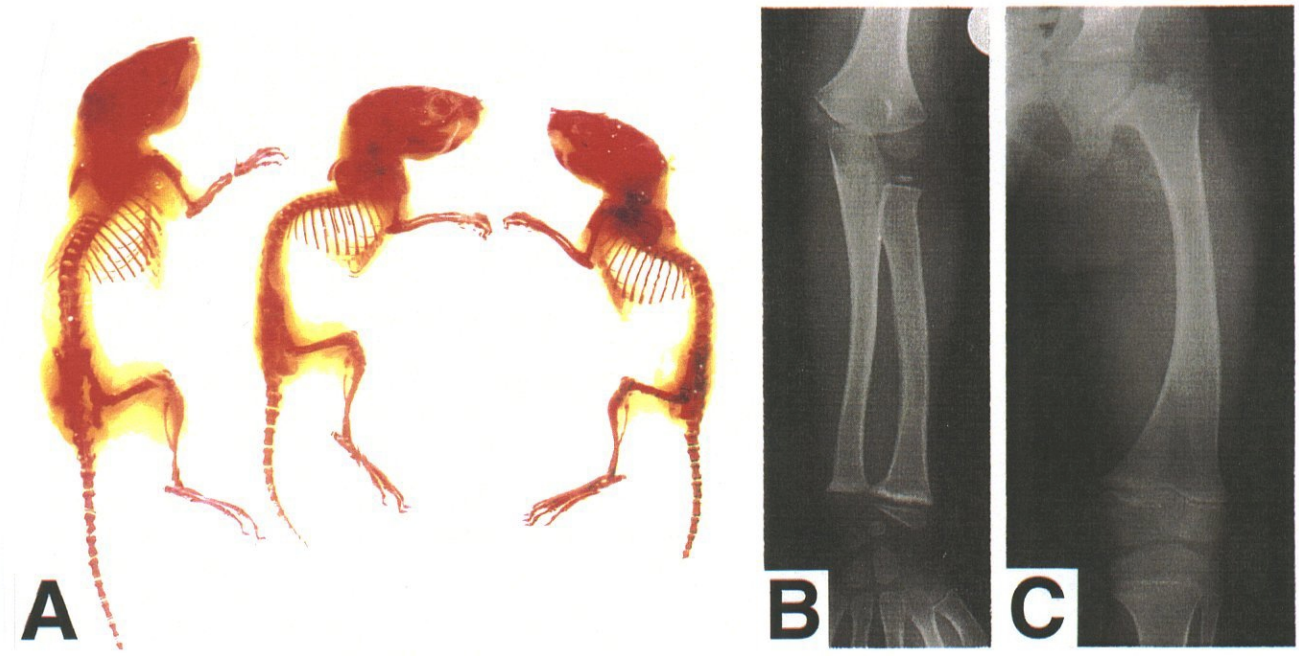

Figure 2. Collagen X mutations result in skeleto-hematopoietic defects Tg mice, and SMCD in humans. (**A**). Alizarin red S stained whole skeletons of day-21 transgene-negative (left), and transgene-positive, perinatal-lethal mutants (center and right). Note thoracolumbar kyphosis and accentuated neck lordosis in mutants. (**B**) and (**C**). X-rays of the arm (**B**) and femur (**C**) from a 5-year old patient with SMCD. Note flaring of bones at the metaphyses.

nodes were undetectable. Interestingly, among the surviving Tg mice, lymphosarcomas were prevalent with age as well as non-healing skin ulcerations, suggesting impaired immune function (Jacenko *et al*., manuscript in preparation #1).

Taken together, the data firstly implicate an interference by the transgene with collagen X function in the murine skeleto-hematopoietic defects. Secondly, the intriguing hematopoietic and immune abnormalities suggest that cartilage substitution by bone and marrow may help establish the marrow stromal microenvironment prerequisite for blood cell differentiation. An example of one possible scenario out of many maintains that perhaps a primary structural defect in hypertrophic cartilage, such as the disruption of the hexagonal lattice-like pericellular matrix, may result in an alteration of the marrow stromal matrix. This, in turn, would lead to an inability of the marrow to foster appropriate differentiation of blood cells, leading to a decrease in marrow-derived hematopoietic cells and ensuing immune deficiency. These findings, thus, underline the intricate relationship between endochondral skeletogenesis and hematopoiesis.

Schmid Metaphyseal Chondrodysplasia (SMCD) Resulting from Collagen X Mutations in Humans

The first human collagen X mutation (Warman *et al*., 1993) was identified in a large Mormon kindred with SMCD (MIM 156500), an autosomal dominant skeletal disorder resulting from growth plate cartilage abnormalities (Stevens, 1943; Lachman *et al*., 1988). The phenotype shows clinical variability, but is characterized by short stature, *coxa vara* and *genu varum* leading to a waddling gait. Radiological findings (Figs. 2b and 2c) include flaring of the metaphysis and a wide irregular growth plate, especially at the knees. The onset of the skeletal defects correlates with weight bearing (Wasylenko *et al*., 1980), similar to the manifestation of abnormalities in Tg mice (Jacenko *et al*., 1996).

Since the identification of the initial mutation, over 25 mutations have been reported in SMCD covering the spectrum of amino acid substitutions, nonsense mutations and deletions resulting in a predicted protein truncation (For review, see Chan and Jacenko, 1998). With the exception of two amino acid substitutions which appear to be at the signal peptide cleavage site (Ikegawa *et al*., 1997), all other mutations are clustered within the highly conserved NC1 domain (Figs. 1 and 3; Bonaventure *et al*., 1995; McIntosh

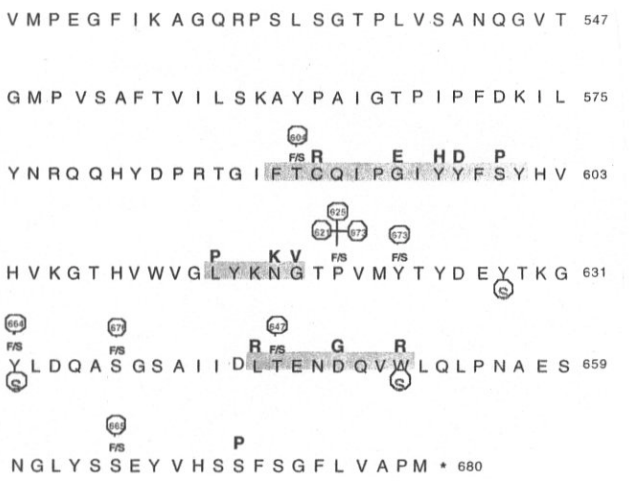

Figure 3. Collagen X NC1 mutations found in patients with SMCD. The amino acid sequence of the human collagen X NC1 domain is listed. The reported missense mutations in SMCD patients are shown in bold letters above the corresponding normal amino acid. The location of stop codon mutations (marked "S" and enclosed in an octagon) and frame-shift mutations (F/S) with predicted termination further along the polypeptide (numbers enclosed in a octagon), are positioned above or below the corresponding amino acid. The proposed 13 amino acid "aromatic zipper" domain (amino acid 589-601; Brass *et al*., 1992), and two other domains (amino acid 614-618 and 644-652) with localized amino acid substitutions are shaded.

et al., 1995; Chan and Jacenko, 1998), suggesting a critical role for this domain in collagen X synthesis, molecular assembly and function. Specifically, the NC1 mutations may prevent assembly of the mutant chains into trimers by disturbing critical sequence domains in the highly conserved regions; this would be consistent with a 50% reduction (haploinsufficiency) in the deposition of collagen X protein in the matrix (Warman *et al*., 1993). Support for haploinsufficiency came from cell-free translation and assembly studies, where the SMCD NC1 mutations including G618V, Y598D, 1963del10 and 1952delC were expressed *in vitro*, and indeed compromised NC1 assembly (Chan *et al*., 1995, 1996a). Furthermore, expression of these constructs in transiently-transfected cells confirmed that the assembly of mutant chains into stable homotrimeric molecules and subsequent secretion were severely impaired (Chan *et al*., 1996b).

Overall, the *in vitro* studies suggested that the common molecular defect underlying SMCD mutations is the compromise of strong interactions that drive NC1 association, preventing the formation of stable collagen X homotrimers, or heterotrimers between normal and NC1 mutant chains. The haploinsufficiency model was further supported by the characterization of a premature termination mutation in an SMCD patient. This mutation resulted in the instability of the mutant mRNA, leading to the complete absence of the mutant mRNA and protein in patient growth cartilage (Chan *et al*., 1998). While the degradation of mRNAs arising from premature termination mutations is a common occurrence in many diseases (Maquat, 1995), not all nonsense mutations will result in the complete degradation of mutant mRNA; furthermore, missense and some frame-shift mutant mRNAs are likely to be stable and expressed as proteins. Along these lines, the *in vitro* assembly studies did not exclude the possibility that weak or transient interactions may occur that could impact on the formation of the final normal collagen X trimer. Indeed, trace amounts of heterotrimer assembly were detected when certain α1(X) NC1 missense mutants were expressed *in vitro* (Chan *et al*., 1996b). These data suggested that while the dominant disease mechanisms underlying SMCD likely involved collagen X reduction due to either the inability of the mutant chain to participate in trimer assembly, or absence of the mutant allele product due to nonsense-mediated mRNA decay, it is also possible that certain NC1 mutations may allow some mutant: normal heterotrimer assembly. Likewise, these mutations may interfere with normal α1(X) interactions, thus exerting a dominant-interference effect on collagen X assembly.

To resolve this important issue, we developed a semi-quantitative *in vitro* co-expression and assembly assay for potential normal and mutant NC1 interactions. Specifically, we wanted to examine in detail the molecular consequences of SMCD mutations, and to dissect systematically the role of NC1 domains in the assembly process. This study confirmed that α1(X) chains containing certain SMCD mutations indeed interfere with the efficiency of normal α1(X) assembly during *in vitro* co-expression, opening the possibility of a dominant-interference phenotype (Chan *et al*., manuscript in preparation #4). Thus, the proposition emerging from these data maintains that the molecular basis of each mutation should to be considered individually. Moreover, these studies underline the need to dissect the disease mechanisms underlying each mutation, preferably through a combination of *in vitro* and *in vivo* analyses.

Disruption of Collagen X Function in Mice through Gene Targeting and Homologous Recombination

Based on the dominant interference Tg murine model as well as on the human mutations resulting in SMCD, one would predict a moderate-to-severe phenotype if collagen X were inactivated. However, Rosati *et al*. (1994) reported no gross phenotypic changes in their knock-out (KO) mice.

The KO chimeras were on the 129/SvEv x C57BL/6J (B6) background, and were crossed with B6 wild type mice for the F1 generation. Homozygous KOs were analyzed for gross and histological/immunohistochemical skeletal changes; screening for ultrastructural differences was by electron microscopy using tannic acid in the fixative rather than RHT.

In contrast, chondro-osseous defects were observed in a second set of collagen X KO mice, generated by Kwan *et al.* (1997). In addition to generating 129/SvJ x B6 hybrids, the null mutation was bred into the 129/SvJ strain. Interestingly, more pronounced phenotypic changes were observed in the 129/SvJ strain than in the hybrid background. Analyses included histomorphometry, which confirmed subtle growth plate compressions primarily in the resting and proliferative chondrocyte zones. Trabeculae appeared more compact, and seemed to reflect the phenotype of the Tg mice. *In situ* hybridization did not detect altered expression of a number of matrix molecules typically found in the chondro-osseous junction, as was also observed by Rosati *et al*. (1994). However, ultrastructural analysis using RHT in the fixative revealed an altered compartmentalization of the growth plates, as was also seen in the Tg mice. This was evidenced as RHT-positive aggregates, likely proteoglycans, as well as matrix vesicles distributed throughout the resting and proliferative zones, but being reduced in the hypertrophic zone where they are typically localized in control mice.

The KO mice described by Kwan *et al*. (1997) also resembled the SMCD phenotype, in that older mice developed *coxa vara*. This was demonstrated by contact microradiography of femurs, which also revealed a decrease of overall bone content, and changes in trabecular bone opacity. While these studies have reconciled some differences in the mouse phenotypes, the later onset of *coxa vara* and an overall milder phenotype than in the SMCD patients is still unresolved. Furthermore, unlike human patients with SMCD, mice heterozygous for the null allele appear phenotypically normal despite being haploinsufficient. Perhaps, the unfolded mutant collagen X chains in humans with SMCD may accumulate or undergo intracellular degradation, and, thus, initiate an aberrant cellular response contributing towards an interference phenotype. Such issues may be addressed through the generation of an SMCD mutation in mice.

Lastly, foremost amidst the unresolved phenotypic differences are the hematopoietic changes described in the collagen X Tg mice, which have not been originally reported in either the KO mice, or in SMCD patients. Interestingly, we have recently observed a variable skeleto-hematopoietic phenotype in about 13% of the collagen X null mice (Rosati *et al*., 1994), that mirrored the defects previously described for a subset of the Tg mice with perinatal lethality (Healy *et al*., manuscript in preparation #2). Further characterization of the murine phenotypes should establish whether all mice with defective collagen X have altered hematopoiesis, if this is strain-specific, an effect of a modifier gene, or a direct result of collagen X disruption. In conjunction, unraveling the mechanisms underlying the murine and human phenotypes should provide insights into other disorders with skeleto-hematopoietic changes.

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Discussion with Reviewers

W.A. Horton: While patients with Schmid chondrometaphyseal dysplasia do not typically have hematopoietic problems, there are a number of human chondrodysplasias in which patients display both metaphyseal dysplasia and immune defects. Is this coincidence, related to primary or secondary problems with type X collagen or to some other abnormality of the microenvironment to which type X collagen and other molecules contribute?

Authors: A number of mammalian chondrodysplasias with skeleto-hematopoietic involvement have been documented, including human, canine, feline, and murine examples (please see answer to first question from Dr. McIntosh below for two examples of the human disorders). We believe that there is a direct link between endochondral skeletogenesis, involving the replacement of hypertrophic cartilage by marrow and trabecular bone, and establishment of the prerequisite marrow stromal environment for blood cell differentiation. Consequently, any number of defects in endochondral ossification, which would effect marrow establishment, may be manifested as a skeleto-hematopoietic phenotype. Thus, strengthening the link between skeletal and blood cell development by unraveling the downstream consequences resulting from the initial changes in collagen X in the transgenic mice, should be invaluable for identifying the molecular basis of certain skeleto-hematopoietic, immunologic, and perhaps metastatic disorders.

At present, we could only speculate as to the involvement of collagen X (please see answer to third

question from Dr. Horton below). Furthermore, we can only surmise from the temporal sequence of events whether a defect in the growth plate alters the marrow, or vise versa. Based on the temporal onset of the histological defects underlying the perinatal-lethal phenotype in the collagen X mice, we hypothesize that the skeletal changes precede the marrow changes.

The skeletal changes likely are due to disruption of collagen X function, leading to a decompartmentalization of the growth plate. Whether collagen X has a direct effect on the marrow through the alteration of the matrix or a cytokine imbalance, or rather represents solely a structural alteration in a unique hypertrophic cartilage environment, remains to be established. We are addressing this issue by manipulating the hypertrophic cartilage zone through other approaches and observing the effects on the marrow. Likewise, we are screening other mouse models with alterations in hypertrophic cartilage for hematopoietic defects.

W.A. Horton: The authors suggest that a few Schmid mutations may act through a dominant interference mechanism. Are there any differences in the clinical phenotypes of patients with such mutations and patients with more common mutations thought to act through haploinsufficiency?

Authors: A correlation between the nature of the mutation and the clinical phenotype requires knowledge of the precise molecular consequences and direct comparison of the clinical phenotypes. In the absence of detailed clinical and radiographic comparisons of SMCD patients with published mutations, this task is even more difficult. Therefore, we can only speculate on the possible outcomes. From our limited observation of two SMCD patients, one where we have demonstrated haploinsufficiency due to nonsense-mediate mRNA decay and another patient with a G628V substitution that has the potential to act in a dominant-interference manner (based on our unpublished *in vitro* interaction study), there appear to be no significant differences in the clinical manifestations.

In contrast, recently Ikegawa *et al*. (1998) have described a collagen X mutation (Gly595Glu) in the NC1 domain that results in Spondylometaphyseal Dysplasia, not SMCD. This phenotype may ensue from a dominant interference mutation. This documentation expands the phenotypic spectrum of pathologies that may result from defects in collagen X.

W.A. Horton: The authors suggest that type X collagen in hypertrophic cartilage somehow influences bone marrow microenvironment even though hypertrophic cartilage appears to be completely degraded at the trailing edge of the growth plate. I would enjoy hearing their speculation on how this might occur.

Authors: There are several scenarios through which hypertrophic cartilage (and perhaps collagen X) may influence the marrow environment. Firstly, the interterritorial matrix of hypertrophic cartilage is not entirely degraded at the trailing edge of the growth plate (or, at least not immediately). Rather, this matrix forms a scaffold which becomes covered by bone lining cells and osteoblasts; the osteoblasts proceed to deposit bone on top of the hypertrophic cartilage core. Thus, all trabecular/ cancellous/ spongy bones initiate as a hybrid tissue, and gradually are remodeled to mature bone. Alterations in the properties of the hypertrophic cartilage core, thus, may affect osteoblast and bone lining cell adhesion, bone deposition, sequestering of growth factors, and cell-matrix interactions prerequisite for establishing the hematopoietic niches. Specifically, we have observed that disruption of the collagen X network does cause an altered distribution of other matrix components, such as proteoglycans and glycosaminoglycans; some of these have been implicated in hematopoiesis. Furthermore, severe trabecular bone reduction may alter the spatial arrangement of marrow into hematopoietic compartments, since the distribution of hematopoietic cells within the marrow may actually be ordered and dependent on such niches. Likewise, trabeculae may sequester factors, such as heparan sulfate or cytokines, which are required for blood cell differentiation. Along these lines, our preliminary data implies that the cytokine metabolism is altered in these mice, which, in turn, could affect signal transduction in a variety of pathways, as well as directly cause osteopenia and depletion of the marrow hematopoietic compartment. These several scenarios may contribute either alone, or in concert, to an altered marrow.

Secondly, collagen X may have a more direct effect on the marrow. To date, we do not have a clear understanding of what constitutes the marrow stroma (please also see answer to Dr. Aszodi below). It is possible that as the hypertrophic chondrocytes are undergoing apoptosis and are being degraded, some of the collagen X territorial matrix may persist and, thus, contribute directly to the stromal matrix network. Alternatively, it has not been conclusively demonstrated that collagen X is not expressed in the marrow (please see answer to Dr. Poole below). Our bias is that changes in hypertrophic cartilage, resulting from collagen X defects, translate to marrow alterations. At present, we cannot exclude the possibility that changes in the marrow environment, perhaps involving a cytokine imbalance, may contribute/cause the skeletal changes. We are currently addressing these possibilities.

Thirdly, it is possible that a second “modifier gene”, which may be strain-specific, may contribute to the murine phenotype. Such modifiers have been linked to human and

murine disorders with variable penetrance (please see answer to first question of Dr. McIntosh below). The actions of the modifier may be linked to those of collagen X. For example, the presence of the modifier may be required in a recessive state, along with either one or two transgene-expressing or inactivated collagen X alleles, to "permit" the severe perinatal-lethal phenotype. Although this is an indirect effect of collagen X, a common pathway is implicated between skeletal development and marrow hematopoiesis.

A.R. Poole: The abnormalities in white blood cell maturation could be explained by the fact that type X collagen is also expressed by bone marrow cells. This possibility should be discussed. For example, have studies been made of wild type bone marrow *in situ* hybridization or immunostaining to see if there is evidence for expression of the type X collagen gene in this tissue?
Authors: A number of attempts were made to address this particular issue by ourselves and others (unpublished information from us and others). We have tried both immunohistochemistry and *in situ* hybridization using both cryosections, and marrow cultures from wild type mice, collagen X transgenic and knock-out mice, and transgenic mice expressing the Lac Z gene via use of the collagen X promoter (unpublished data). Due to excessively high endogenous levels of alkaline phosphatase, peroxidase, as well as β-galactosidase in marrow cells, we could not distinguish any signal above the background level through these approaches. We had to conclude that if any marrow cells express collagen X, they represent only a small fraction of the total marrow stromal cell population.

In an alternate approach, we attempted reverse transcriptase polymerase chain reaction (RT-PCR) on RNA extracted from marrow cultures or marrows from the above-mentioned mice; in all cases, using several sets of specific primers for both mouse collagen X and chick collagen X, we detected a faint signal in the marrow (unpublished observations). Currently, we cannot rule out that this signal is from the trabecular projections that may be encountered in the marrow space (although we cannot pick up collagen II or IX). We are currently trying to resolve this issue. Localization of collagen X in the marrow would directly implicate the involvement of this molecule in the hematopoietic environment.

R.S. Tuan: It would be helpful for the readers if the authors could provide sample immunohistochemical micrographs, either from the authors' or other investigators' work, illustrating the location of collagen Type X expression with respect to the maturation stages in the growth cartilage. A clearly labeled growth cartilage micrograph should go a long way in helping the readership better appreciate the biological specificity of collagen type X.
Authors: We have recently published an extensive review on collagen X which contained a similar figure to the one suggested (Chan and Jacenko, 1998). Furthermore, we are currently preparing a manuscript where we co-localize mouse collagen X with the transgene in the mouse growth plate (manuscript in preparation #2). Thus, to minimize repetition, we have chosen to omit this information from the current review and refer the readers to a few representative references which contain this or similar information (Chan and Jacenko, 1998; Gibson and Flint, 1985; Jacenko *et al*., 1993a; LuValle *et al*., 1989; Poole and Pidoux, 1989; Schmid and Linsenmayer, 1985).

R.S. Tuan: It would also be very helpful to include one or more histological micrographs illustrating the perturbations in growth cartilage as a result of SMCD and/or *COL10A1* transgenic manipulations. These will go very nicely together with the whole-mount and radiographic figures in Figure 2.
Authors: The histological defects in metaphyses of bones from collagen X transgenic mice have been published several times. Furthermore, we are currently preparing additional manuscripts with micrographs documenting the defects in mice with either altered or inactivated collagen X. Rather than duplicate information, we refer the readers to those citations which contain this data (Chan and Jacenko, 1998; Chung *et al*., 1997; Jacenko *et al*., 1993a, b; LuValle *et al*., 1993b).

I. McIntosh: In light of the observations (Wynne-Davies *et al*., 1985) that human autosomal dominant metaphyseal dysplasia (Schmid and Jansen) DO NOT exhibit immunologic or hematologic abnormalities in contrast to the recessive forms (McKusick and Shwachman), can the authors speculate on which types of molecule may be affected in the latter syndromes since type X collagen has been excluded. (Note: McKusick type metaphyseal dysplasia (CHH) maps to 9p13 and *COL10A1* has been excluded as the Shwachman locus).
Authors: The collagen X locus has been excluded in two recessive human disorders with metaphyseal and hematopoietic defects. It is possible though that molecules downstream in the pathway affected by collagen X disruption in the collagen X transgenic mice may contribute to the observed phenotypes. For these reasons, it would be invaluable to further characterize the downstream effects of collagen X disruption in mice, and strengthen the link between skeletal development and blood cell differentiation in the marrow.

At present, we could only speculate as to candidate molecules for the above disorders. We have preliminary unpublished data that cytokine levels (e.g., IL-12, interferon

gamma) are significantly elevated in the collagen X transgenic mice. Furthermore, there are murine models with either altered or overexpressed cytokine levels where both skeletal and hematopoietic changes ensue (e.g., IL-4 overexpression in mice; Lewis *et al*., 1993). Thus, among many possibilities, cytokines, growth factors, and their associated receptors, including interleukins, interferon gamma, G-CSF, may be among candidate molecules. Furthermore, the transgenic mice and often the human disorders manifest a variable phenotype, with variations in immune dysfunction, occasional malignancies, or blood cell proliferation defects. Additional candidate molecules, thus, may include players in signal transduction pathways, cell cycle, and tumor suppressor genes.

Lastly, we cannot rule out the involvement of a modifier gene in either the human or the murine defects due to the variable penetrance of the disease phenotype. This issue was briefly discussed for the transgenic mice (see also the answer to the first question of Dr. Horton). In humans with CHH, a significantly lower penetrance of the disorder has been observed than was expected (Sulisalo *et al*., 1997), supporting this possibility.

I. McIntosh: The observation that a nonsense mutation results in absence of the mutant transcript as described by Chan *et al*. (1998) is of great interest. In all previous examples of this phenomenon, the mutation was always in the penultimate or more 5' exon. Do the authors have any theories to explain how the cell determines that a more 5' stop codon is utilized? Has it been shown that there is not a more 3' non-coding exon?

Authors: To our knowledge, no additional 3' non-coding exon(s) has been identified for the collagen X gene. Therefore, the nonsense-mediated mRNA decay resulting from a premature termination mutation in the penultimate exon (exon 3) of collagen X is of significance and likely represents an alternate nhRNA scanning mechanism. At present, we are not in the position to offer a model mechanism for this finding. However, it is of interest to point out that this observation is not unique to our published example for a SMCD patient; in the two papers (Kwan *et al*., 1997; Rosati *et al*., 1994) describing the use of gene targeting by introducing premature termination in exon 3 of *COL10A1* to "knock out" collagen X in the mouse, little or no collagen X mRNA was detected in the homozygous *COL10*-null mice.

I. McIntosh: Do the authors believe that the difference in phenotype between the strains of knock-out mice is due solely to the genetic background of the strains? Might something have been missed by Rosati *et al*. (1994) since they employed a different staining protocol?

Authors: For ultrastructural analyses, Dr. Cheah's group (Kwan *et al*., 1997) did compare results obtained with two different fixation techniques. The first protocol included ruthenium hexamine trichloride (RHT), as in the analysis of the transgenic mice; the second used tannic acid, as in the study by Rosati *et al*. (1994). With RHT fixation, significant changes were evident in the matrices of knock-out mice on both the C57Bk/6 and the 129 backgrounds. Specifically, an altered distribution of matrix components was observed throughout the growth plate. This was manifested as RHT-positive aggregates, likely proteoglycans, as well as matrix vesicles. In contrast, use of tannic acid resulted in the removal of the granular RHT-positive aggregates and other matrix materials, but exposed the collagen fibrils.

These ultrastructural studies by Kwan *et al*. (1997) bridge both the studies by Rosati *et al*. (1994), as well as our studies on the transgenic mice. Rosati *et al*. (1994) only used tannic acid, and focused primarily on the hypertrophic cartilage; thus, altered proteoglycan distribution was not detected. On the other hand, our data on the transgenic mice are comparable to those of Kwan *et al*. (1997), since we also detected a decompartmentalization of the growth plate. One difference was our visualization of the pericellular lattice-like network in the hypertrophic zone of wild type mice, which was disrupted in the transgenic. Such a network was not described by Kwan *et al*. (1997), however, their analyses were presented at a higher magnification than ours, thus, it is unclear where the micrographs were taken with respect to the cell surface.

In additional to the differences in the staining protocols, Dr. Cheah's group (Kwan *et al*., 1997) also detected subtle differences between the two mouse strains; these subtle phenotypic differences are likely due solely to the genetic background. The C57Bk/6 mouse strain had a milder phenotype than the 129 strain, which is consistent with the work of Rosati *et al*. (1994).

Lastly, we believe that a more intriguing issue is why a variable, perinatal-lethal phenotype was observed by us only in a subset of the KO mice generated by Rosati *et al*. (1994). We are currently investigating whether this is due to genetic or environmental influences.

I.A. Aszodi: The authors suggest that the disruption of the hexagonal lattice-like network of hypertrophic cartilage in transgenic mice leads to formation of altered bone marrow stroma which is unable to provide an appropriate environment for proper blood cell development. Does the marrow stromal matrix of transgenic mice show any structural abnormalities supporting this explanation?

Authors: To date, it has not been possible to visualize the marrow stroma *in vivo*. Thus, it is still unclear as to what constitutes the marrow stroma (e.g., is it a sheet of cells comprising the endosteum that constitutes the inner lining of the bone, or is it a matrix network that permeates the

marrow space and is interspersed between the hematopoietic cells). Thus, for lack of appropriate isolation and visualization methods in viewing intact marrow stroma and cells, we were not able to view any structural differences in the marrow *in vivo*, other than changes in the blood cell lineage composition. We are currently addressing these issues through marrow cultures, as well as by bone marrow transplantations. As discussed above, establishing whether collagen X is a component of the marrow, or whether these changes are more indirect (e.g., through changes in trabeculae) is essential. Furthermore, it is still not confirmed that collagen X indeed forms a hexagonal lattice network in the hypertrophic cartilage pericellular matrix. Knowing the precise structure and localization of this molecule would be invaluable in addressing this critical issue.

Editor: Can you please give further details of your manuscripts in preparation that are mentioned in the text of the paper.

Authors: Most of these papers have not yet been submitted for publication. We will provide interested readers with more information on request, as it becomes available for release. In the following, "#1, #2", etc. refer to the numbers included in the text with each mention of "manuscript in preparation":

#1: Jacenko O, Healy C, Tao Z, Campbell M: Skeleto-hematopoietic defects in collagen X transgenic mice. In preparation.

#2: Healy C, de Crombrugghe B, Jacenko O: Growth plate compressions and altered hematopoiesis in collagen X null mice. In preparation.

#3: Jacenko O, Chan D, Franklin A, Ito S, Bateman J, Olsen BR, Campbell M: A dominant interference collagen X mutation results in growth plate and marrow defects in mice. In preparation.

#4: Chan D, Freddi S, Weng YM, Bateman JF (submitted) Interaction of collagen α1(X) containing engineered NC1 mutations with normal α1(X) *in vitro*: Implication for the molecular basis of Schmid metaphyseal chondrodysplasia. Submitted to J. Biol. Chem.

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