Chicken Collagen X Regulatory Sequences Restrict Transgene Expression to Hypertrophic Cartilage in Mice

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Collagen X is produced by hypertrophic cartilage undergoing endochondral ossification. Transgenic mice expressing defective collagen X under the control of 4.7- or 1.6-kb chicken collagen X regulatory sequences yielded skeleto-hematopoietic defects (Jacenko O, LuValle P, Olsen BR: Spondylometaphyseal dysplasia in mice carrying a dominant-negative mutation in a matrix protein specific for cartilage-to-bone transition. *Nature* 1993, 365:56–61; Jacenko O, Chan D, Franklin A, Ito S, Underhill CB, Bateman JF, Campbell MR: A dominant interference collagen X mutation disrupts hypertrophic chondrocyte pericellular matrix and glycosaminoglycan and proteoglycan distribution in transgenic mice. *Am J Pathol* 2001, 159:2257–2269; Jacenko O, Roberts DW, Campbell MR, McManus PM, Gress CJ, Tao Z: Linking hematopoiesis to endochondral ossification through analysis of mice transgenic for collagen X. *Am J Pathol* 2002, 160:2019–2034). Current data indicate that the hematopoietic abnormalities do not result from extraskeletal expression of endogenous collagen X or the transgene. Organs from mice carrying either promoter were screened by immunohistochemistry, *in situ* hybridization, and Northern blot; transgene and mouse collagen X proteins and messages were detected only in hypertrophic cartilage. Likewise, reverse transcriptase-polymerase chain reaction revealed both transgene and mouse collagen X amplicons only in the endochondral skeleton of mice with the 4.7-kb promoter; however, in mice with the 1.6-kb promoter, multiple organs were transgene-positive. Collagen X and transgene amplicons were also detected in marrow, but likely resulted from contaminating trabecular bone; this was supported by reverse transcriptase-polymerase chain reaction analysis of rat tibial zones free of trabeculae. Our data demonstrate that in mice, the 4.7-kb chicken collagen X promoter restricts transcription temporospatially to that of endogenous collagen X, and imply that murine skeleto-hematopoietic defects result from transgene co-expression with collagen X. Moreover, the

4.7-kb hypertrophic cartilage-specific promoter could be used for targeting transgenes to this tissue site in mice. (*Am J Pathol* 2004, 164:487–499)

During skeletal development, chondrocytes undergo several stages of differentiation. The final stage, chondrocyte hypertrophy, is associated with endochondral ossification (EO), the process by which cartilage is replaced by trabecular bone and marrow.¹ Specifically, as EO initiates, chondrocytes terminate proliferation and begin to swell, while the avascular and noncalcifiable cartilage matrix becomes a calcifiable one that is penetrated by blood vessels. The vasculature imports chondroclasts/osteoclasts for hypertrophic cartilage degradation and also stem cells for establishment of bone and marrow stroma. These changes result in the deposition of trabecular bony spicules on top of hypertrophic cartilage remnants. The porous trabecular bony network then protrudes into the bone marrow cavity and likely provides hematopoietic niches in the marrow for subsequent blood cell differentiation.⁵ The continual replacement of hypertrophic cartilage by trabecular bone and marrow results in the formation of growth plates at the outer ends of the tissue, consequently providing longitudinal skeletal growth until maturity.

A distinctive feature and major biosynthetic product of hypertrophic chondrocytes undergoing EO is the matrix protein collagen X.^{1,2} Its temporo-spatial localization to cells destined for replacement by bone and marrow predicted that this protein may participate in EO-associated events such as mineralization, vascular invasion, matrix stabilization, partitioning of the chondro-osseous junction, and/or establishment of the marrow environment. To test this hypothesis, we had previously generated transgenic (Tg) mice carrying dominant interference mutations in collagen X.^{3–5} Specifically, transgene constructs encoded chicken collagen X variants with in-frame deletions of either 21 (21Δ, eg, SpLX) or 293 (293Δ, eg,

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SpLXH) amino acids within the central triple-helical domain,³ and were shown to have potential for dominant interference.⁴ Expression of the chick collagen X 21 Δ or 293 Δ transgenes in mice was driven by either a 4.7-kb (~4750 bp upstream from transcription start) or a 1.6-kb (~1650 bp upstream from transcription start) chicken collagen X promoter fragment. Constructs also contained the remainder of exon 1 after transcription start, intron 1, and exon 2 up to translation start (~800 bp). Thus, four transgene constructs were generated that contained either the long or the short promoter and regulatory elements, combined with either the 21 Δ or 293 Δ cDNAs,³ and are designated here as: 4.7-21 Δ , 4.7-293 Δ , 1.6-21 Δ , and 1.6-293 Δ .

The resultant murine skeleto-hematopoietic disease phenotype was seen in multiple Tg lines representing all four transgene constructs, and was comparable in all lines. Moreover, confirmation of independent transgene insertion sites in each of the Tg mouse lines indicated that the skeleto-hematopoietic phenotype was a consequence of transgene presence and not a result of endogenous gene inactivation because of transgene insertion.^{3,6} Interestingly, phenotype severity was variable within each line, ranging from perinatal lethality to transient dwarfism, and involved all EO-derived tissues.⁵ Specifically, growth plate compressions, reduced hypertrophy, and a diminished length and number of trabecular bony spicules constituted skeletal defects whereas hematopoietic defects included marrow hypoplasia.^{5,7} In addition, mice displayed an impairment of hematopoiesis throughout life, which manifested as a reduction of marrow and splenic B cells, elevated splenic T cells, and a predisposition to lymphosarcomas and ulcerations.⁵ Mice with the most acute disease phenotype developed cachexia approximately week 3 after birth with ensuing death within 24 hours of the visual onset of the phenotype. This murine subset exhibited marrow hypoplasia, lymphatic organ atrophy, a reduction of thymic T cells and marrow and splenic B cells, and generalized lymphopenia.⁵ Subtle growth plate and hematopoietic abnormalities were also observed in the collagen X null mice; some of these features, in particular the perinatal lethality and marrow hypoplasia in the most severely affected murine subset, mirrored the Tg mouse defects.⁷

The hematopoietic murine defects in the collagen X Tg and null mice were unprecedented, and underscored an unforeseen link between hypertrophic cartilage, EO, and establishment of a marrow microenvironment needed for hematopoiesis. However, to causally link these hematopoietic alterations to a primary defect in hypertrophic cartilage, it was necessary to first exclude any possibility of extraskelatal expression of either endogenous mouse collagen X, or the transgene, especially in the sites exhibiting the unexpected abnormalities. In this study, we demonstrate by immunohistochemistry, *in situ* hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR), as well as Northern blot analysis, that the transgene is co-expressed temporo-spatially with endogenous collagen X in the endochondral skeleton (ES). Moreover, we establish that endogenous collagen X is not expressed in the affected extraskelatal sites. This

strengthens the intriguing probability that the changes in hypertrophic cartilage have led to the hematopoietic abnormalities in both the collagen X Tg and null mice. Furthermore, for the first time, this study identifies a hypertrophic cartilage-specific promoter that could be used for targeting additional transgenes to this tissue site in mice.

Materials and Methods

Mouse Maintenance and Genotyping

Mice were maintained in a virus-free barrier facility in microisolators containing *Pine Dri wood* chips, and were fed autoclaved *Purina mouse chow* (Animal Specialties and Provisions, LLC, Quakertown, PA) and water *ad libitum*. The colony was examined daily for any abnormalities in growth, such as decrease in size; skeleto-hematopoietic defects, such as hunching of the back, sloping of the buttocks, wasting, skin ulcers, and tumors; or behavior, such as lethargy or changes in mobility. At 3 weeks of age, mice were weaned, ear punched, and genotyped using a tail biopsy for DNA isolation,⁸ phenol/chloroform purification, and genomic Southern blotting.³ Mice were euthanized by methoxyflurane exposure (Mallinckrodt Veterinary, Mundelein, IL).

Immunohistochemistry, *in Situ* Hybridization, and Histology

Immunohistochemistry for collagen X was as described² without modifications. Specifically, primary polyclonal antibodies included our recently characterized anti-mouse collagen X,⁴ and chicken collagen X antibodies generously provided by Dr. M. Pacifici (Department of Orthopedics, Thomas Jefferson University, Philadelphia, PA). Longitudinal cryosections (6 to 8 μ m long) of week 3 tibiae and femurs were first screened from all available Tg lines (eg, two lines per transgene construct) with either the 4.7- or the 1.6-kb promoter (eg, 4.7-21 Δ , 4.7-293 Δ , 1.6-21 Δ , or 1.6-293 Δ). Because variable staining intensities were observed in different lines, the epitope-demasking protocol involving treatment with testicular hyaluronidase (1 mg/ml for 45 minutes at 37°C being established as optimal⁴) was modified to range from 15 minutes to 1.5 hours of incubation, with no improvement of staining observed. Subsequently, two lines exhibiting the strongest staining of hypertrophic cartilage and containing the 1.6-293 Δ or 4.7-21 Δ constructs were selected for all further analyses. From these lines, 6- to 8- μ m cryosections of week 3 brain, muscle, eyes, thymus, heart, lung, liver, spleen, kidney, skin, and calvaria were screened for collagen X and transgene protein. In each case, comparable organs from collagen X null mice were stained in parallel to control for nonspecific staining, which was only seen in the white matter and the Purkinje cell layer of the cerebellum and the choroid layer of the eye. This staining was confirmed as nonspecific because several other unrelated antibodies yielded similar results.⁴

In situ hybridization was as described.⁹ Briefly, tibiae and femurs from newborn controls and collagen X 1.6-293Δ or 4.7-21Δ Tg mice, brain, muscle, thymus, lung, spleen, and skin from newborn 1.6-293Δ mice, and sternae from embryonic D-19 chicks were fixed [4% formaldehyde/2×phosphate-buffered saline (PBS); 4°C, rocking gently overnight], washed (1× PBS; 30 minutes, three times), and embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA). Longitudinal cryosections (6 to 8 μm long) were placed on a slide warmer (56°C, 1 hour; Fisher Scientific, Pittsburgh, PA), and treated with 0.2 N HCl (15 minutes). After washes (1× PBS; 5 minutes, two times), sections were treated with Proteinase K (10 μg/ml, pH 7.4, 10 mmol/L Tris/1 mmol/L CaCl₂ at 37°C for 15 minutes; Fisher Scientific;), rinsed (1× PBS; 5 minutes, two times), treated with 4% paraformaldehyde (room temperature for 5 minutes), and rinsed again (1× PBS; 5 minutes, two times). Sections were then acetylated (0.1 mol/L triethanolamine, pH 8, 0.25% acetic anhydride; room temperature for 15 minutes), rinsed (1× PBS; 5 minutes, two times), dehydrated with increasing ethanol concentrations (15 seconds each), and air-dried (30 minutes). Slides were next incubated in the hybridization solution [50% formaldehyde, 10 mmol/L Tris, pH 7.6, 200 μg/ml tRNA, 1× Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 10% dextran sulfate, 600 mmol/L NaCl, 0.25% sodium dodecyl sulfate, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8, 51°C for 2 hours]. A ~900-bp *PvuII* fragment of the chicken SpLX cDNA³ was labeled with a DIG RNA labeling kit (SP6/T7; Roche Applied Science, Indianapolis, IN) according to manufacturer's protocol. The probe was added to the hybridization solution, heated (85°C, 3 minutes), and applied to sections (1.5-μl probe/100-μl hybridization solution/slide) for overnight hybridization (50 to 55°C). Slides were then washed (5× standard saline citrate; 52°C for 5 minutes) and treated with formamide (50% in 2× standard saline citrate; 52°C for 30 minutes). After incubating with 10× TNE (1 mol/L Tris, pH 7.6, 5 mol/L NaCl, 0.5 mol/L EDTA; 37°C for 10 minutes), sections were treated with RNase (10 μg/μl of 10× TNE; 37°C for 30 minutes), and rinsed (10× TNE; 37°C for 10 minutes). Subsequent washes (2× standard saline citrate; 50°C for 20 minutes; twice with 0.2× standard saline citrate; 50°C for 20 minutes) were followed by rinsing with DIG1 buffer (pH 7.5, 1 mol/L Tris, 5 mol/L NaCl; room temperature for 5 minutes) before incubating sections in levamisole (85 mg/35 ml DIG1 buffer; room temperature for 1 hour). After washes in DIG1 buffer (room temperature for 5 minutes), sections were treated with 1.5% blocking reagent (DIG Nucleic Acid Detection Kit, room temperature for 30 minutes; Roche Applied Science), and rinsed (DIG1 buffer, 5 seconds). Incubation with anti-DIG antibody (1:500 dilution; room temperature for 1 hour) was followed by washes [DIG1 buffer: room temperature for 15 minutes, two times; and DIG3 buffer: (1 mol/L Tris, pH 9.5, 5 mol/L NaCl, 1 mol/L MgCl₂) room temperature for 5 minutes]. Color development involved the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stock in DIG3 buffer (1:50 dilution). Slides were then rinsed (deionized water) and coverslips were

mounted using Aquamount (Lerner Laboratories; Fisher Scientific). Visualization of sections occurred using an upright Olympus BX60 light microscope with a Photomicrographic System PM20 (Olympus America, Inc., Lake Success, NY).

For histology, right tibiae from day 22 BBDR/Wor rats (Biomedical Research Models, Inc., Worcester, MA) were fixed in Bouin's (1 week), decalcified (4% formalin, 10% EDTA, 1% sodium acetate), dehydrated through increasing ethanol concentrations, cleared with Propar (Anatech, Battle Creek, MI), and profused and embedding in *paraffin*.⁵ Longitudinal sections (6 μm long) were stained with Alcian blue at pH 1.0 (Lev et al, 1964) to visualize glycosaminoglycans, and with Harris hematoxylin and eosin Y (H&E; Sigma Diagnostics, St. Louis, MO).

RNA Isolation

Brain, muscle, skin, eye, liver, lung, kidney, spleen, thymus, heart, and ES (vertebral column, and growth plates from tibiae, femorae, radiae, and ulnae) were isolated from newborn, week 1, 2, and 3 control, and collagen X Tg mice carrying either the 1.6-293Δ or 4.7-21Δ transgene constructs. As an additional control, ES samples were also obtained from the collagen X null mice. Soft organs were immediately frozen in liquid nitrogen, while the ES was crushed to a powder in liquid nitrogen by mortar and pestle. Trizol (1 ml/0.1 g; Life Technologies Inc., Rockville, MD) was then added, and samples were mixed by a PowerGen 125 homogenizer (Fisher Scientific) and incubated (30°C for 5 minutes). Chloroform was added according to initial Trizol (0.6 ml/ml Trizol), and samples were shaken vigorously, placed at room temperature (2 to 3 minutes), and then centrifuged (12,000 × g, 15 minutes, 4°C). The aqueous layer was mixed with 100% isopropanol (0.5 ml/1 ml Trizol), precipitated (10 to 15 minutes at room temperature), and then centrifuged (12,000 × g, 15 minutes at 4°C). Pellets were washed with 75% EtOH (1 ml/1 ml Trizol), mixed by pipetting, centrifuged (7500 × g, 5 minutes at 4°C), and then resuspended with 1 ml of 75% EtOH. After centrifuging (10,300 × g, 5 minutes at 4°C), pellets were air-dried and resuspended in 0.1% diethyl pyrocarbonate water (Sigma Chemical Co., St. Louis, MO).

Day 22 BBDR/Wor rats (Biomedical Research Models, Inc.) were generously provided by Mrs. Pam Tolomeo and Dr. Ali Naji (University of Pennsylvania Medical School). Left tibiae were processed for histology (see above), whereas each right tibia was cut into five cross-sections (see Figure 7A). The two tibial ends containing growth plates were finely chopped, crushed in liquid nitrogen, and incubated in Trizol (15 minutes at room temperature) before chloroform addition (see above). Marrow from each of the three remaining tibial cross-sections was flushed with Dulbecco's modified Eagle's medium (Life Technologies, Inc.), and incubated with Trizol (3 ml) for RNA isolation as above.

Bone marrow cultures were obtained from 5 week 3-Tg mice carrying the 1.6-293Δ construct, and displaying perinatal lethality. Briefly, marrow from tibia and femur

hind limbs was flushed out with Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 1% L-glutamine (Mediatech, Fisher Scientific), 50 U/ml penicillin-streptomycin (Life Technologies, Inc.), and fetal bovine serum (Hyclone, Logan, UT) using a 22-gauge needle. Cells were pelleted (2500 rpm, 3 minutes, 4°C), and resuspended in fresh media. Cultures were plated at 5×10^7 cells per 2.5 ml, and media was changed every 48 hours until the cells reached confluency at 2 weeks. For RNA isolation, 1.25 ml of Trizol was added to each culture, and plates were swirled on an orbital shaker (30 seconds). RNA isolation then proceeded as above except for the following modifications: centrifugation was at $11,500 \times g$ (10 minutes) after both chloroform and isopropanol additions, and washing with 75% ethanol was omitted.

Chicken bone marrow aspirates were similarly isolated from 36 embryonic day 18 white leghorn chicken (Truslow Farms, Inc., Chestertown, MD) tibia and femur hind limbs with Dulbecco's modified Eagle's medium without L-glutamine (Life Technologies, Inc.). For RNA isolation, cells were resuspended in Trizol (12 ml), and processed as above, using modifications for cell cultures.

DNase Treatment

Fifty μ l of 25- μ g RNA was incubated (37°C for 1 hour) with TE (24 μ l; 10 mmol/L Tris, 1 mmol/L EDTA), $MgCl_2$ /dithiothreitol; (20 μ l; 100 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol), and DNase I (4 μ l; Life Technologies, Inc), before a second incubation (37°C for 1 hour) with more master mix (11 μ l TE, 10 μ l $MgCl_2$ /dithiothreitol, 4 μ l DNase I). After a third addition of DNase (3 μ l; 37°C for 30 minutes), reaction was terminated using DNase STOP solution (37.5 μ l; 50 mmol/L Tetra EDTA, 1.5 mol/L NaOAc, and 1% sodium dodecyl sulfate). After phenol-chloroform extraction and ethanol precipitation, samples were resuspended in diethyl pyrocarbonate-treated water (25 μ l). Before cDNA synthesis, each DNased sample was retested for quality by ethidium bromide-staining of formamide/formaldehyde-containing agarose gels (see Northern Blot Analysis). Likewise, each sample was subsequently included in RT-PCR reactions to ensure lack of DNA contaminants (see below).

cDNA Synthesis-Reverse Transcriptase

cDNA were generated using the Preamplification Kit-Reverse Transcriptase (Life Technologies, Inc.) following the manufacturer's protocol. Briefly, 5 μ g of DNased RNA (11 μ l) that was proven to be free of DNA contaminants (see above) was incubated (70°C for 10 minutes) with an oligo dT primer (1 μ l), then chilled on ice (2 minutes), and mixed with master mix (1 μ l dNTP, 2 μ l $MgCl_2$, 2 μ l 10 $\times$ PCR buffer, 2 μ l dithiothreitol). A preincubation (42°C for 5 minutes) was followed by addition of Superscript II (reverse transcriptase, 42°C for 50 minutes; Life Technologies, Inc.), after which the reaction was terminated (70°C for 15 minutes). DNased RNA control samples,

which were included in all PCR reactions to ensure absence of contaminating DNA, were processed alongside experimental samples in a final treatment with RNase H (1 μ l, 37°C for 30 minutes; Life Technologies, Inc.).

PCR

PCR was run on cDNA and DNased RNA samples using three sets of mouse, five sets of chicken, and one set of rat primers under optimized conditions for each pair. The primer sets were chosen from several sites along the collagen X gene (see Figure 3) based on sequences reported for chicken,¹⁰ mouse,¹¹ and rat.^{12,13}

Using a PCR Core Kit (Roche Applied Science), all reaction mixes consisted of primers (0.5 μ l; 0.5 μ mol/L each), dNTPs (0.2 μ l), *Taq* polymerase (0.1 μ l), 10 $\times$ buffer with or without $MgCl_2$ (1 μ l), distilled water, and cDNA template (1 μ l). Mouse primers M1s (nucleotides 140 to 161, 5'-CCATAAAGAGTAAAGGGATCCC-3') and M1a (nucleotides 560 to 583, 5'-CCATATCCTGTTCCCTTTCC-3') required buffer with $MgCl_2$, and 30 cycles of denaturing at 94°C (1 minute), annealing at 60°C (1 minute), and extending at 72°C (2 minutes), using a PTC-100 Thermal Cycler (MJ Research, Inc., Watertown, MA). Both mouse primer sets M1s and M2a (nucleotides 721 to 740, 5'-CCTGATGGTCCCATTCTCC-3') and M1s and M3a (nucleotides 1570 to 1590, 5'-CTGGCCTGCTTTATGAAGCC-3') produced optimal results using buffer without $MgCl_2$, and 30 cycles of 94°C (40 seconds), 61°C (1 minute), and 72°C (2 minutes).

Chicken primer C1s (nucleotides 78 to 99, 5'-GAAA-CAGTCCAGCATCAAGGG-3') and C1a (nucleotides 725 to 748, 5'-GGATACCAGCTTCTCCACGTG-3') were used at 94°C (40 seconds), 63°C (1 minute), 72°C (2 minutes) for 30 cycles using buffer with $MgCl_2$. Optimal conditions for chicken primers C1s and C2a (nucleotides 1237 to 1255, 5'-CCAGGATGGCCAGCATGGCC-3') and C1s and C3a (nucleotides 1582 to 1604, 5'-CCTGATAGTCTCTAGACTCTCC-3') included buffer with $MgCl_2$, 18 cycles of 94°C (40 seconds), 62°C (1 minute), 72°C (4 minutes), and 13 cycles of 94°C (40 seconds), 60°C (1 minute), and 72°C (4 minutes). Chicken primers C2s (nucleotides 1100 to 1130, 5'-GGATTCCCAGGAGCAAGGGT-3') and C4a (nucleotides 1693 to 1716, 5'-GTCAAATTTGATGGGGACTGTTGC-3') required buffer with $MgCl_2$ (2 μ l), and 30 cycles of 95°C (40 seconds), 68°C (1 minute), and 72°C (2 minutes). The final chicken primer set of C3s (nucleotides 1237 to 1256, 5'-GGC-CATGCTGGCCATCCTGG-3') and C3a, required buffer with $MgCl_2$ and denaturing, annealing, and extending temperatures of 94°C (40 seconds), 64°C (1 minute), and 72°C (2 minutes), respectively, for 30 cycles.

Rat primers R1s (nucleotides 1547 to 1568, 5'-CCCG-GCCAAGCAGTCATGCCTGA-3') and R1a (nucleotides 2035 to 2056, 5'-GGAGCCACTAGGAATCCTGAG-3') used buffer with $MgCl_2$ and 30 cycles of 94°C (1 minute), 59°C (2 minutes), and 72°C (2 minutes, 30 seconds).

RT-PCR products were visualized by electrophoresis on a 2% Seakem GTG agarose gel (BioWhittaker Molecular Applications, Rockland, ME), blotted onto Hy-

bond-N+ transfer membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and hybridized with a ^{32}P -labeled chicken $\alpha 1(\text{X})^3$ or a mouse $\alpha 1(\text{X})^{14}$ probe.

Northern Blot Analysis

RNA (25 μg) was combined with equal volume of buffer [24% formaldehyde, 68% formamide, 8% 10 $\times$ MOPS, pH 7 (0.2 mol/L MOPS, 5 mmol/L disodium EDTA, 50 mmol/L NaOAc)], vortexed, incubated (65°C, 3 minutes), mixed with termination buffer (0.2 g bromophenol, 0.2g xylene cyanol, 12.5 g Ficoll in 50 ml of deionized water), and run on an 0.8% Seakem Agarose GTG gel (BioWhittaker Molecular Applications) containing MOPS and formaldehyde. Duplicate samples on the gel were then transferred onto Hybond N+ and hybridized with a random prime-labeled probe for either chicken (SpLX¹) or mouse (mX¹⁴) collagen X, whereas the other set were stained with ethidium bromide (10 mg/ml; 45 minutes) for UV visualization.

Results

Endogenous Collagen X and Transgene Product Co-Localize to Hypertrophic Cartilage in the ES

Although the skeletal changes seen in the collagen X Tg as well as null mice were anticipated, the hematopoietic consequences were unprecedented. To causally link the marrow and blood cell changes to transgene expression in hypertrophic cartilage and/or to the lack of collagen X function, it became necessary to exclude extraskeletal expression of either endogenous mouse collagen X, or the transgene. To this end, studies using species-specific collagen X antibodies demonstrated that the transgene product co-localized with endogenous collagen X temporo-spatially to hypertrophic cartilage in growth plates and ossification centers, and that proliferative cartilage of the Tg mice also stained diffusely.⁴ These studies were extended here by screening all available Tg lines (eg, two lines per construct) with either the 4.7- or the 1.6-kb promoter (eg, 4.7-21 Δ , 4.7-293 Δ , 1.6-21 Δ , or 1.6-293 Δ Tg mice; Figure 1). In general, in every Tg line, the same localization of the transgene product and mouse collagen X was seen in growth plate hypertrophic cartilage, with a region of less mature hypertrophic chondrocytes staining most intensely, as observed previously.⁴ However, these growth plate regions from mice with 1.6-kb promoter constructs typically appeared to exhibit stronger staining than that seen in most lines containing the 4.7-kb promoter (Figure 1, D and E, *versus* A, B, and C). For all subsequent analyses, the mice exhibiting the strongest staining and containing the 1.6-293 Δ (line 3-2) or 4.7-21 Δ (line 1-2) constructs were used; this first allowed comparison of the specificity of the two promoters, and second, provided different transgene products that could be distinguished by PCR in the studies below.

Cryosections of mouse brain, muscle, eye, thymus, heart, lung, liver, spleen, kidney, skin, calvaria, and ES from these two mouse subsets were also reacted with the species-specific antibodies, which localized either mouse collagen X or the transgene product only to the ES (data not shown).

Collagen X transgene expression was also assessed by *in situ* hybridization of tibial cryosections (Figure 2), as well as of brain, muscle, thymus, lung, spleen, and skin from newborn Tg mice, and of marrow stromal cell cultures from week 3 Tg mice. Specificity of the chicken collagen X probe was established by strong hybridization with hypertrophic cartilage (Figure 2C) but not of proliferative cartilage (Figure 2B) in embryonic day 19 chick sternum. In both 1.6-293 Δ or 4.7-21 Δ Tg mice, hybridization was localized to growth plate hypertrophic cartilage (Figure 2; E, G, and I) when compared to no probe control sections (Figure 2; D, F, and H), or to the uniform, nonspecific light purple staining obtained from hybridization with the sense probe (not shown). Moreover, no signal was observed in the bone marrow cavity (Figure 2I), or in any of the tested extraskeletal organs or stromal cell cultures (not shown). Taken together, the immunohistochemistry and *in situ* hybridization data detected mouse collagen X and transgene mRNA and protein only in hypertrophic cartilage, consistent with the anticipated restricted temporo-spatial co-expression of the transgene with endogenous collagen X. Moreover, both the 4.7- and the 1.6-kb promoters seemed to exhibit comparable tissue specificity at this level of detection.

To further ensure that the observed murine skeleto-hematopoietic defects were not a result of extraskeletal expression of endogenous collagen X and the transgene, nor of transgene misexpression, cDNAs of selected organs from wild-type controls, collagen X null, and collagen X Tg mice containing either the 1.6-293 Δ or 4.7-21 Δ transgene constructs were screened by RT-PCR. Organs were obtained from newborn mice (before any visible histological defect), mice at week 1 (before establishment of secondary ossification centers, which precede growth plate compressions), week 2 (first histologically visible growth plate changes), and week 3 (pronounced growth plate compressions and marrow hypoplasia).⁴ These organs were analyzed for the expression of endogenous mouse collagen X and the transgene product message by use of five chicken and three mouse species-specific primer set combinations that included amplicons ranging from the NC2 amino terminal domain to the NC1 carboxyl terminal domain (Figure 3). The availability of multiple primer sets proved useful in screening for cross-reactivity between species, as well as with other collagens homologous to collagen X, and/or containing triple-helical domains. Because no developmental stage-related differences were observed, data from week 1 samples are summarized (Table 1 and Figure 4).

Endogenous mouse collagen X expression was restricted to the ES in mice by two of three mouse primer sets (M1s $\times$ M1a and M1s $\times$ M3a; Table 1 and Figure 4, A and B; lanes 13 and 14). This was in agreement with the generally accepted association of collagen X with EO in chicken, bovine, and human. In mice however, because

Figure 1. Co-expression of transgene product with endogenous mouse collagen X. Longitudinal tibial sections from week 3 collagen X Tg mice containing the constructs 4.7-21Δ (line 1-2, **A** and **G**), 4.7-293Δ (line 3-1, **B**; line 2-2, **C**), 1.6-293Δ (line 3-2, **D**), or 1.6-21Δ (line 5-1, **E**), and from wild-type (**F** and **H**) and collagen X null (**I**) mice were stained with antibodies against chicken (**A–F**) or mouse (**G–I**) collagen X. Note lack of cross-reactivity between chicken antibodies and mouse proteins (**F**), and mouse antibodies and noncollagen X mouse proteins (**I**). Mouse collagen X and transgene product co-localize to hypertrophic cartilage, where the staining is most concentrated within a strip of less mature hypertrophic cells. Tg mouse lines with the 1.6-kb promoter constructs (**D** and **E**) typically exhibit stronger staining than the lines with the 4.7-kb promoter (**A–C**). Scale bar, 100 μ m.

only limited *in situ* hybridization has been performed on skeletal tissue,¹³ these data for the first time confirmed the restricted expression of collagen X. The primer set (M1s $\times$ M2a) showed weak expression of the amplicon in several tissues, especially after blotting and hybridizing the agarose gels with a mouse collagen X probe (MX3' ⁷, Table 1). Screening of the 4.7-21Δ mice with chicken primers revealed restriction of transgene expression to the ES in two of three primer sets (C1s $\times$ C1a and C3s $\times$ C3a; Table 1 and Figure 4, C and D; lane 13); the third primer set (C2s $\times$ C4a) also appeared to yield an amplicon restricted to the ES, however hybridization with the chick transgene-specific probe (SpLX,³ Table 1) showed faint signals in other tissues. As with the M1s $\times$ M2a primer sets, this weak amplicon presence may represent cross-reaction with other molecules, especially because the other two mouse and chick primer sets yielded an ES-restricted expression pattern. In contrast, screening of 1.6-293Δ mice revealed multiple extraskeletal organs

positive for collagen X expression such as brain, skin, eye, lung, and kidney, depending on the particular primer set, and especially on hybridization (Table 1 and Figure 4, E and F). Moreover, two primer sets (C2s $\times$ C4a; C3s $\times$ C3a) that yielded an ES-specific product in the 4.7-21Δ mice, showed extensive extraskeletal expression in the 1.6-293Δ mice. It is noteworthy though that neither mouse or transgene collagen X transcripts were detected in these extraskeletal sites from 1.6-293Δ mice by *in situ* hybridization or Northern blot analysis, nor was mouse or transgene collagen X protein detected by immunohistochemistry (data not shown). Moreover, because the disease phenotype is indistinguishable in mice with either promoter construct, the possibility of increased promiscuity of the 1.6-kb promoter is not likely to be the cause of the observed murine defects. Taken together, these data identify a hypertrophic cartilage-specific 4.7-kb collagen X promoter, and suggest that the murine skeleto-hematopoietic defects are not a result of extraskeletal expres-

Figure 2. *In situ* hybridization localizing chicken collagen X expression to hypertrophic cartilage in chick sterna and tibiae in collagen X Tg mice. Longitudinal sections of embryonic day-18 chick sterna (A–C) and tibiae from newborn 1.6-293Δ (D–G) or 4.7-21Δ (H and I) mice. A, D, F, H: Control sections where probe was omitted. B, C, E, G, I: Sections hybridized with chicken collagen X anti-sense probe. F and G: Higher magnification view of sections comparable to those shown in D and E. A–C: Specificity of chick collagen X probe is depicted by restriction of hybridization to sternal hypertrophic chondrocytes (C) but not to proliferative chondrocytes (B), or to a hypertrophic chondrocyte control (A). E, G, I: Staining is restricted to hypertrophic chondrocytes (HC) in Tg mice with either 1.6-kb and 4.7-kb promoter, and is not detected in bone marrow (M), nor in comparable controls (D, F, H).

sion of endogenous collagen X and the transgene, nor of transgene misexpression.

Detection of Endogenous Collagen X or Transgene Product Expression in the Marrow

The variable hematopoietic abnormalities observed in all collagen X Tg and null mice involved different degrees of altered lymphocyte development, lymphopenia, and marrow hypoplasia.^{5,7,15} For this reason it was necessary to

Figure 3. Generation of species-specific mouse, chicken, and rat collagen X primers for RT-PCR. The coding region of the collagen X gene is shown with sense (s) and anti-sense (a) mouse (M)-, chicken (C)-, and rat (R)-specific primers indicated by arrows. NC2 = 5' amino terminal domain; Col 1 = central, triple helical collagenous domain; NC1 = 3' carboxyl terminal domain. Nucleotide designations of primers are as follows: M1s, 140 nucleotides; M1a, 560 nucleotides; M2a, 721 nucleotides; M3a, 1570 nucleotides (based on Ninomiya et al¹⁰); C1s, 78 nucleotides; C2s, 725 nucleotides; C3s, 1090 nucleotides; C4s, 1236 nucleotides; C1a, 725 nucleotides; C2a, 1236 nucleotides; C3a, 1581 nucleotides; C4a, 1693 nucleotides (based on Elima et al¹¹); R1s, 1547 nucleotides; R1a, 2035 nucleotides (based on Chung et al¹² and Kong et al¹³). In most cases, s and a primers could be interchanged to generate different size amplicons. Amplicon sizes for different primer combinations are as follows: M1s × M3a = 1.45 kb; M1s × M1a = 446 bp; M1s × M2a = 601 bp; C3s × C4a = 608 bp; C1s × C2a = 320 bp; C1s × C1a = 650 bp; C1s × C3a = 695 bp; C3s × C3a = 375 bp; R1s × R1a = 488 bp. Primer sequences and PCR conditions are listed in Materials and Methods.

establish if either endogenous collagen X or the transgene were expressed in the marrow and directly contributed to these defects, or whether the hematopoietic changes were a consequence of altered hypertrophic cartilage in the Tg and null mice. For this purpose, RNA from marrow stromal cell cultures of 1.6-293Δ mice and chick whole marrow aspirates was analyzed by RT-PCR using the primer sets described above (Figure 3). For marrow stromal cell cultures, only one mouse primer set (M1s × M2a) produced visible amplicon bands on agarose gels (Table 1; Figure 5A), whereas on hybridization with the mouse collagen X probe, all three mouse primer sets amplified mouse collagen X (Table 1 and Figure 5C). Chicken collagen X, corresponding to the transgene product, was detected on agarose gels and on hybridization with the chicken collagen X probe by only one of four chicken primer sets (C2s × C4a; Table 1 and Figure 5, B and D). Primer specificity for collagen X was confirmed by lack of amplicon in the ES from collagen X null mice (Figure 5C, lane 7); moreover, no cross-reactivity between mouse and chicken primers was detected because of the lack of signal from chick aspirate using mouse primers (Figure 5D, lane 10).

Northern blot analysis of the above and comparable samples detected message only in the ES from week 3 Tg mice for the transgene product (Figure 6B; SpLX, lanes 7 and 8) or endogenous collagen X (Figure 6C; MX3', lanes 7 and 8) at the expected molecular weights of 1.8 kb and 2.4 kb, respectively. In contrast to the RT-PCR data (Figure 5), marrow stromal cultures from 4.7-21Δ and 1.6-293Δ mice (Figure 6, lanes 3 and 4), as well as of chick marrow aspirates (Figure 6, lane 5) lacked collagen X expression.

Exclusion of Collagen X Expression in Rat Marrow

Conflicting data indicated both the presence, by RT-PCR (Figure 5), and absence, by Northern analysis (Figure 6),

Table 1. RT-PCR Results Using Species-Specific Collagen X Primers

Construct	1.6–293Δ						4.7–21Δ						1.6–293Δ							
	M1s × M1a		M1s × M2a		M1s × M3a		C1s × C1a		C2s × C4a		C3s × C3a		C1s × C2a		C1s × C3a		C2s × C4a		C3s × C3a	
Primers	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter	Gel	Filter
Endochondral skeleton	+	+	+	++	+	+	+	+	+	++	+	+	+	+	+	++	++	+-	+	++
Brain	–	–	–	+/–	–	–	–	–	–	+	–	–	–	+/–	–	+/–	++	+	+	+
Muscle	–	–	–	+/–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
Eye	–	–	–	–	–	–	–	–	–	–	+/–	–	–	–	–	+/–	+++	+++	+	++
Liver	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+/–	+	–	–
Lung	–	–	+/–	+	–	–	–	–	–	+	–	–	++	+	–	+/–	+	+	+	+
Thymus	–	–	+/–	+/–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+/–	+
Spleen	–	–	–	–	–	–	–	–	–	+/–	–	–	+	–	–	–	–	–	–	–
Kidney	–	–	–	–	–	–	–	–	–	–	–	–	++	+	–	+	+++	++	++	++
Heart	–	–	+/–	+/–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+	+
Skin	–	–	–	+/–	–	–	–	–	–	+/–	–	–	–	+/–	–	+/–	+++	++	+/–	+
Marrow (n = 4)	–	+/–	+	+	–	+	NA	NA	NA	NA	NA	NA	–	–	–	–	+/–	+	–	–
Chicken marrow	–	–	–	–	–	–	NA	NA	NA	NA	NA	NA	+	+	+	+	+++	+++	++	+++

immunohistochemistry (Figure 1),⁴ and *in situ* hybridization (Figure 2) of type X collagen message and protein in the bone marrow. We suspected that the positive collagen X signal resulted from contaminating trabecular bony spicules typically isolated with mouse marrow aspirates. In young mice, these spicules are normal constituents of marrows and are composed of hypertrophic cartilage cores with bone deposited on surfaces. To exclude trabecular contamination, week 3 rat tibia were sectioned and regions of the diaphysis with minimal trabecular bone were selected (Figure 7A; regions A, B, and C). RNA was isolated from aspirates of these isolated cross sections in the parallel tibia and analyzed by RT-PCR using rat-specific primers (Figure 7B).

RT-PCR analysis yielded the expected 488-bp amplicon in rat hypertrophic cartilage (Figure 7, B and C; lane 4), as well as in the ES of 1.6-293Δ Tg mice (Figure 7, B

and C; lane 3). The latter suggested cross-reactivity of the rat primers with mouse collagen X, which was anticipated because of our selection of homologous primers. No amplicons were generated from regions A to C (Figure 7, B and C; lanes 5 to 7). Northern blot analysis confirmed these observations (not shown). These data support the premise that contaminating trabecular spicules isolated with marrow aspirates likely contributed to the positive collagen X/transgene signal in the Tg mice (Figure 5), and that endogenous and transgene collagen X expression is restricted to hypertrophic cartilage. Moreover, these data strengthened our hypothesis linking defects in hypertrophic cartilage, resulting from hypertrophic cartilage-restricted expression of collagen X and transgene product, to the observed abnormalities in the marrow and lymphatic organs in the collagen X Tg and null mice.

Figure 4. Visualization by RT-PCR of endogenous mouse and chicken transgene collagen X in organs from control, collagen X Tg, and null mice. **A, C, E:** Agarose gels visualizing RT-PCR products with primers M1s × M3a (1.45-kb amplicon), C1s × C1a (650-bp amplicon; specific for either transgene construct) and C1s × C3a (695-bp amplicon; specific for transgene construct with 293 aa Δ). In all gels, note presence of amplicons (arrows) only in lanes corresponding to ES from 4.7-21Δ and 1.6-293Δ lines. **B, D, F:** Southern blot analysis of corresponding filters hybridized with the mouse collagen X probe mX3' (**B**),¹⁴ or with the chicken collagen X probe SpLX (**D** and **F**).³ In **B**, note restriction of hybridization signal (arrows) to lanes corresponding to ES from 4.7-21Δ and 1.6-293Δ lines, and in **D**, to ES from 4.7-21Δ line, confirming tissue specificity of the 4.7-kb chicken collagen X promoter fragment. In **F**, multiple transgene-positive organs include brain, skin, eye, lung, kidney, and ES from 1.6-293Δ line, and imply lack of tissue-specific expression in Tg mice carrying the 1.6-kb promoter fragment. Std = molecular weight standard; ES Null = ES from collagen X null mice; ES 4.7-21 = ES from 4.7-21Δ line; ES 1.6-293 = ES from 1.6-293Δ line; ES Wt = ES from wild-type mice.

Figure 5. Visualization by RT-PCR of endogenous mouse and chicken collagen X in whole marrow aspirates and marrow stromal cultures. **A** and **B**: Agarose gels visualizing RT-PCR products amplified with mouse primers M1s × M2a (**A**), or chicken primers C2s × C4a (**B**). **C** and **D**: Southern blot analysis of corresponding filters hybridized with the mouse collagen X probe mX3'¹⁴ (**C**), or with the chicken collagen X probe SpLX³ (**D**). In **A** and **C**, note detection of mouse collagen X in all marrow stromal cultures and ES samples from Tg 4.7-21Δ and 1.6-293Δ mice. Likewise, in **B** and **D**, note presence of chicken collagen X in comparable samples, as well as in chick whole marrow aspirates. Std = molecular weight standard; 4.7-21 marrow stromal cultures = from week 3 4.7-21Δ mice; ES Null, 4.7-21, or 1.6-293 = ES from week 3 collagen X null, 4.7-21Δ, or 1.6-293Δ mice; Ck marrow = embryonic day-18 chick whole marrow aspirates.

Discussion

Collagen X Tg and null mice display a disease phenotype that is variable in severity, but which includes skeleto-hematopoietic defects in all tissues derived by EO.^{1,5,7,15} We had previously established that in Tg mice, these skeleto-hematopoietic abnormalities resulted from Tg presence, because identical defects were seen in multiple lines, each with different transgene insertions.^{3,6} Moreover, our recent data demonstrated that the Tg murine phenotype likely resulted from dominant interference of the transgene product, which co-localized with endog-

enous collagen X in hypertrophic cartilage.⁴ We also observed structural differences in hypertrophic and proliferative cartilage, and identified glycosaminoglycans/proteoglycans (GAGs/PGs) whose growth plate distribution was altered; interestingly, some of these molecules have been implicated in establishing hematopoietic niches by sequestering cytokines and presenting them to hematopoietic stem cells and stromal components. Based on these data we proposed a provocative hypothesis linking the disruption of the collagen X-containing matrix to altered GAG/PG distribution and a potential locus for hematopoietic failure in the Tg and null mice.⁴ Specifically, we maintain that in growth plates, collagen X provides a structural framework surrounding hypertrophic chondrocytes in the form of a lattice-like network that is stabilized by PGs and GAGs. Disruption of collagen X results in the collapse of the lattice network and compartmentalization of the growth plate/marrow junction with respect to PGs/GAGs. We hypothesize that the collagen X/PG/GAG network sequesters hematopoietic cytokines, and that disruption of collagen X results in an imbalance in cytokine metabolism, which may be the cause of subsequent hematopoietic and immune defects. However, to provide credence to this hypothesis and to causally link these hematopoietic alterations to a primary defect in hypertrophic cartilage, it was essential to exclude any possibility of extraskeletal expression of either endogenous mouse collagen X, or the transgene, especially in the sites exhibiting the unexpected abnormalities. The data summarized in this study demonstrate by immunohistochemistry (Figure 1), *in situ* hybridization (Figure 2), Northern blot analysis (Figure 6), and RT-PCR (Figures 4 and 7), that the transgene is co-expressed temporo-spatially with endogenous collagen X in hypertrophic cartilage of the ES, and that neither endogenous collagen X nor the transgene are expressed in the af-

Figure 6. Northern blot analysis of endogenous mouse and chicken collagen X in mouse marrow stromal cultures, ES, and chick marrow aspirates. **A**: Agarose gel depicting total RNA. **B** and **C**: Filter hybridized first with the chicken collagen X probe (**B**, SpLX), and then stripped and rehybridized with the mouse collagen X probe (**C**, MX3'). Hybridization with SpLX yielded a 1.8-kb band (**B**, arrow) corresponding to transgene message, and with MX3' a 2.4-kb band (**C**, arrow) corresponding to full-length mouse collagen X message only in ES from 1.6-293Δ line. Std = molecular weight standard; 4.7-21Δ, 1.6-293Δ = marrow stromal cultures from week 3 mice; Ck marrow = chicken marrow aspirate from embryonic day-18 chicks.

Figure 7. Lack of collagen X expression in rat bone marrow. **A:** H&E and Alcian blue staining of a longitudinal section from week 3 rat tibia. GP, growth plate; TB, trabecular bone; M, marrow. **Dotted lines** corresponding to regions **A–C** represent diaphyseal zones in the parallel tibia from which marrow aspirates were taken for RNA isolation; note minimal trabecular bone in these regions. **B:** Agarose gel visualizing RT-PCR products amplified with rat primers R1s × R1a. **C:** Southern blot analysis of corresponding filter hybridized with the mouse collagen X probe mX3'.¹⁴ Note collagen X restriction to the ES 1.6-293Δ mouse line, and tibial growth plate cartilage in rat (GP), but not to marrow aspirates from tibial regions **A–C** in rat (**A, B, C** zone lanes). Std = molecular weight standard.

ected extraskeletal sites, including the marrow (Figure 7). Moreover, these studies characterize a 4.7-kb chicken collagen X hypertrophic cartilage-specific promoter that could be used for targeting transgenes to this site in mice.

The chicken, mouse, bovine, and human collagen X genes have been characterized, but to date, the mechanisms responsible for the tightly regulated expression of collagen X are only partially understood.^{16–18} The most extensive analyses of the 5'-regulatory elements have been conducted in the chicken, in which collagen X expression was demonstrated to be controlled by transcriptional mechanisms.^{19–21} Specifically, chicken collagen X gene regulation was shown to be under the control of multiple *cis*-elements within both the distal (–4442 to 558 bp from transcription start) and the proximal promoter region (–558 to +1).²¹ Proximal sequences directed high reporter gene activity in three cell types tested (hypertrophic chondrocytes, immature chondrocytes, and fibroblasts), and elements within nucleotides –557 to –513 were subsequently identified to be necessary for cell-specific expression of collagen X by hypertrophic chondrocytes.^{20,22–25} These proximal sequences were included in our transgene constructs containing either the 4.7- or the 1.6-kb promoter region. The distal elements acted in an additive manner repressing the effects of the proximal sequence on reporter gene activity in noncollagen X-expressing cells.²¹ Moreover, distinguishable elements mediating the effects of c-Raf and BMP were identified between nucleotides –2864 and –2410 of the distal promoter;^{26,27} these sequences were present in the transgene constructs containing the 4.7-kb fragment. Based on these data, we anticipated hypertrophic cartilage-specific transgene expression at least in the constructs containing the 4.7-kb chicken collagen X promoter and regulatory elements.

The data generated in this study are consistent with our initial expectations. Specifically, when multiple organs from mice carrying either the 4.7- or 1.6-kb collagen X promoter were screened by immunohistochemistry (Figure 1), *in situ* hybridization (Figure 2), and Northern blot analysis (Figure 6), transgene and endogenous mouse collagen X proteins and messages were detected only in hypertrophic cartilage, implying a tissue-specific expression from both promoters. However, when a more sensitive screening approach involving RT-PCR and Southern blotting was used (Table 1 and Figure 4), only the 4.7-kb promoter exhibited tissue-specificity, revealing transgene and mouse collagen X amplicons only in the ES samples. As predicted by studies of LuValle and co-workers,²¹ several organs were transgene-positive in Tg mice with the 1.6-kb promoter. However, because the collagen X Tg mice with either promoter construct exhibit a disease phenotype that is indistinguishable, it is unlikely that the possibility of increased promiscuity of the 1.6-kb promoter may result in the observed murine hematopoietic defects. A final concern was that both mouse collagen X and transgene amplicons were occasionally detected in marrow aspirates and stromal cell cultures by RT-PCR (Table 1, Figure 5). However, by screening marrow aspirates from rat tibia that were free of trabecular

bone, we demonstrated that collagen X was not expressed in marrow (Figure 7). Taken together, these data demonstrated that despite the species-specific differences in the 5' regulatory elements of the collagen X gene,^{16,17,18} the chicken collagen X promoter was functional in mice. Moreover, the 4.7-kb promoter fragment restricted transgene transcription temporospatially to that of endogenous collagen X. These data imply that the observed murine skeleto-hematopoietic defects result from transgene co-expression with endogenous collagen X in hypertrophic cartilage, and are not a result of extraskeletal expression of endogenous collagen X and the transgene, nor of transgene misexpression. This underscores an intimate but previously unforeseen link between EO and establishment of the marrow environment needed for subsequent hematopoiesis.

Once marrow forms as a result of EO, hematopoiesis occurs almost exclusively within endochondral bone. An understanding of how a marrow environment is established, what makes it preferential toward supporting hematopoiesis, and which skeletogenesis defects may contribute to marrow alterations, is essential for the diagnosis and treatment of hematopoietic diseases. Along these lines, the association of skeletal defects with altered hematopoiesis and immune dysfunction has been recognized, and the number of characterized immuno-osseous disorders is increasing. A few human disorders with potential relevance to the murine collagen X skeleto-hematopoietic disease phenotype include cartilage hair hypoplasia,²⁸ spondylo-mesomelic-acrodysplasia with severe combined immunodeficiency,²⁹ Schmid dysplasia,³⁰ Omenn phenotype with short-limbed dwarfism,³¹ Schwachman-Diamond,³² Dubowitz,³³ and Kyphomelic³⁴ syndromes, as well as aplastic anemia³⁵⁻³⁷ and Kostmann's syndrome involving congenital neutropenia.³⁸ However, despite these examples of immuno-osseous defects, it cannot be overlooked that humans with collagen X mutations, as well as several murine models that have altered EO, hypertrophic cartilage, and perhaps collagen X, have not been reported to have hematopoietic abnormalities.

Over 30 mutations in collagen X had been identified in humans with Schmid metaphyseal chondrodysplasia,^{39,40} an autosomal dominant skeletal disorder characterized by short stature, waddling gait, bowed legs, coxa vara, flared metaphyses of long bones,⁴¹ and occasional spinal involvement (eg, Japanese-type spondylometaphyseal dysplasia.⁴² To date, all reported mutations, with the exception of two, were localized to a highly conserved C-terminal NC1 domain of collagen X,^{4,43} and were proposed to affect collagen X trimerization and secretion (eg, causing haploinsufficiency, possibly with a dominant interference effect).^{4,39,44-46} Although the Schmid metaphyseal chondrodysplasia/spondylometaphyseal dysplasia patients shared specific skeletal defects and dwarfism seen in either the Tg or the null mice, hematopoietic defects or impaired immune function were not described in the affected individuals. Moreover, no null mutations, or defects affecting the triple helical domain, have yet been identified in humans. We speculate that a spectrum of abnormalities may ensue from muta-

tions in different collagen X domains, as is the case with type I collagen and osteogenesis imperfecta.⁴⁷ Based on the Tg mouse phenotype, certain triple-helical mutations might yield a more severe phenotype that may include hematopoietic defects. Along these lines, it is noteworthy that a dominant mutation was recently identified in domestic pigs that was identical to an amino acid substitution within the collagen X carboxyl domain that causes Schmid metaphyseal chondrodysplasia in humans.⁴⁸ The porcine defects mimicked those in Schmid metaphyseal chondrodysplasia patients and in particular, certain features in Tg mice. Specifically, the hypertrophic growth plate zone was hyperplastic with disorganized columns of hypertrophic chondrocytes with pycnotic nuclei. Moreover, areas of hemorrhage and necrosis were observed between trabecular spicules within marrows, consistent with the morphological manifestation of certain marrow changes in the Tg mice.^{5,48}

Likewise, in several existing murine models with defects in hypertrophic cartilage, hematopoietic defects have not yet been reported. One explanation may be that because a direct causal link between EO and hematopoiesis has not yet been established, until now there has been no precedence to look for such changes. Along these lines, we are analyzing selected models for hematopoietic defects resembling those in the collagen X mice, as well as are generating additional Tg mouse models. Specifically, we have used the 4.7-kb collagen X promoter to direct transgenes, including β -galactosidase and the proto-oncogene *c-myc*, to hypertrophic cartilage in mice. Our preliminary analyses of the β -galactosidase Tg mice confirmed the data presented in this study, namely that Lac Z expression was restricted to growth plates in whole mounts and hypertrophic chondrocytes in culture, and that the β -galactosidase Tg mice did not exhibit an obvious disease phenotype (Campbell MR, Tao Z, Beier F, LuValle PA, Jacenko O, manuscript in preparation). Moreover, histology of the *c-myc* Tg mice revealed both skeletal and hematopoietic changes distinct from those seen in the collagen X mice, but also involving the growth plate, trabecular bone, and marrow (Campbell MR, Tao Z, Kowalchuk D, Franklin A, McManus PM, Beier F, LuValle PA, Jacenko O, manuscript in preparation). Based on the data presented here on the collagen X mice, as well as on the above preliminary data, we propose that alterations in either hypertrophic cartilage, or in the molecules regulating the transition process of EO, could result in associated marrow and hematopoietic changes.

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