Linking Hematopoiesis to Endochondral Skeletogenesis through Analysis of Mice Transgenic for Collagen X

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Each skeletal element where marrow develops is first defined by a hypertrophic cartilage blueprint. Through programmed tissue substitution, the cartilaginous skeletal model is replaced by trabecular bone and marrow, with accompanying longitudinal tissue growth. During this process of endochondral ossification, hypertrophic cartilage expresses a unique matrix molecule, collagen X. Previously we reported that transgenic mice with dominant interference collagen X mutations develop variable skeletal-hematopoietic abnormalities, manifested as growth plate compressions, diminished trabecular bone, and reduced lymphatic organs (Nature 1993, 365:56). Here, histology and flow cytometry reveal marrow hypoplasia and impaired hematopoiesis in all collagen X transgenic mice. A subset of mice with perinatal lethality manifested the most severe skeletal defects and a reduction of marrow hematopoiesis, highlighted by a lymphocyte decrease. Thymic reduction is accompanied by a paucity of cortical immature T cells, consistent with the marrow's inability to replenish maturing cortical lymphocytes. Diminished spleens exhibit indistinct lymphatic nodules and red pulp depletion; the latter correlates with erythrocyte-filled vascular sinusoids in marrows. All mice display reduced B cells in marrows and spleens, and elevated splenic T cells. These hematopoietic defects underscore an unforeseen link between hypertrophic cartilage, endochondral ossification, and establishment of the marrow microenvironment required for blood cell differentiation. (*Am J Pathol* 2002, 160:2019–2034)

The vertebrate skeleton forms predominantly by endochondral ossification (EO), where the cartilaginous model of the axial and appendicular skeleton, as well as of certain cranial bones, is replaced by bony trabeculae and marrow. The distinctive feature of this process is

comprised of hypertrophic cartilage where EO initiates and collagen X is predominant.¹ Emergence of hypertrophic cartilage defines each skeletal element where marrow forms. Since the marrow provides niches for blood cell differentiation, alterations in the cartilage-to-bone and marrow transition of EO may affect stromal and hematopoietic constituents. We demonstrate here that mice transgenic (Tg) for collagen X develop both skeletal and hematopoietic abnormalities, and that the latter likely arise as a consequence of disrupted collagen X function. These data reveal an unforeseen link between endochondral skeletogenesis and establishment of the marrow microenvironment prerequisite for hematopoiesis.

During embryogenesis, EO initiates in cartilage with hypertrophy, and progresses by transforming a pre-existing non-calcified avascular cartilage to a calcifiable one permissive to vascularization.¹ Invading blood vessels import mesenchymal cells, hematopoietic precursors, and osteoclasts/chondroclasts. As osteoclasts/chondroclasts degrade hypertrophic cartilage, mesenchymal cells differentiate to primitive marrow cells and osteoblasts; osteoblasts line the hypertrophic cartilage cores and deposit osteoid in this primary ossification center. Replacement of maturing cartilage by bone and marrow, together with establishment of secondary ossification centers at outer (epiphyseal) tissue ends, defines the cartilaginous growth plates which provide bones with longitudinal growth potential until maturity. Thus, EO represents skeletal growth through deposition of bone on pre-existing hypertrophic cartilage; the resultant network of trabecular bony spicules protrudes into the marrow and likely provides hematopoietic niches.¹ The ultimate outcome of EO is the establishment of marrow,² resulting in blood cells colonizing spaces carved out from embryonic cartilage, and hematopoiesis ensuing almost exclusively within the endochondral bone.^{3,4} Thus, the newly formed marrow environment becomes critical for promoting hematopoietic progenitor cell proliferation, differenti-

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Figure 1. Phenotypic variability in collagen X Tg mice. **A** and **B**: Control and Tg mice week 3 after birth with perinatal lethality (**A**), or dwarfism (**B**). **A**: Perinatal lethality manifests in ~25% of Tg mice as thoracolumbar kyphosis, neck lordosis by X-ray, wasting, and death. **B**: Variable dwarfism manifests in the remaining ~75% of Tg mice. **C** and **D**: Dwarfed mice approach normal size by weeks ~8 to 12, but are predisposed to psoriasis-like ulcers (**C**, arrows), lymphatic tumors (**C**), and aggressive lymphosarcomas (**D**).

ation, and controlled egress into the lymphatics or systemic circulation.

The spatio-temporal restriction of collagen X to hypertrophic cartilage associates this matrix protein with fundamental events of EO namely, mineralization, matrix stabilization during remodeling, and vascular invasion.¹ To define its function, Tg mice were generated expressing defective collagen X variants.^{5,6} Transgene constructs for dominant interference contained chicken $\alpha 1(X)$ cDNA with in-frame deletions in regions encoding the central triple-helical domain; transgene expression was driven by different lengths of chicken collagen X promoter fragments.⁵ Transgene design assumed that homotrimeric collagen X subunits assemble through associations at the carboxyl-terminal domain, followed by their trimerization along the central triple-helical region to the amino terminus.^{1,5}

Tissue-specific transgene expression in hypertrophic cartilage⁵ yielded skeleto-hematopoietic defects in 14 Tg mouse lines. Phenotype severity in each line ranged from perinatal lethality to variable dwarfism and involved all EO-derived tissues. Skeletal deformities included growth plate compressions, diminished hypertrophy, and reduced trabecular bone.⁵ Here we describe that mice with the most severe skeletal defects exhibit marrow hypoplasia, lymphatic organ atrophy, altered lymphocyte development, lymphopenia, and perinatal lethality. Moreover, survivors exhibit subtle hematopoietic changes which include altered B and/or T cell profiles throughout life in marrows (Figures 3 and 4), thymuses (Table 1), and spleens (Figure 8), as well as occasional non-healing ulcerations (Figure 1C) and a predisposition to lymphosarcomas (Figure 1D). These skeleto-hematopoietic de-

fects likely result from the disruption of collagen X function, and imply that replacement of hypertrophic cartilage during EO establishes the required hematopoietic marrow environment.

Materials and Methods

Mouse Maintenance

Mice were maintained in a virus-free colony in micro-isolators, fed autoclaved Purina mouse chow (Animal Specialties and Provisions, LLC, Quakertown, PA) and water *ad libitum*, and from birth inspected daily for growth, behavioral, skeletal, or hematopoietic abnormalities. Weaning at day 21 included ear punching for identification and tail biopsy. Genotyping involved tail DNA isolation,⁷ phenol/chloroform purification, and genomic Southern blotting.⁵ Euthanization involved methoxyflurane exposure (Mallinckrodt Veterinary, Mundelein, IL).

Histology

Femur/tibia hind limbs, thymuses, and spleens were dissected from week 3 wild-type and Tg mice from lines 4200–21 and 1600–293⁵ with mild or perinatal-lethal phenotypes (8 to 12 animals per group). Tissues were either fixed for histology in 4% formaldehyde/phosphate-buffered saline (PBS) (pH 7.4) at 4°C for 1 week, or embedded for immunohistochemistry unfixed in Tissue Tek OCT (Miles, Elkhart, IN). Fixed samples were rinsed in deionized water (DI), limbs were decalcified (4% formalin, 1% sodium acetate, 10% EDTA), and dehydrated in ethanols. Following clearing with Propar (Anatech, Battle Creek, MI) and paraffin-embedding, 6- μ m longitudinal sections were heat-fixed onto slides and stained with Harris hematoxylin and eosin Y (H&E; Sigma Diagnostics, St. Louis, MO). Tibia/femur sections were stained with Alcian blue (pH 1.0)⁸ for sulfated glycosaminoglycans before H&E. Giemsa (Sigma Diagnostics) was used to stain hematopoietic cells. To quantitate differences in the length of trabecular bony spicules in the tibial/femoral sections, measurements were taken from the end of the hypertrophic cartilage zone to the end of trabecular bone in the marrow, within the center of each tibial growth plate.

For immunohistochemistry, 6- μ m cryosections of thymuses and spleens were acetone-fixed (1 minute), rinsed in PBS (15 minutes, 3 changes), treated with 0.3% H₂O₂/PBS (10 minutes for spleen, 5 minutes for thymus), rinsed in 4% heat-inactivated newborn calf serum (NCS) (Sigma) in PBS (NCS/PBS; 9 minutes, 3 changes), pre-incubated in NCS/PBS (30 minutes), and reacted with primary anti-mouse antibodies in NCS/PBS (1 hour at 23°C). Primary antibodies (10 μ g μ l⁻¹) from BD PharMingen (San Diego, CA) included: CD3e (hamster IgG; thymocytes and mature T lymphocytes), CD4/L3T4 (rat IgG2a, κ ; thymocytes and T helper cells); CD8a/Ly-2 (rat IgG2a, κ ; thymocytes and T suppressor/cytotoxic cells); CD45R/B220 (rat IgG2a, κ ; all B cells), TER-119 (rat IgG2b, κ ; erythroid lineage), and purified rat IgG2a, κ and IgG2b, κ

isotype controls; MOMA antibodies (supernatant; 1:25 dilution; rat IgG2a; macrophage subpopulations which in spleen surround the periarteriolar lymphatic sheath) were from Serotec Ltd. (Kidlington, Oxford, England). After rinsing with NCS/PBS (4 changes, 8 minutes), sections were reacted (30 minutes at 23°C) with biotinylated secondary anti-rat or anti-hamster IgG antibodies ($5 \mu\text{g } \mu\text{l}^{-1}$; Vector Laboratories, Inc., Burlingame, CA), and rinsed in NCS/PBS (4 changes, 12 minutes). Peroxidase activity was visualized by reacting sections in Vectastain ABC reagent containing avidin and biotinylated horseradish peroxidase (Vector Laboratories, Inc.; 30 minutes), rinsing in PBS (4 changes, 8 minutes), and incubating in diaminobenzidine tetrahydrochloride solution (Pierce, Rockford, IL; 3 to 5 minutes). Sections were rinsed in distilled water, air-dried, and mounted with Aqua-mount (Lerner Laboratories, Pittsburgh, PA). *tnT*-mediated dUTP-X nick end-labeling (TUNEL) for apoptosis involved an *In Situ* Death Detection kit, POD (Roche Molecular Biochemicals, Indianapolis, IN). Exceptions to manufacturer's protocol included omission of Triton X-110 incubation, and extended reaction in Converter-POD (60 minutes). Acridine orange/ethidium bromide staining for necrosis⁹ included minor modifications; cryosections were rinsed in PBS, stained with the dye mix (5 seconds), washed in PBS, and analyzed under fluorescence optics. Sections were viewed with an Olympus BX60 light microscope with a Photomicrographic System PM20 (Olympus America, Inc., Lake Success, NY).

Bone Marrow Cell Isolation and Differential Analysis

Bone marrow was isolated by flushing dissected tibiae and femora with calcium/magnesium-free Hanks' balanced salt solution (HBSS; Gibco BRL, Life Technologies Inc., Grand Island, NY) through a 26G5/8 Becton Dickinson needle. Marrow cells were dispersed first by manual agitation and then by gentle vortexing. For some flow cytometric studies, erythrocyte lysis solution (0.17 mol/L Tris, 0.16 mol/L NH_4Cl) was added to cell suspension (2 ml lysis/5 ml suspension) and incubated at room temperature (2.5 minutes). Cells were then pelleted (1500 rpm for 10 minutes at 4°C) and resuspended in HBSS. Cell concentration for all organs was determined by Bright-Line Reichert-Jung hemocytometer (Hausser Scientific, Hershaw, PA).

Bone marrow suspensions were cytocentrifuged using a Wescor, CytoproTM 7620 and stained with Harleco, Wright-Giemsa stain. Myeloid to erythroid ratios and lymphocyte percentages were determined by enumeration of 500 cells/smear/mouse. A single observer (P.M.M.) performed all counts using an Olympus BX40 light microscope at 1000 $\times$ magnification.

Flow Cytometry

Bone marrow was isolated as detailed above. Spleens and thymuses were cut into ~50-mm pieces, placed into

2 ml of Tenbroeck Tissue Grinders (Wheaton, Millville, NJ) containing HBSS. The plunger was lowered, turned 360°, and lifted twice for spleens and once for thymuses. Cell suspensions were removed, tissue fragments resuspended in HBSS, and subjected to an additional two to three plunges. Twice-pooled cell suspensions were gently vortexed, settled on ice (5 minutes), aliquoted from tissue particles, pelleted twice (1500 rpm for 10 minutes at 4°C), resuspended in HBSS, and counted by hemacytometer.

Cells ($10^6/100 \mu\text{l}$ HBSS) were single-labeled in polystyrene tubes with $20 \mu\text{l}$ of ($10 \mu\text{g ml}^{-1}$) primary antibodies including CD138/Syndecan-1 (IgG2a, κ ; precursor B cells), CD106 (IgG2a, κ ; bone marrow stromal cells and myeloid cells), CD11b (IgG2b, κ ; macrophages, dendritic cells, and granulocytes), IgM and IgD (IgG2a, κ ; immature and mature B cells, respectively), as well as those listed above (all from BD PharMingen), in fluorescence-activated cell sorting (FACS) buffer (0.4% BSA/PBS for 30 minutes at 4°C). After rinsing with FACS buffer and pelleting (1500 rpm for 5 minutes at 4°C), cells were resuspended in $100 \mu\text{l}$ of FACS buffer, and incubated in $20 \mu\text{l}$ of ($5 \mu\text{g ml}^{-1}$) FITC polyclonal anti-rat IgG (BD PharMingen; 30 minutes at 4°C, covered). For IgD/B220 and IgM/B220 double-labeling, $20 \mu\text{l}$ ($10 \mu\text{g ml}^{-1}$) of directly conjugated PE-B220 or PE-rat IgG2a, κ isotype control (BD PharMingen) were incubated with $20 \mu\text{l}$ ($5 \mu\text{g ml}^{-1}$) of the secondary FITC anti-rat IgG (30 minutes at 4°C, covered). Likewise, for CD4/CD8 double-labeling, $20 \mu\text{l}$ ($10 \mu\text{g ml}^{-1}$) of directly conjugated PE-CD8a or PE-rat IgG2a, κ isotype control were incubated with secondary FITC anti-rat IgG. After rinsing and pelleting as above, cells were resuspended in 0.5 ml FACS buffer. Propidium iodide (PI; BD PharMingen; $10 \mu\text{l}$ at $50 \mu\text{g ml}^{-1}$) was added 5 minutes before sorting for dead cell exclusion.

The FACSCalibur was used with CELL Quest 3.1 (Becton Dickinson, Mansfield, MA) for data acquisition and analysis. Live cells were visualized on dot plots where $X = \text{FSC}$ and $Y = \text{PI fluorescence in Log}$; 10,000 PI negative events were acquired for each antibody or corresponding control, and lymphocytes, identifiable by low side-side scatter (SSC) and increasing forward-side scatter (FSC) distribution, were gated. Gates were consistent between antibodies for each organ sample, and corresponded to fluorescence peaks on histogram plots ($\sim 10^2$ to 10^3). For B220, strong ($\sim 10^2$ to 10^3) and weak ($\sim 10^1$) fluorescence peaks were gated. Erythroblasts were calculated within the live cell distribution first as the percentage of lymphocytes, multiplied by the percentage of Ter+ live lymphocytes, and divided by 100. Fluorescence of isotype controls was subtracted, and the percentage of antibody-positive cells was recorded relative to live total cells and lymphocytes. For double-labeling, compensation between FITC, PI, and PE channels was required, following which 10,000 cells were acquired. Data were analyzed on dot plots formatted to include either live total cells or lymphocytes, where $X = \text{FITC}$ and $Y = \text{PE}$.

Results

Phenotypic Variability in Collagen X Transgenic Mice

Five homozygous Tg lines with independent transgene insertions were selected for analyses based on breeding ability. These lines were originally established on C57Bl/6 $\times$ SJL $\times$ D2 strains,⁵ and were subsequently backcrossed several generations into the C57Bl/6 background. The four transgene constructs were represented, and contained chicken $\alpha 1(X)$ cDNA with in-frame deletions encoding either 21 or 293 amino acids (aa) within the triple-helical domain, combined with either a 4.9-kb or 1.6-kb chicken collagen X promoter and upstream elements. Both promoter fragments co-expressed the transgene with endogenous mouse collagen X in hypertrophic cartilage,⁵ with no mis-expression in lymphatic organs (M. Campbell, C. Gress, O. Jacenko, manuscript in preparation). In each line, regardless of transgene copy number,⁵ similar skeleto-hematopoietic abnormalities ensued. These data implied that the observed murine disease phenotype is a consequence of transgene expression in hypertrophic cartilage.

Typically, Tg pups were indistinguishable from chronologically equivalent controls until 2 to 3 weeks after birth, when $>25\%$ (1220 mutants of 4428 mice; 5 lines) developed perinatal lethality. Pups exhibited hunched backs and neck lordosis, low weight, lethargy, wasting, and death within 24 hours of the visible onset of abnormalities (Figure 1A; also see reference 5 for initial description of the skeletal phenotype). Lethality primarily occurred during this age, and corresponded to the pups' increased mobility and weaning. A similar demise was occasionally seen in mice over 3 months of age. Upon dissection, all wasting animals had pronounced red marrows and lymphatic organ atrophy. The surviving $\sim 75\%$ of mice exhibited transient and variable dwarfism, ranging from extreme where mice were approximately one-third the control weight (Figure 1B), to barely distinguishable. Visually, dwarfism appeared to persist until approximately weeks 8 to 12. This murine subset had a normal life span, but by ~ 6 months mice were susceptible to skeletal deformities, along with hematopoietic and suppressed immunity-related changes including chronic hyperplastic dermatitis (Figure 1C) and aggressive lymphosarcomas (Figure 1, C and D). Tumors usually involved mesenteric lymph nodes, small intestine, spleen, liver, kidney, and lungs. Occasional abnormalities also seen in both murine subsets included tooth malocclusion, hydrocephaly, and microphthalmia.

Skeletal Defects

In each transgene-positive mouse, similar skeleto-hematopoietic changes ensued in EO-derived axial, appendicular, and cranial skeletal elements.^{5,10} Through our analysis of a large number of mice over several years, a correlation became evident between the severity of histological defects and the outward murine phenotype (Figure 2). For example, tibial growth plates of all Tg mice were compressed compared to those of controls, periosteal and trabecular bones were osteopenic, and long bones exhibited mild metaphyseal flaring (Figure 2A). Growth plate compressions first occurred 2 to 3 weeks after birth, coinciding with the establishment and maturation of secondary ossification centers and the growth plate, as well as with the animal's increased mobility. Although these compressions involved all growth plate zones, the one most affected was hypertrophic cartilage, where reduction was apparent in cell size and number (Figure 2B), and nuclei were often picnotic. This was contrary to the defects seen in the surviving subset of collagen X null mice, where the growth plates were only slightly thinner than those of controls, and where the zone most affected comprised the proliferative chondrocytes.^{11,12} These growth plate differences, however, could be attributed to the pathogenic mechanisms determined by the collagen X mutations (eg, dominant interference *versus* gene inactivation).^{12,13} Moreover, it is noteworthy that the hypertrophic cartilage zone that was particularly affected in the Tg mice corresponded to the site of co-expression of both the endogenous collagen X and the transgene product⁵ (M. Campbell, C. Gress, O. Jacenko, manuscript in preparation), implying that this likely represents the site of the primary defect in the Tg mice.

Trabecular bony spicules, comprised of hypertrophic cartilage cores with osteoid on surfaces, were also reduced in number and length, with the least trabeculae present in mice with the most severe phenotypes (Figure 2, A and B). Specifically, when measurements were taken within the center of each tibial growth plate, starting with the end of the hypertrophic cartilage zone to the end of trabecular bone in the marrow, trabecular spicules in the wild-type controls (311.11 ± 26 ; $n = 8$) were longer than those in Tg mice with mild (250.00 ± 16 ; $n = 8$) or the severe, perinatal-lethal (168.57 ± 34 ; $n = 7$) phenotypes. Moreover, in the most severely affected mice, generalized osteopenia included the periosteal, intramembranously derived bones as well. It is noteworthy however, that gross perturbations in mineralization were not observed in the Tg mice. Previously we had reported minor alterations, including decreased Alizarin red S staining in Tg sections and whole mounts⁶ and subtle differences in

Figure 2. Tibial longitudinal sections from control and collagen X Tg mice with mild (TG⁺) or severe (TG⁺ perinatal-lethal) phenotypes at week 3 after birth. **A:** Alcian blue/H&E staining reveals growth plate (GP) compressions, trabecular bone (TB) reductions, and metaphyseal flaring in all Tg mice. Marrows (M) of perinatal-lethal mice exhibit erythrocyte (red) predominance and leukocyte (purple) paucity. Scale bar, 1 mm. **B:** Metaphyseal regions from **A**. Note the correlation between outward disease severity and histological defects including pycnotic hypertrophic chondrocyte (HC) nuclei, diminished trabecular bone, and red marrows. Scale bar, 100 μ m. **C:** Giemsa staining of sections in **A** and **B** reveals marrow aplasia in perinatal-lethal mice, manifested as a depleting hematopoietic compartment (blue). Scale bar, 100 μ m.

the quality of the mineralized matrix, as observed by Fourier transform infrared spectroscopy.¹⁴ Most recently we described an occasionally altered mineral distribution around hypertrophic chondrocytes, which is the likely consequence of the loss of structural integrity in hypertrophic cartilage.¹³

Although these skeletal changes were most prominent in the rapidly growing week 3 mice, similar defects also persisted throughout life. For example, histology of long bones and vertebrae from aging Tg mice (~6 months to 1 year) showed persistent osteopenia and marrow hypoplasia (exemplified by a fatty, rather than a red, marrow described below), as well as osteoarthritis-related changes (data not shown).

The last abnormality that involved all EO-derived skeletal elements was immediately obvious upon dissection, and included readily discernible erythrocyte-filled marrows in mice exhibiting perinatal lethality (Figure 2C).

Marrow Defects

Marrow changes were manifested histologically as an erythrocyte predominance and reduction of leukocytes in the most severely affected mice (Figure 2A). This is characteristic of marrow hypoplasia, and was evidenced as a depletion of the hematopoietic compartment in perinatal-lethal mutants (Figure 2C). The temporal onset of marrow hypoplasia correlated with the metaphyseal skeletal defects, which became histologically apparent once the secondary ossification centers formed around weeks 2 to 3 (data not shown). In the surviving Tg mice, marrow changes were not visually obvious in tibial sections (Figure 2C).

In all week 3 Tg mice, a reduction was seen in the overall number of nucleated cells flushed from the marrow (Figure 3A). Moreover, a differential analysis of marrow smears revealed close to a twofold decrease in the percentage of lymphocytes in both the surviving Tg mice, and those exhibiting perinatal-lethality (Figure 3B). In the myeloid:erythroid ratio, there were substantial variations among animals; nevertheless, an overall increase was observed in all Tg mice when compared to controls (Figure 3C). In most marrows of Tg mice exhibiting perinatal lethality, few to moderate numbers of atypical late stage erythroblasts were also detected. Morphological changes were suspicious for apoptosis and included chromatin karyolysis and nuclear fragmentation. Macrophage phagocytosis of intact granulocytes and erythroblasts were noted in association with these changes and likely represent an attempt to remove dying cells. Other morphological abnormalities included asymmetric mitotic figures, siderocytes, and sideroblasts. These alterations suggest disruption in normal maturation and ineffective iron utilization by erythroblasts. Comparable changes were never observed in marrows from wild-type controls or in the surviving subset of Tg mice.

Leukocyte depletion in the marrows of the collagen X Tg mice was further confirmed by flow cytometry (Figure 4). Specifically, decreases in B220-positive B lymphocytes were seen in all Tg mice by around week 3 after birth, and persisted throughout life (Figure 4A). This re-

Figure 3. Differential analysis of marrow aspirates from week 3 wild-type (WT), and collagen X Tg mice with mild (Tg) or perinatal-lethal (Tg Mut) phenotypes. **A:** Comparison of total number of live nucleated cells reveals close to a twofold decrease in both the Tg and Tg Mut bone marrows. **B:** Determination of lymphocyte percentage by enumeration of 500 cells/smear/mouse likewise reveals a similar reduction in the collagen X mice. **C:** A comparable analysis of myeloid: erythroid ratios reveals large variations between animals (note large error bars and *P* values, below), although implies an overall increase in the Tg and Tg Mut marrows. Statistical analysis using the two-tailed *t*-test assuming two-sample unequal variance, indicates Tg and Tg Mut for **A** at *P* ≤ 0.001; for **B** at *P* ≤ 0.01; and for **C** at *P* ≤ 0.4. Error bars, SE; animal number is indicated over error bars.

duction was most pronounced in the perinatal lethal mutants at week 3 where B cell levels were less than half those seen in controls; likewise, another conspicuous decrease was observed in mice at 6 months and older (Figure 4A). The decrease in B220-positive cells corresponded to a diminution of B cell precursors, as indicated by staining for CD138/syndecan-1-positive cells (Figure 4A), and of immature B cells, as indicated by staining for IgM-positive B cells (Figure 4, B and C). Specifically, the temporal profiles of the CD138 and IgM-positive B cells mirrored that of B220, with the greatest impact felt by pups around week 3 after birth, or by mice at 6 months or older. A similar, yet not as pronounced, reduction was seen in the temporal profile of the more mature IgD-positive B cells (Figure 4, B and C); however, since this subset of mature B cells only represents a small percentage of the total marrow B cells, and are only transiently present in the marrow, it is more difficult to assess changes in this population while dealing with a variable phenotype. In general, for most B cell markers, mice at weeks 1 and 2 generally did not show significant B cell reductions (Figure 4, A–C); this was not unexpected since histological changes were also not detected in either the chondro-osseous junctions or marrows before weeks 2 to 3.¹³ However, the fact that marrows of 6 to 12 week mice showed only subtle

changes in B cell levels may be noteworthy; the cause of this is unclear, but may be related to the onset of sexual maturity. Moreover, it should be noted that most mice in this age group were generally healthy, with no overt examples of wasting or tumor formation.

Regarding other marrow cell types, no significant differences were observed by flow cytometry throughout life for the marrow stromal and myeloid cell marker CD106, and for the macrophage/dendritic cell/granulocyte marker CD11b (data not shown). Preliminary flow cytometric staining for TER-119 showed an increase in erythroblasts in week 3 mice, particularly in the perinatal-lethal mutants. However, at this time it is unclear whether the increase in the percentage of TER-119-positive cells is due to altered erythropoiesis, or to the relative decrease of B cells in the marrow compartment.

In summary, the marrow differential analyses and flow cytometry revealed that B lymphocytes at all stages of development remained somewhat lower in marrows of Tg mice throughout most of their life, compared to their chronologically equivalent controls (Figures 3 and 4). Altered lymphopoiesis was also reflected by a reduction of thymic T cells in perinatal-lethal mutants at week 3 (eg, Figure 5 and Table 1), by changes in splenic B and T cells in all Tg mice throughout most of their life (eg, Figure 8), as well as by a lymphocyte reduction in peripheral blood coupled with an elevation of polymorphonuclear leukocytes.¹⁵ Taken together, these data are consistent with the marrow's inability to provide an appropriate environment for lymphocyte differentiation upon the onset of hypoplasia.

Lymphatic Organ Defects

Lymphatic organs of mice exhibiting perinatal lethality depicted gross, histological, and hematopoietic defects. Thymuses and spleens were reduced compared to body and kidney, and spleens were discolored. Specifically, while the ratios of kidney-to-body weight remained constant in all mice, those of thymus-to-kidney (0.23:1) and spleen-to-kidney (0.22:1) weight were reduced in perinatal-lethal mutants, compared to the controls (0.52:1 for thymus; 0.90:1 for spleen). Lymph nodes were undetectable, likely due to dramatic size reduction. In less affected Tg mice, no gross changes other than subtle splenic reduction (0.57:1 spleen-to-kidney ratio) were noticeable. Moreover, in all collagen X Tg mice before weeks 2 to 3, the lymphatic organs were visually indistinguishable from those of controls. With the onset of growth plate compressions and marrow hypoplasia, a systemic reduction in lymphocytes likely caused a depletion of these cells from all lymphoid organs by week 3 (see below).

Thymus

In addition to size reduction, thymic architecture from week 3 mice with perinatal lethality was altered compared to controls, where the extensive cortex was densely populated with immature T lymphocytes (Figure

Figure 4. FACS of marrow aspirates from wild-type (WT), and collagen X Tg mice with mild (Tg) or perinatal-lethal (Tg Mut) phenotypes. **A** and **B**: Single-label FACS of marrow aspirates from mice ranging from 1 week, 2 weeks, 3 weeks, 6 to 12 weeks, 6 months, and 9 months+. **A**: An overall decrease in B220+ B lymphocytes is seen in Tg mice at all ages, as well as a reduction in CD138+ (syndecan-1+) B cell precursors. The most notable B cell lineage decline is seen in the 3 week Tg and Tg Mut mice, as well as in Tg mice at 6 months or older. Statistical analysis using the two-tailed *t*-test assuming two-sample unequal variance, indicates for B220 labeling: 3 weeks, 6 to 12 weeks, 6 months, and 9 to 15 months at $P \leq 0.0001$; 1 week and 2 weeks at $P \leq 0.2$; and for CD138 labeling: 3 week and 9 to 15 months at $P \leq 0.0001$; 6 months at $P \leq 0.01$; 1 week, 2 weeks, and 6 to 12 months at $P \leq 0.3$. **B**: A comparable decrease in early immature IgM+ B lymphocytes is revealed in Tg mice of all ages, but especially in Tg and Tg Mut mice at 3 weeks, and at 6 months and older. Reductions in more mature IgD+ B lymphocytes likewise are most pronounced at these time points, but are comparable to the control levels at 6 to 12 weeks. Statistical analysis for IgM labeling: 1 week and 3 weeks at $P \leq 0.0001$; 9 to 15 months at $P \leq 0.001$; 2 weeks, 6 to 12 weeks, and 6 months at $P \leq 0.04$; for IgD labeling: 6 months and 9 to 15 months at $P \leq 0.002$; 2 weeks at $P \leq 0.03$; 3 weeks and 6 to 12 weeks at $P \leq 0.5$. **C**: Double-label FACS with FITC-conjugated antibodies staining IgM (top panel; lower right quadrant) or IgD (bottom panel; lower right quadrant), and PE-conjugated antibodies labeling B220 (upper left) of week 3 mice. Note a pronounced decrease in the IgM+/B220+ cells (top panel; upper right quadrant) in the Tg and Tg mutant mice, and a subtle reduction in the more mature IgD+/B220+ cells (bottom panel; upper right quadrant). Error bars, SE; animal number is indicated over error bars in **A** and **B**. **C**: *N*, animal number.

Table 1. Week 3 Thymus Flow Cytometry

	Wild type	Col × Tg	Col × Tg mutant
No. of live cells	7.97 E7 ± 0.37 E7 (30)	4.00 E7 ± 0.55 E7 (20)	7.27 E6 ± 1.72 E6 (23)
% Lymphocytes	94.01 ± 0.58 (21)	95.82 ± 0.64 (12)	80.13 ± 1.82 (14)
% CD4 + lymphocytes	91.78 ± 0.79 (19)	91.67 ± 0.52 (21)	61.94 ± 2.70 (21)
% CD8 + lymphocytes	88.37 ± 0.73 (13)	86.80 ± 1.03 (19)	21.09 ± 1.53 (20)
% CD4/CD8 lymphocytes	85.57 ± 0.82 (5)	84.52 ± 1.1 (9)	5.27 ± 1.49 (14)

Numbers in parentheses indicate number of animals tested. Statistical analysis using the two-tailed *t*-test with two-sample unequal variance, indicates for collagen × Tg mutant values $P \leq 0.000001$.

5A). These cells acquire lineage identity in the marrow, and then migrate to the thymic cortex; on maturation, they progress to the medulla and some enter the circulation.¹⁶ In perinatal-lethal mutants, thymuses lacked a distinct cortex, which was virtually devoid of cells. This implied that as T cells progressed from the cortex, they were not replenished by marrow-derived progenitor or immature T cells; thus, as medullar T cells entered the circulation, organ size diminished. This scenario was supported by TUNEL assay (Figure 5B), which indicated reduced apoptosis in thymuses from perinatal-lethal mutants. Moreover, acridine orange/ethidium bromide staining (not shown) concurred that necrosis was not increased in these organs. Thus, in Tg mice exhibiting perinatal lethality, thymic reduction did not result from increased cell death, but likely from lack of progenitor cells/immature lymphocytes emigrating from the hypoplastic marrow. This hypothesis is supported by the reduced lymphocyte levels in bone marrows of these mice (Figures 3 and 4).

Immunohistochemistry and flow cytometry using T cell surface markers (Figure 5C; Table 1) confirmed an altered lymphocyte distribution in thymuses from perinatal-lethal mutants. Specifically, examination of the cortico-medullar junction in wild-type thymuses (Figure 5C, left panels) revealed a densely populated cortex with cells double-positive for CD4 and CD8, while in the medulla, only a subset remained double-positive, as depicted by patchy staining with either antibody. In contrast, in Tg mice with perinatal lethality (Figure 5C, right panels), only a subset of cortical lymphocytes stained for either CD4 or CD8, confirming an increase in single-positive cortical T cells. These data were confirmed by both single- and double-label flow cytometry (Table 1), where, for example, the percentage of CD4/CD8 double-positive T cells was reduced from ~85% seen in controls and Tg mice with milder phenotypes, to ~5% seen in Tg mice with perinatal lethality. As mentioned above, these data underscored the likely lack of replenishment by marrow-derived progenitors of the immature CD4/CD8 double-positive T cells, as they matured to single-positive medullar T lymphocytes.

In less affected Tg mice, the thymus did not appear to be perturbed, and the percentage of CD4 and/or CD8-positive cells was comparable to those of controls (Table

1). This was true for Tg mice at both earlier and later ages (eg, birth through several months of age; data not shown). The only exceptions included the mice that developed acute marrow hypoplasia and lethality at time points other than week 3, or aggressive tumors; T cell profiles in these animals mirrored those of the week 3 perinatal-lethal mutants. However, a decrease in the overall number of thymic cells was seen in all Tg mice at week 3 (Table 1) as well as in younger and older mice (not shown); this decrease was most pronounced in mice with perinatal lethality (Table 1). These data indicated a significant decrease in the overall number of T lymphocytes in all transgene-positive mice, regardless of phenotype severity, with most acute reductions seen in perinatal-lethal mutants.

Spleen

Altered splenic architecture in perinatal-lethal mutants was discernible as poorly defined lymphatic nodules, and a severely diminished red pulp (Figure 6,A and B). Immunohistochemistry (Figure 7) revealed disorderly MOMA-1 staining of macrophages surrounding periarteriolar lymphatic sheaths. CD3⁺, CD4⁺, and CD8⁺ T cell distribution in lymphatic nodules did not differ from controls, however nodule size and intranodular distance in mutant spleens were decreased, likely reflecting red pulp depletion. This implied a relative increase in the percentage of splenic T cells, which likely escaped the hypoplastic marrow effects and migrated to the spleen from the thymus. It is possible that the red pulp depletion may have ensued from splenic release of erythrocytes into vascular sinusoids of hypoplastic marrows (Figure 2), to compensate for the diminishing hematopoietic compartment. Splenic B cells from mutants stained diffusely with B220, and were dispersed throughout the red and white pulp rather than concentrated in lymphatic nodules as in controls (Figure 7). These mature B cells appeared functional, based on IgM (not shown) and IgD (Figure 7) staining. Finally, TER-119 labeling of cells in the erythroid lineage confirmed altered splenic architecture (Figure 7).

Flow cytometry confirmed a relative increase in splenic CD4⁺ T lymphocytes in all week 3 Tg mice, implying that

Figure 5. Histology and immunohistochemistry of longitudinal sections of thymuses from week 3 wild-type (WT) and collagen X Tg mice (TG) with perinatal lethality. **A:** H&E staining depicts differences in organ size, and depletion of cortical T cells in the TG thymus. Scale bar, 1 mm. **B:** Immunohistochemical localization of apoptotic cells by TUNEL in WT (**left**) and TG (**right**) thymus reveals decreased apoptosis in the perinatal-lethal mutant. Scale bar, 100 μ m. **C:** Immunolocalization of T cells in the thymic cortex (**left**)-medulla (**right**) junction reveals in Tg perinatal-lethal mutants a reduction of cortex and cortical lymphocytes in H&E-stained sections, and a depletion of CD4⁺/CD8⁺ immature T cells. Scale bar, 100 μ m.

Figure 6. H&E staining of longitudinal spleen sections from week 3 wild-type (WT) and collagen X Tg (mutant) mice with perinatal lethality. **A:** Low magnification reveals a diminished Tg spleen with reduced red pulp and altered architecture. Scale bar, 1 mm. **B:** Boxed-in zones in (A) show red pulp depletion and poorly distinguishable lymphatic nodules in Tg spleen. Scale bar, 100 μ m.

this cell population was likely least affected; in contrast, only slight increases in CD8⁺ cells indicated their reduction concomitant with that of the entire organ (Figure 8A). Similar trends were seen in spleens of Tg mice at younger and older ages (Figure 8A). Moreover, B cell reduction was seen in all Tg mice compared to controls from birth, and persisting throughout life (Figure 8B). This temporal profile of B220-positive cells reflected that seen in the marrow, especially with the most pronounced reductions seen in the week 3 mice, including the perinatal-lethal mutants, as well as in mice at 6 months and older (Figures 4 and 8B). Taken together, these data underscored the dependence of B cells on the marrow environment, and were consistent with the possibility that less B cells

were being released from marrows of Tg mice, leading to reduced splenic and peripheral B cell levels.¹⁵

Significant elevations in the relative number of splenic TER-119⁺ erythroblasts were seen in week 3 Tg mice with milder phenotypes ($70.38 \pm 1.62\%$, $n = 15$ vs. $46.31 \pm 2.29\%$, $n = 18$ in control), supporting extramedullary hematopoiesis. In perinatal-lethal mutants, these cells were reduced to control levels (40.15 ± 3.94 , $n = 12$). Only subtle increases in the erythroblast profiles were observed between Tg and control mice throughout various developmental ages (data not shown).

Finally, in all collagen X transgene-positive mice, spleen size and cell number were decreased (an approximate threefold reduction in Tg mice with mild pheno-

type and an approximate ninefold reduction in perinatal-lethal mutants when compared to wild-type controls; data not shown), as was the percentage of lymphocytes in perinatal-lethal mutants (an approximate threefold reduction). This was consistent with differential analysis of peripheral blood¹⁵ which confirmed lymphopenia in perinatal-lethal mutants.

Discussion

Our data demonstrate both skeletal defects and altered marrow and hematopoiesis in all Tg mice with dominant interference mutations for collagen X. Furthermore, growth plate^{11,12} and hematopoietic¹² abnormalities were also observed in the collagen X null mice (see reference 1 for review); some of these features, in particular the perinatal lethality and marrow hypoplasia in a subset of the null mice, mirrored the Tg mouse defects described here.¹² These two murine models directly implicate the disruption of collagen X function in hypertrophic cartilage to the observed skeleto-hematopoietic defects. Furthermore, the apparent interdependence between endochondral bone and hematopoiesis⁴ can now be extended to include hypertrophic cartilage, and thus underscores an intimate and unforeseen link between EO and marrow establishment.

The hematopoietic niches in the marrow are likely both spatial and chemical in nature.³ For example, the porous trabecular spicule network protruding into the marrow, the endosteal network lining the inner surfaces of the periosteal and trabecular bones that interface with the marrow, as well as the stromal cells, vascular cells, and various matrix components (eg, collagens, glycosaminoglycans (GAGs), proteoglycans (PGs), glycoproteins) may physically compartmentalize the marrow space. Likewise, associated bioactive components may include growth factors and cytokines, synthesized by resident cells or made elsewhere, and sequestered by the matrix. Moreover, interactions between the physical and diffusible factors may contribute toward the establishment of a marrow microenvironment supportive for hematopoiesis. For example, heparan sulfate PGs (HSPGs) have been proposed to orchestrate hematopoietic niches by sequestering cytokines and juxtaposing them with the marrow stroma to which they bind, and to hematopoietic progenitor cells.^{17–20} Below, four mechanistic scenarios are summarized that could explain how a physical alteration in collagen X and hypertrophic cartilage may ultimately affect either one or both the spatial and chemical components within the chondro-osseous junction, and thus contribute toward the observed hematopoietic abnormalities in the collagen X mice.

First, our data indicate that the tissue-specific co-localization of the transgene product with endogenous collagen X in hypertrophic cartilage¹³ is coincident with the histological defect observed in that zone (Figure 2). The overall growth plate phenotype is manifested as a compression, primarily involving hypertrophic cartilage, but also affecting proliferative cartilage, as seen in the collagen X null mice.^{11,12} Moreover, ultrastructural and immu-

nohistochemical analyses indicate that disruption of a network in the pericellular space around hypertrophic chondrocytes, likely comprised of collagen X, results in growth plate decompartmentalization.^{11,13} Although the *in vivo* configuration of collagen X supramolecular aggregates is still uncertain, abundant evidence implies that collagen X can assemble into a hexagonal lattice-like array.^{1,13,21,22} Thus, loss of structural integrity in the hypertrophic cartilage zone may represent the primary locus of the collagen X defect, and result in a re-distribution of matrix components such as the GAGs/PGs.¹³ These molecules likely associate with and stabilize the lattice, and may thus impact the spatial and chemical microenvironments of both the chondro-osseous junction and the marrow. Along these lines, we detected a modified growth plate distribution for free hyaluronan and HSPG.¹³ Both of these molecules have been implicated as key components of marrow niches required for hematopoiesis, and likely mediate their effects by regulating cytokine bioactivity and availability.^{17–20} It remains to be established whether collagen X supramolecular aggregates in the hypertrophic chondrocyte pericellular matrix require associations with specific classes of GAGs/PGs for stability and function, and if these interactions are needed for hematopoiesis.

Second, our histology (Figure 2) indicates a reduction in trabecular bone in the Tg mice, with the least amount of bony spicules present in mice with the most acute phenotype. The physical decrease in the length and/or amount of the highly porous trabecular spicules may directly alter the spatial arrangement of marrow into hematopoietic compartments, as well as decrease the surface area of bone-hematopoietic stem cell interactions.²³ Specifically, both the trabecular and periosteal bone surfaces are lined with a continuous membrane of bone-lining (endosteal) cells. These endosteal cells first provide a physical barrier between the osseous and the extracellular compartments, and second, have been directly implicated in regulating hematopoiesis.²⁴ Specifically, several reports describe an intimate association between generative hematopoietic stem cells and the endosteal surfaces.²⁴ The trabecular spicule reductions seen in the Tg mice may ensue as a direct consequence of a decrease in the hypertrophic cartilage matrix scaffold on which osteoblasts deposit bone; along these lines, our TRAP assays (unpublished) suggest that osteoclast activity is reduced, rather than increased.

An alternate explanation for trabecular spicule reduction and generalized osteopenia, which is most pronounced in the perinatal-lethal mutants, involves a chemical imbalance in these mice, and represents the third scenario that could lead to a skeleto-hematopoietic phenotype. Regulation of growth factor and cytokine activity and availability by PGs may be an important mechanism for locally controlling hematopoietic cell development.^{17–20} Moreover, our data suggest that interactions of growth factors and hematopoietic cytokines with GAGs/PGs in hypertrophic cartilage and/or trabecular bone may be impaired.¹³ Along these lines, our preliminary data (unpublished) show elevated levels of certain cytokines associated with cachexia or wasting in both the

Figure 8. Single-label FACS of splenic lymphocytes and erythroblasts from wild-type (WT), and collagen X Tg mice with mild (TG) or perinatal-lethal (TG Mut) phenotypes. Ages range from newborn (NB), 1 week, 2 weeks, 3 weeks, 6 weeks, 3 months, to 6 months+. **A:** An increase in CD4⁺ and CD8⁺ lymphocytes is seen in Tg mice at 3 weeks and older, with the largest increases in perinatal-lethal mutants and 6 week mice. Statistical analysis using the two-tailed *t*-test assuming two-sample unequal variance, indicates for CD4 labeling: 3 weeks at $P \leq 0.0001$; 6 weeks at $P \leq 0.001$; 3 months at $P \leq 0.01$; 2 weeks at $P \leq 0.06$; 1 week at $P \leq 0.5$; for CD8 labeling: TG Mut 3 weeks at $P \leq 0.0001$; 6 weeks at $P \leq 0.001$; 1 week, TG 3 weeks, 3 months at $P \leq 0.05$; 2 weeks, 6 months at $P \leq 0.15$. **B:** A reduction of B220⁺ B cells is seen in all Tg mice, with most pronounced reductions at 3 weeks. Statistical analysis for B220 labeling: 3 weeks, 6 months at $P \leq 0.0001$; 2 weeks at $P \leq 0.001$; 6 weeks, 3 months at $P \leq 0.05$. Error bars, SE; animal number is indicated over error bars.

collagen X Tg and knockout (KO) mice. These osteolytic cytokines may directly induce osteopenia, inhibit hematopoiesis, and cause marrow hypoplasia.^{23,25–28} Likewise, fluctuations in cytokine levels, or their overproduction, may be indicative of an inappropriate and overwhelming immune response to opportunistic infections, (eg,^{29,30}) and may contribute toward the acute phenotype in the collagen X mice. We are currently in-

vestigating the basis for the inappropriate cytokine production, whether it can be linked to the altered GAG/PG distribution in the chondro-osseous junction, and if it may lead to the variable perinatal-lethal disease phenotype. Phenotypic variability may also result from a modifier gene³¹ whose actions may be linked to collagen X; however, persistence of the variable phenotype after inbreeding the collagen X mice over 10 generations into several pure mouse strains strongly argues against this possibility (unpublished data).

Each of these three scenarios, either on their own or in conjunction with others, could compromise marrow-derived hematopoietic precursors, leading to impaired blood cell maturation, immune dysfunction, and in extreme cases, marrow aplasia. Interestingly, aplastic anemia has been associated with an abnormal marrow environment and a cytokine-mediated destruction of progenitor cells.^{26–28} The fourth scenario which may contribute to a skeleto-hematopoietic defect involves the unlikely possibility that collagen X may normally persist in the marrow stroma. If so, the extracellular collagen X supramolecular aggregates, which may bind and sequester hematopoietic GAGs/PGs,¹³ may persist after the demise of the chondrocyte and thus provide a compartmentalized hematopoietic environment in the marrow. Along these lines, we have detected collagen X in adherent marrow cultures by RT-PCR, although our data also indicated that this likely resulted from contaminating hypertrophic cartilage and trabecular bone remnants during marrow flushes. Moreover, we have ruled out transgene mis-expression, or presence of endogenous collagen X in any extra-skeletal sites (M. Campbell, C. Gress, O. Jacenko, manuscript in preparation). This implies that the hematopoietic abnormalities in the collagen X mice result as a consequence of the changes in hypertrophic cartilage.

One other possibility noteworthy of consideration is that the murine hematopoietic phenotype is a secondary consequence of the smaller skeletal size, and that other dwarfs, if analyzed comparably to the collagen X mice, would have similar hematopoietic changes. From literature review it is apparent that in mice, smaller skeletal size on its own will not lead to hematopoietic defects. Several examples of dwarfed mice that appear to have normal life spans with no obvious hematopoietic defects include mice null for membrane-bound metalloproteinase (MT1-MMP),³² collagen receptor DDR2,³³ hepatocyte nuclear factor-1 α ,³⁴ as well as aggrecan-defective (cartilage matrix deficiency (*cmd*)) heterozygotes.³⁵ Interestingly, in these mice, the hypertrophic cartilage zone has not been documented to be affected. Alternatively, a number of dwarfed mice have been reported to have variable disease phenotypes; several examples include mice null for ATF-2,³⁶ FGFR3,³⁷ natriuretic peptide,³⁸ Igf-1 and Igf1r,³⁹ cyclin D1 and p27^{Kip140} Tg mice for

Figure 7. Immunohistochemical localization of macrophages, T, B, and erythroid cells in longitudinal spleen cryosections from week 3 wild-type (WT) and collagen X Tg mice (TG) with perinatal lethality. Note in the Tg mutant: reduced MOMA staining for macrophages; unaltered distribution of CD3⁺, CD4⁺ and CD8⁺ cells, but a reduction in nodular size and intranodular space; diffuse B220 staining for B cells unlike their concentration in lymphatic nodules as in WT; presence of mature IgD-producing B cells; and altered splenic architecture by TER-119 staining of erythrocytes. Scale bar, 1 mm

COL2A1-directed SV40 T antigen,⁴¹ MAD,⁴² and the osteochondrodystrophy (*ocd*) mice.⁴³ Interestingly, most of these mice have alterations in their hypertrophic cartilage; we would speculate that they may also have hematopoietic alterations. Finally, it is also relevant that some of the best characterized dwarfed mice include the anterior pituitary dwarf mutants (eg, little, Snell-Bagg, Ames dwarf mice).^{44–46} Hematopoietic and immune defects in these animals have been extensively documented^{45,47,48} and in many aspects appear to mirror those seen in the collagen X mice. Moreover, several reports indicate a deficiency in thyroid-stimulating hormone production of thyroxine in the Snell dwarf mice.^{49,50} It is particularly interesting that thyroxine is required for proliferation of marrow B cells,⁵¹ and also acts directly on growth plate chondrocytes to promote hypertrophy.⁵² We are thus looking into the possibility that the Snell-Bagg dwarf mice represent an animal model with defects affecting the same cascade of interacting molecules as that altered in the collagen X mice.

Additional links between altered hematopoiesis and skeletal defects were also observed in mice overexpressing interleukin-4, where the defects appeared to mirror those seen in the collagen X mice, resided in the stroma, affected marrow-derived T cell precursors, and resulted in trabecular reductions and osteopenia.^{53,54} Other animal mutants with altered skeleto-hematopoiesis include mice with null alleles for *c-fos*,⁵⁵ *c-src*,⁵⁵ *TGFβ1*,^{56,57} *Bcl-2*,^{58,59} *NF-κB* subunits p50 and p52,⁶⁰ *NF-κB* inhibitors *IκBα* and *IκBβ*,⁶¹ *RANK*,⁶² and *Abl*,⁶³ as well as in Alaskan Malamute Dwarf dogs.⁶⁴ Moreover, in humans, the association of inborn defects of skeletal development and dysfunction of the immune system has been recognized, and the number of characterized immuno-osseous disorders is increasing. A few of potential relevance to our murine phenotype include spondylo-mesomelic-acrodysplasia with severe combined immunodeficiency,⁶⁵ Schimke dysplasia,⁶⁶ ADA deficiency with osseous changes,⁶⁷ Omenn phenotype with short-limbed dwarfism,⁶⁶ cartilage hair hypoplasia,^{68,69} Schwachman-Diamond,⁷⁰ Dubowitz,⁷¹ Kyphomelic⁷² and Kostmann's⁷³ syndromes, and aplastic anemia.²⁶

Despite these examples of immuno-osseous defects, it cannot be overlooked that several murine models have altered EO, hypertrophic cartilage, and perhaps collagen X, but no hematopoietic defects have been reported. One explanation would be that since a direct causal link between the endochondral skeleton and hematopoiesis has not yet been established, until now there has been no precedent to look for such changes. We are analyzing selected models for hematopoietic defects resembling those in collagen X mice, as well as generating additional Tg models with altered hypertrophic cartilage. It is noteworthy that we have already observed skeleto-hematopoietic changes in a few of these models, further supporting the skeleto-hematopoietic link (unpublished data). Moreover, we would speculate that alterations in either hypertrophic cartilage, or in the molecules regulating the transition process of EO, would result, at least to a certain extent, in associated marrow and hematopoietic changes.

The predominant phenotype of dwarfism with spondylometaphyseal involvement in collagen X Tg mice has already assisted in identifying COL10A1 mutations in humans with Schmid metaphyseal chondrodysplasia¹ as well as spondylometaphyseal dysplasia.⁷⁴ Neither of these disorders is associated with hematopoietic abnormalities, however both result from mutations predominantly in the COL10A1 carboxyl domain.^{1,74–76} Based on differences in the severity of the murine and human phenotypes (likely resulting from different disease mechanisms, including dominant interference in mice versus haploinsufficiency in humans with SMCD^{1,77–79}), we predict a phenotypic spectrum of skeleto-hematopoietic disorders caused by collagen X mutations. Specifically, it is conceivable that mutations in the triple-helix, or in molecules affecting the same cascade of events either upstream or downstream from collagen X, may yield skeleto-hematopoietic defects similar to those in collagen X Tg mice. Such disorders involving hematopoietic and immune systems have not been linked to defects in a molecule or pathways involved in skeletogenesis.

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