SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIALS AND METHODS

Chondrocyte RNA extraction

Fresh cartilage tissue was dissected free from hind limbs of 18.5 dpc embryos, flash frozen, and stored at -80°C. RNA extraction was carried out using a modified version of the RNeasy Mini Kit (Qiagen) protocol. Samples were thawed on ice and incubated in 600 µl of RLT buffer (provided in kit) for 30 minutes on ice. Cartilage fragments were disrupted and lysed by passing samples repeatedly through 18- and 21-gauge needles and mixing vigorously. The remainder of the preparation was carried out according to the manufacturer's protocol. Total RNA was resuspended in nuclease-free water and analyzed on a Nanodrop spectrophotometer for purity and RNA concentration determinations. cDNA was generated according to the manufacturer's instructions using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit.

Mouse and Chick Aggrecan Gene ExpressionAnalysis

Prior to performing quantitative real-time RT-PCR (qRT-PCR), a preliminary experiment was performed to select an appropriate endogenous control transcript by running samples from each genotype through a panel of commonly used "housekeeping genes", which revealed β-actin as the most reliable control transcript for analysis. qRT-PCR was carried out using SsoAdvanced Universal SYBR green Supermix (Bio-Rad) in the CFX96 Touch system (Bio-Rad). Each 20 ng cDNA sample was amplified in duplicate using either a chick-specific primer set (sequences below) or mouse-specific primer set (purchased from Bio-Rad) for the aggrecan coding sequence. At least five independent

embryos (or samples) were analyzed for each genotype. Data were analyzed by the $2^{-\Delta\Delta Ct}$ relative quantification method using β-actin as the normalizing control gene. Relative amounts of mouse and chick aggrecan transcripts were determined in five independent Agc^{17} transgenic mouse cartilage mRNA samples by qRT-PCR and standardized with respect to the expression of β-actin ($2^{-\Delta Ct}$) transcripts using the software LinReg PCR (Ruijter JM, et. al. Nucleic Acids Research 37(6): e45, 2009) to check for differences in efficiency of the individual primer pairs; none were detected. Chick-specific aggrecan primers: Forward 5'-CTCTCAGCGAGACCATCTGC-3' Reverse 5'-TGGGTGCTCTCCAGACTCAG-3'

Histological Calcium Detection

Von Kossa calcium staining was performed on dissected sterna fixed in 4% paraformaldehyde that was paraffin embedded and sectioned. Consecutive slides containing serial 5 μm sections were processed for von Kossa staining (counterstained with neutral red) and hematoxylin & eosin staining by the University of Chicago Clinical Pathology Facility.

FIGURE LEGENDS FOR SUPPLEMENTARY FIGURES

Supplemental Figure 1

Growth plate expression patterns of mouse aggrecan transcripts by mRNA *in situ* hybridization. Representative expression analysis of mouse aggrecan mRNA in 18.5 dpc tibia sections from (A) wild-type (+/+), (B) *cmd-bc* heterozygotes (cmd^{bc}/+), and (C) chick aggrecan transgenic embryos (Agc^{17}/+). *In situ* hybridizations with DIG-labeled probes were performed on 40 μm sections; cartilage elements are outlined for clarity.

Supplemental Figure 2

Gene expression analysis of mouse and chick aggrecan mRNA in embryonic growth plate cartilage. Quantitative real-time RT-PCR performed on RNA harvested from 18.5 dpc wild-type, cmd/+, Agc^{17}/+, cmd/+; Agc^{17}/+, and rescue (cmd/cmd; Agc^{17}/+) embryonic limb cartilage. RT-PCR was performed in duplicate for three experimental repetitions (n=5-10 embryos/genotype), using either chick-specific aggrecan (A) or mouse-specific aggrecan (B) primer sets across genotypes. Expression was normalized to β-actin and displayed as $2^{-\Delta\Delta Ct}$ relative to Agc^{17}/+ for chick aggrecan, and to wild type for mouse aggrecan. Data not shown for cmd^{bc}/cmd^{bc} embryos, which do not express any mouse or chick aggrecan mRNA transcripts. (C) mRNA gene expression of chick and mouse aggrecan transcripts ($2^{-\Delta Ct}$) normalized to β-actin in cartilage-derived RNA from five independent 18.5 dpc Agc^{17}/+ mice. *$p<0.004$, n= 5; paired t-test.

Supplemental Figure 3

Chondroitin-4-sulfate expression pattern analysis in embryonic femurs. Representative immunohistochemistry experiments using a monoclonal antibody against proteoglycan-4-sulfate (clone 2-B-6) are shown for distal femurs of 18.5 dpc embryos of each genotype from the same litter. The genotype of each embryo is labeled in the upper right corner of each panel and growth plates are outlined in white for clarity.

Supplemental Figure 4

Histological examination of mutant and rescue sternum growth plates. Representative serial sections of 18.5 dpc sterna stained with hematoxylin and eosin (H&E, top row) and von Kossa (bottom row) demonstrating chondrocyte morphology and calcium deposition for wild-type (+/+), mutant (cmd^{bc}/cmd^{bc}), and rescue (cmd^{bc}/cmd^{bc}; Agc^{17}/+) embryos. Asterisks (*) in the top row panels indicate cartilage segments remaining between sternebrae growth plates in wild-type and rescue embryos, which are absent in mutants (denoted by brackets, top middle panel). Images in the bottom row were taken following von Kossa staining for calcium deposits. Wild-type (left) and rescue (right) embryonic sterna show mineralization and calcification of cartilage in the centers of sternebrae III, IV, and V, indicating the commencement of bone formation. Note the decreased amount and irregular pattern of calcium deposition

in mutant embryonic sterna (middle panel), as well as abnormal cartilage tissue with negligible histologically visible matrix (top row, middle panel).