Appendix I

The Apparatus and Its Function

Compression across the tibial physes of the hind limbs of the animals was applied by means of an external loading apparatus (Fig. E-1) (see figure legend and article text for more detail) that was similar to that used by Stokes et al.[32]. Two parallel surgical pins (1.6-mm Kirschner wires) were passed percutaneously through the tibiae, proximal and distal to the physis. Each end of each pin was secured in a hole at the midpoint of a stainless steel block by means of a set screw. Stainless steel machine screws (4/40 × 44.5 mm) were passed through symmetric clearance holes in each block. A stainless steel compression spring, with an unloaded length of 9.5 mm (nominal) and a stiffness of 1.8 N/mm (nominal), was placed around the threaded end of each machine screw distal to the distal block and was held in place by two stainless steel nuts with an intervening lock washer. Compression of the springs, set by positioning the nuts and lock washer, was transferred by the blocks to the tibiae as compression across the physis.

Compressed lengths (L_c) of the four springs on each limb that were needed to apply the desired loads of 10 N and 30 N across the physis were determined from the stiffness of the springs (K = 1.8 N/mm [nominal]) and their unloaded length (L_o = 9.5 mm) [L_c = (Desired load/4K) – L_o]. Compressed spring lengths of 8.1 mm and 5.4 mm were used to apply the 10-N and 30-N nominal compressive physeal loads, respectively. Gauge blocks in these lengths were used to set the compressed length of each spring, and the spring length was checked with the gauges and reset at least twice weekly to negate any effects of nut loosening or creep deformation of the bone, bone-pin junction, or machine screw assembly. To assess any change in spring stiffness or creep deformation of the springs during the test periods, the stiffness and unloaded length of a small sample (n = 12) of the springs were determined before and after six weeks of constant compression to lengths of 8.1 mm and 5.4 mm, corresponding to the experimental 10-N and 30-N physeal loads, respectively. The analysis of covariance indicated that the spring stiffness (1.8 N/mm) was not significantly altered during these loadings ($p = 0.71$), but the length of the springs significantly decreased by 0.2 mm ($p = 0.04$), resulting in a 1.0-N decrease in the total compressive load across the physis over the six-week period of compression.

Appendix II

Additional Details of Specimen Preparation

Additional details of methodology for this study are described next.

Sample Preparation

Proximal tibial samples fixed in neutral buffered formalin were washed in phosphate buffered saline, decalcified in 20% buffered formic acid for approximately one week, and embedded in paraffin. A rotary microtome (RM2255; Leica Microsystems, Richmond,

Illinois) was used to section the blocks at 6-µm thickness. Sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania), deparaffinized, and rehydrated for subsequent histological staining or immunohistochemistry.

Immunohistochemistry

The Bond Intense R Detection System (Leica Microsystems) consists of stages of specimen treatment incorporating a peroxidase block, an open secondary, a streptavidin horseradish peroxidase conjugate to 3,3′-diaminobenzidine (DAB), and Mayer hematoxylin counterstain. Type-II collagen (1:1000 dilution) was processed according to a standard Leica protocol. The open secondary used was a MaxTag biotinylated anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania). A standard Leica protocol was revised to optimize binding of the type-X collagen antibody to rabbit experimental sections. The primary mouse anti-pig monoclonal antibody was diluted 1:500 and incubated for two hours at room temperature. Epitope retrieval followed the manufacturer protocol. Hematoxylin stain Gill 3 (Ricca Chemical, Arlington, Texas) was utilized as counterstain following DAB in type-X collagen processing. After counterstaining, sections were dehydrated and mounted with use of DPX (Sigma-Aldrich). All experimental staining procedures were accompanied by control slides omitting primary antibody binding to assess the level of nonspecific staining. Sections were examined and photographed as previously described. To compare DAB binding between sections of different specimens, all images were white-balanced for background and were maintained constant in hematoxylin staining intensity.

Gene Expression Analysis

Rabbit-specific primers for polymerase chain reaction analysis of aggrecan, MMP-13, type-II and X collagen, and β-actin were designed with use of the Primer Express Software program (Applied Biosystems) and (when possible) primers spanning at least one intron of the respective known gene sequences (Entrez Gene; National Center for Biotechnology Information [NCBI]). Designed primer sets listed in Table E-2 were verified with use of the BLAST (Basic Local Alignment Search Tool) program (NCBI). Appropriate negative controls, samples containing no reverse transcriptase, and buffer blanks were analyzed to confirm the absence of genomic DNA or DNA contamination in general[26,29-31].

An ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) was used for quantitative polymerase chain reaction analysis following defined parameters[29]. Amplicons were initially electrophoresed on DNA chips with use of an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) to verify single bands at correct molecular weights. Expression levels of aggrecan, MMP-13, and type-II and X collagen were normalized to the housekeeping reference gene, β-actin. Gene expression levels of compressed physes at two and six-week harvest time points were normalized to respective sham surgeries and means plotted with the respective standard errors[26,31].

Statistical analyses of gene expression of comparisons between loaded samples and unloaded shams were performed with use of analysis of variance and Tukey post hoc test. Significance was set at $p \leq 0.05$.

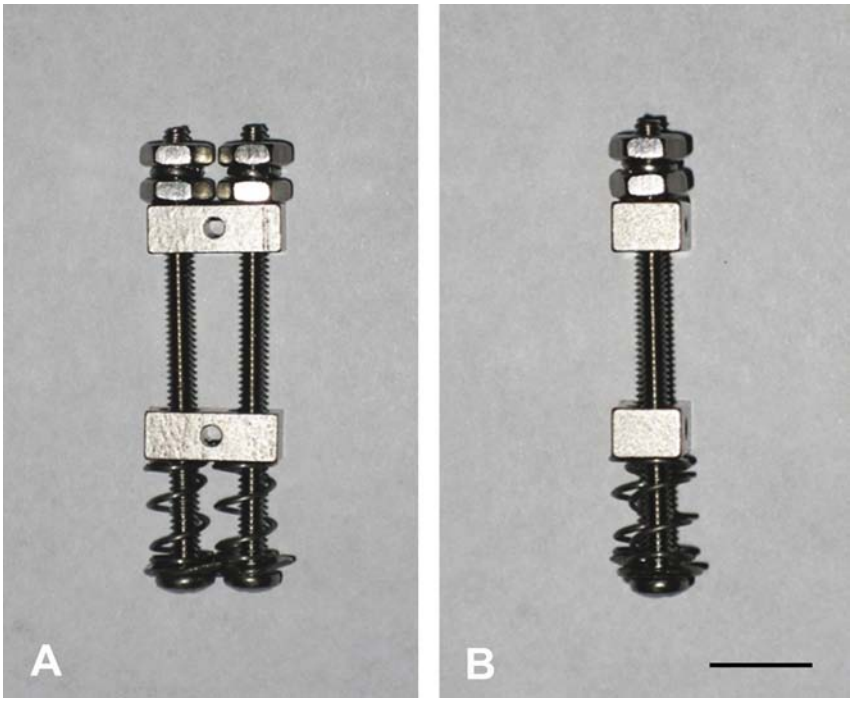

Fig. E-1

Lateral (**Fig. E-1A**) and anteroposterior (**Fig. E-1B**) photographs showing the external fixator device in detail. Spring compression was set by adjusting positions of nuts and lock washers along the length of the stainless steel screws. Bar = 1 cm for both panels at the same enlargement.

TABLE E-1 Experimental Design Outlining the Number of Rabbits Utilized and Proximal Tibial Samples Obtained by Group (Two or Six Weeks), Limb, and Mechanical Load Applied

		No. of Proximal Tibial Samples Obtained*		
		Limb 1		Limb 2
Group	No. of Rabbits	10 N	30 N	Sham
Two weeks	8	4	4	8
Six weeks	9	5	4	9

*Limbs 1 and 2 of the animals were randomized with respect to the left or right tibia. Sample sizes were chosen to allocate at least four rabbits for each treatment group to provide a reasonable estimate of the within-treatments variance. Because this was initial pilot research, no estimates of the variance in gene expression were available to allow a formal sample size determination by means of a power analysis.

TABLE E-2 Rabbit-Specific Primer Sequences

Oligo Name	Accession (Genbank)	Sequence 5′ to 3′	Melting Temperature *(°C)*	Amplicon Length (Base Pairs)
Aggrecan	L38480	F245 TGGAGGTCGTGGTGAAAGGT R305 AGGGTGTATCGCGTGGAGAT	82	61
β-actin	NM_001101683	F261 TGGCATCCTGACGCTCAA R317 TCGTCCCAGTTGGTCACGAT	84	57
Type-II Collagen	D83228	F362 ATGGCGGCTTCCACTTCAG R424 TCTGGACGTTGGCAGTGTTG	84	63
Type-X Collagen	AF247705	F186 CCACTGGGAAGCCAGGTTT R267 GGCCCAATGTCTCCTTTCG	84	82
MMP-13*	NM_001082037	F1472 GAGATGAAGACCCCAACCCTAA R1548 GGTAATGGCATCAAGGGATAAGG	79	77

*MMP-13 = matrix metalloprotease-13.