Osteoarthritis and Cartilage (2010) **18,** 88–96

doi:10.1016/j.joca.2009.11.006

Osteoarthritis and Cartilage

Phenotypic characterization of epiphycan-deficient and epiphycan/biglycan double-deficient mice[1]

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Summary

Objective: To characterize the *in vivo* role epiphycan (*Epn*) has in cartilage development and/or maintenance.

Methods: *Epn*-deficient mice were generated by disrupting the *Epn* gene in mouse embryonic stem cells. *Epn*/biglycan (*Bgn*) double-deficient mice were produced by crossing *Epn*-deficient mice with *Bgn*-deficient mice. Whole knee joint histological sections were stained using van Gieson or Fast green/Safranin-O to analyze collagen or proteoglycan content, respectively. Microarray analysis was performed to detect gene expression changes within knee joints.

Results: *Epn*-deficient and *Epn/Bgn* double-deficient mice appeared normal at birth. No significant difference in body weight or femur length was detected in any animal at 1 month of age. However, 9-month *Epn/Bgn* double-deficient mice were significantly lighter and had shorter femurs than wild type mice, regardless of gender. Male *Epn*-deficient mice also had significantly shorter femurs than wild type mice at 9 months. Most of the deficient animals developed osteoarthritis (OA) with age; the onset of OA was observed earliest in *Epn*/*Bgn* double-deficient mice. Message RNA isolated from *Epn*/*Bgn* double-deficient knee joints displayed increased matrix protein expression compared with wild type mice, including other small leucine-rich proteoglycan (SLRP) members such as asporin, fibromodulin and lumican.

Conclusion: Similar to other previously studied SLRPs, EPN plays an important role in maintaining joint integrity. However, the severity of the OA phenotype in the *Epn/Bgn* double-deficient mouse suggests a synergy between these two proteins. These data are the first to show a genetic interaction involving class I and class III SLRPs *in vivo*.

Key words: Osteoarthritis, Cartilage, Mouse, Microarray, Small leucine-rich proteoglycans.

Introduction

Epiphycan (*Epn*), a chondroitin/dermatan sulfate proteoglycan preferentially expressed in epiphyseal cartilage during embryonic development[1], is a member of the small leucine-rich proteoglycan (SLRP) family. SLRPs are extracellular matrix (ECM) molecules that contain a tandem array of leucine-rich repeat (LRR) motifs flanked by N-and C-terminal cysteines. Many SLRP core proteins are also substituted with glycosaminoglycan (GAG) chain(s), although the presence, nature, and size of the GAGs appear to be tissue-specific and age-dependent[2].

SLRPs are divided into three classes based upon the spacing of their N-terminal cysteines, the number of LRRs they contain, and their corresponding gene structures. Class I (decorin, biglycan, and asporin) and class II SLRPs

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Received 15 May 2008; revision accepted 15 May 2008.

(fibromodulin, proline/arginine-rich end leucine-rich repeat protein (PRELP), keratocan, lumican, and osteoadherin/osteomodulin) contain 10–12 LRRs, whereas class III SLRPs (EPN/PG-Lb/DSPG3, opticin, and osteoglycin/mimecan) contain 6–8 LRRs. Chondroadherin, NYX/nyctalopin, ECM2, podocan, and nephrocan contain both the N-terminal cysteine cluster and the LRR domain characteristics of SLRPs but are not assigned to a class.

SLRPs interact with a variety of ECM proteins. Reported binding partners include fibronectin[3], thrombospondin[4], fibrinogen[5], heparin cofactor II[6], complement component 1 q (C1q)[7–9], mannose-binding lectin[10], collectin 43[10], conglutinin[10], pulmonary surfactant D[11], and transforming growth factor-beta (TGF-beta)[12,13]. The most commonly reported binding partner for SLRP proteins is collagen[14]. Moreover, the targeted disruption of individual SLRPs can lead to obvious collagen fibril formation defects *in vivo*. However, single SLRP-deficient mice display relatively mild phenotypes when considering their tissue distributions; an observation that suggests some SLRPs may compensate for the loss of others. For example, lumican-null mice exhibit corneal clouding and skin laxity, but do not show significant alterations in tendon stiffness[15]. However, lumican loss-of-function enhances the reduced tendon stiffness seen in fibromodulin-null mice, suggesting that lumican compensates for the loss of fibromodulin[16]. This putative genetic interaction is particularly interesting in light of the fact that fibromodulin and lumican bind to a similar site on collagen[17].

Many different SLRPs are expressed in cartilage, including asporin, biglycan, chondroadherin, decorin, EPN, fibromodulin, keratocan, lumican, opticin, and PRELP[18–23]. However, the ability of SLRPs to influence osteoarthritis (OA) progression is not understood. Furthermore, increased SLRP degradation has been detected in human osteoarthritic cartilage samples[24]. Such degradation presumably leads to cartilage matrix destabilization. Recent reports have implicated one particular SLRP, asporin, with hip and knee OA in Asian populations[25,26]. A specific human polymorphism in the asporin gene, resulting in an N-terminal region that contains 14 aspartic acid residues rather than 13 aspartic acid residues, is significantly associated with individuals that have OA.

Very little is reported about class III SLRPs, particularly *Epn*. *Epn* expression appears to be restricted to cartilage and testis[27]. The unique tissue expression of *Epn* in chick[28] and mouse[27] cartilage suggests that this SLRP may play a key role in chondrocyte differentiation and, hence, cartilage stability. To further investigate this possibility, we generated and characterized *Epn*-deficient mice. These mice exhibit a very mild skeletal phenotype. Since biglycan (*Bgn*) is also expressed in mouse cartilage, and may compensate for the loss of *Epn*, *Epn/Bgn* double-deficient animals were also generated and characterized.

Materials and methods

GENERATION OF *Epn*-DEFICIENT AND *Epn*/*Bgn* DOUBLE-DEFICIENT MICE

An 8 kb fragment of the 129S6 mouse *Epn* gene was identified with a rat *Epn* probe and used to construct the targeting vector, which contained a *LacZ* expression cassette followed by a neomycin (*neo*)-resistance gene flanked by loxP sites. A MC-1 *thymidine kinase* cassette was included immediately outside the homologous mouse sequence of the targeting vector.

Mouse AB2.2 embryonic stem cells (ESC; a gift from Allan Bradley) were electroporated with the linearized targeting vector described above using a BioRad Gene Pulser and grown in M15 media containing G418 to select for cells containing the *neo*-resistance gene. The targeting vector was designed to insert the *LacZ* cassette six base pairs downstream of and in frame with the starting ATG codon. ESC clones containing the correct insertion were identified by Southern blot hybridization and injected into C57BL/6J albino blastocysts according to standard procedures. High percentage coat-color-contribution chimaeras were crossed with C57BL/6J albino mice to obtain germline transmission of the targeted allele. Southern blot and PCR analyses were used to genotype the mice.

Double heterozygous *Epn*/*Bgn* mice (F_1 generation) were produced by intercrossing homozygous *Bgn*-deficient females ($Epn^{+/+}Bgn^{-/-}$) with homozygous *Epn*-deficient males ($Epn^{-/-}Bgn^{+/0}$). The production and characterization of *Bgn*-deficient mice was previously described[29]. *Epn*/*Bgn* knockout mice (F_2) were obtained by intercrossing F1 double heterozygous *Epn*/*Bgn* mice. All of the mice were maintained in a hybrid 129S6:C57BL/6J albino genetic background. Each animal experiment was approved by the Institute of Biosciences and Technology Institutional Animal Care and Use Committee.

SOUTHERN BLOT ANALYSIS

Mouse ESC genomic DNA was digested with ScaI or BglII. Southern blot hybridization was performed according to the procedure of Ramírez-Solis *et al.*[30] with radiolabeled 5′ or 3′ probes that hybridized to regions of the *Epn* gene lying outside of the targeting vector homology.

POLYMERASE CHAIN REACTION (PCR)

The PCR scheme for the *Bgn* allele was performed as described previously[29]. To identify the *Epn* genotype of each mouse, the following primers were used in separate PCR reactions: a forward primer (Epn1fw) corresponding to sequences in *Epn* exon II (5′GGTCAGGGGCAAATACCAAGGACTCT), a reverse primer (Epn2rv) corresponding to sequences in *Epn* exon III (5′-CTCTACATGG TTGTCAGGAATGTG), and a second forward primer (Bpa) corresponding to a sequence contained within the bovine growth hormone polyadenylation signal sequence of the *neo* cassette (5′-GCTTCTGAGGCG-GAAAGAACCAGCTA). The amplified PCR product for the wild type *Epn* allele with primer pair Epn1fw/Epn2rv was 2.6 kb, whereas the product for the disrupted *Epn* allele with primer pair Bpa/Epn2rv was 0.4 kb.

BODY WEIGHT AND FEMUR LENGTH DETERMINATION

The body weights and femur lengths of each mouse was measured at 1 and 9 months of age. Femur length was measured under a dissecting microscope using calibers. The data from 13–40 different mice per genotype of each gender were analyzed using the Kruskal–Wallis test followed by a Dunn's multiple comparison post-test.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STAINING

All tissues were fixed in either 4% paraformaldehyde or 10% formalin, decalcified at room temperature in 0.5 M ethylenediaminetetraacetic acid (EDTA) for $\geq$1 week, dehydrated in ascending concentrations of alcohol, and paraffin-embedded. Serial sections were cut from the paraffin block using a microtome and thereafter dewaxed and rehydrated. For histological analysis of collagen content, tissue sections were stained with hematoxylin for 2 min followed by immersion in van Gieson stain for 5 min. The remaining sections were stained with hematoxylin for 2 min, briefly differentiated in acidified alcohol, and stained with 0.2% Fast green for 2 min. After washing with 1% acetic acid, the sections were stained with Safranin-O for 3 min to examine tissue morphology and proteoglycan content.

Immunohistochemical staining was performed using polyclonal antibodies that detect either mouse BGN (LF-159) or mouse EPN (R561). The rabbit polyclonal anti-BGN antibody, LF-159[31], was a gift from Dr. Larry Fisher (NIH/NIDCR, Bethesda, MD). The polyclonal anti-EPN antibody, R561, was raised in rabbits against purified recombinant EPN protein (Ala_{18}-Thr_{111} fused at the C-terminus to glutathione S-transferase; Alpha Diagnostics International, San Antonio, TX). R561 antibody characterization demonstrated that it is specific for EPN. Tissue sections were digested with 4 mg/ml hyaluronidase in phosphate buffered saline (PBS) at 37°C for 30 min prior to immunostaining. The sections were then incubated with R561 (1:1000) or LF-159 (1:2000) overnight at 4°C. Antibody staining was visualized with the Vectastain Elite ABC immunoperoxidase kit (Vector Laboratories, Hercules, CA). Hematoxylin was used as a counterstain.

OA GRADING AND SCORING

Serial sagittal histological sections of individual mouse knee joints were examined for signs of OA in a double-blind fashion. The slides were scored for OA severity used a grading system based upon the criteria of Maier and Wilhelmi[32]. This system uses the following scale to quantify OA severity: grade 0 indicates no apparent changes, grade 1 indicates slight fibrillation of the articular cartilage, grade 2 specifies defects limited to the uncalcified cartilage, grade 3 specifies defects penetrating into the calcified cartilage, and grade 4 indicates subchondral bone exposure at the joint surface. Scores for each genotype were averaged and analyzed using an unpaired Student's *t*-test with Welch's and Bonferroni corrections.