Evaluation of a fibrillin 2 gene haplotype associated with hip dysplasia and incipient osteoarthritis in dogs

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Objective—To determine whether a mutation in the fibrillin 2 gene (FBN2) is associated with canine hip dysplasia (CHD) and osteoarthritis in dogs.

Animals—1,551 dogs.

Procedures—Hip conformation was measured radiographically. The FBN2 was sequenced from genomic DNA of 21 Labrador Retrievers and 2 Greyhounds, and a haplotype in intron 30 of FBN2 was sequenced in 90 additional Labrador Retrievers and 143 dogs of 6 other breeds. Steady-state values of FBN2 mRNA and control genes were measured in hip joint tissues of fourteen 8-month-old Labrador Retriever–Greyhound crossbreeds.

Results—The Labrador Retrievers homozygous for a 10-bp deletion haplotype in intron 30 of FBN2 had significantly worse CHD as measured via higher distraction index and extended-hip joint radiograph score and a lower Norberg angle and dorsolateral subluxation score. Among 143 dogs of 6 other breeds, those homozygous for the same deletion haplotype also had significantly worse radiographic CHD. Among the 14 crossbred dogs, as the dorsolateral subluxation score decreased, the capsular FBN2 mRNA increased significantly. Those dogs with incipient hip joint osteoarthritis had significantly increased capsular FBN2 mRNA, compared with those dogs without osteoarthritis. Dogs homozygous for the FBN2 deletion haplotype had significantly less FBN2 mRNA in their femoral head articular cartilage.

Conclusion and Clinical Relevance—The FBN2 deletion haplotype was associated with CHD. Capsular gene expression of FBN2 was confounded by incipient secondary osteoarthritis in dysplastic hip joints. Genes influencing complex traits in dogs can be identified by genome-wide screening, fine mapping, and candidate gene screening. (Am J Vet Res 2011;72:530–540)

Canine hip dysplasia has been the subject of intensive study because some large dog breeds have > 50% incidence. The trait has a systemic component because multiple joints can be involved, yet its most conspicuous manifestation is in the hip joint. Debilitating osteoarthritis, which occurs in affected dogs, adversely affects hip function and gait.

Several investigators have explored the origins of hip joint laxity and instability in the supporting structures of the hip joint, especially the hip joint capsule and round ligament of the femoral head, because interruption of such support may be a critical initiating event.
feature of dysplastic hip development.3 Lust et al, Fa-
rese et al,1 and Smith6 reported that hip joint laxity, ac-
accompanied by synovial effusion and hypertrophy of the
round ligament of the femoral head that signal incipient
osteoarthritis, contributed to the pathogenesis of CHD.
On the basis of previous genome-wide linkage anal-
ysis and fine mapping,9 we hypothesized that a can-
didate gene in the QTL interval on CFA11 at 18.5 to 21 cM
influenced hip joint laxity, as measured by the DI, in
dogs. One gene at 20.3 to 20.5 megabases on CFA11,
FBN2, was an attractive positional candidate gene be-
because it encodes for a microfibrillar component of ex-
tacellular matrix that is present in fibrous joint capsule
and articular cartilage.10 Mutations in FBN2 are asso-
ciated with congenital contractual arachnodactyly in
humans,11 and some affected patients have had joint
laxity. The purpose of the study reported here was to
determine whether a mutation in FBN2 is associated
with CHD and osteoarthritis in dogs.

Materials and Methods
Cornell DNA Hip Archive—Study dogs were from
closed breeding colonies at the Baker Institute for Ani-
mal Health at Cornell University and Guiding Eyes for the
Blind (Yorktown Heights, NY), and dogs admitted to
the Cornell University Hospital for Animals. There
was DNA available from 210 Labrador Retrievers bred
from 1989 over 8 generations. More recently, the au-
thors developed a crossbreed pedigree for QTL map-
ping of CHD founded with 7 unaffected Greyhounds
(2 males and 5 females) and 8 Labrador Retrievers (4
males and 4 females) with CHD and secondary osteo-
arthritis.12 The pelvis of dogs from the Baker Institute
for Animal Health underwent radiography between 8
and 12 months of age, whereas the dogs from Guiding
Eyes for the Blind underwent radiography between 14
and 18 months of age. The pedigrees of all available
dogs were used to derive breeding values for hip trait
mapping. The final data set contained Labrador Ret-
rievers, Greyhounds, their crossbred offspring, and 14
other breeds, totaling 1,551 dogs that had at least 1 of
the 4 radiographic hip measurements. Ancestry of these
1,551 dogs was traced back as far as 17 generations,
resulting in a total of 2,716 dogs, including 1,498 dogs
from Guiding Eyes for the Blind, 571 from the Baker
Institute for Animal Health, 423 from the Cornell Uni-
versity Hospital for Animals, and 222 ancestors traced
from the Orthopedic Foundation for Animals semiopen
database.13 The study was approved by the Cornell Uni-
versity Institutional Animal Care and Use Committee.

Phenotyping—The severity of CHD was measured
from pelvic radiographs by use of the NA,14 DI,15,16 DLS
score,15 and EHR projection score adopted by the Or-
thropedic Foundation for Animals and by evaluation of
hip osteoarthritis (new bone formation on the femo-
ral head and neck and acetabular osteophytes) on the
EHR. Although these radiographic measurements were
correlated,18 none of the measurements completely re-
placed the other so that at least 2 of them are required
to represent a dysplastic hip joint.

Narrowing the QTL interval on CFA11—A QTL
for CHD on 12 chromosomes in a Greyhound–Labra-
dor Retriever pedigree was previously identified.19 To
improve genome coverage, these dogs were later gen-
yotyped at 225 additional microsatellites of minimal
screening set 2 on 16 chromosomes.9,11–13 Linkage anal-
ysis confirmed the presence of a QTL for CHD segregat-
ing in the crossbreed pedigree with a maximum loga-
rithm of odds peak score of 3.45 on CFA11 at 36 cM for
the second principal component of the DLS score and
DI. Genotyping was performed on 257 dogs, including
Labrador Retrievers, Greyhounds, and their crossbred
offspring, at 111 SNPs in this QTL region on CFA11.
Multipoint linkage analysis25 revealed substantial evi-
dence for 2 QTLs contributing to the DI between 15.7
to 17 cM and 18.5 to 21 cM (95% posterior probability
interval), which explained approximately 15% to 18%
of the total variance in the DI. FBN2, a candidate gene
at 20.3 to 20.5 megabases, was within this interval.

FBN2 screening—The exon structure of FBN2 was
identified by use of an online genome browser21 and
National Center for Biotechnology Information22 and
University of California-Santa Cruz23 genome databases
of the second release of the Canis familiaris genome se-
quence. Primer sequences were designed by use of soft-
ware24 and ordered from a commercial source24 and are
available from the primary author (SGF). Exons were
amplified from genomic DNA via PCR under standard
conditions. Amplicons were purified. Each exon was
sequenced initially in the forward direction only, then
in the reverse direction if necessary. Sequencing was
performed on a commercial sequencer.4 In addition,
introns 1 and 2 and 372 bp upstream of exon 1 were
sequenced from genomic DNA to identify regulatory re-
regions that might affect gene expression. Sequence was
analyzed by use of commercial software.5

Sequencing and association study—FBN2 was first
sequenced in 21 Labrador Retrievers and 2 Greyhounds.
Intron 30 of FBN2 was then sequenced in a larger number
of dogs selected from the dogs in the CHD DNA archive.
Dogs were chosen by use of an algorithm to maximize ge-
etic diversity and minimize relatedness.25 Dog selection
based on phenotype was performed by use of the principal
components of the DI, DLS score, EHR score, and NA,
weighted by their eigenvalues. Intron 30 of FBN2 was se-
quenced in 90 additional Labrador Retrievers, 18 Border
Collies, 47 German Shepherd Dogs, 29 Golden Retriev-
ers, 29 Newfoundlands, 19 Rottweilers, and 1 Great Dane.
The PCR primers (forward, 5′-ggccaatgtaccaacattcc; re-
verse, 5′-taaggtcatcctgtgtc) were designed to amplify
intron 30 of FBN2 and included at least 50 bp in either
direction by use of commercial software.25 M13 universal
primers were added to the 5′ end of each primer to facil-
tate sequencing.

Characterization of FBN2 expression—Specimens
of the dorsal fibrous joint capsule, round ligament of
the femoral head, and full-thickness noncalcified articular
cartilage from the dorsal surface of the femoral head
but not including the perifoveal area were collected from
fourteen 8-month-old Labrador Retriever–Grey-
hound crossbred dogs at necropsy. Six of the dogs were
from one litter (three-quarter Labrador Retriever and
one-quarter Greyhound [litter 1]), and the remaining 8 dogs
were from another litter (seven-eighth Labrador
Retriever and one-eighth Greyhound [litter 2]. The joints were evaluated grossly for evidence of osteoarthritis as the traditional indication of hip dysplasia. Tissue was harvested within 2 hours of euthanasia. Samples from the right hip joint of each dog were used for mRNA isolation. Samples from litter 1 were flash frozen in liquid nitrogen and stored at −80°C, and samples from litter 2 were preserved for later RNA extraction and stored at −20°C. The RNA was isolated by use of a commercial kit designed to extract RNA from fibrous tissues. Briefly, approximately 150 mg of each frozen sample was placed in a stabilizing buffer and homogenized 2 or 3 times for 30 to 45 seconds on ice. The homogenate was incubated at 55°C for 20 minutes with protease K and was then pelleted by centrifugation at 4,500 X g to remove cellular debris. The supernatant solution was collected, a 0.5 volume of 100% ethanol was added, and the solution was filtered via centrifugation at 4,500 X g through a silica column designed to bind RNA. DNase I was added to the bound RNA to remove any contaminating DNA. The sample was washed and eluted in nuclease-free water. Concentrations and purity were assessed spectrophotometrically.

Expression of FBN2 was measured via quantitative reverse transcription PCR assay. First, cDNA was prepared from each RNA sample. One microgram of RNA was added to a standard mixture of deoxynucleotide triphosphates (4X), random primers (1X), recombinant Moloney murine leukemia virus reverse transcriptase (1X), and reaction buffer (1X). Reactions were incubated in a thermal cycler, annealment was performed at 25°C for 10 minutes, copying was performed at 37°C for 2 hours, and inactivation was performed at 85°C for 5 seconds. Custom primers and probes were designed to amplify portions of FBN2 cDNA at the junctions of exons 4-5, 30-31, and 59-60 (Appendix 1). Glucose phosphate isomerase was used as an endogenous control with a 1:1 (vol/vol) RNA:mixture ratio. Reactions were incubated in a thermal cycler, annealment was performed at 25°C for 20 minutes, copying was performed at 37°C for 2 hours, and inactivation was performed at 85°C for 5 seconds. Each PCR reaction was carried out in quadruplicate and activated at 95°C for 10 minutes, followed by 40 cycles of 15-second denaturation at 95°C, followed by annealing-extension at 60°C for 1 minute. Relative quantification was performed by use of the mean difference in threshold cycle values of all the samples for a particular probe as the calibrator; difference in threshold cycle values were read midway into the linear phase of the amplification curve. Expression of control genes FBN1, kinesin 11 (KIN11), and topoisomerase 1 (TOP1) was similarly measured.

Alternative splicing—Two or 3 mRNA samples isolated from fibrous joint capsule, round ligament, and articular cartilage from dogs of each intron 30 FBN2 genotype (unaffected homozygous, heterozygous, and affected or deletion homozygous) were selected for further analysis. One-step reverse transcription PCR amplification of these samples was performed in regions adjacent to intron 30 in 2 experiments. In the first experiment, 1 upstream primer was selected within exon 26 and downstream primers were chosen within exons 28, 29, 30, 31, and 33 (Appendix 2). In the second experiment, 1 upstream primer was chosen within exon 34 and downstream primers were chosen within exons 36, 37, 39, 40, 41, 42, and 43. The reverse transcription PCR amplification was performed by use of a 1-step reverse transcription PCR system. Briefly, 250 ng of template RNA was mixed with 2 U of reverse transcriptase and 0.2 μM of each primer and reaction buffer and incubated. Thermal cycler temperatures were 55°C for 30 minutes, 94°C for 2 minutes, 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and 68°C for 5 minutes. The PCR reaction products were analyzed on a 2% agarose gel to detect amplicons shorter than expected.

Immunohistochemical analyses—Formalin-fixed sections of dorsal hip capsule from affected and unaffected crossbred dogs from which the mRNA was isolated and amplified were also examined by use of H&E stain; Vierhoff von Giesen stain; and elastin fibers was also used. The elastin stain revealed sparse numbers of short fibrils concentrated primarily beneath the synovial lining in the loosely arranged connective tissue. For immunohistochemical analyses, sections were deparaffinized, blocked with 3% H2O2 in methanol, and washed with distilled H2O and Tris-buffered saline solution (pH 7.6). Sections were blocked with 10% normal goat serum with 2% casein. After washing with Tris-buffered saline solution, biotinylated goat anti-rabbit IgG was applied at 1:200 for 20 minutes at 20°C, followed by Tris-buffered saline solution washes and incubation for 20 minutes with streptavidin peroxidase. The substrate was prepared as directed and applied for 10 minutes. Slides were counterstained with hematoxylin and mounted. Controls were included, substituting normal rabbit IgG in place of primary antibody. Immunohistochemical staining for FBN2 expression was compared with serial sections treated with normal rabbit serum. The intensity of staining was subjectively graded.

Statistical analysis—The effect of the FBN2 haplotype on each hip joint measurement was evaluated in cumulative distribution plots. The cumulative distribution plots were compared with results of the Kolmogorov-Smirnov test. The linear model used to test the effect of the FBN2 haplotype on hip dysplasia was as follows:

Radiographic trait = Mean for the trait + sex + breed (Labrador Retriever or non-Labrador Retriever) + body weight + age + FBN2 haplotype + interactions

where the interaction term included all possible pairwise interactions. Variable selection strategy was performed to find the final linear model with significant (P < 0.05) terms only. This model was applied to each of the 4 CHD traits. Given any randomly sampled dog of known breed and body weight and knowing its FBN2 haplotype, the mean radiographic trait could be calculated using the final model.
haplotype, a prediction interval for the hip trait was calculated.

Values for expression of mRNA were log transformed and tested as a function of the presence or absence of secondary osteoarthritis or CHD phenotypes (DLS score, NA, and EHR score) in a mixed linear model that included the covariates of the dog's genotype at the FBN2 locus, sex, age, and body weight, with a random effect for litter. To test the effect of hip joint phenotype on the expression of the control genes, FBN1, TOP1, and KIN11 in fibrous joint capsule, the FBN2 genotype was not included in the model. The effect of the hip joint phenotype on the expression of FBN1 and FBN2 only was tested in the femoral head articular cartilage. For all comparisons, values of P < 0.05 were considered significant.

Results

Dogs and phenotypes—Twenty-three dogs were first selected for FBN2 sequencing of genomic DNA. One group of 12 affected Labrador Retrievers had EHR scores ≥ 5, NAS < 100°, and DLS scores < 45° for both hip joints. The other group of 11 unaffected dogs included 9 Labrador Retrievers and 2 Greyhounds with EHR scores ≥ 2, NAS > 100°, and DLS scores > 55° in both hip joints. The hip joint phenotypes of an additional 90 Labrador Retrievers and 143 non–Labrador Retrievers used to sequence intron 30 of FBN2 were determined (Figure 1). Of the 257 dogs originally used in SNP fine mapping that localized the QTL to 18 to 21 cM, only 8 Labrador Retrievers from the Baker Institute for Animal Health and 11 German Shepherd Dogs satisfied the criteria for FBN2 sequencing of genetic and phenotypic diversity. Of the 14 Labrador Retriever cross-bred dogs used for quantitative reverse transcription PCR analysis of FBN2, FBN1, TOP1, and KIN11, 6 dogs had early hip joint osteoarthritis at necropsy characterized by perivascular articular cartilage fibril- lation, and 8 dogs had no gross signs of hip joint osteoarthritis. Only 1 dog had unilateral (right) hip joint osteoarthritis and no signs of osteoarthritis in the opposite hip joint. For these dogs, mean ± SD right hip joint DLS score was 48 ± 6.3 (range, 41% to 62%), mean ± SD EHR score was 2.4 ± 1.2 (range, 1 to 5), and mean ± SD NA was 99.3 ± 5 (range, 90° to 105°).

Sequencing—A haplotype was identified within the first 100 bp of intron 30 in FBN2 that segregated in 12 Labrador Retrievers affected with CHD (AGC haplotype) and 9 Labrador Retrievers and 2 Greyhounds unaffected with CHD (GAT haplotype; Appendix 3). The GAT haplotype extended downstream (TACCTTTA) between bp 189 and 195 where the 7-bp deletion for the AGC haplotype occurred) within intron 30. Additional sequencing revealed that the GATTACTTTA haplotype in intron 30 extended further downstream, with A at intron 37 base 27, C at intron 40 base 32, and T at exon 41 base 143; the latter caused no change in amino acid sequence. In these same positions, the haplotype continued downstream as follows: G-A-C for the AGC (deletion) haplotype, and R-M-Y for the heterozygous haplotype. Via sequencing of 372 bp upstream of FBN2 exon 1, an SNP was found that was not associated with the 2 groups that were either affected or unaffected with CHD. It was not possible to sequence farther upstream. In the same group of dogs, intron 2 was also sequenced and none of the 3 SNPs discovered was associated with CHD in the same dogs.

The DNA from an additional 90 Labrador Retrievers and 143 non–Labrador Retrievers was sequenced for the same FBN2 haplotype in intron 30. The cumulative trait distributions for the effect of the 3 genotypes on each hip joint trait on the Labrador Retrievers were determined (Figure 2). The cumulative distribution plots of the Labrador Retrievers homozygous for the AGC haplotype were significantly different, compared with either of the other 2 haplotypes for all traits except the left NA (P = 0.08), the right DI (P = 0.08), and both left and right EHR scores (largest P = 0.29). However, via analysis in a linear model with covariates, dogs homozygous for the AGC (deletion) haplotype had worse hip joint conformation characterized by a lower DLS score, higher DI, lower NA, and higher EHR score than did those with the GAT or heterozygous haplotype (Figure 3).

An example of the effect of the FBN2 genotype on a CHD trait (the ANOVA table for the effect of haplotype, body weight, and breed on the left NA) was developed (Table 1). Other hip joint traits were similarly affected.
Figure 2—Cumulative distribution (proportion) plots for Labrador Retrievers genotyped for the *FBN2* haplotype illustrating the cumulative effect of the *FBN2* haplotype on each dog's hip joint measurement (the left [L] and right [R] DLS score [n = 101 dogs], NA [102], DI [101], and EHR score [102]). AGC indicates homozygosity for the *FBN2* deletion. GAT indicates homozygosity for the unaffected haplotype. Het = Heterozygote. See Figure 1 for remainder of key.
Because the dog’s sex, age when the radiograph was taken, and all other interaction terms except body weight by breed were not significant, these variables were dropped from the model. This linear model explained approximately 14% of the total variation in the left NA.

For any randomly sampled Labrador Retriever with body weight of 30 kg that was homozygous for the deletion AGC haplotype, the equation for the estimated left NA was as follows:

$$\text{LNALR}_{\text{AGC,weight}} = 76.15 + 0.79 \times \text{body weight} = 76.15 + 0.79 \times 30 = 99.9°$$

where LR is Labrador Retriever breed. For a similar dog homozygous for the GAT haplotype, the equation for the estimated left NA was as follows:

$$\text{LNALR}_{\text{GAT,weight}} = 76.15 + 6.84 + 0.79 \times \text{body weight} = 106.7°$$

For a similar but heterozygous Labrador Retriever, the equation for the estimated left NA was as follows:

$$\text{LNALR}_{\text{het,weight}} = 76.15 + 5.91 + 0.79 \times \text{body weight} = 76.15 + 5.91 + 0.79 \times 30 = 105.8°$$

where het is heterozygous. However, the interaction of breed and body weight contributed significantly to the variation of the NA. Heavy Labrador Retrievers had higher NAs than did heavy non-Labrador Retrievers. Similar interactions of body weight occurred with the other hip joint traits (data not shown).

**FBN1, FBN2, KIN11, and TOP1 expression and immunohistochemical analysis**—In the mixed linear model, the effects of sex, age, and body weight on gene expression were not significant and these terms were dropped from the model. As the DLS score decreased by 1%, FBN2 expression in the fibrous joint capsule increased by approximately 5% (Tables 2–5). The model including the DLS score and hip joint osteoarthritis explained approximately 40% of FBN2 expression. Dogs that had incipient osteoarthritis at necropsy as the traditional indicator of prior CHD had approximately 50% greater FBN2 mRNA in their hip joint capsule than did dogs with nonaffected hip joints. Dogs with the AGC homozygous genotype had less FBN2 expression in hip joint capsules, although the difference was not significant. In the femoral head articular cartilage, the GAT homozygous dogs had significantly higher FBN2 expression, compared with the heterozygotes and dogs homozygous for the AGC (deletion) haplotype. There was no evidence of alternatively spliced FBN2 transcripts resulting from the deletion in intron 30 in mRNA transcript isolated from the joint capsule.
Most immunostaining for FBN2 was seen in the sub-synovial region of the hip joint capsule (Figure 4). No qualitative difference was observed in immunostaining of FBN2 in the hip joint capsule of affected hip joints, compared with unaffected hip joints. There was no significant difference in mRNA expression in the fibrous joint capsule of FBN1 and TOP1 of dogs with osteoarthritis, compared with those without osteoarthritis. However, KIN11 mRNA expression was significantly greater in the joint capsules of osteoarthritic hip joints (Tables 2–5). For each unit increase (worsening) of the EHR score, the KIN11 expression increased by 34%. There was no effect of litter or tissue preparation on gene expression.

### Discussion

The candidate gene, FBN2, was identified through genome-wide, microsatellite-based screening of a Labrador Retriever–Greyhound pedigree, followed by narrowing of the QTL region on CFA11 through multipoint linkage analysis with SNP genotypes. An association between a mutation-deletion haplotype in FBN2 and CHD was detected. This is the first gene reported to be associated with all 4 CHD radiographic measurements, an important orthopedic trait that leads to debilitating hip joint osteoarthritis. The FBN2 mutation was identified in an initial set of 23 dogs and in additional Labrador Retrievers and dogs of other breeds. Only 19 of the 257 dogs in which the QTL for the DI was originally identified were later subjected to FBN2 sequencing. The authors made the a priori decision not to include crossbred dogs from our colony in the analysis. We reasoned that testing the mutation in unrelated purebred dogs, most of which were not included in prior linkage analyses, would strengthen support for any association discovered. In hindsight, it is probably wise to screen for segregation of a candidate gene mutation in the subset of the individuals originally used in the linkage study prior to validation of a mutation discovery in a new population. The AGC allele had recessive characteristics because the heterozygous and homozygous GAT individuals were similar phenotypically. Clearly, other genes must contribute to CHD because the FBN2 locus does not explain all the genetic trait variation in CHD. This is consistent with the results of QTL mapping in Labrador Retriever–Greyhound crossbred dogs,19 Labrador Retrievers,22 German Shepherd Dogs,7 and Portuguese Water Dogs,27 in which no locus for CHD was identified that contributed >18% of trait variance.

Expression of FBN2 in the joint capsule was related inversely with the DLS score; that is, as the right DLS score decreased (a worse hip joint conformation), the FBN2 expression increased in the right hip joint capsule. It was expected that FBN2 expression would be lower in dogs with lower DLS scores because the dogs that were homozygous for the deletion (AGC) haplotype had significantly lower FBN2 expression in their femoral head articular cartilage. One explanation is that the experiments were confounded by the presence of secondary osteoarthritis in dysplastic hip joints. The...
The fibrillins belong to a family of structurally related glycoproteins that include the latent transforming growth factor-β-binding proteins and fibulins. Fibrillins are the major structural components of extracellular microfibrils, which are either associated with elastin or act as elastin-free assemblies. Mutations in the gene encoding FBN2 have been associated with human congenital contractual arachnodactyly, a disorder characterized by arachnodactyly (elongated distal long bones), dolichostenomelia (elongated limbs), scoliosis, multiple congenital contractures, and abnormalities of the external portion of the ears. A similar phenotype was produced in mice through a deletion of the FBN2 gene. Most of the mutations discovered in FBN1 and FBN2 occur in the same region (ie, upstream or downstream of exon 30) as for the mutation discovered in dogs with CHD in the present study. The CHD phenotype is genetically complex, and the effect of any FBN2 haplotype would not be expected to be as severe as the FBN2 knockout phenotype in the mouse. The joint contractures in humans with congenital contractual arachnodactyly disappear with aging. Mice heterozygous for the FBN2 mutation do not have an abnormal phenotype unless they are also heterozygous for a mutation in bone morphogenetic protein 7. Some human patients with congenital contractual arachnodactyly have joint laxity including hypermobile thumbs. However, the mechanism by which such an intron deletion in FBN2 may contribute to the CHD phenotype remains unclear.

Fibrillins are present in the chondroepiphysis of the developing joint. Because congenital contractual arachnodactyly is characterized by deformation of the pinnae, Yanagino et al examined the regulation of FBN2 in chondrocytic differentiation of murine ATDC5 cells. The FBN2 was highly expressed in the early stage of differentiation and later declined. The 5′ flanking region of FBN2 contains potential binding sites for the E2 factor (F1) family, Runx, AP-2, and Sox transcription factors. Overexpression of E2F1 results in a marked increase in FBN2 promoter activity. Because the dogs homozygous for the FBN2 deletion mutation had lower FBN2 expression in femoral head cartilage, examination of FBN2 expression in chondroepiphyseal cartilage of the developing hip joint might further elucidate the functional importance of the FBN2 mutation. However, biopsy of femoral chondroepiphyseal tissue during development would damage the hip joint and result in osteoarthritis.

The mechanism by which altered concentrations of FBN2 protein might interrupt the mechanical integrity of the hip joint capsule or femoral head articular cartilage contributing to hip joint laxity and dysplasia...
microfibril assembly can result in excess local TGF-β of TGF-β molecules affects the function of surrounding fibrillin concentration may be indirect. The location of fibrillin, sequester TGF-β cause extracellular matrix proteins, such as the fibril- lins, to floc, and condense to form the nucleus, which can develop into a new cell. The nucleus is surrounded by the cytoplasm, which contains the organelles and the cell membrane. The cell membrane is composed of a lipid bilayer and proteins, and it provides the barrier between the inside and outside of the cell.

The cross-sectional collagen fibrillar diameter in the hip joint capsule of a Labrador Retriever without CHD and with tight hip joints was uniform. Flexor digitorum longus tendons from mice null for FBN2 have the same collagen content as control mice but contain fewer intermolecular collagen cross-links of hydroxylysyl and lysyl pyridinoline.36 The collagen of femoral heads of dysplastic Labrador Retrievers is more soluble than the collagen of femoral heads of unaffected puppies. Electron micrographs of articular cartilage of the same femoral heads reveal disruption of collagen fibrils in young dogs with CHD.37,38 The etiology of this disruption, which was discovered through an electron microscopic and biochemical evaluation decades ago, has never been elucidated, but a plausible explanation may now be possible to achieve through positional cloning of contributing genes.

Understanding the genetic basis of CHD will help identify similar biochemical pathways that lead to developmental dysplasia of the human hip joint, for which there is evidence of a genetic basis. Previous studies that examined the underlying biochemical mechanism for hip joint laxity in humans examined the capsular collagens. Increased collagen type III-to-type I ratios in hip joint capsules of dysplastic human neonates have been reported.39,40 Similar findings of increased ratios of type III to type I collagen propeptides in synovial fluid have been reported in the hip joints of dysplastic dogs, compared with unaffected dogs.41 The action of the maternal hormones estrogen, relaxin, and testosterone on genetically susceptible hip joint tissues may contribute to hip joint laxity in the perinatal period,42 but whether this effect persists is unknown. Fibrillin 2 is the preferred target for relaxin activity and FBN2 expression, compared with fibrillin 1, as measured in canine osteoarthritis. KIN11 protein is a major component of intracellular machinery.43 Gross evidence of osteoarthritis was chosen in the present study as the traditional indicator of concomitant CHD because there is no precisely accurate method to assess functional or dynamic subluxation of a dysplastic hip joint that results in osteoarthritis. The DLS score alone has approximately 80% accuracy for predicting osteoarthritis of the hip joint as measured via combined sensitivity and specificity.44 All the radiographic methods of hip joint assessment are performed with the dog passively positioned and recumbent, and radiographs do not reveal the incipient stages of osteoarthritis in the hip joint. Besides those genes that influence the expression of CHD, other genes likely influence the onset and progression of hip joint osteoarthritis in dysplastic hip joints.

The mapping and identification of FBN2 as the first gene reported to be associated with CHD indicate that complex traits such as CHD can be evaluated in dogs at the molecular genetic level. The strategy of coarse genome screening with microsatellites or SNPs followed by fine mapping with SNPs can resolve a QTL to a small interval suitable for candidate gene screening.45 The association between the FBN2 deletion haplotype and CHD was detected in Labrador Retrievers and non–Labrador Retriever breeds, indicating that some of the genes that contributed to CHD were conserved across breeds. Further studies are needed to elucidate the biochemical pathway through which mutations in FBN2 might influence in the developmental pathogenesis of CHD.
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Appendix 1

Primer sequences for amplification of FBN2, FBN1, and GPI (glucose phosphate isomerase) mRNA from hip joint capsule tissue of dogs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBN2</td>
<td>Exon 4-5</td>
<td>CAGTGGCCAGAAGGGATATATTGGAA</td>
<td>CGTCCACCATTCTGACATCCATTT</td>
</tr>
<tr>
<td>FBN2</td>
<td>Exon 30-31</td>
<td>TGGCTTCATGGCTTCAATGGATACATGCT</td>
<td>CTCACATTCCCCAAACATACAGATGT</td>
</tr>
<tr>
<td>FBN2</td>
<td>Exon 59-60</td>
<td>GCTATGTCCTACAAGAGGATGCAAA</td>
<td>GCAGTTGTGTTGTTTGGATTCA</td>
</tr>
<tr>
<td>GPI</td>
<td>Exon 2-3</td>
<td>ACACCGTGATGCAGATGCT</td>
<td>TGATCTTCTCGCCACTGAACATG</td>
</tr>
<tr>
<td>FBN1</td>
<td>Exon 6-7</td>
<td>GCCGAGGCTTCATTCCAAATATC</td>
<td>GGGATGGCCTGGCATTCAT</td>
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</tbody>
</table>

Appendix 2

FBN2 primer sequences for exon amplification to test for alternative transcripts in a study of hip dysplasia in dogs.

<table>
<thead>
<tr>
<th>Primer location</th>
<th>Direction</th>
<th>Sequence</th>
<th>Expected amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 26</td>
<td>Forward</td>
<td>agctacgaaagtgggttca</td>
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<td>Exon 28</td>
<td>Reverse</td>
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<td>364</td>
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<td>Exon 34</td>
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<td>gtctcaatcgcaggtgagt</td>
<td>—</td>
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<td>Exon 42</td>
<td>Reverse</td>
<td>ggtatgctcaaggtggaatcag</td>
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</tr>
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<td>Exon 43</td>
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</table>

— = Not applicable.

Appendix 3

Haplotype structure of intron 30 of FBN2 where a deletion mutation occurred, in dogs with CHD.

<table>
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<th>Haplotype structure</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
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<td>Unaffected homozygous</td>
<td>CATCT G</td>
<td>TGCAC...</td>
<td>GGGAA A</td>
<td>GAGGCTTT T</td>
<td>TCAGT...</td>
<td>AGATTA</td>
<td>TACCTTA...</td>
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<tr>
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<td>TGCAC...</td>
<td>GGGAA G</td>
<td>GAGGCTTT C</td>
<td>TCAGT...</td>
<td>AGATTA</td>
<td>ACTTTA...</td>
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<td>TGCAC...</td>
<td>GGGAA R</td>
<td>GAGGCTTT Y</td>
<td>TCAGT...</td>
<td>AGATTA</td>
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</tr>
</tbody>
</table>

Base position 41 68 77 189–195

N = Unknown nucleotide sequence.