Reduced perlecan in mice results in chondrodysplasia resembling Schwartz–Jampel syndrome

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Perlecan knock-in mice were developed to model Schwartz–Jampel syndrome (SJS), a skeletal disease resulting from decreased perlecan. Two mouse strains were generated: those carrying a C-to-Y mutation at residue 1532 and the neomycin cassette (C1532Yneo) and those harboring the mutation alone (C1532Y). Immunostaining, biochemistry, size measurements, skeletal studies and histology revealed Hspg2 transcriptional changes in C1532Yneo mice, leading to reduced perlecan secretion and a skeletal disease phenotype characteristic of SJS patients. Skeletal disease features include smaller size, impaired mineralization, misshapen bones, flat face and joint dysplasias reminiscent of osteoarthritis and osteonecrosis. Moreover, C1532Yneo mice displayed transient expansion of hypertrophic cartilage in the growth plate concomitant with radial trabecular bone orientation. In contrast, C1532Y mice, harboring only the mutation associated with SJS, displayed a mild phenotype, inconsistent with SJS. These studies question the C1532Y mutation as the sole causative factor of SJS in the human family harboring this alteration and imply that transcriptional changes leading to perlecan reduction may represent the disease mechanism for SJS.

INTRODUCTION

Perlecan is among the most ubiquitous proteoglycans in the mammalian extracellular matrix (1). It is conserved across species, with localization to basement membranes, cartilage and bone marrow stromal cells (2–4). In mammals, perlecan is associated with endochondral ossification (EO), whereby cartilage is replaced by trabecular bone and marrow. Localized to interterritorial and pericellular matrices of articular and growth plate chondrocytes, perlecan contains both heparan sulfate and chondroitin sulfate side chains (5–7), suggesting, among perlecan’s many purported activities, an important role in cell–matrix communication within cartilage undergoing EO.

Perlecan is encoded by the heparan sulfate proteoglycan-2 gene [HSPG2 (MIM 142461)], producing a ~14 kb mRNA product (8). Although alternative splicing has been predicted (9), HSPG2 splice variants have only been identified in patients with perlecan mutations (10,11), indicating the full-length transcript to be the only functional product. The ~450 kDa perlecan core protein consists of seven protein modules within five domains (6,12), two of which (I and V) possess attachment sites for glycosaminoglycan chains (13–15) giving fully glycosylated perlecan a size of ~800 kDa.

Functional null mutations of the perlecan gene in humans and mice confirm a role for perlecan in skeletogenesis. Patients with dyssegmental dysplasia of the Silverman–Handmaker type [DDSH (MIM 224410)] and perlecan-null mice develop severe chondrodysplasia characterized by disorganized growth plate, defective EO, flat face, cleft palate and perinatal death from respiratory distress (11,16,17). Although knock-out mice appear to model DDSH, the early lethality prevents the study of postnatal skeletal development.

Schwartz–Jampel syndrome [SJS (MIM 255800)], a non-lethal, autosomal-recessive, progressive disorder resulting in reduced stature, short tubular bones, distinguishable facial features, malformed hip structures, pigeon breast and other skeletal defects (18–20), is a second human disease linked to HSPG2 mutations (21), resulting in reduced perlecan (10).
Myotonia and ocular defects also characterize SJS. Most SJS patients are of type 1A and have childhood onset with moderate skeletal dysplasia. SJS type 1B is recognizable at birth with more pronounced skeletal dysplasia. SJS type 2 is similar to type 1B, with more severe leg bowing and myotonia from birth, but has recently been re-categorized as Stuve–Wiedemann syndrome (MIM 601559); it is caused by mutations in leukemia inhibitory factor receptor.

To date, SJS has been linked to 29 documented HSPG2 mutations (22), which include insertions, deletions, missense, nonsense and splice site mutations, found throughout the HSPG2 transcript excluding only the region encoding perlecan domain I (22). Altered transcription has been observed in every reported case of SJS in which RNA was analyzed, including mutations in regions not predicted to alter transcription (10,22). One HSPG2 mutation, however, was described in a Turkish family with SJS type 1A, where a tyrosine was substituted for a conserved cysteine because of a G-to-A mutation at nucleotide 4595 (Table 1). This exonic mutation was assumed to result in misfolded protein, as it would abolish the C5–C6 disulphide bond in the third L4 module of perlecan domain III, a module conserved in perlecan across species. However, this predicted disease mechanism has never been explored, as thorough transcript analysis on patient mRNA was not performed.

To better understand perlecan’s role in the skeleton, we introduced the G4595A exonic mutation modeled in this study. *, compound heterozygous mutation.

### RESULTS

#### Expression of Hspg2 C1532Y alteration in cells impairs secretion of mutant perlecan

The G-to-A substitution at Hspg2 nucleotide 4595 was introduced into the pRC/CMV mammalian expression vector containing perlecan domain III-3 (plnIII-3) cDNA (23) and sequence verified. The nucleotide change altered one amino acid from C to Y at residue 1532, which was suspected to disrupt disulfide bond formation within plnIII-3 and thus affect perlecan conformation (Fig. 1A). Although the disease mechanism has never been established, this particular mutation has been associated with SJS disease in humans (Table 1).

To investigate the consequence of this mutation prior to mouse generation, a human cell line, human embryonic kidney (HEK) 293, was transfected with either wild-type or mutated vector and plnIII-3 secretion evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining. Protein corresponding to plnIII-3 was detected in the culture media of cells transfected with wild-type vector; however, cells transfected with mutant vector showed only trace amounts of plnIII-3 secretion (Fig. 1B). To exclude processing differences between humans and mice, murine NIH/3T3 cells were subsequently transfected with wild-type and mutant constructs. Comparable effects of the mutation were observed on plnIII-3 secretion, as confirmed by immunoblotting with anti-plnIII-3 antibody (23), which detected ample plnIII-3 secretion from cells transfected with wild-type construct, whereas cells transfected with mutant construct secreted reduced levels (Fig. 1C). plnIII-3 was not detected in significant quantities within the cellular fraction following either wild-type or mutant transfection (data not shown), nor was it detected in non-transfected cells (Fig. 1B and C). These data are consistent with the premise that the C1532Y alteration likely destabilizes plnIII-3 structure in both human and mouse cells, resulting in decreased plnIII-3 secretion, and possibly protein degradation. These data provided precedence for generating mice with the Hspg2 ntG4595A mutation.

#### Targeted Hspg2 mutagenesis in mice

The same ntG4595A mutation that inhibited plnIII-3 secretion in cells (Fig. 1B and C) was introduced into exon 36 of murine

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### Table 1. The first eight identified mutations linked to SJS

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genomic location</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splicing</td>
<td>IVS64 + 4A-to-g intron 64</td>
<td>Frameshift</td>
<td>Nicole et al. (21)</td>
</tr>
<tr>
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<td>Unknown</td>
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</tr>
<tr>
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<td>4595G-to-A exon 36</td>
<td>Misfolding?</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
<tr>
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<td>*7374 + 4a-to-g intron 56</td>
<td>Frameshift</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
<tr>
<td>Splicing</td>
<td>*Exon 60 fused to exon 61</td>
<td>Frameshift</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
<tr>
<td>Silent</td>
<td>*8544G-to-A exon 64</td>
<td>Frameshift</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
<tr>
<td>Deletion</td>
<td>*N-3del9 intron 66/exon 67</td>
<td>Frameshift</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
<tr>
<td>Deletion</td>
<td>N + 21del1708 exon 96 to 3' flanking region</td>
<td>Frameshift</td>
<td>Arikawa-Hirasawa et al. (10)</td>
</tr>
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Note that all but two mutations result in transcriptional alterations. Text in italics indicates the G4595A exonic mutation modeled in this study.
Hspg2. A neomycin selection cassette (neo), flanked by frt sites, was inserted into intron 36 at a region predicted to exclude splice acceptor, donor and loop sites (Fig. 1D). Linearized construct was transfected into embryonic stem cells and targeted mutagenesis assessed by Southern analysis of EcoRI-digested genomic DNA using an external probe (Fig. 1E). Two independently targeted embryonic stem cell clones were sequenced for mutation retention and injected into C57Bl/6 blastocysts. Both clones produced male chimeras that were backcrossed to obtain F1 mice with germ-line transmission, which were either intercrossed to generate homozygous knock-in mice (C1532Yneo) directly or mated with FLPe deletor mice (24) to eliminate neo (C1532Y mice). Mice were genotyped either by Southern hybridization or by polymerase chain reaction (PCR) (see Materials and Methods). Genomic DNA was isolated from homozygous mutant mice following neo removal and sequence-verified to retain the mutation (Fig. 1F, shaded region). Reproduction for all mice followed Mendelian ratios.

No obvious SJS phenotype in C1532Y mice
Northern blot analyses of total RNA isolated from newborn endochondral skeleton revealed comparable amounts of full-length Hspg2 transcript in wild-type and C1532Y mice, with no indication of alternate transcripts (Fig. 2A). Likewise, the
Figure 2. C1532Y mice have no apparent SJS disease phenotype. (A) Northern blot of newborn skeletal RNA from WT, HET and homozygous C1532Y mice showing comparable levels ($P > 0.2669$, based on Student’s $t$-test) of HSPG2 expression in WT and C1532Y mice with no alternate splice product. A β-actin probe was used for loading control; two bands appear as the probe also hybridizes with 18S rRNA. Reproduced with equivalent results using three sets of mice. (B) Longitudinal sections of newborn tibia from WT and C1532Y mice, showing comparable perlecan localization and staining intensity for perlecan domain V in the territorial matrix, but a subtle reduction in staining in the interterritorial matrix of chondrocytes in the C1532Y mice. Repeated on three sets of mice. (C) Higher magnification of newborn WT and C1532Y growth plates showing large, round hypertrophic chondrocytes and increased matrix-to-cell ratio in C1532Y mice. (D) Growth chart of C1532Y mice when compared with WT littermates shows no indications of growth retardation ($P > 1.0$ at all stages). Error bars represent SD. Data represent four litters of mice. (E) Whole mount skeletal staining of newborn mice using Alizarin red S for mineralized tissue and Alcian blue for cartilage. No significant differences were measured in size of six skeleton preparations ($P > 0.1063$), nor were obvious differences observed in bone structure, or mineralization between WT and C1532Y mice. (F) Longitudinal sections of newborn tibias stained with H&E (top) and safranin orange/fast green (bottom) show no overt differences in growth plates from C1532Y and WT mice, although hypertrophic chondrocytes appear larger and the interterritorial matrix appears more abundant in C1532Y growth plates. There is a similar proteoglycan content in WT and C1532Y mice. (G) Higher magnification of newborn WT and C1532Y growth plates stained for H&E and Alcian blue shows slight disorganization of hypertrophic chondrocytes in C1532Y mice. All stains were produced in triplicate.
expression of perlecan mRNA in other C1532Y newborn tissues (kidney, lung, heart and skeletal muscle) was indistinguishable from that of wild-type (data not shown). These data suggest that the ntG4595A mutation does not affect Hspg2 transcription; this was not unexpected, because the C1532Y alteration was anticipated to affect perlecan conformation. Surprisingly, however, immunostaining of C1532Y newborn long bones (Fig. 2B and C), skeletal muscle, heart and kidney (data not shown) with antibodies against perlecan domains I and V (25,26) showed strong reactivity in all wild-type and C1532Y samples. Thus, contrary to the in vitro transfection data, the C1532Y mutation does not significantly alter the secretion and deposition of full-length perlecan in vivo.

Heterozygous and homozygous C1532Y mice displayed no obvious anatomical or behavioral abnormalities. Weighing and measuring homozygous C1532Y and wild-type mice every second day from postnatal day (P) 4 to P 14 showed no significant size discrepancies, although C1532Y mice tended to be slightly larger (Fig. 2D). Skeletal staining and measurement of long bones likewise showed no obvious differences in shape or mineralization between C1532Y and wild-type mice (Fig. 2E), but again, long bone size was always equal to or greater than that of wild-type controls (data not shown). Histological examination of newborn long bones revealed subtle changes in both the proliferative and the hypertrophic zones of C1532Y mice. Specifically, there appeared to be an increase in the interterritorial matrix versus cell ratio in the proliferative zone of the C1532Y growth plate, hypertrophic cells appeared rounder and larger, on average, than the wild-type cells and stacking of cells within both zones was somewhat disorganized (Fig. 2C, F and G). No abnormalities were noticed in the proteoglycan content of C1532Y growth plates based on safranin orange staining (Fig. 2F, lower panel).

Taken together, these data indicate that the ntG4595A mutation has little effect on perlecan secretion in vivo (Fig. 2B), which is in contrast to the results obtained using domain III alone in vitro (Fig. 1B and C). Moreover, the ntG4595A mutation does not dramatically affect skeletal development and is not sufficient to recapitulate SJS disease in mice.

**Altered Hspg2 transcription in C1532Yneo mice**

Transcriptional aberrations resulting in altered HSPG2 splicing have been identified as the molecular mechanism of disease in all SJS patients examined, resulting in reduced, or hypomorphic, perlecan levels. Because neo retention has been linked with hypomorphic protein levels in knock-out mice and in cultured cells (27), C1532Yneo mice were analyzed for Hspg2 transcriptional changes. Northern blot hybridization of total RNA isolated from newborn endochondral skeleton revealed reduced levels of full-length Hspg2 transcript in homozygous C1532Yneo mice and, to a lesser extent, in heterozygous C1532Yneo mice (Fig. 3A, arrow). In addition, a novel truncated transcript was apparent in both homozygous and heterozygous C1532Yneo mice, but absent in wild-types (Fig. 3A, arrowhead). Analyses of newborn kidney, lung, heart and skeletal muscle RNA preparations showed similar findings (data not shown), indicating all C1532Yneo tissues to have the same truncated transcript, concomitant with a reduced level of full-length perlecan. Re-probing these blots for neo resulted in a single ~2 kb band in the heterozygous and homozygous C1532Yneo lanes with no hybridization to the truncated perlecan transcript (data not shown), indicating that neo is indeed spliced out and the additional C1532Yneo product is not a hybrid transcript. Thus, the retention of neo in these mice alters Hspg2 transcription, suggesting that the C1532Yneo mice may be comparable with SJS patients where the disease mechanism has been established (22). Unfortunately, there are no data regarding perlecan transcription in patients harboring the ntG4595A mutation.

**Reduced perlecan secretion in C1532Yneo mice**

To examine the effect of altered Hspg2 transcription on protein level, primary skin fibroblasts isolated from wild-type, heterozygous and homozygous C1532Yneo mice were labeled with Na$_2^3$SO$_4$, and proteins were isolated from both medium and cell layer, followed by proteoglycan enrichment by diethylaminoethyl (DEAE) chromatography. Non-digested and heparatinase-digested samples were fractionated by SDS–PAGE, and the gels were evaluated by phosphorimaging. Although Na$_2^3$SO$_4$-labeled protein larger than 206 kDa was apparent in media extracts of wild-type and heterozygous C1532Yneo cells (Fig. 3D), it was nearly absent in media from homozygous C1532Yneo cells. Moreover, all smears disappeared upon heparatinase digestion (data not shown), consistent with the likelihood that the high molecular weight (MW) bands contained perlecan. In support, immunoprecipitation of medium from primary fibroblasts, followed by western blot analysis, showed very little perlecan secretion from C1532Yneo fibroblasts (Fig. 3E), and immunostaining of these cells confirmed increased perlecan retention within C1532Yneo cells (data not shown). Immunostaining additionally revealed only trace levels of perlecan in C1532Yneo long bones (Fig. 3F and G), skeletal muscle, heart and kidney (data not shown), further signifying neo to interfere with perlecan processing. Together, these data support a mechanism whereby perlecan transcription is altered in C1532Yneo mice, resulting in reduced perlecan secretion.
Reduced perlecan in C1532Yneo mice results in SJS phenotype

Gross analysis revealed heterozygous C1532Yneo mice to appear phenotypically normal. Homozygous C1532Yneo mice, however, were smaller and walked with exaggerated hip movement by 4 weeks, as supported by footprint analysis (data not shown). By 8 weeks, C1532Yneo mice had a flat face and small eyes. Overt differences were not noted in relative organ size, color or placement.

Homozygous C1532Yneo mice exhibited a ∼20% reduction in whole-body weight (data not shown) and length...
(Fig. 4A), as well as in lengths of individual long bones (Fig. 4D and data not shown) when compared with wild-types. Plotting the published heights of SJS patients against the CDC stature-for-age percentiles revealed the reduced size of C1532Yneo mice to be consistent with the relative size of SJS patients, who are 84% of average height (Fig. 4B).

Skeletal staining revealed thickened and irregularly shaped long bones and sternal malformations in C1532Yneo mice at all stages (Fig. 4C, D and data not shown). Moreover, rib and sternal skeletal elements did not lie in a single plane (Fig. 4D), a diagnosis consistent with pigeon breast in SJS patients; delayed ossification in the spine was also noted (data not shown). Length-to-width ratios of skulls from wild-type and C1532Yneo mice revealed no difference at newborn, but a reduction in C1532Yneo mice by 8 weeks (Fig. 4E), consistent with progressive flattening of the face reported in SJS patients.

Histology of long bones revealed a transiently expanded hypertrophic zone with altered stacking of hypertrophic chondrocytes in C1532Yneo growth plates from approximately E 16.5 through newborn (Fig. 5A and B), with normalization by 2 weeks (Fig. 5C). The number of hypertrophic cells per area was reduced by ~30% when compared with wild-type, with matrix filling the acellular spaces (Fig. 5B). Subtle disorganization of proliferative chondrocyte stacking was also observed, predominantly in the central region of the growth plate, which appeared more hypocellular (Fig. 5B, asterisk). Tartrate-resistant acid phosphatase (TRAP) staining revealed ~20% more osteoclasts in newborn C1532Yneo versus wild-type long bones; these cells also appeared larger (Fig. 5D). Endomucin staining revealed distended blood vessels at the chondro-osseous junction of newborn C1532Yneo mice (data not shown), whereas von Kossa identified mis-oriented trabecular bones aligning radial to the longitudinal axis of the long bone (Fig. 5E). This was also reflected by alkaline phosphatase (AP) staining in newborn C1532Yneo mice (Fig. 5F). Establishment of secondary ossification centers was also delayed (data not shown), and marrows appeared hypocellular in C1532Yneo mice (data not shown).

Hips of SJS patients are grossly abnormal, often with complete radiographic loss of the femoral head (19). Skeletal staining and radiography (Fig. 6A) showed incorrect positioning of adult C1532Yneo innominate bones, bilaterally, and newborn femoral necks appeared short and thick (Fig. 4D). By 4 weeks, proper femoral head and neck structures of C1532Yneo mice were absent (Fig. 6B and C), with the femoral head region lacking proper consistency and shape (Fig. 6B), indicative of changes in matrix integrity and possibly degrading tissue.

Thick and bent humeri were also observed in C1532Yneo mice (Fig. 6D), with skeletal staining revealing a rough humeral head surface at 4 weeks (Fig. 6E), indicative of severe osteoarthritis. Furthermore, histology showed

Figure 4. C1532Yneo mice mimic the human SJS disease phenotype. (A) Four litters of mice were measured, revealing C1532Yneo mice to be ~20% smaller than WT littermates (left panel, P < 0.0524 at all stages). Error bars represent SD. (B) Average height of male (left side) and female (right side) SJS patients, which measures at 84% of the 50% average height of unaffected individuals, is consistent with that observed in C1532Yneo mice. (C) Whole-mount skeletal staining using Alizarin red S/Alcian blue shows decreased size of C1532Yneo newborn mice when compared with WT littermates. (D) Skeletal staining showing malformations in newborn C1532Yneo sternum and innominate bones. Dark lines represent typical alignment of sternal vertebrae (red). Hip structures and femurs were notably shorter, thickened and mis-shaped in C1532Yneo mice. (E) Bar graph showing decreased length:width ratio in 8-week-C1532Yneo skulls (P = 0.0067), zindicating a flattened face by adulthood. Error bars represent SD. Data represent measurements from four sets of mice for each time point.
asymmetric articular cartilage zones at the epiphyses that appeared thinner when compared with wild-type (Fig. 6F, arrowhead) and were absent in certain regions (Fig. 6F, asterisk). Because many SJS patients suffer from myotonia (the failure of muscles to relax following contraction), cross and longitudinal sections of skeletal muscle from C1532Yneo mice were examined. There was no indication of muscle dystrophy or hypertrophy in that muscle fiber size was consistent with no centrally located nuclei observed at any stage examined. However, 4-week-C1532Yneo skeletal muscle appeared hyperplastic, showing more muscle fibers/field than controls. These changes in muscle tone were transient, since by

**Figure 5.** Newborn C1532Yneo mice have an expanded growth plate, aberrant trabecular bone arrangement. (A) Longitudinal tibial sections of newborn mice show the hypertrophic zone of C1532Yneo mice to be expanded at this stage. (B) Higher magnification of proliferative and hypertrophic zones from (A) show decreased hypertrophic chondrocyte cell number, lack of organized stacking of cells into vertical rows and an increased matrix-to-cell ratio in both proliferative (asterisk) and hypertrophic zones of the C1532Yneo growth plate. These differences are largely resolved by 2 weeks (C). Longitudinal tibial sections from newborn WT and C1532Yneo mice (D, E, F). (D) TRAP staining reveals osteoclasts (pink) on the surface of trabecular bone spicules (T), which, in WT, are parallel to the longitudinal axis of the long bone. In C1532Yneo, osteoclasts are significantly more pronounced and line trabecular spicules which are perpendicular to the longitudinal axis of the tibia. (E) von Kossa/safranin orange staining highlights in WT the parallel orientation of trabecular bone spicules (dark brown) to the longitudinal axis of tibia, with bright red staining for proteoglycans in growth plate cartilage. In the C1532Yneo mice, irregular trabecular bone orientation and reduced staining for proteoglycans are revealed. (F) AP staining is consistent with aberrant trabecular bone arrangement in C1532Yneo tibia when compared with WT. Histology was performed on at least three sets of mice.
8 weeks, C1532Yneo muscle was indistinguishable from controls (data not shown).

Taken together, these data show that C1532Yneo mice mimic the disease phenotype of SJS patients as they are smaller than wild-types and have multiple comparable skeletal defects. Furthermore, mice harboring only the human G4595A mutation, without neo, lack symptoms of SJS, indicating that this mutation alone may not cause SJS disease.

**DISCUSSION**

The initial goal of this study was to generate a mouse model for SJS, a human skeletal disorder resulting from decreased, but not absent, perlecan levels, in order to study perlecan function during skeletal development. The *HSPG2* ntG4595A mutation chosen to develop our model abolishes the C5–C6 disulfide bond in plnIII-3 (Table 1 and Fig. 1A). This mutation

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Figure 6. Perlecan depletion in C1532Yneo mice leads to loss of femoral head reminiscent of osteonecrosis and humeral defects reminiscent of osteoarthritis. (A and D): 8 weeks; (B, C and E): 4 weeks and (F): 2 weeks. (A–F) X-ray analysis reveals hip deformations in the ishium (asterisk) and pubus, and apparent loss of the femoral head and neck regions in C1532Yneo mice, n = 4. Dissection of femurs reveals a softened, deformed femoral head region in C1532Yneo mice, n = 6. Skeletal staining reveals the lack of femoral neck (arrow) and lack of mineralized femoral head in C1532Yneo mice, n = 3. X-ray analysis reveals short, bent humeri in C1532Yneo mice with additionally bent ulna (arrow), n = 4. By 4 weeks, a rough surface is apparent on the C1532Yneo humeral head with possible osteoarthritic clefs (asterisk), n = 3. Longitudinal sections of shoulders. Note the abnormal cartilaginous region (asterisk) and absent articular cartilage (arrowhead) in C1532Yneo mice, n = 3.
has been linked to a family with SJS (21) and has been presumed to lead to an altered perlecan protein product that causes the disease. In vitro, the C1532Y alteration indeed resulted in decreased plnIII-3 protein secretion (Fig. 1B and C), consistent with a conformational change. In mice, however, the C1532Y alteration led only to subtle growth plate changes, but did not cause SJS disease. The C1532Y mice did not present with any of the disease characteristics seen in SJS patients. In contrast, immunoreactivity against perlecan domain I and V antibodies was strong in all tissues examined, and the C1532Y mice exhibited no signs of dwarfism, no flattened face, no hip dysplasia and no observed muscle abnormalities. To reconcile with the absence of an SJS-like disease manifestation in the C1532Y mice, it is conceivable that deletion of C1532 in the context of full-length perlecan may result in alternate disulfide bond formation that maintains the overall structural integrity of the protein.

It is noteworthy that the hypomorphic perlecan C1532Yneo mice do present with a skeletal disease phenotype mimicking that of SJS patients. All SJS patients in whom the disease mechanism has been studied show transcriptional changes that result in decreased perlecan protein (10,22). As in human SJS patients, the C1532Yneo mice have reduced full-length Hspg2 transcript with the appearance of an alternate truncated transcript that is not due to fusion of Hspg2 with neo (Fig. 3A). Although the mechanism by which this truncated transcript is produced remains elusive, there is precedence for both exon skipping and intron retention in SJS patients where splicing defects have been defined as the disease mechanism (10,21,22). Skipping of exons 38 to 78 would create the ∼9 kb product seen by northern blot in C1532Yneo RNA (Fig. 3A), as would skipping an unknown number of exons combined with retention of one or more introns. Similar scenarios have been documented in the COLIA1 gene, where an intronic splice-donor mutation caused osteogenesis imperfecta. Specifically, Byers and coworkers (28) demonstrated that the order and rate of intron removal in the COLIA1 gene do not proceed unhindered from the 5′ end to the 3′ end of the initial transcript. Rather, there are rapid and slow splicing events that impact the order of intron removal in the normal gene, and these are important determinants in the outcome of splice-site intronic mutations. This may likewise be the case for HSPG2. It is possible that placement of the neo cassette disrupted unidentified splice enhancers or suppressors or led to the use of cryptic splice or branch sites, producing the truncated transcript in C1532Yneo RNA. This scenario has further precedence with another basement membrane glycoprotein, laminin, in which retention of the neo cassette in the gene encoding laminin α5 led to alternate splicing upstream of the neo insertion, resulting in a mouse model for polycystic kidney disease (29). Given the tremendous size of HSPG2 (spanning 110 kb of genomic DNA), the elucidation of transcriptional mechanisms for this gene has not yet been undertaken; thus, the identification of specific transcriptional changes in our mice and in SJS patients remains largely unresolved. It is also possible that the C1532Yneo mice have a compound phenotype due to the presence of both the mutation and the neo cassette; mice harboring only the neo cassette and not the mutation may provide an additional SJS model.

The net result of reduced full-length perlecan transcript in C1532Yneo RNA is reflected by the near-absence of perlecan secretion and localization in tissues of the C1532Yneo mice (Fig. 3D–F). Immunoblotting and immunostaining tissues from C1532Yneo mice revealed reduced secretion and deposition of the entire perlecan protein, as both N- and C-terminal-domain-specific antibodies were used for protein detection. This is consistent with that described in SJS patients, who have decreased perlecan C-terminal domain immunoreactivity. Although truncated perlecan products have been widely assumed to account for some cases of SJS, only decreased levels of the entire protein have been presented in the literature, indicating that reduced levels of full-length perlecan, and not partially functional truncated perlecan, lead to SJS pathology. In support, the Nicole lab (22) recently identified 21 new SJS mutations and report no genotype–phenotype correlation, suggesting a common disease mechanism. All data support degradation, and not secretion, of mis-translated protein, as the low level of full-length perlecan transcript in C1532Yneo mice correlates well with the low level of protein deposition in these mice and with the intracellular perlecan immunostaining seen in C1532Yneo hypertrophic chondrocytes (Fig. 3) and reported in fibroblasts from SJS patients harboring the C1532Y alteration (22).

Availability of mouse models for comparable human diseases involving perlecan permits us to speculate about perlecan’s function within skeletal development. At least minimal levels of full-length perlecan are required for the maintenance of life, as both DDSH patients and mice harboring functional null mutations in perlecan have severe skeletal defects and die either during embryonic development or just after birth (16,17,30). In contrast, the point mutation (ntG4595A) altering a disulfide bond in plnIII-3 is practically silent in mice. C1532Yneo mice and SJS patients lie intermediate to these two cases. Although they have a near-depletion of full-length perlecan protein, this is not incompatible with a full life. Severely bent and distorted long bones are observed in DDSH patients (30) and perlecan-null mice (16,17), whereas C1532Yneo mice show growth plate abnormalities at the newborn stage that predominantly resolve over time, indicating a critical temporal role for baseline levels of perlecan during skeletal development. In support, the specific sulfation pattern of chondroitin sulfate GAG chains attached specifically to perlecan has recently been shown to be critical for proper collagen II fibril formation (31).

The size discrepancy between C1532Yneo mice and controls, and between SJS patients and non-affected persons, might be attributed to the transiently expanded hypertrophic zone, as described in several other knock-out and transgenic mice (32–34). Perhaps, the content and organization of the pericellular matrix influence growth, orientation and removal of hypertrophic chondrocytes, particularly during intense growth periods. This is also plausible, as the hypertrophic zone has a higher GAG:DNA ratio than other growth plate zones (35), suggesting a particular role for proteoglycans in this zone. In further support, a comparison of the growth plate disease phenotype in the perlecan-null and the C1532Yneo mice reveals many similarities, including a
disorganization of the columnar arrangement of growth plate chondrocytes, fewer hypertrophic chondrocytes and disrupted EO with respect to a delay in the formation of primary and secondary ossification centers (16,17). These alterations within the growth plate and EO are pronounced in the null mice, but more subdued in the SJS mouse model; regardless, they underscore a key role for perlecan in cartilage extracellular matrix undergoing EO.

It is also likely that low levels of perlecan may directly impact growth factor signaling. Perlecan promotes chondrocyte attachment in vitro (36) and modulates FGFR-2 binding to chondrocytes in growth plate extracts (37). Furthermore, FGFR3 signaling has been implicated in the phenotype of perlecan-null mice, because FGFR-1, a potential activator of FGFR3 signaling, accumulated on the chondrocyte cell surface of these mice (16). Moreover, mutations in a mediator of FGFR signaling, leukemia inhibitory factor receptor, lead to Stuve–Wiedemann syndrome, formerly categorized as SJS type 2 because of phenotypic similarities (38). Perlecan may also affect the endocrine system, as other dwarf phenotypes are linked to mutations affecting metabolism, in which size discrepancies are never resolved (39,40).

The striking femoral and humeral head phenotypes in C1532Yneo mice pose further similarities to SJS patients. Already by 1 week, the femoral head and neck of C1532Yneo mice are undetectable by skeletal staining. However, at 4 weeks, a non-mineralized matrix is present at the most proximal end of the femur (Fig. 6B), indicating that either instability of the matrix or dislocation due to malformation causes the femoral head to degrade in a manner consistent with osteonecrosis, making this strain useful for therapeutic trials. The pronounced osteoarthritic clefts seen in C1532Yneo humeral heads (Fig. 6E, asterisk) may also prove useful for therapeutic intervention.

Perlecan is a ubiquitous member of basement membranes; for this reason, its relatively benign role outside the skeletal system in perlecan-null and hypomorphic mice is unexpected. It is possible that the complete role(s) of perlecan will not be realized under physiological conditions. Studies have recently shown perlecan to be involved in wound healing (41) and in glomerular filtration under conditions of high protein load (42). Perhaps another proteoglycan is capable of compensating for the loss of perlecan under normal circumstances, but perlecan itself is essential under stressed conditions as indicated in the earlier cases, as growth factors have been shown to bind specifically to perlecan core protein or to heparan sulfate side chains attached specifically to perlecan (23,37,43). The array of perlecan ligands may also be tissue-specific and/or temporal-specific. Additionally, it is possible that perlecan cleavage products are essential molecules under certain circumstances such as angiogenesis (44).

In conclusion, we demonstrated that mice harboring the ntG4595A mutation identified in an SJS family, as well as a neomycin selection cassette, present with all expected skeletal disease features of SJS. The C1532Yneo mice have an alternate, truncated transcription product, reduced perlecan secretion, a dwarfed stature, a flat face and hip dysplasia. In addition, these mice present with growth plate defects similar to, but less severe than, DDSH patients and perlecan-null mice; these specific characteristics have not yet been explored in SJS patients. In contrast, we have shown mice harboring the human ntG4595A mutation alone to exhibit only a mild skeletal phenotype, but to lack all disease symptoms of SJS. It would be informative to examine the mechanistic consequence of the HSPG2 ntG4595A mutation in SJS patients. Whether or not the lack of C1532 may have altered perlecan tertiary structure, it is likely that these patients may have an additional, possibly intrinsic mutation that would lead to altered transcription. If this were the case, these patients would support the same overall SJS disease mechanism as the C1532Yneo mice, as well as all reported SJS patients thus far investigated. As only exons and their splice junctions were screened for mutations in the C1532Y SJS family, presumably due to the tremendous size of HSPG2, this intriguing issue is currently outstanding.

MATERIALS AND METHODS

Transfection of 293 and NIH/3T3 cells

The G4595A mutation was introduced into perlecan domain III-3 (23) using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA). Mutant and wildtype vectors were transiently transfected into HEK 293 cells and mouse embryonic fibroblasts NIH/3T3 (American Type Culture Collection, Manassas, VA, USA). Pooled medium (1 ml) was precipitated with trichloroacetic acid (10%) and Triton X-100 (0.1%; ice, 30 min). Pelleted samples were washed (1 ml acetone, 4°C), resolved by SDS–PAGE under reducing conditions and visualized with Coomassie Brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA, USA) or transferred to nitrocellulose and probed with rabbit-anti-mouse IgHIII-3 antibody 1030 (23). Membranes were washed, incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare, Piscataway, NJ, USA) and developed using enhanced chemiluminescence detection (GE Healthcare).

Generation of perlecan knock-in mice

A 10 kb C-terminal fragment of mouse perlecan was isolated from a 129SV/SvevTACfBr mouse PAC library (MRC Geneculture, Cambridge, UK) using 660 bp mouse Hspg2 cDNA. The targeting construct contained a 4 kb 5′ arm and a 6 kb 3′ arm separated by a phosphoglycerate kinase-neomycin cassette flanked by frt sites. Prior to assembly, the human G4595A mutation was introduced to the 5′ fragment and sequence verified. Linearized construct was electroporated into R1 embryonic stem cells (45) and G418-resistant homologous recombinant clones were confirmed by Southern blotting (Fig. 2A). Two independent clones were injected into blastocysts and implanted into pseudo-pregnant females. Resultant male chimeras were backcrossed with 129Sv females, confirming germ-line transmission.

Mice were genotyped by Southern blotting or by PCR with the following primers: 5′-ATGAGATCATCTTCCGAGA-3′ (S) and 5′-ggttaggacaaacagactttgg-3′ (AS) to detect 400 bp wild-type and 500 bp C1532Y mutant amplicons and 5′-TGCTCCTGCGAGAAAGTATCCATGTCGC-3′ (S) and 5′-CGCCAAGCTTTCGCAATATCACCGGGTAG-3′ (AS).
(AS) to detect neo, using 2 mM MgCl₂, 30 cycles, denaturing 94°C, 30 s, annealing 55°C, 30 s and extending 72°C, 1 min.

RNA isolation, northern hybridization and RT–PCR
RNA was isolated from newborn mouse endochondral skeleton, kidney, lung, heart and skeletal muscle using TRIZol® (Invitrogen). RNA (30 µg) was fractionated (0.8% agarose, 2.2 M formaldehyde, 1 × MOPS gel) and quality assessed by ethidium bromide staining. Duplicate lanes were transferred to Hybond-N+ (GE Helathcare) and hybridized with β²⁸-labeled pBlIII-3, neo and β-actin probes. Message levels were quantified using the Storm860 PhosphorMager and ImageQuant software (GE Healthcare). For RT–PCR, 1 µg RNA was reverse transcribed using SuperScript III (Invitrogen), and perlecain transcript analyzed semi-quantitatively using cycle numbers; expression of glyceraldehyde 3-phosphate dehydrogenase provided an internal standard. Primers: GAPDH 5'-GGTGAAGGTCCAGTCACCG-3' (S) and 5'-GGTCA TGATGCTTCCACGAT-3' (AS); exon 34 5'-CTCTTGG ACCCTGATATCCAGA-3' (S); exon 36 5'-GAGTCGGG GGGGCCAGAT-3' (S) and 5'-CTGCGAGGCAAAGCGCTACATA-3' (AS); exon 37 5'-GACTGTGCCC GGCTACA CT-3' (S) and 5'-CGACAGGTCTGAGTCTCCGG-3' (AS) and exon 38 5'-ACACAGCTCACGAATCTCACA-3' (AS) with 2 mM MgCl₂ for 15, 20, 25 and 30 cycles; control samples excluded cDNA.

Cell culture, metabolic labeling and protein analyses
Skin fibroblasts from newborn mice were isolated (0.1% trypsin/EDTA, 30 min, 37°C), pelleted (2,200 g) and plated onto 75 cm² flasks with Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 37°C and 5% CO₂. Upon ~80% confluency, Na₂SO₄ (35 µCi/ml) was added for 24 h, after which cells were scraped and pelleted (2,200 g), and media fractions were extracted in 6 M urea with protease inhibitor cocktail (AEBSF, EDTA, Bestatin, E-64, Leupeptin and Aprotinin; Sigma-Aldrich, St Louis, MO, USA) and extracted (24 h, 4°C). Proteoglycan enrichment from cell and media extracts was by DEAE chromatography. Columns were washed with low salt buffer (0.1 M NaCl, 6 M urea, 50 mM Tris, pH 7.0) and eluted with high salt buffer (1.5 M NaCl, 6 M urea, 50 mM Tris, pH 7.0). Half of the eluates were dialyzed (12 h, 4°C) against 50 mM Tris, pH 7.5, 5 mM calcium acetate and 0.5 mg/ml bovine serum albumin, followed by heparanase digestion (0.3 U, 3 h, 37°C; Sigma-Aldrich). Samples (40 000 c.p.m.) were compared by SDS–PAGE and phosphorimaging (GE Healthcare).

For immunoprecipitation, 1.5 × 10⁸ primary fibroblasts were extracted (10 mM EDTA, 12 h, 4°C). Media and cellular extracts were preclearced with protein A–Sepharose (12 h, 4°C), and supernatants were incubated with affinity-purified anti-mouse perlecain polyclonal antibodies 1042 (26) or 1056 (25). Immune complexes were precipitated with protein A–Sepharose (3 h, 4°C), washed in extraction buffer and pellets solubilized in Laemmli sample buffer containing 5% 2-mercaptoethanol for SDS–PAGE. Proteins were transferred onto nitrocellulose and immunolabeled with antibody 1042 or 1056.

Histology, immunohistochemistry and skeletal staining
Tissues were fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline pH 7.4 or 0.5% PFA, 1 week. Postnatal samples were decalcified (10% EDTA, 0.5% PFA, 1 week). Samples were dehydrated through graded ethanol, paraffin embedded and 6 µm longitudinal sections were stained with either hematoxylin and eosin with and without Alcian blue, safranin orange/fast green, TRAP (46), AP or von Kossa (46,47). Antibodies for immunodetection were against mouse perlecain domain I (1042) and domain V (1056). Alizarin red S and Alcian blue 8GS skeletal staining was as described previously (48).

ELECTRONIC DATABASE INFORMATION

Conflict of Interest statement. The authors have no conflict of interest.

REFERENCES


