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Model systems for studying skeletal dysplasias caused by TSP-5/COMP mutations

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Abstract. Cartilage oligomeric matrix protein, also known as thrombospondin-5 (TSP-5), is an extracellular matrix protein found primarily in cartilage and musculoskeletal tissues. TSP-5 is of interest because mutations in the gene cause two skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED/EDM1). Both PSACH and EDM1 have a characteristic chondrocyte phenotype distinguished by giant rough endoplasmic reticulum (rER) cisternae containing TSP-5 and other extracellular matrix proteins such as type IX collagen and matrilin-3. The accumulation of proteinaceous material in the rER compromises cellular function and leads to premature chondrocyte death. Both *in vitro* and *in vivo* models have been generated with varying degrees of success to study the cellular mechanisms of the disease process. Here we review and discuss *in vitro* and *in vivo* PSACH and MED model systems and describe two transgenic mouse lines expressing human mutant TSP-5 protein. These model systems have revealed several important features of the PSACH cellular pathology: unfolded protein response activation, upregulation of apoptosis and inappropriate assembly of matrix network in the rER. Some of these models are valuable reagents that may be of use in testing therapeutic interventions. (Part of a Multi-author Review)

Keywords. TSP-5, PSACH, pseudoachondroplasia, mouse model, chondrocyte, growth plate, COMP, MED, multiple epiphyseal dysplasia.

Introduction

Cartilage oligomeric matrix protein (COMP/TSP-5) is one of the many low-abundance proteins that comprise the complex extracellular matrix (ECM) networks of cartilage, tendon and ligament that cushion and stabilize skeletal elements [1–3]. It is the fifth member of the thrombospondin gene family (TSP-5) and belongs to the pentameric subgroup [4–7]. TSP-5 is a multi-domain protein comprised of an N-terminal pentamerization domain, a epidermal growth factor (EGF)-like domain, a highly conserved type 3 (calcium-binding) repeat domain and a C-terminal globular region [8, 9]. Recent work suggests that TSP-5 may have various functions, including 1) regulation of collagen fiber assembly/diameter [10], 2) interaction with other ECM molecules [11–13], 3) regulation of chondrocyte proliferation [14, 15], 4) regulation of apoptosis [16–18] and 5) enhancement of cellular attachment [19]. Mutations in TSP-5 significantly impact skeletal growth and produce a characteristic chondrocyte pathology [20]. The focus of this article is to review the model systems that have been developed and used to define the pathophysiology of the disease process.

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Clinical phenotype

Mutations in TSP-5 cause two skeletal dysplasias: a severe disproportionate dwarfing condition, pseudoachondroplasia (PSACH) and a milder condition, multiple epiphyseal dysplasia (MED/EDM1 subtype) [13, 21–23]. Many novel TSP-5 mutations have been identified with the majority found in the highly conserved type 3 calcium-binding repeat domain [22–25]. One common mutation, D469del, accounts for approximately 30% of all PSACH cases [24, 25]. PSACH is a dwarfing condition first described in 1959 [26], and subsequent publications have delineated the natural history of the condition [20, 21, 25, 27–31]. Babies with PSACH have a normal birth length and weight and are first diagnosed when their linear growth decelerates between 1 and 2 years of age [25, 32]. Radiographic studies show metaphyseal irregularity, epiphyseal dysplasia and platyspondyly [33]. Characteristic PSACH clinical findings include waddling gait beginning in early childhood, widened and lax joints, brachydactyly, windswept, knock knee or bowing deformities of the lower extremities, exaggerated lumbar lordosis and early onset osteoarthritis [25, 32]. The head circumference is normal, and the face is angular and very attractive [32]. Together, the clinincal and radiographic findings establish the diagnosis of PSACH. Replacement of the weight-bearing joints, particularly knees and hips, is often required in early adulthood [25]. Sequencing of the TSP-5 gene is commercially available to confirm the diagnosis. In contrast to PSACH, MED is diagnosed around 5 years of age when the children present with abnormal gait and/or stiff, painful hips [25]. Mild short stature and osteonecrosis of the hips (Legg-Perthes disease) are also frequent findings in MED [25]. Hip replacement is often needed in early adulthood [25]. TSP-5 mutations cause a specific type of MED (EDM1) and account for 25–35% of cases with brachydactyly [21, 34]. However, there are five other types of MED caused by mutations in type IX collagen [21, 35], matrilin-3 (MATN3) [36–38] and diastrophic dysplasia sulfate transporter (DTDST) genes [39–42]. Molecular diagnostic testing is used to establish the exact type of MED; genetic counseling and prognostic information depend on an accurate diagnosis.

Human PSACH growth plate pathology

Electron microscopy studies of PSACH iliac crest biopsies in 1973 showed a characteristic lamellar appearing material retained in the rough endoplasmic reticulum (rER) of chondrocytes [20]. Following the identification that TSP-5 mutations cause PSACH, TSP-5 was found to be retained in the rER [7, 43]. Recent studies show that in addition to TSP-5, type IX collagen and MATN-3 are also retained and form an ordered intracellular matrix network [44]. This retention disrupts export of these matrix proteins and results in a disorganized ECM [45, 46]. Detailed analysis of a PSACH growth plate with a D511Y mutation shows an extended resting zone with fewer chondrocytes in the proliferative and hypertrophic zones [18]. There was generalized disorganization with the chondrocytes found in clusters rather than an orderly columnar pattern. Additionally, there were many dead chondrocytes and cell remnants throughout the growth plate [18]. All of the chondrocytes had enlarged rER cisternae containing TSP-5 and other ECM proteins [18]. Mutant TSP-5 protein has been shown to fold incorrectly and is recognized as abnormal by chaperone proteins in the chondrocyte rER, thereby disrupting the export of mutant TSP-5 to the ECM (Fig. 1A) [9, 13, 43, 47–52]. Accumulation of mutant TSP-5 results in rER stress, because the retained material is not degraded with sufficient rapidity to clear the rER and maintain normal rER function [7, 20, 43, 48]. Premature chondrocyte death is observed in the PSACH growth plate [7, 16–18]. Apoptosis is upregulated in cultures of PSACH chondrocytes [16] and in several cell types overexpressing mutant TSP-5 [53]. This suggests that prolonged rER stress causes apoptosis in PSACH/MED chondrocytes and could be one mechanism responsible for the loss of growth plate chondrocytes [54]. A consequence of chondrocyte depletion is diminished linear growth, resulting in the disproportionate short stature associated with PSACH (Fig. 1A) [32]. In addition to depleting the pool of viable chondrocytes, TSP-5 mutations also affect the export of other ECM proteins, such as type IX collagen, MATN3 and to a lesser extent type II collagen [7, 18, 45, 55–57]. The resulting matrix has a disorganized network comprised of type II collagen fibrils (Fig. 1B) [45]. The abnormal matrix, particularly in the articular cartilage, is easily eroded even with normal activity. PSACH and MED patients develop early-onset osteoarthritic changes, and the joint erosion necessitates hip replacement in early adulthood (Fig. 1B). Other joints are also affected and may require replacement. Natural history studies suggest that overall health and longevity in PSACH is normal and that TSP-5 mutations specifically affect the musculoskeletal system [30].

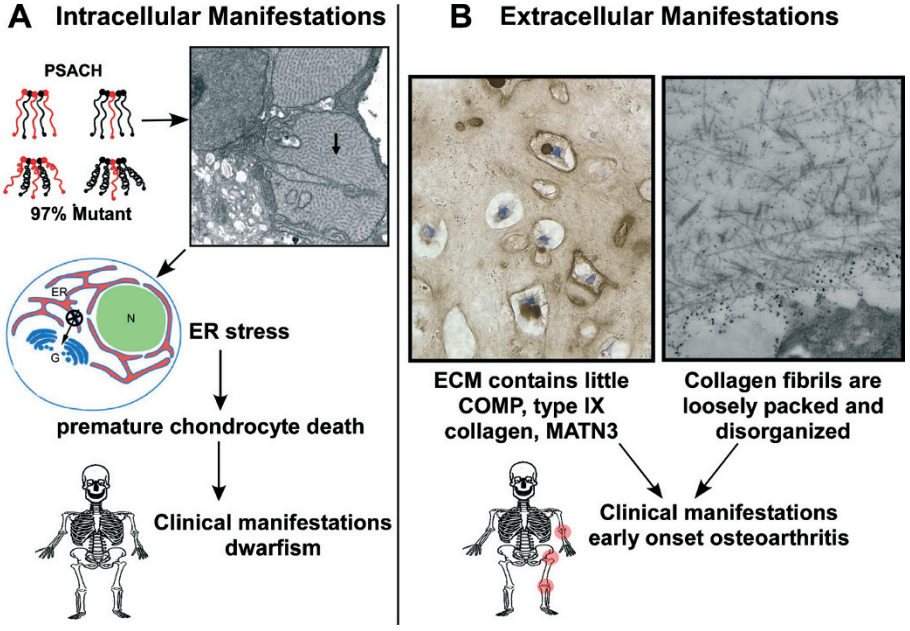

Figure 1. Effects of COMP mutations. (*A*) Intracellular manifestations of TSP-5 mutations. Mutant TSP-5 protein is unable to fold properly and is retained within the rER, causing ER stress and premature chondrocyte death, which causes short stature. (*B*) Extracellular outcomes of TSP-5 mutations. There is diminished TSP-5, type IX collagen and MATN3 in the ECM, and collagen fibrils appear disorganized and sparse. This poorly formed ECM is easily eroded, leading to early onset osteoarthritis.

In vitro model systems

Several *in vitro* systems have been developed to recapitulate the PSACH chondrocyte phenotype in order to study mutant and wild-type TSP-5 protein trafficking [31, 48, 49, 57, 58]. These systems have been developed with the goal of studying the pathophysiology of the disease, which cannot be observed in cross-sectional human growth plate samples.

Bovine articular cartilage model

Primary bovine articular cartilage chondrocytes infected with an adenovirus expressing rat D469del TSP-5 attached to a BMP-40 signal peptide were used to study protein trafficking [48]. These chondrocytes exhibited decreased cell viability and delayed TSP-5 secretion. The protein retained in the rER was granular and did not have the lamellar or punctate pattern, and the rER were not massively enlarged [48]. Two rER chaperone proteins, Grp78 and calreticulin, were found to be associated with mutant TSP-5, which is similar to results found in the human PSACH chondrocyte [43, 48]. Despite this delay in trafficking in the rER, mutant TSP-5 protein was processed through the Golgi without an increase in degradation [48]. Mutant TSP-5 was found in the matrix, which was disorganized. Collagen fibrils were thin, and there was an irregular pattern of proteoglycan nodules with amorphous aggregates containing TSP-5 throughout the pericellular space [48], similar to that observed in human PSACH tissue [49]. The presence of increased cell death and disorganized matrix correlates with the human PSACH growth plate observations (Fig. 1B).

In subsequent studies, the bovine chondrocyte system was used to evalute D475N, D469del, and H587R PSACH and D361Y MED mutations. The H587R mutation is in the C-terminal domain (CTD), while all the others are in the type 3 repeat domain [46]. The D469del mutation was used as the trafficking standard. rER retention was observed with D469del and D475N mutant proteins. Only slightly delayed protein secretion was observed with D361Y and H587R mutant proteins. Decreased chondrocyte viability and disorganized ECM were associated with all TSP-5 mutant proteins [46]. These results led to the suggestion that extracellular mutant TSP-5 makes a significant contribution to the pathogenesis of PSACH and MED. The use of the BM40 signal peptide complicates interpretation of the results, as it may artificially promote more efficient secretion [46, 48, 59, 60]. The applicability of this model system is limited, because the PSACH cellular phenotype was not recapitulated [46] and the alginate bead redifferentiation system may alter collagen fibrils and cause differential ECM gene expression [61, 62].

COS-7 (African green monkey SV40-transformed kidney fibroblast cells) model

A transformed kidney cell line (COS-7) was used to overexpress the D472Y TSP-5 protein using an adenovirus; the D472Y mutation is in the type 3 repeat domain [17]. D472Y TSP-5 protein was retained in the rER, and some characteristics of the PSACH cellular phenotype were recapitulated. This is

particularly interesting because kidney cells do not express other ECM proteins, such as MATN3 and type IX collagen, which are involved in human chondrocyte pathology [17]. Enlarged and gnarled rER were associated with mutant TSP-5 expression. Immunohistochemistry localized mutant TSP-5 to the rER, whereas the wild-type TSP-5 was detected in the Golgi [17]. The ER stress marker, phosphorylated eukaryotic initiation facter 2 α (eIF2α), was induced, and apoptosis increased with expression of D472Y mutant protein [17]. These studies demonstrate that induction of the rER stress response and apoptosis was associated with mutant TSP-5 expression regardless of the cell type.

Swarm rat chondrosarcoma cells (RCS) model

RCS cells were used to study D469del TSP-5 protein trafficking [58, 63]. One study compared protein secretion in RCS to non-chondrocytic COS-1 cells [58]. Cells were transiently transfected with a plasmid expressing human FLAG-tagged D469del TSP-5 protein [49, 58]. Cellular localization was assessed using both biochemical and immunohiostochemical methods. In RCS cells, wild-type TSP-5 protein was exported to the extracellular space, while D496del TSP-5 was retained in the cell lysate [58]. In contrast, both D469del and wild-type TSP-5 proteins localized to the Golgi compartment and were equally exported to the cell culture media in COS-1 cells [58]. This indicates that rER retention of mutant COMP may depend on the cell type but does not agree with the findings observed in the non-chondrocytic COS-7 cells (see discussion above) [17]. The inconsistency between COS-1 and COS-7 cells may be due to differences in these cell lines. In RCS cells, type IX collagen was retained with TSP-5 and both proteins were markedly diminished in the ECM [58]. Interestingly, aggrecan trafficking was not significantly altered [58]. In subsequent experiments, this same RCS model system was used to characterize the trafficking behavior of 11 mutant TSP-5 proteins [63]. The aim of this study was to determine whether the cellular phenotype correlated with the genotype. Three mutations were located between the type 2 and type 3 repeat domains (D271H-PSACH, L272P-PSACH, P276R-MED), and three mutations were in the first type 3 calcium-binding repeat (G299R-PSACH, D302V-MED and G309R-PSACH) [63]. The D469del mutation, which has been extensively studied, was included along with two different mutations that occur at the same residue (D473N-PSACH and D473G-PSACH) [63]. Three mutations were located in the CTD (T585M-MED, T585R-MED and H587R-PSACH) [63]. For mutations in the calcium-binding domain of TSP-5, the extent of the TSP-5 protein-trafficking defect was correlated with the severity of the clinical phenotype. MED mutations (P276R-MED and D302V-MED) showed the least trafficking abnormality, while PSACH mutations (G299R-PSACH, L272P-PSACH and G309R-PSACH) exhibited a moderate to severe trafficking defect [63]. No correlation was observed for mutations in the CTD, most likely related to the domain-dependant context of the mutation [63]. These data support a disease model based on impaired trafficking for mutations in the calcium-binding domain. For mutations in the CTD, the clinical phenotype of patients is likely caused by both the mutations and other undefined factors.

Tendon cell system

Most of the PSACH/MED research has focused on chondrocyte pathology because of the disproportionate short stature caused by TSP-5 mutations; however, other musculoskeletal systems are also affected. Joint laxity, particularly in PSACH, is of clinical importance because it contributes to joint abnormalities that occur in childhood [21, 25, 32]. The limited *in vivo* studies with tendons suggest that TSP-5 mutations may affect tendon cells differently than chondrocytes [58]. Ultrastructural analysis and immunohistochemical studies on different tissues from a PSACH patient, with a D346N mutation, illustrate this difference [64]. Chondrocytes with this mutation had enlarged rER that were immunopositive for TSP-5 and type IX collagen proteins [64]. In contrast, there was pronounced TSP-5 immunostaining in the ECM of tendons, indicating that the mutant D346N protein was secreted by the tendon cells [64]. Similar results were found in tendons with the D469del mutation [58]. The results from both of these studies suggest that mutant TSP-5 protein can be exported by tendons and is not significantly retained. This may reflect a different secretory pathway in two cell types and/or, more likely, the absence of other interacting ECM proteins in tendon cells [65].

In vitro studies were undertaken to gain a better understanding of these cell-specific differences. Primary bovine tenocytes were infected with an adenovirus containing either D469del, D473N, H587R or wild-type TSP-5 [53]. Two mutations were in the type 3 repeat domain (D469del and D473N), and the third (H587R) was in the CTD of TSP-5 [53]. Secretion of D469del and D473N TSP-5 proteins was significantly delayed, whereas export of H587R was similar to wild-type TSP-5 protein trafficking [53]. Wild-type TSP-5 and H587R proteins co-localized with a Golgi-specific marker, whereas the D469del and D473N proteins co-localized with an rER-specific marker [53]. This is in contrast to the *in vivo* study, which found D473N TSP-

5 protein in the ECM of tendon [64]. All tendon cells expressing mutant TSP-5 protein had abnormal matrix deposition, with fewer type I collagen fibrils and large amorphous TSP-5 immunopositive deposits [53]. Higher levels of apoptosis were observed in tenocytes expressing mutant TSP-5 protein [53]. The differences between the *in vivo* and *in vitro* observations may be attributed to the overexpression of mutant TSP-5 from an adenovirus compared to lower endogenous expression levels. Additional *in vivo* studies are necessary to assess this model system and to understand TSP-5 protein trafficking in tendon.

Human chondrocyte model system

Human chondrocytes have been used to assess how mutant TSP-5 protein affects the cellular secretory pathway and matrix production [16, 43, 49, 57]. This approach has been employed because human chondrocytes are more likely to mimic the *in vivo* condition. However, human chondrocytes are a terminally differentiated cell type and rapidly lose the chondrocyte phenotype when cultured in monolayer [66, 67]. To overcome this problem, chondrocytes are cultured either in alginate beads or in nonadherent 3-D conditions [16, 43, 49, 57]. Both systems induce the production of chondrocyte-specific proteins, but the matrix produced by the chondrons in the 3-D culture system is more similar to that observed *in vivo* [7, 18, 43, 49, 61, 62].

PSACH chondrocytes obtained from iliac crest biopsies, with D469del, G427E and D511Y mutations, and chondrocytes overexpressing D469del TSP-5 protein from an adenovirus show the characteristic large rER cisternae and disorganized matrix [16, 43, 49, 57]. These findings are comparable to those seen in PSACH growth plates [7, 18]. Studies with PSACH chondrocytes cultured for 3–6 months were the first to show that mutant TSP-5 protein also affected the secretion of type IX collagen, MATN3 and, to a lesser degree, type II collagen [43]. Furthermore, four chaperone proteins, calreticulin (CRT), protein disulfide isomerase (PDI), Grp94 and BiP all co-localized with intracellular TSP-5 protein [43], suggesting that retained mutant TSP-5 induces the ER unfolded protein response (UPR) [54].

Long-term cultures (20 weeks to 1 year) of PSACH chondrons/nodules in alginate demonstrated cellular trafficking abnormalities causing massive intracellular protein retention, which compromises cellular function and cartilage production [16]. The number of apoptotic chondrocytes and nodule size were not significantly different at 20 weeks [16]. However, after 1 year in culture, nodule size, chondrocyte number and percent of cartilage per alginate bead were significantly less in the PSACH samples compared to the controls [16]. Apoptosis was increased 1.6-fold in the PSACH samples [16]. These results suggested that, over time, retention of mutant TSP-5 protein had a toxic affect on the chondrocytes.

A model system utilizing human chondrocytes overexpresseing D469del TSP-5 protein from adenovirus grown in 3-D culture were also used to assess the effect of mutant TSP-5 proteins on MATN3 and type IX collagen [57]. This model system closely recapitulated that observed *in vivo* and confirmed many of the earlier observations that the other ECM protein export was also affected by TSP-5 mutations [7, 18]. Deconvolution microscopy was used to define the architecture of the proteinous material retained in the chondrocyte rER [44]. Chondroctyes with D469del, G427E and D511Y TSP-5 mutations were maintained in 3-D culture for 56 days. All of the chondrocytes showed a similar pattern of protein retention even though the mutations were different. An ordered 'intracelluar matrix' composed of a type II procollagen core encircled by a network composed of MATN3, type IX collagen and TSP-5 was observed [44]. This was the first study to establish that stalling of mutant TSP-5 protein in the secretory pathway can catalyze the production of an ordered matrix. This finding may, in part, explain why this material is not efficiently degraded. The human chondrocyte model systems have yielded important insights into chondrocyte pathology associated with TSP-5 mutations. However, human chondrocyte model systems require long periods of time to obtain results and special culturing conditions to maintain the chondrocyte phenotype.

Mouse models

Animal models of human disease are useful because they provide sufficient experimental materials for complex experiments and a system that can be manipulated and observed throughout development. Both transgenic and knock-in approaches have been used to develop mouse models with the knock-in approach having better success.

TSP-5 BAC transgenic mouse

A BAC (Research Genetics BAC 2102B03) containing the human TSP-5 gene and native promoter was used to generate a murine transgenic TSP-5 model. The D469del mutation was introduced using the two-step 'hit and fix' oligonucleotide-based BAC recombinant technique employing the bacteriophage lambda Red recombination system [68–70].

The purified D469del TSP-5 BAC was microinjected into C57BL6/DBA2 mice blastocysts. Two founders

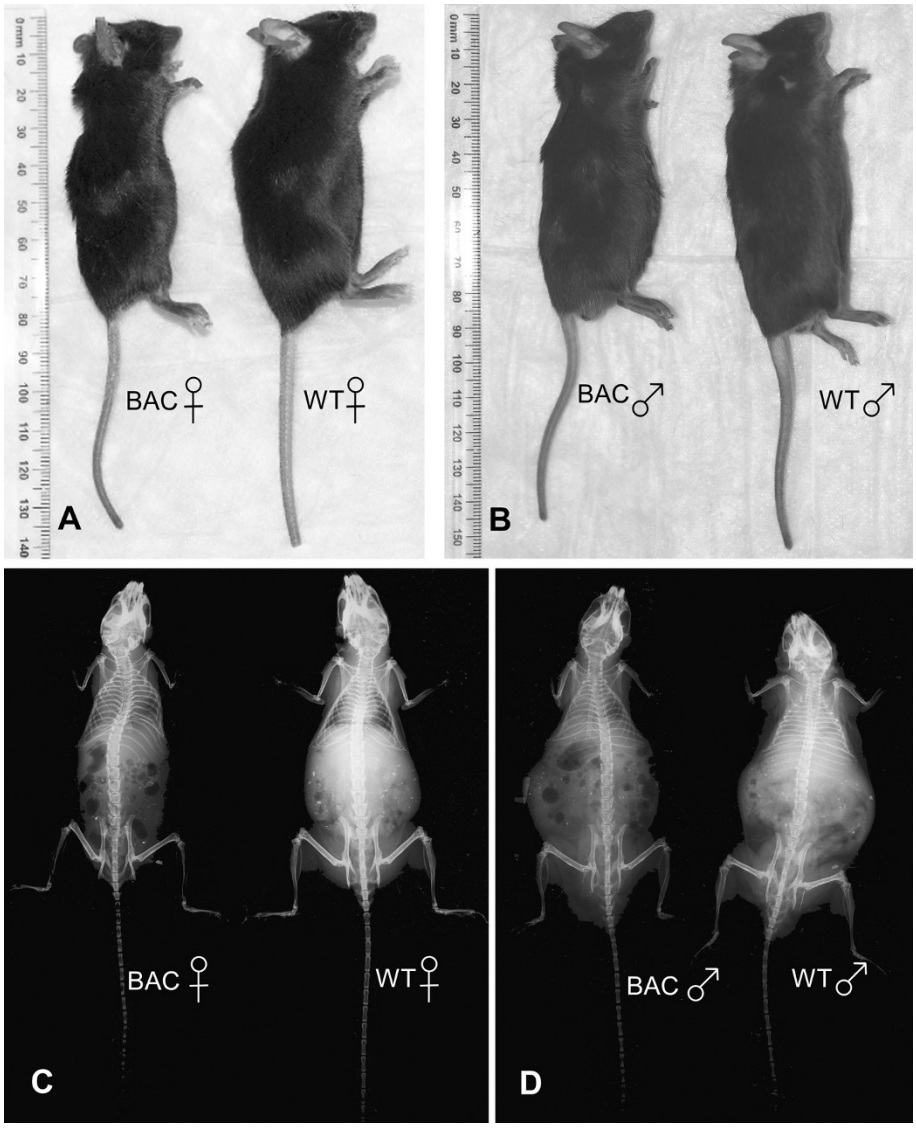

Figure 2. Wild-type and D469del transgenic mice. (*A* and *B*) Lateral views of founder D469del mice (on left in all panels) and wild-type (WT) controls (right); females are shown in (*A*) and males in (*B*). D469del mice have shorter crown-to-rump length compared to wild-type siblings. AP radiographs of D469del mice and wild-type siblings (*C* and *D*). Females are on the left and males on the right. Long bones appear shorter and more gracile.

(one male and one female) resulted, and only the female had a clinical phenotype. The female was lethargic, exhibited a gait disturbance and was sterile. The male founder was generally robust and produced 53 offspring, but only two carried the transgene. One pup died in infancy and the other was harvested as a 15-dpc embryo. Both founders died prematurely, the female at age 4.5 months of age and the male at 5.5 months. At the time of death, radiographs were taken, and the long bones of the BAC mice were gracile compared to wild-type litter mates (Fig. 2). No viable F1 progeny were available for study.

The most striking abnormalities in the founders were observed in the growth plates, which were disorganized compared to the wild type littermates (Fig. 3A, panels A–H). Growth plate chondrocyte organization was assessed by counting chondrocytes arranged in a columnar pattern or clustered arrangement divided by the total number of chondrocytes. Significantly fewer D469del BAC chondrocytes were organized in columns (12–18 %) compared to the wild-type mice (73–74 %) ($P < 0.0000001$) (Fig. 4A). Additionally, growth plates from the D469del BAC mice were thinner (data not shown; five measurements were taken along the growth plate of three different sections using Image Pro software, Media Cybernetics) and contained 40–50 % fewer chondrocytes than wild-type mice (data not shown).

TSP-5 distribution was evaluated in growth plate and articular cartilage by immunohistochemistry using a TSP-5 polyclonal antibody (Kamiya Biomedical Company #PC-140; dilution 1 : 100). This antibody recognizes both mutant human and wild-type mouse TSP-5 proteins. The distribution of TSP-5 in the D469del BAC growth plate was patchy and did not have the expected pericellular and territorial chondrocyte dis-

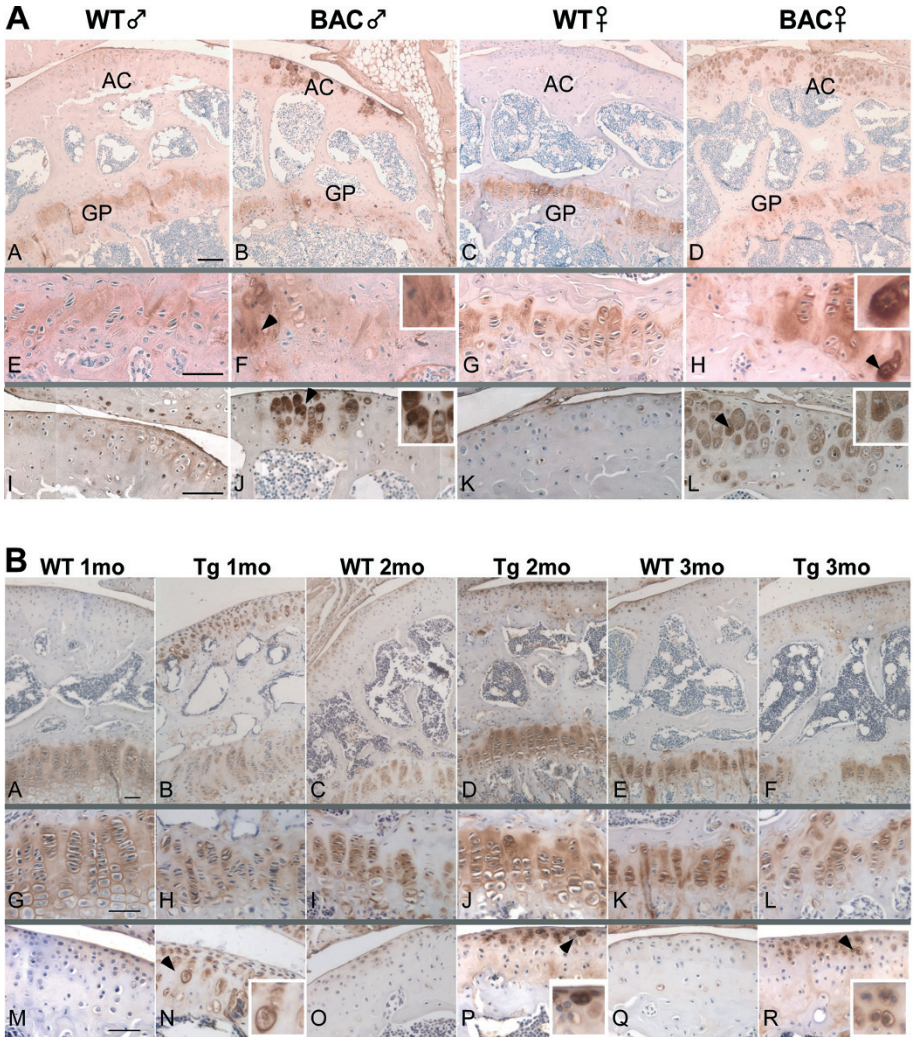

Figure 3. Growth plate (GP) and articular cartilage (AC) of hind limbs from D469del and wild-type mice. (*A*) TSP-5 immunolocalization in BAC transgenic mice growth plate is shown in panels A–D (low magnification) and panels E–H (high magnification). TSP-5 distribution in the D469del mice growth plates is patchy and diminished overall compared to the wild type (WT). A few chondrocytes appear to have intracellular staining (insets). The D469del growth plate is disorganized and has fewer chondrocytes. The articular cartilage is shown in panels I–L. D469del articular chondrocytes show heavy TSP-5 staining (arrowheads) which is absent in the wild-type sibling controls. (*B*) TSP-5 immunolocalization in the transgenic (Tg) D469del mice is shown at 1, 2, and 3 months of age with WT controls (panels A–F). The growth plate appears disorganized compared to controls (panels G–L). Heavy staining in the articular cartilage (arrowheads) is observed and is similar to the staining pattern of the BAC transgenic D469del articular cartilage (panels M–R).

tribution seen in the wild-type littermates (Fig. 3A, panels E–H). Intracellular retention of TSP-5 was observed in some D469del BAC chondrocytes (Fig. 3A, inset F and H). The D469del BAC articular chondrocytes also showed a distinctive distribution of TSP-5 with dark immunopositive staining surrounding the chondrocytes; this was not observed in the wild-type animals (Fig. 3A, panels I–L). These areas of intense immunostaining in the articular cartilage may arise from an individual chondrocyte overexpressing TSP-5 creating an immunoreactive halo around each cell. Alternatively, these immunoreactive areas in the articular cartilage may represent sites of cartilage damage that are being repaired, since higher levels of TSP-5 expression have been associated with joint injury [4, 71–74]. The observed staining pattern is consistent with TSP-5 distribution in human osteoarthritic articular cartilage [75]. A mild articular cartilage irregularity was observed in the BAC female (Fig. 3A, panels I and L).

The distribution of two ECM proteins involved in the PSACH cellular phenotype, MATN3 (antibody provided by Drs. Hanson and Eyre; dilution 1:500) and type IX collagen (antibody obtained from Iowa Hybridoma bank; dilution 1:200), was also examined. Both co-localized with TSP-5 in the D469del BAC growth plates but did not show the patchy staining observed with TSP-5 (data not shown). However, immunopositive staining for type IX collagen and MATN3 surrounding the articular chondrocytes (data not shown) was very similar to the pattern observed for TSP-5 in the articular cartilage (Fig. 3A I–L).

Studies of this BAC mouse model were constrained by the lack of transgenic offspring. However, the limited findings do suggest that the mutant COMP protein affected the growth plate and articular cartilage and showed some similarity to the human PSACH growth plate phenotype [7, 18].

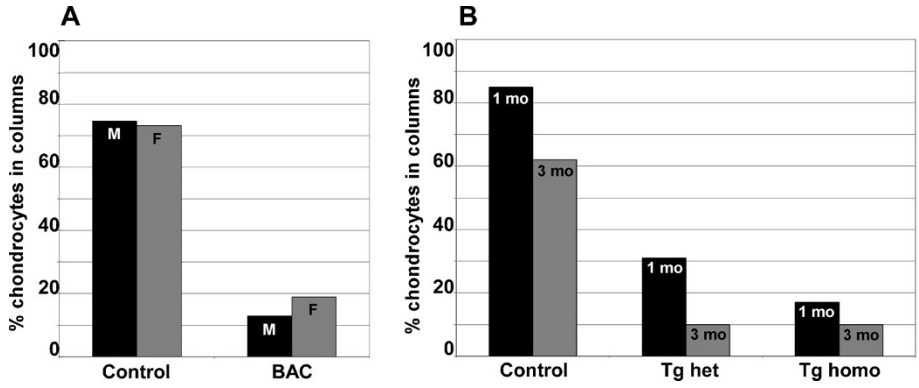

Figure 4. Growth plate chondrocyte organization. (*A*) BAC mice compared to littermate controls at the time of death (4.5 months for female and 5.5 months for male). (*B*) Heterozygous and homozygous Tg D469del mice at 1 and 3 months of age compared to wild-type mice. In both cases, control animals have significantly more growth plate chondrocytes organized in columns rather than in clusters compared to both strains of D469del mice. (P value is ≤ 0.0000001, two-tailed T test). M = male, F = female, mo = month, Het = heterozygous, Homo = homozygous.

Transgenic TSP-5 mice with type II collagen promoter and human COMP

A second approach was used to express D469del TSP-5 protein using a cDNA driven by a mouse type II collagen promoter and enhancer [76]. The human TSP-5 cDNA sequence was amplified from D469del TSP-5 cDNA pBS KS with the addition of flanking *Not* I sites. This amplified fragment was digested with *Not* I and ligated into pKN 185, a vector containing a type II collagen promoter and enhancer [76]. This subcloning procedure effectively replaced the β-galactosidase cassette with D469del TSP-5 cDNA.

The progeny of three positive male founders were tested for transgenic expression using real-time quantitative RTPCR (data not shown), and only the line expressing at the highest level was analyzed. FISH analysis showed that 10–20 copies of the transgene were inserted into chromosome 10.

The F1 mice were evaluated for growth abnormalities. Three week male and female mice were weighed and measured from the tip of the nose to the end of the tail and compared to age- and sex-matched wild-type controls. The transgenic mice weighed 16–18% less than controls and were 11–12% shorter. Limb measurements were taken from radiographs of 10 animals for both the control and transgenic animals. Tibias from 1-month-old homozygous male mice were 5.8% ± 0.6 shorter than wild type. Growth plate abnormalities were very similar to those observed with the BAC transgenic line (Fig. 3B). There was growth plate disorganization in the transgenic animals, with fewer chondrocytes organized into columns in both heterozygous and homozygous mice at 1 and 3 months of age (Fig. 4B). Patchy TSP-5 immunostaining was observed in the growth plate of 3-month-old mice, and dark pericellular staining was found in the articular cartilage at all ages (Fig. 3B M–R). Intracellular retention of TSP-5 was not frequently observed in these mice (Fig. 3B G–L). Type IX collagen and MATN3 immunostaining co-localized with TSP-5 in the growth plate and articular cartilage (data not shown).

While this transgenic mouse model displayed shortened limbs and disorganized growth plate, both of which are consistent with the PSACH phenotype, only a few chondrocytes showed intracellular TSP-5. It is not clear if the articular cartilage findings in weight-bearing joints of these animals match the human condition because there is little information about human PSACH articular cartilage owing to tissue not being readily available for study.

Transgenic TSP-5 mice with type II collagen promoter, rat COMP and BM40 signal peptide

A transgenic mouse, expressing rat D469del TSP-5-myc driven by a type II collagen promoter with a BM40 signal peptide, was generated [60]. Two lines were produced, but only the higher expressing line was analyzed. The transgenic mice were crossed onto the TSP-5 knockout background, and the resulting mice had a more severe phenotype. Nose-to-rump measurements were obtained from 1 to 9 months. The females showed no difference in body length; males on the wild-type and TSP-5 null backgrounds were 8 and 10% shorter, respectively. Femur lengths were also shorter in males, and sternebral fusion was observed in approximately 40% of the transgenic mice compared to 3% of the controls. The growth plate was irregularly shaped, showed mild disorganization, and gaps were noted between the chondrocyte columns. These gaps were attributed to loss of chondrocytes, because apoptosis was detected in the transgenic growth plate but not in the controls. Mutant TSP-5 was discriminated from the endogenous mouse

TSP-5 by the presence of a myc tag. Mutant TSP-5 had a patchy pericellular distribution, whereas the endogenous TSP-5 was found in the interterritorial matrix. Intracellular retention of TSP-5 and dilated rER were found by electron microscopy and were more evident in the mice on the TSP-5 knockout background. MATN-1 expression was markedly upregulated, MATN-3 was minimally affected and aggrecan immunostaining was reduced. The distribution and quantity of MATN-4, type II collagen, type IX collagen and type X collagen was not obviously perturbed. ECM proteins were more easily extracted from control mice than from the D469del TSP-5 matrix, suggesting an altered integration of mutant TSP-5 in the matrix. The male transgenic mice show similar findings to those observed in the human PSACH condition and in transgenic mice with mutant human TSP-5. It is unclear why the female transgenic mice do not show any cellular phenotypic or growth abnormalities.

Knock-in mouse model

A knock-in mouse model with the T585M TSP-5 mutation was generated [77]. Humans with this mutation were diagnosed in one case with mild PSACH [78], and the other was classified as MED [40]. The T585M mutation will be referred to here as a MED mutation since patients do not fit the diagnostic criteria for PSACH as no reported patient had dwarfism.

The heterozygous T585M mice had minimal phenotypic abnormalities [77]. However, the homozygous T585M mice had a slower growth rate, shortened limbs and articular cartilage erosion late in life [77]. Mutant TSP-5 was not observed in the rER, indicating that it was not being retained. However, there was a brief transient upregulation of the UPR [77]. The growth plates showed disorganization, with fewer chondrocytes in columns, and chondrocyte proliferation was reduced in conjunction with an increase in apoptosis that was spatially misregulated. Changes in cell morphology were also observed [77]. At the ultrastructural level, collagen fibrils predominated and there was a deficiency of proteoglycan-like material [77].

Body length, bone mineralization, histology of tibias and growth plate organization were assessed at birth. All of these parameters were normal in the mutant mice [77], which is similar to PSACH and MED patients who are also normal at birth [30, 32, 33]. However, by 9 weeks of age, when skeletal growth ceases, homozygous mutant mice weighed 6% less than wild type, tibias were 4% shorter and hip dysplasia was observed [77]. Loss of articular cartilage was observed in 16-month-old mice but not in controls [77]. This observation was consistent with the natural history of MED; osteoarthritis and joint erosion are common problems [33].

The distribution of mutant TSP-5 in the growth plate was patchy and more pericellular than the uniform distribution of TSP-5 in the wild-type littermate growth plates [77]. No intracellular mutant TSP-5 was observed in the T585M mouse chondrocytes [77]. A moderate trafficking defect was observed in RCS cells with this mutation and suggests that it may have a differential effect depending on cell type and/or the level of protein expression [63].

The homozygous T585M mouse model correlates with the MED phenotype and as such is a good model for studying the effects of this mutation on skeletal growth. It differs from the human condition, which is caused by a heterozygous TSP-5 mutation [56]. The differences between this mouse model and the human disease is most likely related to the different time required for skeletal maturation between humans (average 16 years) and mice (9 weeks) and/or differential skeletal stress between a biped and quadruped species. Nevertheless, this knock-in model will provide important reagents needed to study MED.

Discussion

A model system that completely replicates the human PSACH or MED phenotypes is not available, but the knock-in mouse and some of the tissue culture models provide close approximations [16–18, 43, 44, 48, 49, 57, 58, 63, 77]. Ideally, the characteristic PSACH and MED chondrocyte phenotype of enlarged rER chondrocyte cisternae, disorganized growth plate and diminished TSP-5, type IX collagen and MATN3 in the ECM should be present in a system modeling TSP-5 mutations [18]. Each model system described to date has unique strengths and limitations.

Various *in vitro* systems have been developed and are summarized in Table 1. Biochemical and morphological studies of cellular behavior have yielded different results depending on the system used. Human chondrocytes exhibit the PSACH cellular phenotype with either exogenous or endogenous mutant TSP-5 expression [43, 49, 57]. However, limitation of patient chondrocyte materials and maintenance of chondrocyte phenotype are significant challenges. Moreover, most of the results are cross-sectional, providing little information on the progression of the disease during human development. Nevertheless, this system has provided significant insights into the development of cellular pathology and ECM abnormalities. In contrast, bovine chondrocytes are readily available and may recapitulate the cellular phenotype. However,

Table 1. In vitro model systems.

Mutation	Model system	Dysplasia	TSP-5 source	TSP-5 species	Signal peptide	Secretion delay	rER Enlg	ECM disturb	rER stress	Cell death	TSP-5 domain	Ref.
D271H	RCS	PSACH	pcDNA 3.1	human	native	Y-severe	–	–	–	–	T3	[63]
L272P	RCS	PSACH	pcDNA 3.1	human	native	Y-moderate	–	–	–	–	T3	[63]
P276R	RCS	MED	pcDNA 3.1	human	native	Y-mild	–	–	–	–	T3	[63]
G299R	RCS	PSACH	pcDNA 3.1	human	native	Y-moderate	–	–	–	–	T3	[63]
D302V	RCS	MED	pcDNA 3.1	human	native	Y-mild	–	–	–	–	T3	[63]
G309R	RCS	PSACH	pcDNA 3.1	human	native	Y-moderate	–	–	–	–	T3	[63]
D361Y	PBC	MED	AdEasy vir.	rat	BM40	Y-moderate	N	mild	–	N	T3	[46]
G427E	PHC	PSACH	native	human	native	Y	Y	Y	–	–	T3	[49]
	PHC	PSACH	native	human	native	Y	Y	Y	Y	–	T3	[43]
	PHC	PSACH	native	human	native	Y	Y				T3	[44]
D469del	PBC	PSACH	AdEasy vir.	rat	BM40	Y	Y	Y	Y	Y	T3	[48]
	PBC	PSACH	AdEasy vir.	rat	BM40	Y-severe	N	Y	Y	Y	T3	[46]
	RCS	PSACH	pcDNA 3.1	human	native	Y-severe	–	Y	–	–	T3	[58]
	RCS	PSACH	pcDNA 3.1	human	native	Y-severe	–	–	–	–	T3	[63]
	PBT	PSACH	AdEasy vir.	rat	BM40	Y-severe	–	Y	–	Y	T3	[53]
	PHC	PSACH	native	human	native	Y	Y	Y	–	–	T3	[49]
	PHC	PSACH	native	human	native	Y	Y	Y	Y	–	T3	[43]
	PHC	PSACH	native	human	native	Y	Y	Y	–	Y	T3	[16]
	PHC	PSACH	native	human	native	Y	Y	–	–	–	T3	[44]
	PHC	PSACH	AdEasy vir.	human	native	Y	Y	–	–	–	T3	[57]
D472Y	COS-7	PSACH	pCMV	human	native	Y	Y	NA	Y	Y	T3	[17]
D473G	RCS	PSACH	pcDNA 3.1	human	native	Y-severe	–	–	–	–	T3	[63]
D473N	RCS	PSACH	pcDNA 3.1	human	native	Y-severe	–	–	–	–	T3	[63]
	PBT	PSACH	AdEasy vir.	rat	BM40	Y-severe	–	Y	–	Y	T3	[53]
D475N	PBC	PSACH	AdEasy vir.	rat	BM40	Y-severe	N	Y	–	Y	T3	[46]
D511Y	PHC	PSACH	native	human	native	Y	Y	–	–	–	T3	[44]
T585R	RCS	MED	pcDNA 3.1	human	native	Y-severe	–	–	–	–	CTD	[63]
T585M	RCS	MED	pcDNA 3.1	human	native	Y-moderate	–	–	–	–	CTD	[63]
H587R	PBC	PSACH	AdEasy vir.	rat	BM40	Y-moderate	N	Y	–	Y	CTD	[46]
	RCS	PSACH	pcDNA 3.1	human	native	Y-moderate	–	–	–	–	CTD	[63]
	PBT	PSACH	AdEasy vir.	rat	BM40	N ≈ WT	–	Y	–	Y	CTD	[53]

A comparison of *in vitro* model systems and data obtained from with each system. CTD = C-terminal domain, disturb = disturbance, Enlg = enlarged, N = no, NA = not applicable, PBC = primary bovine articular chondrocytes, PBT = primary bovine tendonocytes, PHC = primary human chondrocytes, RCS = rat chondrosarcoma cells, Ref. = reference, T3 = type 3 calcium-binding domain, vir. = virus, WT = wild-type, Y = yes, – = not assayed.

mutant TSP-5 protein must be introduced to produce the PSACH cellular phenotype. Bovine chondrocytes must also be cultured in a 3-D environment to maintain the phenotype and thus may not be more useful than human chondrocytes. In contrast, RCS cells are easy to culture and maintain a chondrocyte phenotype even when grown in a monolayer. Matrix production is abundant; however, the matrix only contains cross-linked type II N-procollagen instead of the mature type II collagen fibrils normally found in ECM [79]. This difference in phenotype restricts the use of the RCS system to specific questions related to protein trafficking. The absence of ECM proteins that contribute to the PSACH chondrocyte pathology also limits the use of COS-7 cells as a model system. Lastly, lack of *in vivo* tendon studies restricts the assessment of tenocytes as a model system. In summary, human chondrocyte model systems most closely replicate the PSACH cellular pathology *in vivo* and have yielded many important observations.

In vivo models using knock-in and transgenic approaches have recently produced mouse models [60, 77, and data presented in this review]. All of these models show some features of the PSACH and MED cellular phenotypes but none completely mimics the human disease. A big challenge with these models is to correlate a complex disease process that takes years to develop in a bipedal human compared to a murine model that lives only about 2 years and walks on all fours, generating different stresses on the skeletal elements. However, growth plate abnormalities were consistently observed and provide materials to study the development of the phenotype throughout skeletal development and aging. The classic hallmark of giant rER cisternae was not consistently observed; mild rER dilation was observed in one study. In the human mutant TSP-5 transgenic mice, the expression levels may not be sufficient to create a trafficking abnormality. This is also illustrated in the TSP-5 rat transgenic mice in which two lines were generated and only the line with the highest expression showed some intracellular retention of TSP-5 [60]. That study demonstrates that the presence of some normal TSP-5 may ameliorate the chondrocyte pathology because the phenotype in the TSP-5 null background was worse than the control background also expressing normal endogenous TSP-5 protein [60].

The T585M MED knock-in mutation produces mild abnormalities because this TSP-5 mutation is clinically less severe [40, 78]. Phenotypic findings were only observed in the homozygous T585M mouse, whereas the human disease is observed in heterozygotes. Other knock-in mutations may produce more severe cellular and skeletal phenotypes. Nevertheless, the murine models provide important reagents for systematically studying the disease process. Both *in vitro* and *in vivo* models discussed here are valuable tools for understanding disease pathology and for development of therapeutic modalities despite the deficiencies which are inherent to all model systems.

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