

Published in final edited form as:

Arch Oral Biol. 2013 September ; 58(9): 1092–1099. doi:10.1016/j.archoralbio.2013.02.008.

## Osteoarthritis in Temporomandibular Joint of *Col2a1* Mutant Mice

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### Abstract

**Objective**—*Col2a1* gene mutations cause premature degeneration of knee articular cartilage in disproportionate micromelia (*Dmm*) and spondyloepiphyseal dysplasia congenita (*sedc*) mice. The present study analyzes the temporomandibular joint (TMJ) in *Col2a1* mutant mice in order to provide an animal model of TMJ osteoarthritis (OA) that may offer better understanding of the progression of this disease in humans.

**Design**—*Dmm*/+ mice and controls were compared at two, six, nine and 12 months. Craniums were fixed, processed to paraffin sections, stained with Safranin-O/Fast Green, and analyzed with light microscopy. OA was quantified using a Mankin scoring procedure. Unfolded protein response (UPR) assay was performed and immunohistochemistry (IHC) was used to assay for known OA biomarkers.

**Results**—*Dmm*/+ TMJs showed fissuring of condylar cartilage as early as 6 months of age. Chondrocytes were clustered, leaving acellular regions in the matrix. Significant staining of HtrA1, Ddr2 and Mmp-13 was observed in *Dmm*/+ mice ( $p < 0.01$ ). We detected upregulation of the UPR in knee but not TMJ.

**Conclusions**—*Dmm*/+ mice are subject to early-onset OA in the TMJ. We observed upregulation of biomarkers and condylar cartilage degradation concomitant with OA. An upregulated UPR may exacerbate the onset of OA. The *Dmm*/+ mouse TMJ is a viable model for the study of the progression of OA in humans.

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#### Authors' Contributions

RES, LCB and DLK wrote the grants that supported this work. They and PRR oversaw experimental design, execution and interpretation of data. MLR, DWH and DJF also assisted in experimental design, performed experimental procedures, and interpreted data. All authors contributed to writing the manuscript. JTF, MR, JC, TW and BAN conducted key experiments and assisted in the interpretation of results.

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## Keywords

Disproportionate micromelia (*Dmm*); Temporomandibular joint; osteoarthritis; *Col2a1*; *Ddr2*

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## Introduction

Temporomandibular joint (TMJ) growth occurs through regulated synthesis and degradation of various components in the extra cellular matrix (ECM) (1). Osteoarthritis is reported as the most common disease associated with the TMJ (2) and occurs when articular cartilage remodeling is disturbed. We and others have shown that OA in the knee follows a pathway of articular cartilage degeneration involving *HtrA1*, *Ddr2*, and *Mmp13* (3, 4).

The Disproportionate micromelia (*Dmm*) mouse has a mutation that causes mild dwarfism in the heterozygote. *Dmm* mice have a three-nucleotide deletion of the *Col2a1* gene (human homologue is *COL2A1*) (5). These genes code for type II collagen, the most abundant protein in hyaline cartilage (6, 7). It has been reported that in *Dmm*<sup>+</sup> cartilage the ECM has a significant reduction in type II collagen. (8).

*Dmm*<sup>+</sup> mice have decreased proteoglycan in the interterritorial matrix (ITM), yielding early - onset OA of the knee joint (7) and decreasing the ability of chondrocytes to maintain articular cartilage integrity. Mechanical forces applied to damaged joints lead to pain and dysfunction (9). We recently reported a mechanism of OA in another *Col2a1* deficient mouse model, *sedc*, with progressive expression of *HtrA1*, *Ddr2* and *Mmp13* (4).

Research on TMJ OA involving animal models is still developing (2, 10–14). Currently, TMJ disorder diagnoses are based on non-specific symptoms. Treatments cover these symptoms rather than reversing degradation (9, 15–17).

An OA animal model is an important tool in understanding TMJ disease (2, 10, 15, 18). Human studies can only describe TMJ OA, whereas animal models may elucidate a cause and effect relationship that might allow for TMJ disease reversal and a universal understanding of TMJ OA pathobiochemistry that is not currently available (14).

Xu et al. have shown such a relationship in a *Col11a1* mutant chondrodysplasia (*cho*) mouse TMJ OA (19). The degradation pathway includes increased proteoglycan production to compensate for destruction, proteoglycan loss, fibrillation, and eventual degradation of cartilage (20–25). Chondrocyte clustering and increased proteoglycan production in the pericellular matrix (PCM) have also been identified as early OA indicators (7). Furthermore, articular cartilage degradation associated with knee OA in genetic and mechanical models has also been reported (3, 13).

Important mechanistic insight into OA pathogenesis has been provided by published data linking increased expression of *HtrA1*, *Ddr2* and *Mmp13* and knee OA in both genetic and mechanical models (3, 4). The unfolded protein stress response (UPR) has also been implicated in OA progression (26). We have reported that *Dmm* mice exhibit distended ER and Golgi, consistent with the misfolded type II collagen being trapped in the ER rather than secreted to the ECM (7). Although OA degradation is absent in newborn *Dmm*<sup>+</sup> mice, we suspected that intracellular accumulation of misfolded type II collagen might trigger perpetual UPR. If present, it could predispose mice to early-onset OA. The present study suggests that these pathways can initiate OA in the TMJ as well as knee joint.

## Materials and Methods

### Facilities, Animals and Genotyping

Mice were maintained in the animal care facility of Brigham Young University under an approved IACUC protocol. They were provided Harlan Teklad rodent diet 8604, a standard nutritional, grained-based diet, and kept in paper-bedded 5.7 L cages in a room with 12-hr-light/12-hr-dark cycles.

Heterozygous mice (*Dmm/+*) of a C3H strain were crossed to produce wildtype (+/+) and *Dmm/+* mice. Homozygotes (*Dmm/Dmm*) die immediately after birth due to dwarfism that inhibits the ability to respire (6, 7). Approximately 21 days postpartum pups were isolated from dams and numbered using earmark identification. Tail samples were obtained for genotyping.

DNA was isolated from tail samples and polymerase chain reaction (PCR) was performed according to previously published methods (5) (7).

### Tissue Processing

TMJs from *Dmm/+* mice and controls ages two months (n= three each genotype), six months (n=three each genotype), nine months (n= four *Dmm/+* and three +/+), and 12 months (n=three each genotype) were collected. Mice were asphyxiated with CO<sub>2</sub> and heads were removed from bodies. Brain and skin were removed and heads were fixed in 4% paraformaldehyde. Heads were decalcified as previously described (4) and dissected, leaving only a section containing the right TMJ.

Samples were processed and embedded in paraffin wax, sectioned and placed on slides as previously described (4).

Every fifth slide was stained with Safranin-O and Fast Green (SO&FG). Stained sections were viewed under an Olympus BX51 light microscope and photographed by a computer-operated Olympus DP72 digital camera.

### Determination of OA Severity

SO&FG-stained tissues were used to assess the osteoarthritic state of condylar cartilage using a modified Mankin scoring system (9, 20, 21, 27) see also Table 1 in supplemental material. The modified Mankin scoring system is based on three criteria: interterritorial matrix staining (IMS) of de-calcified cartilage with SO, pericellular matrix (PCM) staining (PMS) with SO, and spatial arrangement of chondrocytes (SAC) (See Fig. 1). Scores from the three criteria were summed providing an overall score. Higher scores indicate more severe OA. Each joint provided five scores, and values were averaged with scores of mice of similar age and genotype, providing an average score for each age and genotype.

### Immunohistochemical Analysis

Immunohistochemistry was performed on sections from every sixth slide from three wildtype and three *Dmm/+* animals at six months. Slides were stained with antibodies against HtrA1, Ddr2, and Mmp13 as previously described (4). Differences in staining intensity were measured qualitatively with comparison to wild type control. The qualitative results of the immunohistochemical staining for the *Dmm/+* and wild type samples were analyzed statistically using Chi-Squared tests to evaluate differences between the two samples in staining patterns for key OA biomarkers.

## UPR Analysis

Articular cartilage was isolated from knee joints of 13 *+/+* and 17 *Dmm/+* newborn (0–4 days) mice. TMJ fibrocartilage disc tissue was isolated from 1 *+/+* and 3 *Dmm/+* 2-month-old mice. Individual samples were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and Thermo Scientific Protease Inhibitor Cocktail (product #78410). Protein concentration was measured by Bradford assay, and 10 µg protein per lane were resolved on polyacrylamide gels and analyzed by western blot.

## Statistical Analysis

A two-way analysis of variance was used to compare the means of *Dmm/+* data (i.e., scores in relation to age and in comparison with the means of *+/+* data). The data were blocked to account for multiple measurements obtained from each mouse. T-tests were used to compare IMS sub-scores, PMS sub-scores, and SAC sub-scores from *Dmm/+* and age-matched *+/+* cartilage. P-values less than 0.01 were considered statistically significant for this study. The UPR results were analyzed using an unpaired Student's t-test. P-values less than 0.05 were considered significant.

## Results

### Increased Mankin Scoring in *Dmm/+* Mice

We observed an increase in proteoglycan staining and fissuring of condylar cartilage at 6 months in *Dmm/+* mice compared to wildtype (Fig. 1). At six, nine and 12 months, *Dmm/+* mice exhibited significantly elevated modified Mankin scores compared to wildtype (Fig. 2). *Dmm/+* mice exhibited significantly more erosion than *+/+* (data not shown). The *+/+* mice of all ages showed normal staining of the ITM and PCM. *Dmm/+* cartilage at nine and twelve months displayed a lack of columnar arrangement of chondrocytes with multiple areas of hypocellularity caused by clustering. Areas of the ITM showed little or no staining and the PCM showed enhanced staining. Two month *Dmm/+* mice showed little deviation from wildtype, but older mice show greater sub-scores for each of the criteria (IMS, PMS, SAC) at all age groups when compared to wildtype.

IMS sub-scores stayed consistent for *+/+* mice at all ages. *Dmm/+* cartilage showed proteoglycan deficiency in areas of fissuring as early as 6 months, which increased throughout the condyle with age until no staining was observed at nine and twelve months. PMS sub-scores showed that *+/+* cartilage had enhanced proteoglycan staining starting at around nine months. *Dmm/+* mice showed this kind of staining as early as six months, increasing with age. Sub-scores for the SAC show normal cartilage in *+/+* mice, while *Dmm/+* scores show cartilage irregularities at as early as two months. *+/+* mice showed only slight hypercellularity and minimal points of hypocellularity in older mice, whereas *Dmm/+* mice became intensely hypocellular throughout the articular cartilage.

### Known biomarkers of OA present in TMJ

Immunohistochemical staining of HtrA1, Ddr2 and Mmp13 was virtually undetectable in TMJs from six month old wild type mice. Conversely, we observed significant expression of these biomarkers in age matched *Dmm/+* mice (See Fig. 3) ( $p < 0.01$ ).

### UPR is activated in articular cartilage of newborn *Dmm/+* mice

Initial experiments with TMJ from 2-month-old mice revealed that dissections primarily yielded TMJ disc fibrocartilage with very little condylar cartilage. Fibrocartilage, however, expresses only low levels of *Col2a1* and is therefore less likely than condylar cartilage to

exhibit accumulation of misfolded type II collagen in the ER. Indeed, neither BiP (an ER chaperone protein that increases during early UPR) nor phospho-eIF2 $\alpha$  (a translation initiation factor that is required for synthesis of several UPR proteins) were elevated in TMJ disc fibrocartilage (data not shown). BiP expression in *Dmm*<sup>+/+</sup> newborn articular cartilage showed a 1.66-fold increase compared to wildtype (p=0.01) (see Fig. 4). In addition, the ratio of phosphorylated to unphosphorylated eIF2 $\alpha$  was increased 4.3-fold in *Dmm*<sup>+/+</sup> mice (n=6) compared to wildtype (n=5) (p<0.05) (see Fig. 4).

## Discussion

A large number of human chondrodysplasias are caused by mutations in *COL2A1*(28–33). Most of these mutations, however, are in the triple helical region of the gene. Since such mutations lead to the synthesis of truncated chains that do not participate in trimer assembly due to the absence of the C-propeptide, their molecular consequences are different from the mutation described here. One common human chondrodysplasia described (34) as a C-propeptide mutation in *COL2A1* in a Stickler Syndrome family may represent the closest human counterpart to the *Dmm* mutation. In this human case, a single nucleotide deletion in exon 50 of *COL2A1* causes a frameshift in translation and a premature stop codon in exon 51. The mutant C propeptide consists of only the amino-terminal 30% of the normal sequence. This region contains five of the eight cysteines in the type II C-propeptide (including the four residues involved in interchain disulfide bonds) but is lacking the region that contains the *Dmm* mutation. Heterozygous *Dmm* that we used in the present study may therefore represent a good murine model for this condition and is therefore useful for further studies of pathogenesis and testing of novel therapeutic approaches.

It is of interest to note that at least one of the five reported cases of COL2A1C propeptide mutation resulted in distended RER (35). This human mutation caused shortened limbs, shortened trunk, and midface hypoplasia reminiscent of the *Dmm* phenotype we describe in the present study. The heterozygous *Dmm* mouse thus is a relevant model for human COL2A1 C-propeptide mutations that result in congestion of the RER (discuss distended RER below with regard to UPR and reference distended RER in previous *Dmm* papers), with abnormal pro- $\alpha$ 1(II) chains that cannot assemble into normal trimers.

We have demonstrated that *Dmm*<sup>+/+</sup> mice, which harbor a *Col2a1* C-propeptide mutation, show condylar cartilage abnormalities in the TMJ at early ages. The disorganization of chondrocytes and abnormalities in proteoglycan development appear to alter articular cartilage integrity and cause degradation. This is consistent with our previous report that aggrecan levels are lower in *Dmm*<sup>+/+</sup> mice as compared to WT controls (36). Thus, specific components of the ECM are increased and decreased in this mouse model of OA. Scoring these characteristics confirmed that *Dmm*<sup>+/+</sup> cartilage has qualities of early-onset OA, with six, nine, and twelve month mice showing statistically significant differences compared to controls.

Our previous ultrastructural analyses of articular cartilage from *Dmm*<sup>+/+</sup> knee joints showed distended ER and Golgi in chondrocytes (36). Likewise, the ECM of knee articular cartilage from *Dmm*<sup>+/+</sup> mice showed a significant reduction in type II collagen (8). These data are consistent with the intracellular accumulation rather than secretion of misfolded type II collagen. Such an accumulation would be expected to trigger the UPR stress response, which causes a global reduction in protein synthesis while selectively upregulating proteins that aid in protein folding (37). Under ordinary circumstances, a temporary reduction in new protein synthesis allows clearance of the unfolded protein backlog. In the case of *Dmm*<sup>+/+</sup> mice, however, the *Col2a1* mutation together with the constant demand for type II collagen in cartilage would ensure that new mutant protein was continually synthesized and UPR was

perpetually activated. This would likely result in chondrocytes that were functionally compromised due to perpetual suppression of protein synthesis. In the most severe cases of UPR, apoptosis would be triggered. It seems likely that perpetual activation of the UPR pathway in articular and condylar chondrocytes would compromise articular and condylar structure, contributing to earlier onset OA.

In the present study we found that the TMJ disc fibrocartilage, which expresses low levels of type II collagen, does not show activation of the UPR, but knee articular cartilage from newborn *Dmm/+* mice does. This result is interesting given that the UPR is detectable in newborns even though histological evidence of OA does not appear in the *Dmm/+* knee joint until after 3 months of age, consistent with UPR contributing to the earlier onset of OA. TMJ condylar cartilage is more like knee articular cartilage as far as type II collagen expression levels, suggesting that OA in the TMJ of *Dmm/+* mice might also be exacerbated by UPR.

We observed a significant decrease in proteoglycan shown by decreased SO staining throughout condylar cartilage in *Dmm/+* mice. *Dmm/+* joints showed many areas lacking stain, diminished stain intensity, and a disorganization of chondrocytes. Another sign of OA includes proteoglycan upregulation to combat breakdown caused by inflammatory cytokines. Hypercellularity and cell clustering also occur in the knee of *Dmm/+* mice (7). Our data are consistent with this in that the *Dmm/+* TMJ PCM had areas of SO-positive staining, while the ECM had areas void of both cells and staining.

Fibrillation in six-month *Dmm/+* mice TMJs was also noted. By nine and twelve months, degradation was so severe that parts of the condylar cartilage were completely severed, while wildtype mice maintained healthy cartilage. SO-positive staining was less intense by this stage because damaged chondrocytes stopped producing proteoglycans (38).

Our present work demonstrates that OA progression in the *Dmm* TMJ follows the same pathway as *cho* TMJs and *Dmm* knees, i.e. presence of key biomarkers of OA, consistent with what has only been demonstrated in knee joints (3, 4, 14, 16, 17). The *Dmm/+* TMJ model also mimics human TMJ dysfunction associated with OA, and is an acceptable model for the pathogenesis of this disorder. We also confirm the involvement of HtrA1, Ddr2, and Mmp13 in its progression. Because a degenerative signaling axis that involves HtrA1, Ddr2, and Mmp13 may be universal to OA, elucidation of these biomarkers in OA of the TMJ may lead to notable studies of future clinical significance. In fact, despite increasing emphasis on the burden of TMJ-related disease, the discovery that *Dmm/+* mice model TMJ pathology makes possible additional study into targets potentially useful in the treatment or attenuation of TMJ OA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors thank Dr. Dennis Eggett (Department of Statistics, Brigham Young University) for conducting statistical analyses, and William Hale, Michael Henderson, Shaela Avery, Joshua Lloyd, David Robinson, Karen Xue, Alexandra Wink and Christopher Stockdale for procedural help.

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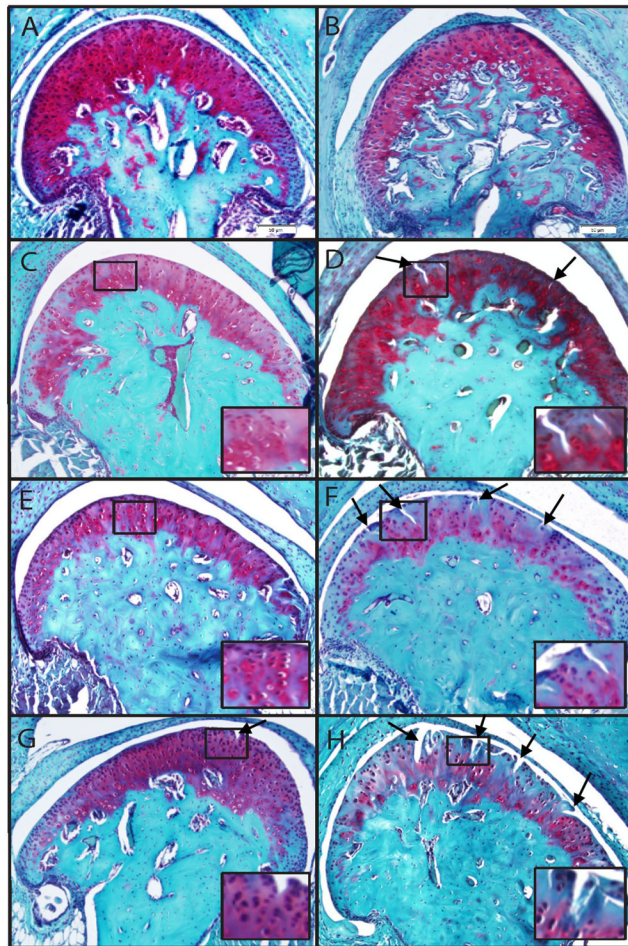
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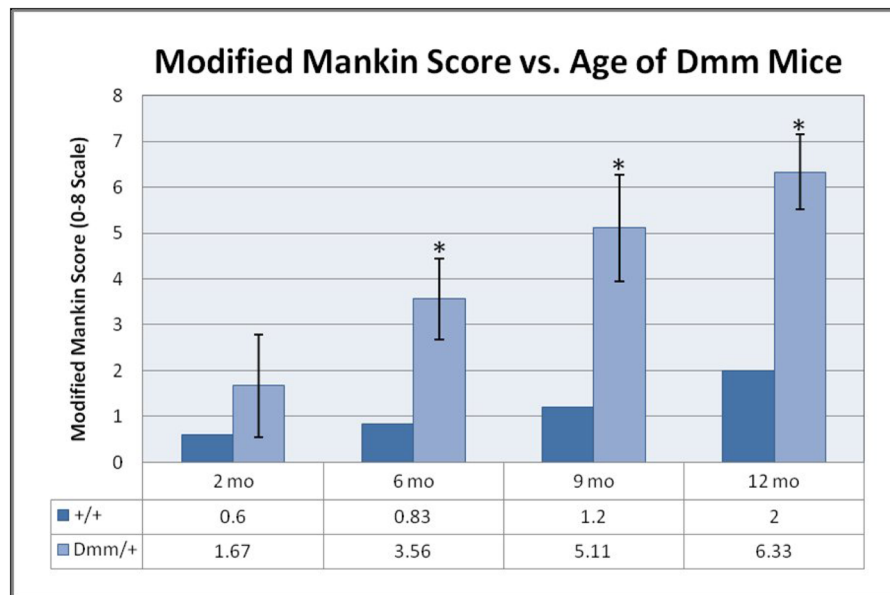


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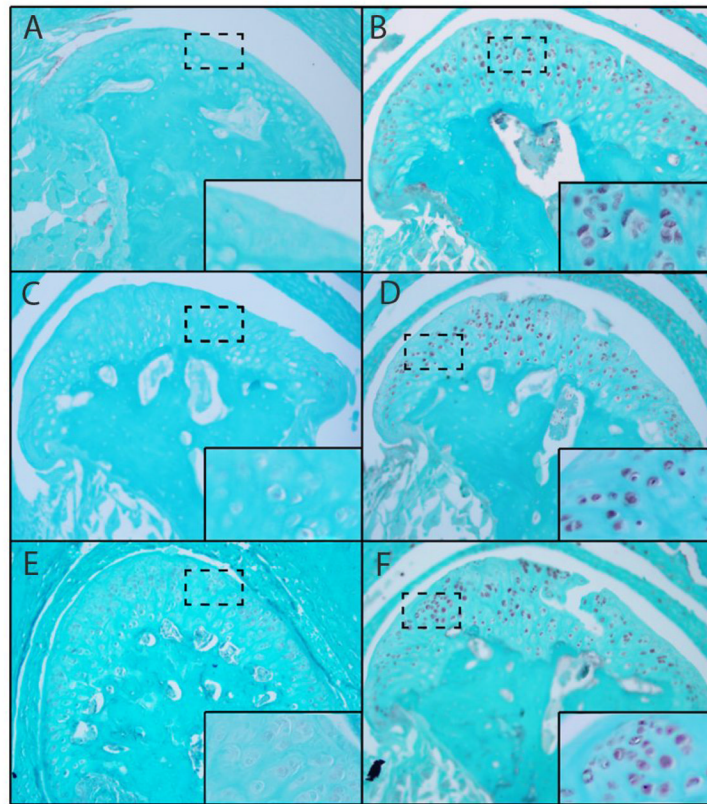
**Figure 1.**

Proteoglycan prevalence observed by SO staining was diminished in *Dmm/+* mice compared to wild type. Proteoglycan prevalence was observed in *Dmm/+* mice at 2, 9, and 12 months (B, F, H) compared to wild type mice (A, E, G); however, an increase in proteoglycan staining was observed in *Dmm/+* mice at 6 months (D) compared to wildtype controls (C). In addition, common fissuring of articular cartilage as a result of the loss of columnar arrangement of chondrocytes was observed in 6, 9, and 12 month old *Dmm/+* mice (D, F, H, arrows) but not until 12 months in wildtype animals (G, arrow). Images are representative and insets indicate areas of irregular SAC and degradation in *Dmm/+* mice or normal cartilage in wildtype.

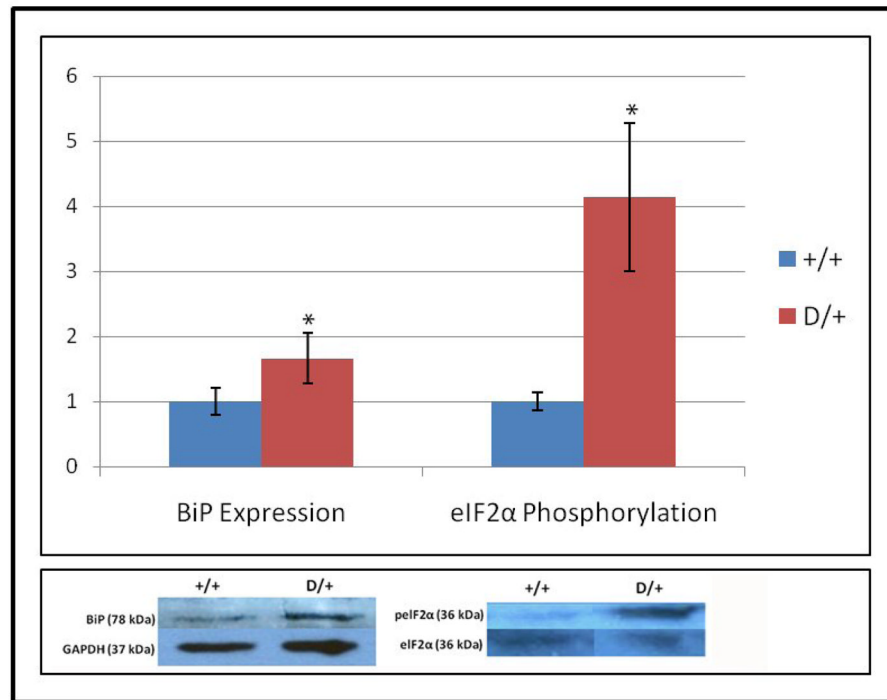


**Figure 2.**

The TMJ in the *Dmm*<sup>+/+</sup> mice displayed premature articular cartilage erosion and higher Mankin scores due to decreased cellularity, decreased IMS staining and increased PMS staining. *Dmm*<sup>+/+</sup> mice had significantly greater defects in chondrocyte arrangement and proteoglycan production and distribution, evidenced by significantly greater Mankin scores for the 6, 9 and 12 month *Dmm*<sup>+/+</sup> mice. ( $P < .01$ )



**Figure 3.** Significantly increased antibody staining of Ddr2, Mmp13, HtrA1 in *Dmm/+* mice. Significant staining of OA biomarkers HtrA1 (B,A); Ddr2 (D,C) and Mmp13 (F,E) was observed in *Dmm/+* compared to six month, aged matched, wildtype mice respectively ( $p < 0.01$ ). This suggests that the Ddr2 degradative pathway plays an active role in the articular cartilage of *Dmm/+* mice. Insets show virtually no staining in WT mice and greatly increased staining of biomarkers in *Dmm/+*.



**Figure 4.** UPR is activated in *Dmm/+* animals. BiP levels were elevated 1.66-fold in *Dmm/+* (n=11) compared to wildtype (n=8) newborn knee articular cartilage (p=0.01). In addition, the ratio of phosphorylated to unphosphorylated eIF2α was increased 4.3-fold in *Dmm/+* mice knee articular cartilage (n=6) compared to wildtype (n=5) (p<0.05). The lower panel shows representative western blots for each antibody.