The Anti-Inflammatory Role of GPNMB in Post-Traumatic Osteoarthritis

Authors: Asaad A. Al-Adlaan[1,2,3], Bryson Cook [1,3], Ernesto Solorzano Z.[1,3,6], Nazar J. Hussein [1,2,3], Fatima A. Jaber [1,2,3], Trinity Kronk[1,3,6], Salvatore Frangiamore[5], Hope C. Ball[4], Fayez F. Safadi[1,2,3,4,7*]

Affiliations:
[1] *Department of Biomedical Sciences, Northeast Ohio Medical University, Rootstown, OH, USA*
[2] *Biomedical Sciences Program, Kent State University, Kent, OH, USA*
[3] *Musculoskeletal Research Group, NEOMED, Rootstown, OH, USA*
[4] *Rebecca D. Considine Research Institute, Akron Children's Hospital, Akron, OH, USA*
[5] *Cleveland Clinic Foundation, Cleveland, OH, USA*
[6] *Basic and Translational Biomedicine Program, Northeast Ohio Medical University, Rootstown, OH, USA*
[7] *University Hospitals, Cleveland, OH, USA*
** Correspondence should be addressed to fsafadi@neomed.edu*

Abstract: Osteoactivin (GPNMB) is a transmembrane protein expressed in multiple cell types with known functions in muscle, bone, and neurons, but the role of GPNMB in chondrocytes and cartilage homeostasis remains unknown. Here we show GPNMB is expressed in human and mouse primary chondrocytes, and that its expression is increased in damaged human cartilage and under pro-inflammatory conditions. We report that recombinant GPNMB treatment inhibits the expression of Mmps, Adamts-4 and Il-6 following IL-1β-stimulation *in vitro*. *In vivo*, GPNMB function was assessed in a post-traumatic osteoarthritis model, destabilization of the medial meniscus (DMM). Transgenic animals lacking functional GPNMB protein (DBA/2J) developed severe cartilage damage and demonstrated significant increases in pro-inflammatory cytokine expression following DMM. To elucidate the mechanism of action, we demonstrate that GPNMB regulates the MAPK signaling pathway in primary murine chondrocytes. Taken together, our results identify a novel anti-inflammatory role for GPNMB in cartilage and chondrocytes and identify GPNMB as a potential therapeutic modality for inflammatory joint diseases.

Keywords: GPNMB, Osteoarthritis, Cartilage, MAPK, DMM, Inflammation

Introduction

Osteoactivin, also known as glycoprotein non-metastatic melanoma protein B (GPNMB), is a transmembrane glycoprotein first identified in a rat model of osteopetrosis (Safadi et al., 2002). GPNMB has subsequently been found in many cell types including osteoblasts, osteoclasts, macrophages, hepatocytes, dendritic cells and cancer cells and is known to be involved in a plethora of functions including proliferation, differentiation, migration, angiogenesis, osteogenesis, and apoptosis (Arosarena et al., 2018; Rose et al., 2010; Schwarzbich et al., 2012; Selim et al., 2003; Sheng et al., 2008; Yu et al., 2016). GPNMB encodes a 574 amino acid protein that includes an arginine-glycine-aspartic acid (RGD) domain, a polycystic kidney disease domain (PKD) and a proline rich repeat (PRRD) domain (**Supplemental 1**) (Owen et al., 2003). The majority of the biological function, however, is conducted by the extracellular domain of the protein, shed from the surface by furin-like proteases and extracellular proteases, such as a disintegrin and metalloproteinase with thrombospondin motifs-10 (Adamts-10) (Kawahara et al., 2016; Murata et al., 2015; Oyewumi et al., 2016; Rose et al., 2010).

The role of GPNMB in fracture repair and bone regeneration is well studied. Upregulated in response to skeletal damage, GPNMB promotes bone formation through either direct or indirect regulation of osteoblast proliferation and adhesion through multiple signaling pathways (Abdelmagid et al., 2010; Abdelmagid et al., 2008; Abdelmagid et al., 2015; Frara et al., 2016). GPNMB also functions as a negative regulator of osteoclast differentiation and survival (Abdelmagid et al., 2015; Sheng et al., 2008; Sondag et al., 2016). Indeed, transgenic GPNMB mice exhibit increased bone volume, mineralization and thickness,

increased osteoblast number and decreased osteoclast survival compared to C57BL/6 control animals (Abdelmagid et al., 2015; Frara et al., 2016; Sondag et al., 2016).

GPNMB is emerging as a crucial factor in the inflammatory response following tissue injury (Li et al., 2010). GPNMB levels were elevated in both mouse and rat models after myocardial infarction (Järve et al., 2017). GPNMB has also been shown to have an anti-inflammatory role in macrophages, reducing the expression of pro-inflammatory signaling molecules such as interleukin-6 (IL-6), interleukin-12p40 (IL-12p40) and nitric oxide (NO) (Li et al., 2010; Ripoll et al., 2007; Zhou et al., 2017). Furthermore, GPNMB has been shown to play a role in the synthesis of extracellular matrix (ECM) components and its expression is increased with inflammatory stimuli and ECM damage, conditions common in the pathophysiology of degenerative joint diseases such as osteoarthritis (OA) (Jiang et al., 2011; Owen et al., 2003; Safadi et al., 2002; Sun et al., 2015). Despite the above roles, the biological function of GPNMB in normal or damaged cartilage and chondrocyte homeostasis have not yet been studied.

To address this knowledge gap, we first determined GPNMB expression in human primary chondrocytes and cartilage. GPNMB expression was increased in damaged cartilage from osteoarthritic patients compared to normal (no history of rheumatic disease) and following pro-inflammatory stimulus in human primary chondrocytes and cell-lines. Furthermore, recombinant GPNMB treatment significantly lowered expression of catabolic markers in human primary chondrocytes and reduced ECM degradation in cartilage explants. We subsequently validated these results in murine primary chondrocytes and examined endogenous GPNMB expression in a mouse model: DBA/2J mice, which produce a truncated and non-functional GPNMB protein (Abdelmagid et al., 2014). To better understand the role of GPNMB *in vivo*, we induced post-traumatic OA via destabilization of the medial meniscus (DMM) surgery in DBA/2J mice (Culley et al., 2015; Glasson et al., 2007). The results demonstrated functional GPNMB is essential for the reduction of inflammation and protection against post-traumatic cartilage damage. Collectively, our results are the first to show the extracellular domain of GPNMB serves an anti-inflammatory function in primary chondrocytes and cartilage and reduces catabolic gene expression both *in vitro* and *in vivo* and suggests GPNMB may be a potential anti-inflammatory therapy for inflammatory joint diseases.

Results

GPNMB expression is elevated in damaged human cartilage

We first assessed the expression levels of GPNMB in human OA cartilage samples. To confirm we had accurately harvested cartilage from regions of both low- and high-grade OA damage, we began by first macroscopically evaluating the cartilage using India Ink, where particles adhere to fibrillated tissue indicative of cartilage damage (Schmitz et al., 2010). Cartilage was isolated from low- and high-grade cartilage samples for mRNA analyses. We examined expression levels of a key cartilage ECM component, aggrecan (ACAN). Expression levels of ACAN were significantly decreased in high-grade OA cartilage compared to low-grade regions supporting our assessment of cartilage damage (**Figure 1A**). We then determined expression levels of GPNMB in cartilage harvested from the low-grade and high-grade OA cartilage. Results demonstrated a significant increase in GPNMB expression in high-grade cartilage versus low-grade cartilage levels (**Figure 1B**). This data and previous findings regarding the anti-inflammatory role of GPNMB in other cell types led us to postulate that GPNMB has an anti-inflammatory role in primary chondrocytes.

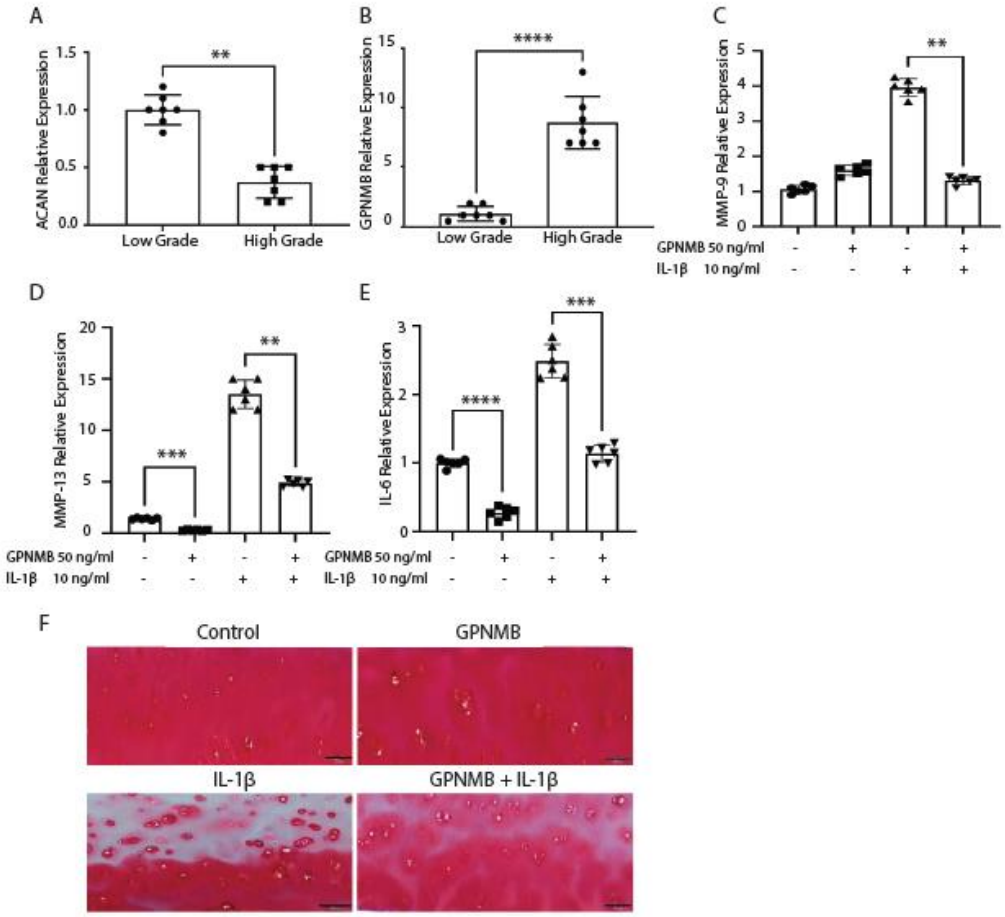


Figure 1: GPNMB expression is increased in high-grade OA cartilage and recombinant GPNMB reduces catabolic gene expression in human HTB-94 cell culture and cartilage damage in human cartilage explants. Human articular cartilage was isolated from low-grade and high-grade OA joints and mRNA expression of (**A**) ACAN and (**B**) GPNMB were determined via RT-qPCR. HTB-94 cells treated with combination of recombinant GPNMB and IL-1β demonstrated significantly decreased expression of (**C**) Mmp-9 (**D**) Mmp-13 and (**E**) IL-6 mRNAs. Combined recombinant GPNMB and IL-1β treatment significantly reduced cartilage degradation in human explant cultures compared to IL-1β treatment alone in Safranin-O-stained human cartilage explants (**F**). Scale bar= 200µm in top panels and 100µm in magnified lower images. Data presented as Mean ± SEM (n=7). **= $p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

<u>GPNMB treatment reduces catabolic gene expression in IL-1β stimulated HTB-94 chondrocytes</u>

One of the hallmarks of OA is increased inflammation within the joint and an increase in the production of inflammatory chemokines and cytokines such as interleukin- 1 beta (IL-1β), interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase -13 (MMP-13) (Gauci et al., 2017; Goldring, 2012; Goldring & Goldring, 2004; Robinson et al., 2016). Since GPNMB was shown to be elevated in more damaged regions of human cartilage, we theorized that increased GPNMB levels may serve a protective anti-inflammatory role. To test this, we utilized human chondrosarcoma cells (HTB-94) which maintain a chondrocyte phenotype in culture and have been established as an appropriate model for examinations of inflammation and inflammatory signaling pathways (Mengshol et al., 2001). Cells were serum starved overnight and then either left untreated or treated for 24 hours with either recombinant

GPNMB alone, IL-1β alone or a combination of recombinant GPNMB and IL-1β. As expected, IL-1β treatment significantly increased expression of Mmp-9, Mmp-13 and Il-6 (**Figure 1C-E**). Interestingly, treatment with recombinant GPNMB alone significantly decreased gene expression of both Mmp-13 and Il-6 and inclusion of GPNMB with IL-1β reduced catabolic expression of all target genes (**Figure 1C-E**). Together, this suggests GPNMB has an anti-inflammatory role in human primary chondrocytes.

Human explant cultures treated with GPNMB display low ECM degradation after IL-1β stimulation

Having determined catabolic gene expression was reduced with recombinant GPNMB treatment in HTB-94 chondrocytes, we next determined if GPNMB pharmacological treatment could reduce ECM degradation following IL-1β stimulation *ex vivo* in human explant cultures. Undamaged human explants were serum starved overnight then left untreated or treated for 24 hours with recombinant GPNMB alone, IL-1β alone or a combination of recombinant GPNMB and IL-1β. Safranin-O-stained explant results showed severe ECM degradation in cultures treated with IL-1β alone compared to untreated controls (**Figure 1F**). Additionally, the severity of the degradation was reduced with the inclusion of recombinant GPNMB (**Figure 1F**). These results establish that recombinant GPNMB treatment decreases IL-1β-induced inflammation in human chondrocytes and cartilage.

IL-1β stimulation induces Gpnmb expression in murine primary chondrocytes

Examinations of endogenous expression of GPNMB in human cartilage and chondrocytes demonstrated that GPNMB expression was upregulated in high-grade damaged OA cartilage and following IL-1β proinflammatory stimulation in human primary chondrocytes. Next, we confirmed these results in murine primary chondrocytes. Primary chondrocytes were isolated from 5-6 day old pups (n=6) as previously described and serum starved overnight (Gosset et al., 2008; Jonason et al., 2015). The chondrocytes were then treated, in triplicate, with either regular culture media (control) or IL-1β for 24 hours and the expression of Gpnmb was determined via RT-qPCR. Results showed a significant increase in GPNMB expression following IL-1β stimulation compared to untreated controls (**Figure 2A**).

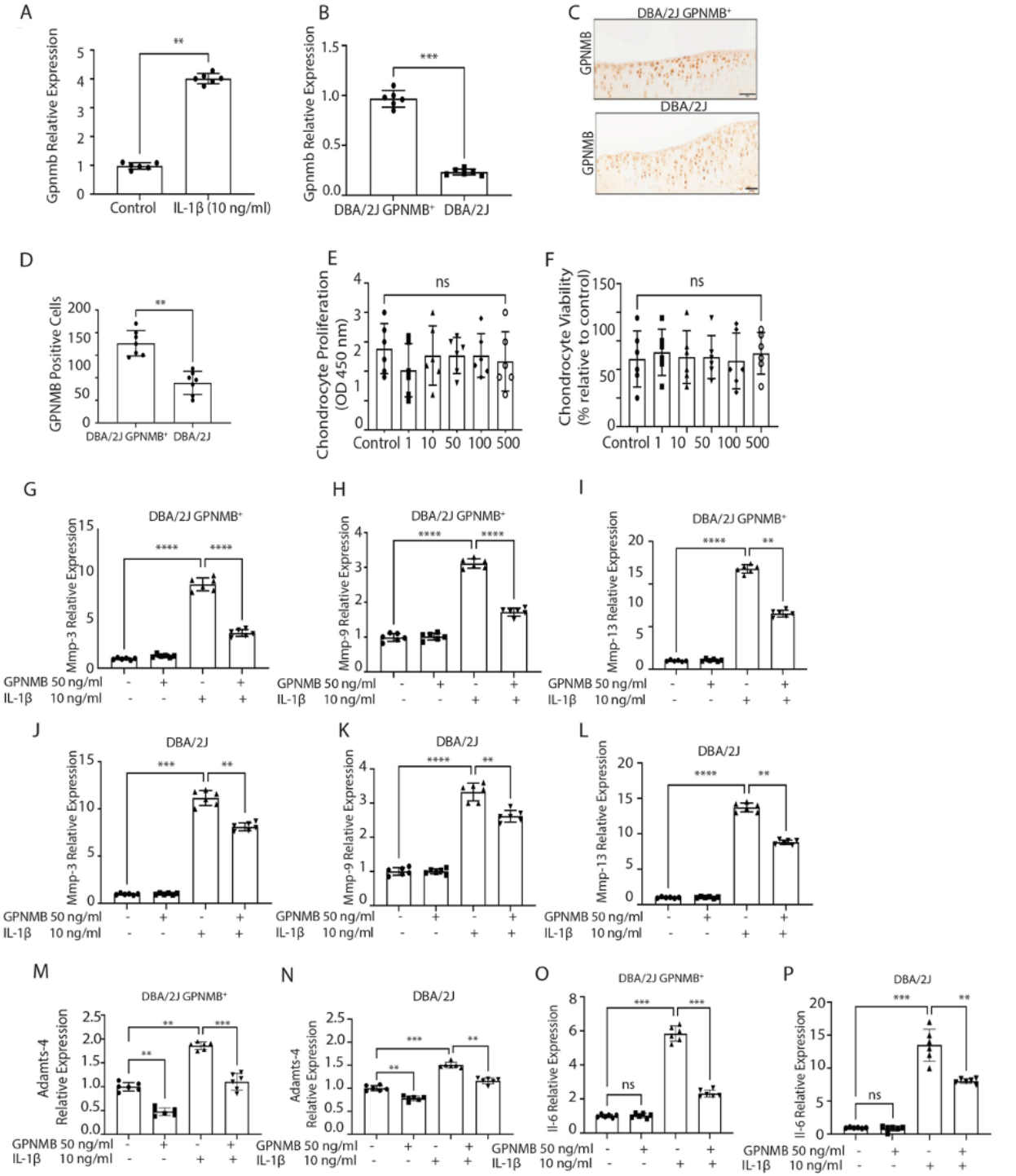


Figure 2: Gpnmb expression is induced with IL-1β proinflammatory stimulation, reduced in DBA/2J mice and recombinant GPNMB inhibits catabolic expression in DBA/2J GPNMB$^+$ and DBA/2J chondrocytes. (**A**) Gpnmb expression is increased with pro-inflammatory IL-1β treatment. Gpnmb expression (**B**) and protein (**C, D**) is significantly reduced in DBA/2J mice. Recombinant GPNMB treatment does not affect chondrocyte proliferation (**E**) or viability (**F**). Recombinant GPNMB inhibits Mmp-3, Mmp-9, Mmp-13 expression in DBA/2J GPNMB$^+$ (**G-I**) and DBA/2J (**J-L**) chondrocytes and

156 reduces Adamts-4 (**M,N**) and IL-6 (**O,P**) expression. Scale bar= 200µm. Data presented as Mean ± SEM
157 (n=6). *=p<0.05, **= p<0.01, ***=p<0.001, ****=p<0.0001.
158
159 Endogenous Gpnmb is reduced in DBA/2J mutant mice
160
161 To study the role of GPNMB in chondrocytes, we utilized a mouse model with a natural mutation
162 in GPNMB (DBA/2J). This global mouse model produces a truncated and non-functional GPNMB protein
163 (Abdelmagid et al., 2014). For this experiment, we determined endogenous Gpnmb levels in primary
164 chondrocytes of DBA/2J GPNMB$^+$ (control) and DBA/2J mice. Results revealed Gpnmb mRNA levels in
165 the mutant DBA/2J mice (n=6) were significantly lower than those found in DBA/2J GPNMB$^+$ (n=6)
166 chondrocytes (**Figure 2B**). We next confirmed these results via immunohistochemical (IHC) analyses of
167 DBA/2J GPNMB$^+$ and DBA/2J articular cartilage using an anti-GPNMB antibody. Joint sections from
168 DBA/2J GPNMB$^+$ and DBA/2J mice were used to visualize and quantify the presence of GPNMB in joints
169 of both mouse models and results showed significantly fewer GPNMB positive cells in DBA/2J mice
170 (**Figure 2C, D**).
171
172 Recombinant GPNMB treatment does not affect murine chondrocyte viability or proliferation
173
174 Before investigating the effects of GPNMB on murine chondrocytes *in vitro*, we first determined
175 if pharmacological treatment with recombinant GPNMB would affect primary chondrocyte viability or
176 proliferation. Chondrocytes were cultured with increasing doses of GPNMB for 72 hours, and cell viability
177 and proliferation were assessed with MTT and CyQuant assays, respectively. Data revealed no significant
178 changes in chondrocyte viability or proliferation with increasing concentrations of recombinant GPNMB
179 (**Figure 2E, F**). These results suggest that treatment has no adverse or toxic effects on chondrocytes.
180
181 GPNMB treatment inhibits Mmp, Adamts-4 and Il-6 expression in DBA/2J GPNMB$^+$ and DBA/2J
182 chondrocytes
183
184 To determine if treatment with recombinant GPNMB would alter catabolic gene expression in
185 murine primary chondrocytes, we isolated femoral and tibial articular cartilage from 5-6 day old male
186 control DBA/2J GPNMB$^+$ (n=6) and mutant DBA/2J mouse pups (n=6) and plated the primary
187 chondrocytes of each mouse model in triplicate. Before treatment, we examined endogenous mRNA levels
188 of Mmp-3, Mmp-9 and, Mmp-13in control DBA/2J GPNMB$^+$ and DBA/2J primary chondrocytes. Results
189 found no significant differences in endogenous mRNA expression in these strains (**Supplementary 2**). The
190 isolated chondrocytes were serum starved overnight and either left untreated (control) or treated with
191 recombinant GPNMB alone, IL-1β alone or a combination of IL-1β and recombinant GPNMB for 24 hours.
192 Expression levels of cartilage-degrading metalloproteinases Mmp-3, Mmp-9 and Mmp-13 were determined
193 via RT-qPCR analysis.
194 In DBA/2J GPNMB$^+$ mice, results showed mRNA levels of Mmp-3, Mmp-9 and Mmp-13 were
195 not significantly different between untreated chondrocytes and those treated with recombinant GPNMB
196 alone (**Figure 2G-I**). Whereas chondrocytes treated with IL-1β alone showed significant upregulation of
197 Mmp-3, Mmp-9, and Mmp-13 (**Figure 2G-I**). Interestingly, when DBA/2J GPNMB$^+$ chondrocytes were
198 treated with a combination of IL-1β and recombinant GPNMB results demonstrated expression of all three
199 catabolic markers were significantly reduced, suggesting that GPNMB might play a role as an anti-
200 inflammatory factor in chondrocytes (**Figure 2G-I**).
201 Mutant DBA/2J primary chondrocyte results showed no significant differences in Mmp-3, Mmp-9
202 or Mmp-13 expression between untreated cells and those treated with GPNMB alone (**Figure 2J-L**).
203 Chondrocytes treated with IL-1β alone demonstrated significant increases in Mmp-3, Mmp-9 and Mmp-13
204 mRNA levels (**Figure 2J-L**). Interestingly, these increases were significantly higher in DBA/2J animals
205 compared to fold changes seen in chondrocytes from DBA/2J GPNMB$^+$ controls (**Figure 2G-I**). Primary

DBA/2J chondrocytes treated with the combination of IL-1β and recombinant GPNMB showed significant reduction in IL-1β-stimulated increased in Mmp-3, Mmp-9 and Mmp-13 expression (**Figure 2J-L**). Together, these results indicate GPNMB has anti-inflammatory functions and reduces expression of IL-1β-stimulated matrix metalloproteinases.

In addition to Mmps, which target and degrade ECM collagens, we also evaluated whether recombinant GPNMB would affect expression of Il-6 and an ACAN degrading enzyme, Adamts-4, in DBA/2J GPNMB$^+$ and DBA/2J primary chondrocytes. First, we evaluated endogenous levels of Adamts-4 and Il-6 in DBA/2J GPNMB$^+$ and DBA/2J primary chondrocytes and found no significant differences in mRNA expression between the strains (**Supplemental 2**). Then, to study the effects recombinant GPNMB treatment on Adamts-4 and Il-6 expression, primary chondrocytes from both strains were serum starved overnight prior to treatment with recombinant GPNMB alone, IL-1β alone or a combination of IL-1β and recombinant GPNMB for 24 hours.

First, we examined Adamts-4 mRNA expression in DBA/2J GPNMB$^+$ chondrocytes (n=6). Results showed that treatment with recombinant GPNMB alone was sufficient to significantly reduce Adamts-4 mRNA expression in treated chondrocytes compared to untreated controls (**Figure 2M**). Treatment with IL-1β alone significantly increased expression of Adamts-4, while treatment with recombinant GPNMB and IL-1β significantly reduced Adamts-4 expression in DBA/2J GPNMB$^+$ chondrocytes (**Figure 2M**). In chondrocytes derived from DBA/2J mice (n=6), treatment with recombinant GPNMB alone significantly reduced Adamts-4 expression compared to untreated controls, while IL-1β treatment alone significantly increased Adamts-4 expression (**Figure 2N**). Treatment with the combination of recombinant GPNMB and IL-1β significantly reduced Adamts-4 levels in treated chondrocytes (**Figure 2N**).

Next, we assessed mRNA expression of Il-6 in primary chondrocytes isolated from DBA/2J GPNMB$^+$ and DBA/2J mice (n=6, each strain). In both strains, recombinant GPNMB treatment alone resulted in no significant difference in Il-6 mRNA compared to expression untreated controls (**Figure 2O, P**). Similarly, IL-1β treatment alone significantly increased Il-6 expression in chondrocytes of both mouse strains (**Figure 2O, P**). Results also revealed that IL-1β-induced expression of proinflammatory Il-6 was significantly higher (a 9-fold) in mutant DBA-2J mice than in DBA/2J GPNMB$^+$ control animals. Furthermore, when treated with IL-1β and recombinant GPNMB Il-6 expression was significantly reduced in DBA/2J GPNMB$^+$ chondrocytes (4-fold) and in those from DBA/2J (9-fold) mice with reduction significantly higher than those of controls (**Figure 2O, P**). Together, these results indicate GPNMB has an anti-inflammatory effect in murine primary chondrocytes by reducing expression of both Adamts-4 and Il-6.

<u>Recombinant GPNMB treatment does not alter anabolic gene expression</u>

Having determined that recombinant GPNMB treatment lowered IL-1β-induced catabolic gene expression in human and mouse primary chondrocytes, we next assessed if GPNMB would affect anabolic gene expression. We began by first determining endogenous mRNA levels of anabolic genes SRY-Box Transcription Factor 9 (Sox-9), type-II collagen (Col2α1) and Acan in DBA/2J GPNMB$^+$ and DBA/2J chondrocytes. Results determined no significant difference in gene expression (**Supplemental 3**). To determine the effect of recombinant GPNMB on primary chondrocytes, the cells were serum starved overnight and then treated for 24 hours with either media (control), recombinant GPNMB alone, IL-1β alone or a combination of IL-1β and recombinant GPNMB. mRNA expression was assessed via RT-qPCR.

Results in DBA/2J GPNMB$^+$ chondrocytes (n=6) showed recombinant GPNMB treatment alone did not significantly alter anabolic gene expression compared to untreated control cells (**Figure 3A, B, C**). Chondrocytes treated with IL-1β alone demonstrated a significant decrease in Sox-9, Col2a1 and ACAN mRNA expression **(Figure 3A, B, C**). Interestingly, the combined treatment of recombinant GPNMB and IL-1β showed no significant effects on anabolic gene expression (**Figure 3A, B, C**).

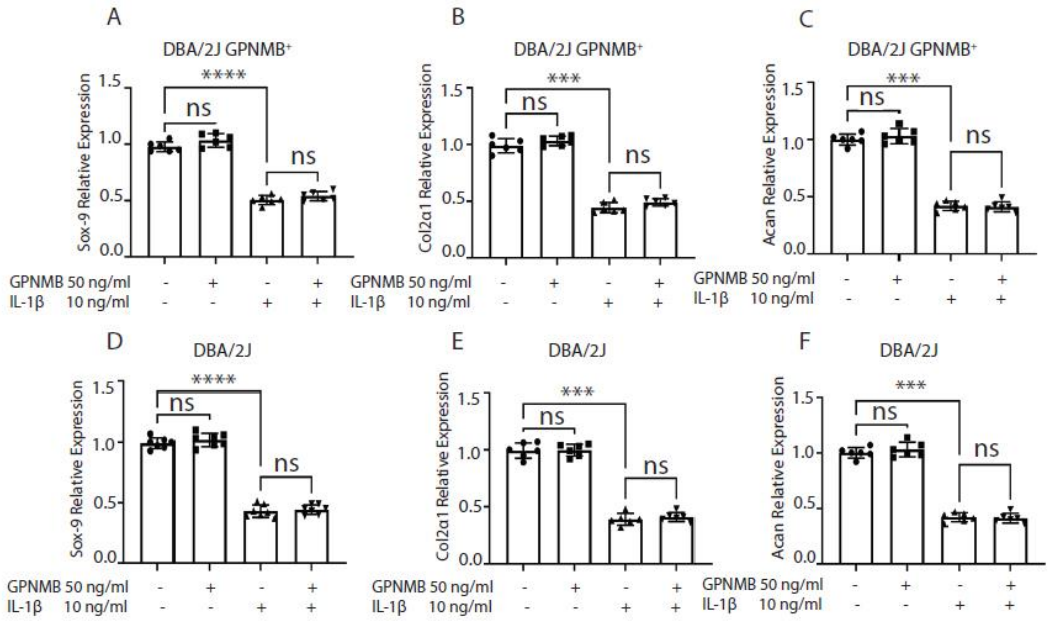


Figure 3: Recombinant GPNMB treatment does not alter anabolic gene expression. Anabolic gene expression was measured in DBA/2J GPNMB$^+$ and DBA/2J chondrocytes by RT-qPCR. Sox-9 mRNA was decreased with IL-1β treatment in DBA/2J GPNMB$^+$ (**A**) and DBA/2J (**D**) chondrocytes and was not rescued following treatment with recombinant GPNMB. Col2α1 expression was decreased with IL-1β treatment and was not increased significantly upon addition of recombinant GPNMB in DBA/2J GPNMB$^+$ (**B**) or DBA/2J (**E**) chondrocytes. Acan mRNA expression decreased with IL-1β treatment and was not rescued with the addition of recombinant GPNMB in DBA/2J GPNMB$^+$ (**C**) or DBA/2J (**F**) chondrocytes. Data presented as Mean ± SEM (n=6). ns= not significant, **= $p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

Data from the DBA/2J chondrocytes (n=6) also showed no significant differences between untreated control cells and those treated with recombinant GPNMB alone (**Figure 3D, E, F**), while treatment with IL-1β alone significantly reduced expression of Sox-9, Col2α1 and ACAN (**Figure 3D, E, F**). The combination treatment of IL-1β and recombinant GPNMB also failed to rescue anabolic gene expression in DBA-2J chondrocytes (**Figure 3D, E, F**). These results confirm that IL-1β stimulation strongly inhibits anabolic gene expression and demonstrates that recombinant GPNMB treatment alone is not sufficient to rescue anabolic gene expression in DBA/2J GPNMB$^+$ or DBA/2J primary chondrocytes.

<u>DBA/2J mice display an accelerated progression of articular cartilage degeneration</u>

The *in vitro* experiments demonstrated recombinant GPNMB treatment inhibited IL-1β-induced inflammation. To understand the possible role GPNMB may play in chondrocytes *in vivo*, we utilized a post-traumatic OA surgical mouse model, destabilization of the medial meniscus (DMM) (Culley et al., 2015; Glasson et al., 2007). First, we confirmed successful DMM surgery in C57BL/6J mice (n=7). Mice were subjected to DMM joint surgery to induce OA on the right joint and sham surgery on the left, which served as internal controls. The mice were sacrificed at 12-weeks post-surgery and joints were histologically analyzed via Toluidine Blue and Safranin O staining (**Figure 4 A-B**). The severity of the damage was analyzed following the Osteoarthritis Research Society International (OARSI) (**Figure 4C**) (Moskowitz, 2000; Pritzker et al., 2006). After 12 weeks, cartilage was intact in sham operated joints and in DMM operated joints fibrillation and erosion was visible in superficial and intermediate layers indicating our DMM surgery was successful.

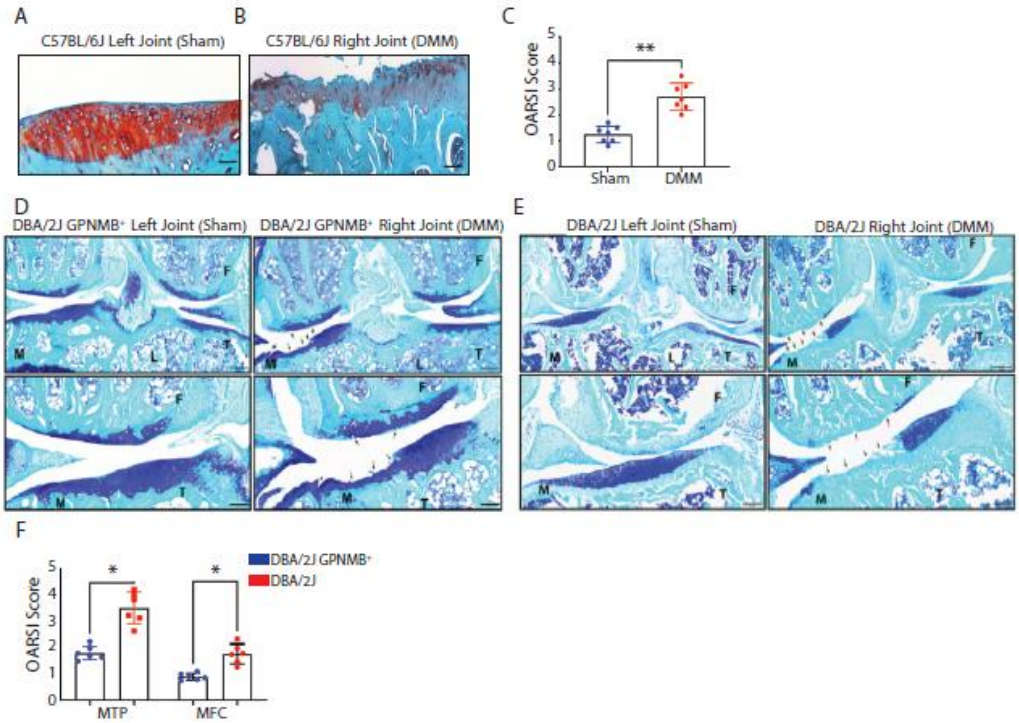


Figure 4: DMM surgery resulted in cartilage fibrillation after 12 weeks in C57BL6/J mice and damage was accelerated in DBA/2J mice following DMM. DMM surgery was validated in C57BL/6J mice. Cartilage was intact in sham operated joints (**A**) while DMM joints displayed superficial and intermediate cartilage damage (**B**) (tibia shown). (**C**) OARSI scores for sham and DMM operated joints. DBA/2J GPNMB$^+$ (**D**) and DBA/2J (**E**) mice, which lack functional GPNMB protein, were subjected to sham and DMM surgery and joints were stained with Toluidine blue. Black arrows indicate damaged areas. (**F**) OARSI scores for femur (MFC) and tibia (MTP) cartilage damage of DBA/2J GPNMB$^+$ and DBA/2J mice. Scale bar= 200µm in C57BL/6J joints and top panels and 100µm in magnified lower images (**D, E**). Images shown are representative and OARSI scorings from multiple images of n=7 each C57BL/6J, DBA/2J GPNMB$^+$ and DBA/2J mice. Data presented as Mean ± SEM (n=6). *$p<0.05$, **= $p<0.01$.

Next, DBA/2J GPNMB$^+$ and DBA/2J (n=7 each strain) mice were subjected to DMM surgery as described above. Cartilage damage caused by joint instability was observed in the articular cartilage of the medial compartment of the femur and tibia in both DBA/2J GPNMB$^+$ and DBA/2J mice (**Figure 4D, E**). Compared to DBA/2J GPNMB$^+$ control mice, the DBA/2J mutant mice demonstrated more severe cartilage damage (**Figure 4D, E**). The severity of the damage was analyzed following OARSI guidelines. DBA/2J GPNMB$^+$ mice showed max scores of 1.629 ± 0.2616 for the medial tibial plateau (MTP) and 0.7571 ± 0.1043 for the femoral medial condyle (MFC) (**Figure 4F**). The DBA/2J mice were significantly higher at 2.656 ± 0.355 for the medial tibial plateau and 1.473 ± 0.202 for the femoral medial condyle (**Figure 4F**). These results show the GPNMB mutant mice, which lack a fully functional GPNMB protein, displayed more severe cartilage damage upon DMM surgery compared to the controls.

<u>DBA/2J exhibit reduced ACAN and enhanced IL-6 expression compared to DBA/2J GPNMB$^+$</u>

Given the severity of the cartilage damage seen in DBA/2J mice following DMM surgery, we next determined if ACAN expression was negatively affected in the mutant mice via immunohistochemistry. ACAN was chosen for analysis since it is the major cartilage proteoglycan responsible for bearing compressive loads during perambulation (Roughley, 2001; Roughley & Mort, 2014). Sham operated joints

showed intact cartilage and intact ACAN contents with no significant differences detected between the groups (**Figure 5A, B**). While the ACAN expression in DBA/2J GPNMB$^+$ DMM joints was also relatively high, DMM joint levels were much lower relative to sham operated controls and much higher than ACAN levels in the DBA/2J DMM mouse joints that showed severe cartilage damage (**Figure 5A, B**). We then quantified percent positive cells and found no significant differences in ACAN positive cells in sham operated joints between strains (**Figure 5C**), but a significant difference in ACAN production between DMM joints of DBA/2J GPNMB$^+$ and DBA/2J mice (**Figure 5D**). These results revealed that DBA/2J mice exhibited severe cartilage damage and that the damage is due, in part, to degradation of critical ECM components such as ACAN.

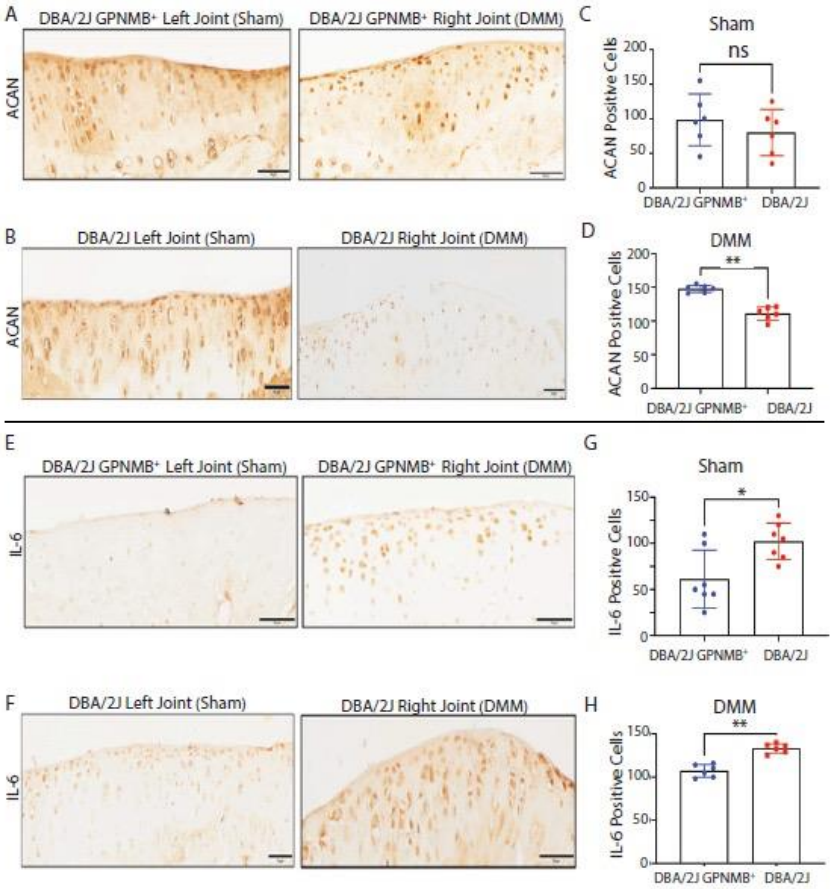


Figure 5: ACAN expression is severely depleted, and IL-6 expression elevated following DMM in DBA/2J mice. ACAN expression was not significantly altered in sham surgery mice (**A-C**) but was reduced following DMM in DBA/2J GPNMB$^+$ (**A, D**) and was severely depleted in DBA/2J (**B, D**) cartilage. Endogenous IL-6 expression is increased in DBA/2J animals without DMM (**E-G**). DBA/2J GPNMB$^+$ (**E, H**) and DBA/2J (**B, H**) cartilage showed an increase in IL-6 following DMM surgery. Scale bar= 200µm top panel and 100µm in magnified lower images. Images shown are representative and OARSI scores are taken multiple images of n=5-7 each DBA/2J GPNMB$^+$ and DBA/2J mice. Data presented as Mean ± SEM. *$p<0.05$, **$p<0.01$.

In addition to the anabolic marker ACAN, we also evaluated the expression of the inflammatory marker IL-6 following DMM surgery in both DBA/2J GPNMB$^+$ and DBA/2J mice (n=7 each strain). Sham and DMM joint sections were subjected to immunohistochemistry staining using an anti-IL-6 antibody. Both DBA/2J GPNMB$^+$ and DBA/2J mice expressed IL-6 as an inflammatory response to the post-

traumatic joint damage caused by DMM (**Figure 5E, F**). Quantification of IL-6 expression showed significantly higher number of IL-6 positive cells in DBA/2J mice following sham surgery compared to DBA/2J GPNMB$^+$ controls (**Figure 5G**), suggesting DBA/2J mutant mice produce higher endogenous levels of proinflammatory IL-6 in the absence of functional GPNMB protein than control animals. This significance is further enhanced upon DMM surgery, where there is a significant increase in IL-6 positive cell numbers in the DBA/2J DMM joints compared to DBA/2J GPNMB$^+$ control DMM results (**Figure 5H**).

GPNMB inhibits MAPK Kinase signaling in mouse primary chondrocytes

Previous literature has shown that GPNMB might play a role in the mitogen-activated protein kinase (MAPK) pathway in a variety of cell types such as mesenchymal stem cells, fibroblasts, and osteoclasts (Abdelmagid et al., 2015; Furochi et al., 2007; Ono et al., 2016). In this study we determined the effects of recombinant Gpnmb on MAPK singling in primary chondrocytes. Primary chondrocytes were isolated from C57BL/6J male mice (n=9), treated with recombinant GPNMB with or without IL-1β and terminated at 0, 10, 30 and 50 minutes after treatment. Protein lysates were probed for extracellular signal-related kinase (ERK), phospho-ERK (pERK), and GAPDH for loading control. Data showed the pERK was increased following IL-1β stimulation and decreased upon recombinant GPNMB treatment (**Figure 6A, B**). Recombinant GPNMB treatment inhibited pERK after 30 minutes. These results were semi-quantified by densitometry analyses (**Figure 6C, D**). These results indicate recombinant GPNMB plays a role in pERK inhibition following inflammation induced by IL-1β stimulation.

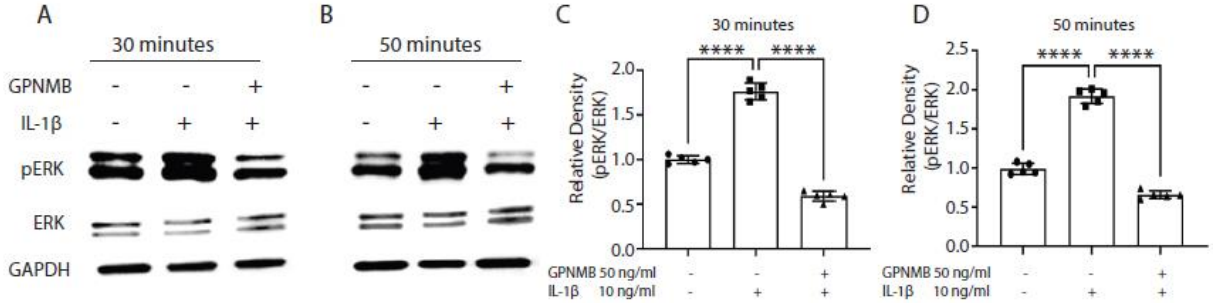


Figure 6: Recombinant GPNMB inhibits the MAPK signaling pathway in primary chondrocytes. Immunoblotting of primary chondrocytes following IL-1β stimulation with and without recombinant GPNMB show GPNMB inhibits pERK at (**A**) 30 and (**B**) 50 minutes post-treatment. Densitometric analyses of pERK were done at (**C**) 30 and (**D**) 50 minutes. Experiments were repeated three times with similar results. Data presented as Mean ±SEM. ****=p<0.0001, ns= not significant.

Discussion

While the role of GPNMB (osteoactivin) in fracture repair and bone regeneration is well characterized, nothing is known of its biological function in the joint or in chondrocytes, the principle cell type present in cartilage (Akkiraju & Nohe, 2015; Hall, 2019). Furthermore, while GPNMB expression is increased under inflammatory conditions in human and porcine chondrocytes, it is unclear what role GPNMB plays in the pathogenesis of cartilage degenerative disorders such as OA (Conde et al., 2015; Jiang et al., 2011; Owen et al., 2003; Schlichting et al., 2014; Sun et al., 2015). OA is characterized by articular cartilage degeneration, abnormal bone formation (osteophytes) and chronic inflammation of the joint that results in pain and loss of mobility in affected individuals (Goldring et al., 2008; Goldring & Otero, 2011; Goldring et al., 2011; Goldring & Goldring, 2016; Loeser et al., 2016; Loeser et al., 2012). GPNMB has been shown to have anti-inflammatory effects in lymphocytes, macrophages, periodontal ligament cells and

384 in microglia and astrocytes (Budge et al., 2020; Li et al., 2010; Neal et al., 2018; Ripoll et al., 2007; Song
385 & Lin, 2019; Zhou et al., 2017). Given these findings, we examined the biological role of GPNMB on
386 inflammation and cartilage damage in primary chondrocytes *in vitro* and in articular cartilage *in vivo*. We
387 provide the first evidence that GPNMB protects against cartilage damage in a post-traumatic mouse model
388 and inhibits the expression of inflammatory cytokines and chemokines without altering anabolic gene
389 expression in human and mouse primary chondrocytes.
390 In humans, GPNMB was increased in damaged high-grade osteoarthritic cartilage, primary
391 chondrocytes, and cell lines *in vitro* and expression was increased with IL-1β proinflammatory stimulation.
392 Additionally, treatment with recombinant GPNMB significantly reduced catabolic gene expression in
393 primary human and mouse chondrocytes without altering anabolic gene expression, suggesting GPNMB
394 does not affect chondrogenesis.
395 Expanding on our *in vitro* findings, we next determined the role of GPNMB *in vivo* via a DMM
396 model of induced post-traumatic OA in mice (Culley et al., 2015; Glasson et al., 2007). Surgeries were
397 performed on DBA/2J GPNMB^{+} controls and in DBA/2J mice, which produce a non-functional truncated
398 version of the GPNMB protein (Abdelmagid et al., 2014; Chung et al., 2007; Gutknecht et al., 2015).
399 Inactive GPNMB leads to increased immune function and inflammation in these animals, with
400 proinflammatory cytokine levels consistently higher than DBA/2J GPNMB^{+} controls (Fan et al., 2010;
401 Wilson et al., 2015; Zhou et al., 2009). When cartilage integrity was assessed post-surgery, DBA/2J mice
402 displayed severe cartilage damage, suggesting GPNMB is beneficial for cartilage homeostasis.
403 To determine the signaling pathway responsible for the GPNMB-mediated reduction of
404 inflammation in cartilage and primary chondrocytes, we evaluated the effects of GPNMB on the MAPK
405 signaling pathway which is required for chondrocyte expression of Mmps, inflammatory cytokines, and
406 Adamts in cartilage (Cui et al., 2020; Goldring, 1999; Tetsunaga et al., 2011). GPNMB is known to function
407 via the ERK pathway in osteoclasts, motor-neuron-like cells, and fibroblasts (Furochi et al., 2007; Ono et
408 al., 2016; Sondag et al., 2016). Also, ERK pathway activation has been shown to regulate osteoblast
409 proliferation and differentiation (Bellido & Plotkin, 2011; Jessop et al., 2002; Yan et al., 2012). However,
410 in chondrocytes, activation of the ERK pathway has been shown to be detrimental resulting in
411 chondrodysplasia and contributing to cartilage degradation in the pathogenesis of OA (Appleton, 2018;
412 Appleton et al., 2010; Lin et al., 2021; Yang et al., 2008). Here, we are the first to show that the anti-
413 inflammatory action of GPNMB in chondrocytes occurs via MAPK in primary chondrocytes with IL-1β-
414 induced inflammation (**Figure 7**). Furthermore, our results suggest the strong anti-inflammatory function
415 of GPNMB may be a result of concurrent inhibition of multiple catabolic markers (MMP-3, MMP-9, MMP-
416 13, ADAMTS-4, and IL-6).

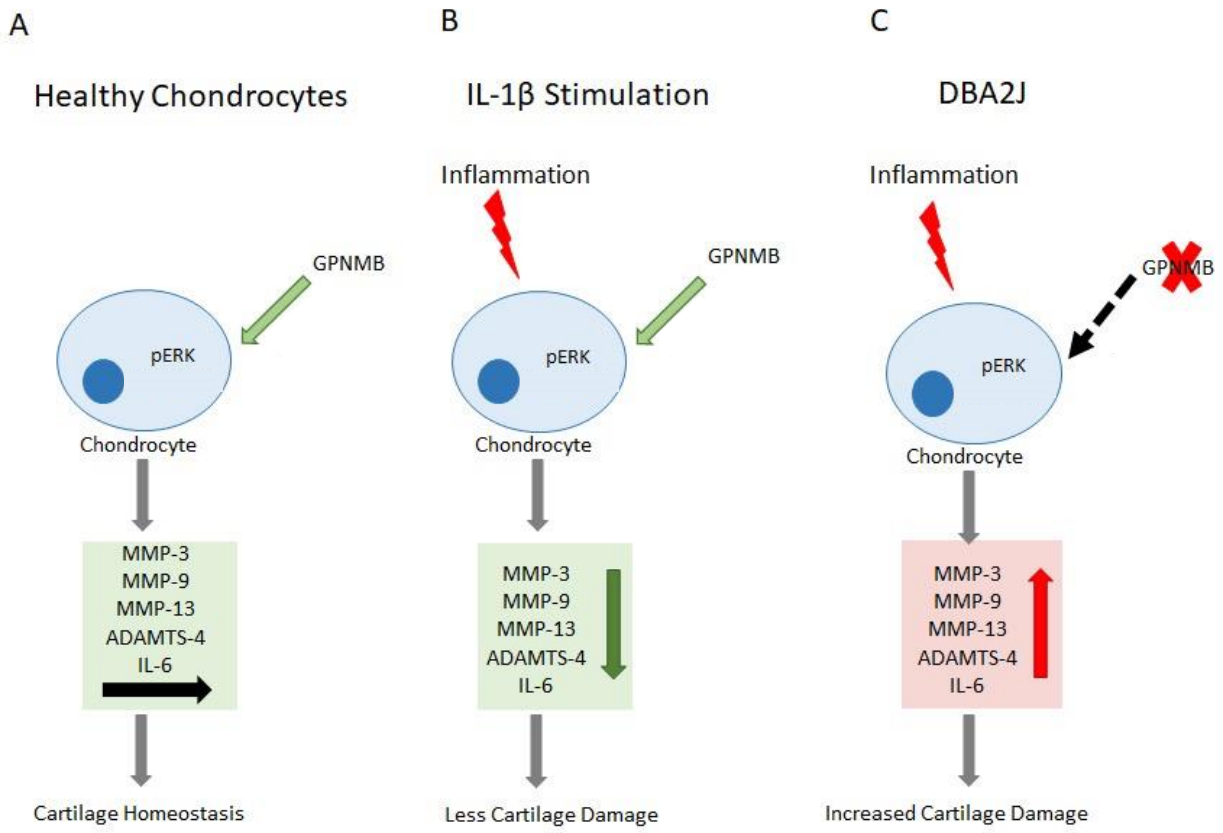


Figure 7: Schematic of the proposed mechanism of GPNMB anti-inflammatory function in chondrocytes. (**A**) In healthy chondrocytes, GPNMB functions without inflammatory stimuli to modulate catabolic chemokines and cytokines and maintain cartilage homeostasis. (**B**) In the presence of inflammatory stimuli (IL-1β), GPNMB acts to modulate MAPK to reduce expression of catabolic chemokines and cytokines and reduce cartilage damage. (**C**) In DBA/2J mice with non-functional GPNMB, there is no inhibition of inflammatory molecules leading to increased catabolic expression and increased cartilage damage.

In conclusion, the data presented here demonstrates a novel anti-inflammatory role for GPNMB in cartilage and primary chondrocytes. While more work is needed to fully elucidate the mechanism *in vitro* and *in vivo*, these novel findings show GPNMB reduces catabolic gene expression and lays the foundation for future studies to assess the use of GPNMB as a possible alternative therapeutic agent in the treatment of OA.

Materials and Methods

HTB-94 Cell Line Culture and Treatment Conditions

HTB-94 cells were cultured in DMEM/F12 (1:1, Cellgro Technologies, Lincoln, NE) culture medium supplemented with Penicillin/Streptomycin (Penicillin: 100 IU/ml, Sigma Aldrich, St. Louis, MO; Streptomycin: 100 µg/ml, Sigma Aldrich) and 10% fetal bovine serum (FBS, GE Healthcare, Chicago, IL) at 37°C and 5% CO_2. Once established in culture, monolayer HTB-94 cells were rinsed with sterile PBS and serum-starved overnight prior to stimulation with 10 ng/ml of recombinant interleukin-1β (IL-1β; R&D Systems, Niskayuna, NY) with or without recombinant GPNMB (R&D Systems) in DMEM/F 12 (1:1)

443 medium supplemented with Penicillin (100 IU/ml, Sigma-Aldrich), Streptomycin (100 µg/m, Sigma-
444 Aldrich). HTB-94 experiments were set up in triplicate and results shown are from three separate
445 experiments.

446 Human Explant Culture and GPNMB/IL-1β Treatment
447
448 Explants were from undamaged regions of cartilage harvested from the discarded and de-identified
449 patients (n=7). Undamaged cartilage regions were again identified using India Ink and then washed twice
450 in sterile PBS. Cartilage explants (2 x 2 mm) were excised using a biopsy punch and maintained for 24
451 hours in supplemented DMEM/F12 culture medium as described above at 37°C and 5% CO_2. After 24
452 hours, the medium was replaced with serum free DMEM overnight and then treated with 10 ng/ml IL-1β
453 with or without recombinant 50 ng/ml GPNMB for 24 hours. Following pharmacological treatment, the
454 explants were fixed in 4% paraformaldehyde (Sigma Aldrich) for 24 hours, decalcified using 14%
455 Ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific, Waltham, MA), and processed.
456 Explants were sectioned (5µm) and stained with Safranin-O stain (Thermo Fisher Scientific) (Schmitz et
457 al., 2010).
458
459 Mouse Strains
460
461 The following mouse strains were utilized in the completion of this study: C57BL/6J (wild type,
462 WT, Stain #: 000664), DBA/2J (GPNMB mutant, Strain #: 000671), and DBA/2J $GPNMB^+$ (GPNMB
463 control, Strain #: 007048). Mice were purchased from Jackson Laboratories and were housed at NEOMED
464 in accordance with protocol guidelines approved by the NEOMED Institutional Animal Care and Use
465 Committee (IACUC).
466
467 Murine Primary Chondrocyte Isolation and Culture and Treatment Conditions
468
469 Primary articular chondrocytes isolated from hind limb dissections of 5-6-day-old mouse pups as
470 previously described (Gosset et al., 2008; Jonason et al., 2015). In brief, skin and soft tissues were removed
471 from the hindlimb bones and the femoral head, femoral condyle and tibial plateau cartilage were removed
472 and placed in 1X sterile PBS on ice. Cartilage was washed twice with cold 1X sterile PBS and incubated
473 twice (45 minutes per incubation) in a digestion solution consisting of Collagenase D (3 mg/ml, Sigma
474 Aldrich) in serum free Dulbecco's modified Eagle's medium (DMEM) supplemented with Penicillin (100
475 IU/ml) and Streptomycin (µg/ml) at 37°C and 5% CO_2. Then, cartilage digestion solution was diluted with
476 serum free culture medium to a final concentration of 0.5 mg/ml and the cells were incubated overnight.
477 After digestion, the cell solution was re-suspended and filtered using a sterile 40 µm cell strainer (Thermo
478 Fisher Scientific). The filtered cell solution was centrifuged at 400g for 10 minutes, rinsed twice with
479 sterile 1X PBS (centrifuging after each rinse), and re-suspended in culture medium consisting of DMEM
480 supplemented with Penicillin (100 IU/ml), Streptomycin (100 µg/m) and 10% FBS. Mouse primary
481 chondrocytes were counted via hemocytometer and seeded at high density in a 6-well culture dish for four
482 days. Chondrocytes were serum stared overnight on day 5 and treated on day 6 with recombinant IL-1β
483 (10 ng/ml) and recombinant GPNMB (50 ng/ml). On day 7, cells were harvested for immunoblotting and
484 total RNA extraction.
485
486 Cell Viability and Proliferation Assays
487
488 Cell viability in response to different doses of recombinant GPNMB was assessed using the MTT
489 assay (Thermo Fisher Scientific). One day prior to treatment, primary chondrocytes were seeded into 96-
490 well culture plates. Chondrocytes were treated for 72 hours with varying doses of recombinant GPNMB
491 (R&D systems) and the culture media was aspirated and 20 µl of MTT solution was added to the cells for
492 4 hours at 37°C and 5% CO_2. Then, the supernatant was aspirated, dimethyl sulphoxide was added with

493 gentle agitation for 15 minutes and absorbance was measured at 570 nm on an ELISA BioTek plate reader
494 (BioTek, Winooski, VT). Cell viability assays were conducted 3 times (3 replicates per group) and
495 treatment results were compared to untreated control cells.
496 Cellular proliferation of primary chondrocytes was determined with the CyQuant NF cell
497 proliferation assay (Life Technologies, Carlsbad, CA). Mouse primary chondrocytes were harvested from
498 45-day-old pups and isolated as previously described. Cells were seeded in a 96-well culture plate for 24
499 hours and then treated with different concentrations of recombinant GPNMB for 72 hours. The media was
500 then aspirated and replaced with 100 µl 1X binding solution and incubated for 1 hour at 37°C. Fluorescent
501 intensity was measured on a BioTek plate reader (excitation at 485 nm and emission at 530 nm).
502 Proliferation assays reported are the results of 3 separate experiments (3 replicates per group) and results
503 of treated cells were compared to those of untreated control cells.
504
505 RNA Isolation and RT-qPCR Analysis of Gene Expression
506
507 Total RNA was isolated from primary chondrocytes using Qiazol (Qiagen, Hilden, Germany) and
508 purified using the RNeasy Mini Kit (Qiagen) following the manufacturer recommended protocols. RNA
509 was quantified on a Nanodrop 2000 spectrophotometer (Nanodrop, Thermo Fisher Scientific). cDNA was
510 synthesized from 1 µg total RNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher
511 Scientific).
512 RT-qPCR analyses were completed in triplicate using in-house designed primers (**Table 1**) and
513 SYBR green PCR Master Mix (Thermo Fisher Scientific) on a Step One Plus Machine (Applied
514 Biosystems, Waltham, MA). Expression of target genes was determined utilizing the delta-delta-Ct method
515 and GAPDH was employed for normalization as an endogenous control (Livak & Schmittgen, 2001;
516 Schmittgen et al., 2008; Schmittgen & Livak, 2008).
517
518 **Table 1: qPCR primers**

Primer	Species	Sequence (5'–3')
Acan	Human	F: CTCTGAAACCAGCACAACGC R: CAGCAATTCCGTGGCTATGC
Acan	Mouse	F: AGGAGGTGGTACTGCTGGTG R: TCTCACTCCAGGGAACTCGT
Adamts-4	Mouse	F: GAGTCCCATTTCCCGCAGAA R: ATAACCGTCAGCAGGTAGCG
Cd44	Mouse	F: CCACAGCCTCCTTTCAATAACC R: TGCCCATCACACTGTCACTGT
Col2a1	Mouse	F: TTCTGCTGCTAATGTTCTTGACC R: GGGATGAAGTATTGTGTCTTGGG
Gapdh	Human	F: ACCACTGTCCACGCCATCAC R: TCCACCACCCTGTTGCTGTA
Gapdh	Mouse	F: CGACTTCAACAGCAACTCCCACTCTTCC R: TGGGTGGTCCAGGGTTTCTTACTCCTT
Gpnmb	Human	F: TCCCCGAGGCCCTGTATTTA R: CCTCTGACCATGCTGTCCAG
Gpnmb	Mouse	F: AATGGGTCTGGCACCTACTG R: GGCTTGTACGCCTTGTGTTT
Il-6	Human	F: GGTACATCCTCGACGGCATCT R: GTGCCTCTTTGCTGCTTTCAC
Il-6	Mouse	F: CCAATTTCCAATGCTCTCCT R: ACCACAGTGAGGAATGTCCA
Mmp-3	Mouse	F: GGAGGCAGCAGAGAACCTAC

		R: ATGGGTAGGATGAGCACACA
Mmp-9	Human	F: CGCAGACATCGTCATCCAGT R: GGATTGGC CTTGGAAGATGA
Mmp-9	Mouse	F: GCATACTTGTACCGCTATGGT R: TGTGATGTTATGATGGTCCC
Mmp-13	Human	F: TGTTGCTGTACAAAAGCAATGGTT R: TGCTCTCATTGACAGACCATGTG
Mmp-13	Mouse	F: TGATGGACCTTCTGGTCTTCTGGC R: CATCCACATGGTTGGGAAGTTCTG
Sox-9	Mouse	F: TCTCCTAATGCTATCTTCAAGGCG R: TGCTCAGTTCACCGATGTCCAC

Immunoblotting

Total cellular protein was isolated from primary chondrocytes using radioimmunoprecipitation buffer (RIPA, Millipore, Burlington, MA) with added phosphatase inhibitor (Pierce, Rockford, IL). Protein concentrations were established using the Pierce BCA Protein Assay Kit (Pierce). Equivalent protein concentrations (20 µg) were loaded with 2X sample buffer (BioRad, Hercules, CA) into 12% SDS-PAGE gels. The Trans-Blot Turbo System (BioRad) was used to transfer proteins to a polyvinylidene fluoride membrane (PVDF, BioRad). Membranes were then blocked with 5% bovine serum albumin (BSA, Cytiva, Marlborough, MA) in 0.1% Tri-Buffered Saline with Tween 20 (TBST) for 1 hour at room temperature and then incubated as described at 4°C overnight with primary antibodies listed in **Table 2**. Membranes were rinsed twice in TBST (5 minutes per wash) with gentle shaking and a Horseradish peroxidase (HRP)-linked secondary in TBST (Cell Signaling Technologies, Danver, MA) was applied to the membranes for 1 hour. Immunoblots were developed with chemiluminescent substrate (Pierce). Bands images and intensity were determined using a Pxi imaging system and Syngene software (Syngene, Bangalore, India).

Table 2: Table of Primary Antibodies

Source	Primary Antibodies	Company
Rabbit	ACAN	Bioss
Rabbit	ERK	Cell Signaling Technologies
Rabbit	pERK	Cell Signaling Technologies
Rabbit	GAPDH	Cell Signaling Technologies
Rabbit	GPNMB	LSBio
Rabbit	IL-6	Abcam

DMM Surgery

DMM was performed in 12-week-old male DBA/2J GPNMB^{+} (control, n=7) and DBA/2J (mutant, n=7) mice to induce OA. DMM was performed as previously described and surgical procedures were approved by the NEOMED IACUC committee (Culley et al., 2015; Glasson et al., 2007). Only male mice were studied at this time. Although recent literature has shown that female mice do in fact develop post traumatic-OA, due to the preliminary nature of this study and resource constrains, only male mice were studied at this time (Hwang et al., 2021; Ma et al., 2007). Female mice will be included in future studies. The right knee joint was subjected to microsurgery under a sterilized microscope. The medial menisci tibial ligament (MMTL), which anchors the medial meniscus (MM) to the tibial plateau, was transected to destabilize the joint. The left knee joint was subjected to sham surgery as a control. The knee was surgically opened but the MMTL was not transected. Mice were sacrificed 12-weeks post-surgery.

DMM Tissue Isolation

The sham and DMM surgery mouse knee joints were harvested as previously described (Culley et al., 2015). Soft tissues were removed and the joints were fixed for 24 hours in 4% paraformaldehyde with gentle agitation. The fixed joints were rinsed for 1 hour in PBS and then decalcified for 10 days in 14% EDTA. The buffer was changed three times per week during the decalcification process. Decalcified joints were washed for 6 hours in PBS, processed, and embedded in paraffin and cut into 6 µm sections.

Histological Assessment of Mouse Sham and DMM Knee Joints

To assess cartilage damage in the sham and DMM surgery mouse joints, the sections were stained with Toluidine blue/fast green stain. Three sections were selected at 90 µm intervals for histological evaluation following the Osteoarthritis Research Society International (OARSI) scoring system (Pritzker et al., 2006). Results were presented as ± mean of the maximum joint score.

Immunohistochemistry

Histological sections of sham and DMM mouse knee joints were subjected to immunohistochemical analysis to measure expression of catabolic and anabolic proteins in damaged and undamaged regions of the cartilage. Histological sections were de-paraffinized for 1 hour and cleared in xylene (Thermo Fisher Scientific) before they were rehydrated in a graded ethanol series. For antigen retrieval, the sections were treated with 0.01 M citrate buffer at 60°C overnight in a water bath. Activity of endogenous peroxidase was quenched using 3% hydrogen peroxide (H_2O_2) solution for 15 minutes before blocking with 5% BSA for 1 hour. Sections were then treated overnight at 4°C with primary rabbit anti-Mmp-13 (Abcam, Cambridge, England), rabbit anti-Il-6 (Abcam), rabbit anti-Gpnmb (LSBio, Lynnwood, WA) or rabbit anti-Acan (Bioss, Woburn, MA). Biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Newark, CA) was added for 1 hour and the Vectastain Elite ABC HRP kit was sued with 3, 3'-Diaminobenzidine (DAB, Vector Laboratories) was used for signal development. The sections were imaged using a BX61VS microscope (Olympus Life Science, Center Valley, PA) and positive cells were quantified with ImageJ software (Schneider et al., 2012).

Statistical Analyses

For all data generated, differences between individual groups were analyzed using GraphPad Prism software (GraphPad, La Jolla, CA). All cell culture experiments were repeated a minimum of three times with similar results. DMM experiments were repeated twice with 6 individuals included in each trial. In comparisons of multiple groups, One-way analysis of variance (ANOVA) tests were employed followed with Tukey's post hoc tests. Comparisons of two groups employed unpaired *T*-tests. For histological scoring, MannWhitney and KruskalWallis tests were performed with Dunn's post hoc analyses were conducted as the nonparametric equivalents for the independent samples t-test and One-way ANOVA. Differences were considered significant where $p<0.05$ and graphs depict group means + standard error of the average (±SEM).

Acknowledgements: Research was funded by a generous gift from the Cook Family Fund for Orthopaedic Research and the Graduate School of Kent State University.

Competing Interests: The authors declare no competing interests.

Data and Materials Availability: All data supporting the findings of this study are available within the article and its Supplementary Information Files and from the corresponding author on reasonable request.

602 **References:**
603

604 Abdelmagid, S. M., Barbe, M. F., Hadjiargyrou, M., Owen, T. A., Razmpour, R., Rehman, S., Popoff, S.
605 N., & Safadi, F. F. (2010). Temporal and spatial expression of osteoactivin during fracture repair.
606 *Journal of Cellular Biochemistry*, *111*(2), 295-309.
607 Abdelmagid, S. M., Barbe, M. F., Rico, M. C., Salihoglu, S., Arango-Hisijara, I., Selim, A. H., Anderson,
608 M. G., Owen, T. A., Popoff, S. N., & Safadi, F. F. (2008). Osteoactivin, an anabolic factor that
609 regulates osteoblast differentiation and function. *Experimental cell research*, *314*(13), 2334-2351.
610 Abdelmagid, S. M., Belcher, J. Y., Moussa, F. M., Lababidi, S. L., Sondag, G. R., Novak, K. M., Sanyurah,
611 A. S., Frara, N. A., Razmpour, R., & Del Carpio-Cano, F. E. (2014). Mutation in osteoactivin
612 decreases bone formation in vivo and osteoblast differentiation in vitro. *The American journal of*
613 *pathology*, *184*(3), 697-713.
614 Abdelmagid, S. M., Sondag, G. R., Moussa, F. M., Belcher, J. Y., Yu, B., Stinnett, H., Novak, K., Mbimba,
615 T., Khol, M., Hankenson, K. D., Malcuit, C., & Safadi, F. F. (2015). Mutation in Osteoactivin
616 Promotes Receptor Activator of NFkappaB Ligand (RANKL)-mediated Osteoclast Differentiation
617 and Survival but Inhibits Osteoclast Function. *J Biol Chem*, *290*(33), 20128-20146.
618 https://doi.org/10.1074/jbc.M114.624270
619 Akkiraju, H., & Nohe, A. (2015). Role of chondrocytes in cartilage formation, progression of osteoarthritis
620 and cartilage regeneration. *Journal of developmental biology*, *3*(4), 177-192.
621 Appleton, C. T. (2018). Osteoarthritis year in review 2017: biology. *Osteoarthritis Cartilage*, *26*(3), 296-
622 303. https://doi.org/10.1016/j.joca.2017.10.008
623 Appleton, C. T. G., Usmani, S. E., Mort, J. S., & Beier, F. (2010). Rho/ROCK and MEK/ERK activation
624 by transforming growth factor-α induces articular cartilage degradation. *Laboratory Investigation*,
625 *90*(1), 20-30.
626 Arosarena, O. A., Barr, E. W., Thorpe, R., Yankey, H., Tarr, J. T., & Safadi, F. F. (2018). Osteoactivin
627 regulates head and neck squamous cell carcinoma invasion by modulating matrix metalloproteases.
628 *Journal of Cellular Physiology*, *233*(1), 409-421.
629 Bellido, T., & Plotkin, L. I. (2011). Novel actions of bisphosphonates in bone: preservation of osteoblast
630 and osteocyte viability. *Bone*, *49*(1), 50-55.
631 Budge, K. M., Neal, M. L., Richardson, J. R., & Safadi, F. F. (2020). Transgenic overexpression of GPNMB
632 protects against MPTP-induced neurodegeneration. *Molecular Neurobiology*, *57*(7), 2920-2933.
633 Chung, J.-S., Sato, K., Dougherty, I. I., Cruz Jr, P. D., & Ariizumi, K. (2007). DC-HIL is a negative
634 regulator of T lymphocyte activation. *Blood*, *109*(10), 4320-4327.
635 Conde, J., Scotece, M., Abella, V., Gómez, R., López, V., Villar, R., Hermida, M., Pino, J., Gómez-Reino,
636 J. J., & Gualillo, O. (2015). Identification of novel adipokines in the joint. Differential expression
637 in healthy and osteoarthritis tissues. *PLoS ONE*, *10*(4), e0123601.
638 Cui, A., Li, H., Wang, D., Zhong, J., Chen, Y., & Lu, H. (2020). Global, regional prevalence, incidence and
639 risk factors of knee osteoarthritis in population-based studies. *EClinicalMedicine*, *29*, 100587.
640 Culley, K. L., Dragomir, C. L., Chang, J., Wondimu, E. B., Coico, J., Plumb, D. A., Otero, M., & Goldring,
641 M. B. (2015). Mouse models of osteoarthritis: surgical model of posttraumatic osteoarthritis
642 induced by destabilization of the medial meniscus. *Methods Mol Biol*, *1226*, 143-173.
643 https://doi.org/10.1007/978-1-4939-1619-1_12
644 Fan, W., Li, X., Wang, W., Mo, J., Kaplan, H., & Cooper, N. (2010). Early involvement of
645 immune/inflammatory response genes in retinal degeneration in DBA/2J mice. *Ophthalmology and*
646 *eye diseases*, *2*, 117917211000200005.
647 Frara, N., Abdelmagid, S. M., Sondag, G. R., Moussa, F. M., Yingling, V. R., Owen, T. A., Popoff, S. N.,
648 Barbe, M. F., & Safadi, F. F. (2016). Transgenic expression of osteoactivin/gpnmb enhances bone
649 formation in vivo and osteoprogenitor differentiation ex vivo. *Journal of cellular physiology*,
650 *231*(1), 72-83.
651 Furochi, H., Tamura, S., Mameoka, M., Yamada, C., Ogawa, T., Hirasaka, K., Okumura, Y., Imagawa, T.,
652 Oguri, S., & Ishidoh, K. (2007). Osteoactivin fragments produced by ectodomain shedding induce

653 MMP-3 expression via ERK pathway in mouse NIH-3T3 fibroblasts. *FEBS letters*, *581*(30), 5743-
654 5750.
655 Gauci, S. J., Stanton, H., Little, C. B., & Fosang, A. J. (2017). Proteoglycan and collagen degradation in
656 osteoarthritis. *Cartilage: Volume 2: Pathophysiology*, 41-61.
657 Glasson, S., Blanchet, T., & Morris, E. (2007). The surgical destabilization of the medial meniscus (DMM)
658 model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis and Cartilage*, *15*(9), 1061-1069.
659 Goldring, M., Otero, M., Tsuchimochi, K., Ijiri, K., & Li, Y. (2008). Defining the roles of inflammatory
660 and anabolic cytokines in cartilage metabolism. *Annals of the Rheumatic Diseases*, *67*(Suppl 3),
661 iii75-iii82.
662 Goldring, M. B. (1999). The role of cytokines as inflammatory mediators in osteoarthritis: lessons from
663 animal models. *Connective Tissue Research*, *40*(1), 1-11.
664 Goldring, M. B. (2012). Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in
665 health and osteoarthritis. *Therapeutic advances in musculoskeletal disease*, *4*(4), 269-285.
666 Goldring, M. B., & Otero, M. (2011). Inflammation in osteoarthritis. *Current opinion in rheumatology*,
667 *23*(5), 471.
668 Goldring, M. B., Otero, M., Plumb, D. A., Dragomir, C., Favero, M., El Hachem, K., Hashimoto, K., Roach,
669 H. I., Olivotto, E., Borzi, R. M., & Marcu, K. B. (2011). Roles of Inflammatory and Anabolic
670 Cytokines in Cartilage Metabolism: Signals and Multiple Effectors Converge Upon Mmp-13
671 Regulation in Osteoarthritis. *European Cells & Materials*, *21*, 202-220. https://doi.org/DOI
672 10.22203/eCM.v021a16
673 Goldring, S. R., & Goldring, M. B. (2004). The role of cytokines in cartilage matrix degeneration in
674 osteoarthritis. *Clinical Orthopaedics and Related Research®*, *427*, S27-S36.
675 Goldring, S. R., & Goldring, M. B. (2016). Changes in the osteochondral unit during osteoarthritis:
676 structure, function and cartilage–bone crosstalk. *Nature Reviews Rheumatology*, *12*(11), 632-644.
677 Gosset, M., Berenbaum, F., Thirion, S., & Jacques, C. (2008). Primary culture and phenotyping of murine
678 chondrocytes. *Nature Protocols*, *3*(8), 1253-1260.
679 Gutknecht, M., Geiger, J., Joas, S., Dörfel, D., Salih, H. R., Müller, M. R., Grünebach, F., & Rittig, S. M.
680 (2015). The transcription factor MITF is a critical regulator of GPNMB expression in dendritic
681 cells. *Cell Communication and Signaling*, *13*, 1-15.
682 Hall, A. C. (2019). The role of chondrocyte morphology and volume in controlling phenotype—
683 implications for osteoarthritis, cartilage repair, and cartilage engineering. *Current Rheumatology*
684 *Reports*, *21*, 1-13.
685 Hwang, H., Park, I., Hong, J., Kim, J., & Kim, H. (2021). Comparison of joint degeneration and pain in
686 male and female mice in DMM model of osteoarthritis. *Osteoarthritis and Cartilage*, *29*(5), 728-
687 738.
688 Järve, A., Mühlstedt, S., Qadri, F., Nickl, B., Schulz, H., Hübner, N., Özcelik, C., & Bader, M. (2017).
689 Adverse left ventricular remodeling by glycoprotein nonmetastatic melanoma protein B in
690 myocardial infarction. *The FASEB Journal*, *31*(2), 556-568.
691 Jessop, H., Rawlinson, S., Pitsillides, A., & Lanyon, L. (2002). Mechanical strain and fluid movement both
692 activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling
693 pathways. *Bone*, *31*(1), 186-194.
694 Jiang, S. S., Chen, C.-H., Tseng, K.-Y., Tsai, F.-Y., Wang, M. J., Chang, I.-S., Lin, J.-L., & Lin, S. (2011).
695 Gene expression profiling suggests a pathological role of human bone marrow-derived
696 mesenchymal stem cells in aging-related skeletal diseases. *Aging (Albany NY)*, *3*(7), 672.
697 Jonason, J. H., Hoak, D., & O'keefe, R. J. (2015). Primary murine growth plate and articular chondrocyte
698 isolation and cell culture. In *Osteoporosis and Osteoarthritis* (pp. 11-18). Springer.
699 Kawahara, K., Hirata, H., Ohbuchi, K., Nishi, K., Maeda, A., Kuniyasu, A., Yamada, D., Maeda, T., Tsuji,
700 A., & Sawada, M. (2016). The novel monoclonal antibody 9F5 reveals expression of a fragment of
701 GPNMB/osteoactivin processed by furin-like protease (s) in a subpopulation of microglia in
702 neonatal rat brain. *Glia*, *64*(11), 1938-1961.

703 Li, B., Castano, A. P., Hudson, T. E., Nowlin, B. T., Lin, S.-L., Bonventre, J. V., Swanson, K. D., &
704 Duffield, J. S. (2010). The melanoma-associated transmembrane glycoprotein Gpnmb controls
705 trafficking of cellular debris for degradation and is essential for tissue repair. *The FASEB Journal*,
706 *24*(12), 4767-4781.
707 Lin, Z., Miao, J., Zhang, T., He, M., Wang, Z., Feng, X., & Bai, L. (2021). JUNB-FBXO21-ERK axis
708 promotes cartilage degeneration in osteoarthritis by inhibiting autophagy. *Aging Cell*, *20*(2),
709 e13306.
710 Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time
711 quantitative PCR and the 2− ΔΔCT method. *Methods*, *25*(4), 402-408.
712 Loeser, R. F., Collins, J. A., & Diekman, B. O. (2016). Ageing and the pathogenesis of osteoarthritis. *Nature*
713 *Reviews Rheumatology*, *12*(7), 412-420. https://doi.org/10.1038/nrrheum.2016.65
714 Loeser, R. F., Goldring, S. R., Scanzello, C. R., & Goldring, M. B. (2012). Osteoarthritis: A disease of the
715 joint as an organ. *Arthritis and Rheumatism*, *64*(6), 1697-1707. https://doi.org/10.1002/art.34453
716 Ma, H.-L., Blanchet, T., Peluso, D., Hopkins, B., Morris, E., & Glasson, S. (2007). Osteoarthritis severity
717 is sex dependent in a surgical mouse model. *Osteoarthritis and Cartilage*, *15*(6), 695-700.
718 Mengshol, J. A., Vincenti, M. P., & Brinckerhoff, C. E. (2001). IL-1 induces collagenase-3 (MMP-13)
719 promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation
720 by p38 MAPK and JNK pathways. *Nucleic acids research*, *29*(21), 4361-4372.
721 Moskowitz, R. W. (2000). Role of collagen hydrolysate in bone and joint disease. Seminars in arthritis and
722 rheumatism,
723 Murata, K., Yoshino, Y., Tsuruma, K., Moriguchi, S., Oyagi, A., Tanaka, H., Ishisaka, M., Shimazawa, M.,
724 Fukunaga, K., & Hara, H. (2015). The extracellular fragment of GPNMB (Glycoprotein
725 nonmelanosoma protein B, osteoactivin) improves memory and increases hippocampal GluA1
726 levels in mice. *Journal of Neurochemistry*, *132*(5), 583-594.
727 Neal, M. L., Boyle, A. M., Budge, K. M., Safadi, F. F., & Richardson, J. R. (2018). The glycoprotein
728 GPNMB attenuates astrocyte inflammatory responses through the CD44 receptor. *Journal of*
729 *neuroinflammation*, *15*(1), 1-14.
730 Ono, Y., Tsuruma, K., Takata, M., Shimazawa, M., & Hara, H. (2016). Glycoprotein nonmetastatic
731 melanoma protein B extracellular fragment shows neuroprotective effects and activates the
732 PI3K/Akt and MEK/ERK pathways via the Na+/K+-ATPase. *Scientific Reports*, *6*(1), 23241.
733 Owen, T. A., Smock, S. L., Prakash, S., Pinder, L., Brees, D., Krull, D., Castleberry, T., Clancy, Y., Marks
734 Jr, S. C., & Safadi, F. F. (2003). Identification and characterization of the genes encoding human
735 and mouse osteoactivin. *Critical Reviews™ in Eukaryotic Gene Expression*, *13*(2-4).
736 Oyewumi, M. O., Manickavasagam, D., Novak, K., Wehrung, D., Paulic, N., Moussa, F. M., Sondag, G.
737 R., & Safadi, F. F. (2016). Osteoactivin (GPNMB) ectodomain protein promotes growth and
738 invasive behavior of human lung cancer cells. *Oncotarget*, *7*(12), 13932.
739 Pritzker, K. P., Gay, S., Jimenez, S., Ostergaard, K., Pelletier, J.-P., Revell, P., Salter, D., & Van den Berg,
740 W. (2006). Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis and*
741 *Cartilage*, *14*(1), 13-29.
742 Ripoll, V. M., Irvine, K. M., Ravasi, T., Sweet, M. J., & Hume, D. A. (2007). Gpnmb is induced in
743 macrophages by IFN-γ and lipopolysaccharide and acts as a feedback regulator of proinflammatory
744 responses. *The Journal of Immunology*, *178*(10), 6557-6566.
745 Robinson, W. H., Lepus, C. M., Wang, Q., Raghu, H., Mao, R., Lindstrom, T. M., & Sokolove, J. (2016).
746 Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nature Reviews*
747 *Rheumatology*, *12*(10), 580-592. https://doi.org/10.1038/nrrheum.2016.136
748 Rose, A. A., Annis, M. G., Dong, Z., Pepin, F., Hallett, M., Park, M., & Siegel, P. M. (2010). ADAM10
749 releases a soluble form of the GPNMB/Osteoactivin extracellular domain with angiogenic
750 properties. *PLoS ONE*, *5*(8), e12093.
751 Roughley, P. J. (2001). Articular cartilage and changes in arthritis: noncollagenous proteins and
752 proteoglycans in the extracellular matrix of cartilage. *Arthritis Research & Therapy*, *3*(6), 1-6.

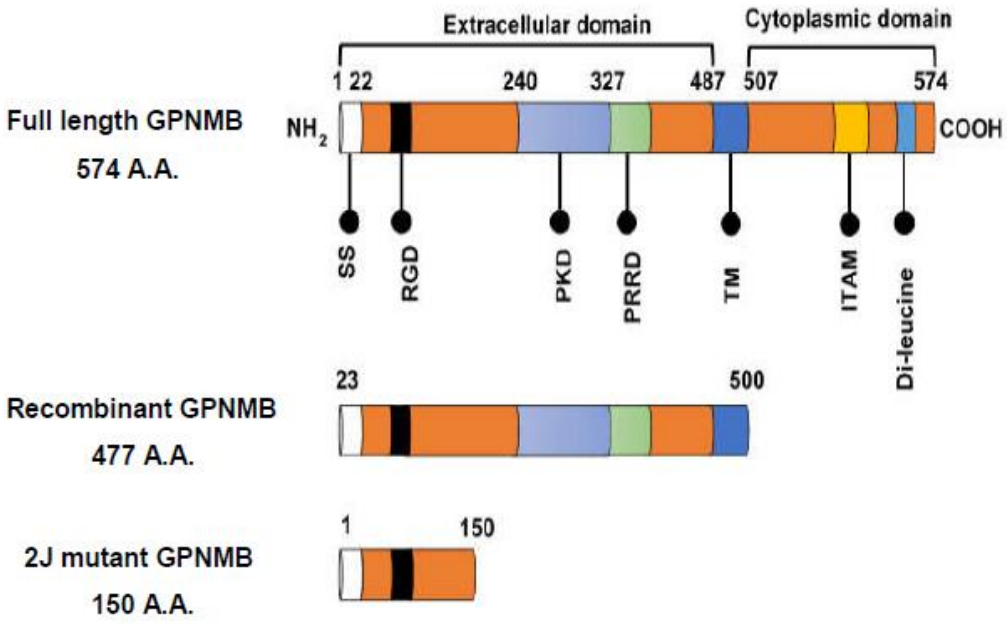
753 Roughley, P. J., & Mort, J. S. (2014). The role of aggrecan in normal and osteoarthritic cartilage. *Journal*
754 *of Experimental Orthopaedics*, *1*(1), 1-11.
755 Safadi, F. F., Xu, J., Smock, S. L., Rico, M. C., Owen, T. A., & Popoff, S. N. (2002). Cloning and
756 characterization of osteoactivin, a novel cDNA expressed in osteoblasts. *Journal of cellular*
757 *biochemistry*, *84*(1), 12-26.
758 Schlichting, N., Dehne, T., Mans, K., Endres, M., Stuhlmüller, B., Sittinger, M., Kaps, C., & Ringe, J.
759 (2014). Suitability of porcine chondrocyte micromass culture to model osteoarthritis in vitro.
760 *Molecular pharmaceutics*, *11*(7), 2092-2105.
761 Schmittgen, T. D., Lee, E. J., Jiang, J., Sarkar, A., Yang, L., Elton, T. S., & Chen, C. (2008). Real-time
762 PCR quantification of precursor and mature microRNA. *Methods*, *44*(1), 31-38.
763 https://doi.org/10.1016/j.ymeth.2007.09.006
764 Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method.
765 *Nature Protocols*, *3*(6), 1101-1108.
766 Schmitz, N., Laverty, S., Kraus, V., & Aigner, T. (2010). Basic methods in histopathology of joint tissues.
767 *Osteoarthritis and Cartilage*, *18*, S113-S116.
768 Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image
769 analysis. *Nature methods*, *9*(7), 671-675.
770 Schwarzbich, M.-A., Gutknecht, M., Salih, J., Salih, H. R., Brossart, P., Rittig, S. M., & Grünebach, F.
771 (2012). The immune inhibitory receptor osteoactivin is upregulated in monocyte-derived dendritic
772 cells by BCR–ABL tyrosine kinase inhibitors. *Cancer Immunology, Immunotherapy*, *61*(2), 193-
773 202.
774 Selim, A. A., Abdelmagid, S. M., Kanaan, R. A., Smock, S. L., Owen, T. A., Popoff, S. N., & Safadi, F. F.
775 (2003). Anti-osteoactivin antibody inhibits osteoblast differentiation and function in vitro. *Critical*
776 *Reviews™ in Eukaryotic Gene Expression*, *13*(2-4).
777 Sheng, M. H.-C., Wergedal, J. E., Mohan, S., & Lau, K.-H. W. (2008). Osteoactivin is a novel osteoclastic
778 protein and plays a key role in osteoclast differentiation and activity. *FEBS letters*, *582*(10), 1451-
779 1458.
780 Sondag, G. R., Mbimba, T. S., Moussa, F. M., Novak, K., Yu, B., Jaber, F. A., Abdelmagid, S. M.,
781 Geldenhuys, W. J., & Safadi, F. F. (2016). Osteoactivin inhibition of osteoclastogenesis is mediated
782 through CD44-ERK signaling. *Experimental and Molecular Medicine*, *48*. https://doi.org/ARTN
783 e257
784 10.1038/emm.2016.78
785 Song, R., & Lin, L. (2019). Glycoprotein nonmetastatic melanoma protein B (GPNMB) ameliorates the
786 inflammatory response in periodontal disease. *Inflammation*, *42*(4), 1170-1178.
787 Sun, J., Yan, B., Yin, W., & Zhang, X. (2015). Identification of genes associated with osteoarthritis by
788 microarray analysis. *Molecular Medicine Reports*, *12*(4), 5211-5216.
789 Tetsunaga, T., Nishida, K., Furumatsu, T., Naruse, K., Hirohata, S., Yoshida, A., Saito, T., & Ozaki, T.
790 (2011). Regulation of mechanical stress-induced MMP-13 and ADAMTS-5 expression by RUNX-
791 2 transcriptional factor in SW1353 chondrocyte-like cells. *Osteoarthritis and Cartilage*, *19*(2), 222-
792 232.
793 Wilson, G. N., Inman, D. M., Dengler-Crish, C. M., Smith, M. A., & Crish, S. D. (2015). Early pro-
794 inflammatory cytokine elevations in the DBA/2J mouse model of glaucoma. *Journal of*
795 *neuroinflammation*, *12*, 1-13.
796 Yan, Y.-x., Gong, Y.-w., Guo, Y., Lv, Q., Guo, C., Zhuang, Y., Zhang, Y., Li, R., & Zhang, X.-z. (2012).
797 Mechanical strain regulates osteoblast proliferation through integrin-mediated ERK activation.
798 *PLoS ONE*, *7*(4), e35709.
799 Yang, X., Harkins, L. K., Zubanova, O., Harrington, A., Kovalenko, D., Nadeau, R. J., Chen, P.-Y., Toher,
800 J. L., Lindner, V., & Liaw, L. (2008). Overexpression of Spry1 in chondrocytes causes attenuated
801 FGFR ubiquitination and sustained ERK activation resulting in chondrodysplasia. *Developmental*
802 *Biology*, *321*(1), 64-76.

Yu, B., Sondag, G. R., Malcuit, C., Kim, M. H., & Safadi, F. F. (2016). Macrophage-Associated Osteoactivin/GPNMB Mediates Mesenchymal Stem Cell Survival, Proliferation, and Migration Via a CD44-Dependent Mechanism. *Journal of Cellular Biochemistry*, *117*(7), 1511-1521.

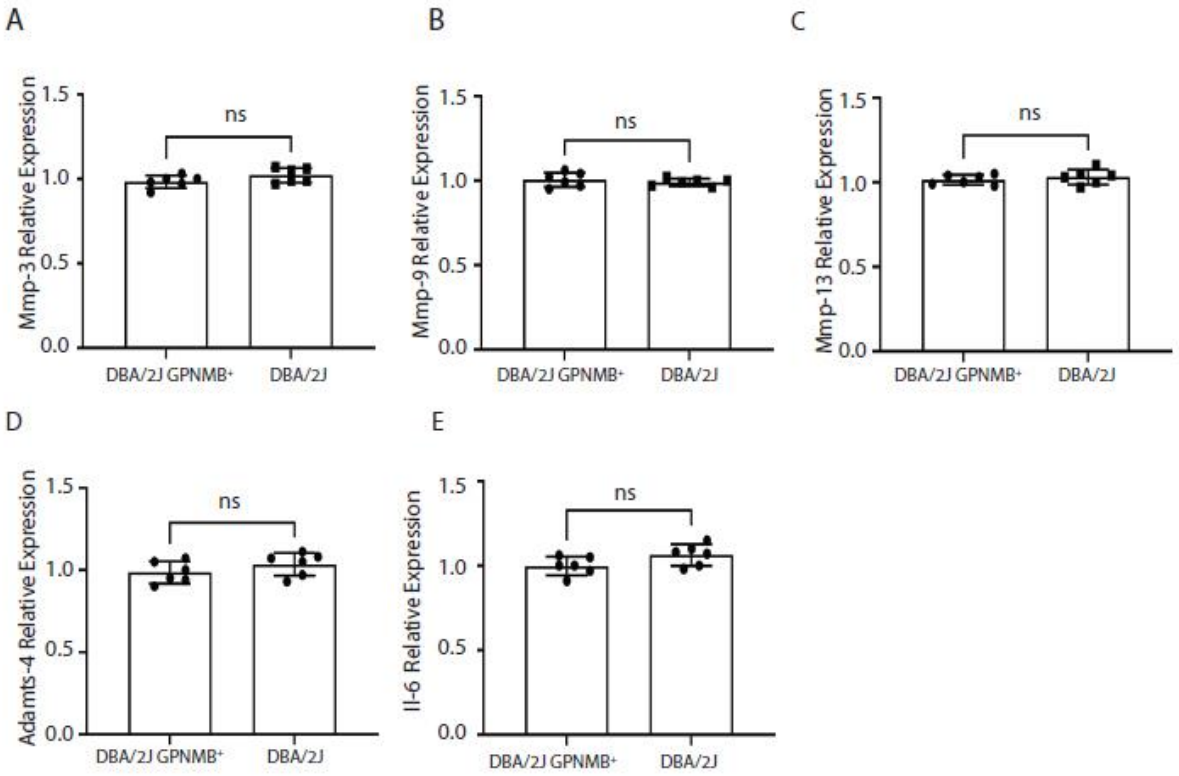
Zhou, L., Zhuo, H., Ouyang, H., Liu, Y., Yuan, F., Sun, L., Liu, F., & Liu, H. (2017). Glycoprotein non-metastatic melanoma protein b (Gpnmb) is highly expressed in macrophages of acute injured kidney and promotes M2 macrophages polarization. *Cellular immunology*, *316*, 53-60.

Zhou, X., Li, F., Kong, L., Chodosh, J., & Cao, W. (2009). Anti-inflammatory effect of pigment epithelium-derived factor in DBA/2J mice. *Molecular vision*, *15*, 438.

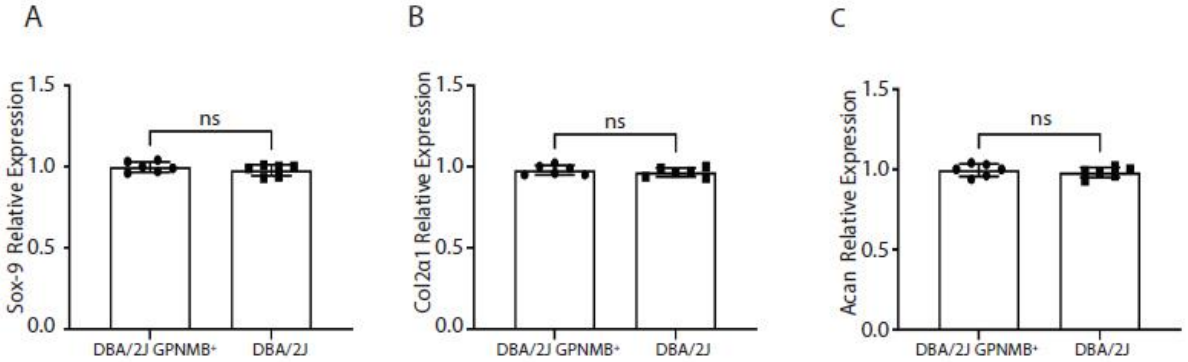
Suppmentary Figures



Supplemental 1: The structure of full length, recombinant and mutant GPNMB proteins. The full length GPNMB is 574 amino acids and has several domains including a N-terminal extracellular domain signal peptide (SS, 1-22), an arginine-glycine-aspartic acid (RGD), a polycystic kidney disease (PKD), and a proline rich repeat (PRRD) domain. The transmembrane domain (TM) of GPNMB is 20 amino acids (503-523). The C-terminal intracellular domain holds an ITAM motif and Di-leucine signal. The recombinant form of GPNMB represents the N-terminal portion of the molecule (23-502) and is derived from a mouse myeloma cell line. The DBA/2J mutant GPNMB is a truncated version of the protein (1-150).



Supplemental 2: Endogenous levels of catabolic genes are not significantly different between DBA/2J GPNMB$^+$ and DBA/2J primary chondrocytes. RT-qPCR analyses of (**A**) Mmp-3, (**B**) Mmp-9, (**C**) Mmp-13, (**D**) Adamts-4 and (**E**) Il-6 expression detected no significant differences in endogenous mRNA expression prior to treatment. Experiments were repeated three times in triplicate with similar results. Data presented as Mean ± SEM; ns = not significant.



Supplemental 3: Endogenous levels of anabolic genes are not significantly different between DBA/2J GPNMB^{+} (control) and DBA/2J primary chondrocytes. RT-qPCR analyses of endogenous anabolic gene expression found no significant differences in (**A**) Sox-9, (**B**) Type-II collagen (Col2α1) or (C) ACAN expression. Experiments were repeated three times in triplicate with similar results. Data presented as Mean ± SEM; ns = not significant.