MOLECULAR RESPONSES TO BONE LOADING IN A PERLECAN/HSPG2- DEFICIENT MOUSE MODEL

by

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ABSTRACT

Perlecan/HSPG2 (PLN), a large Heparan Sulfate Proteoglycan found residing in the osteocytic pericellular matrix (PCM) is a major component of the transverse tethering elements present in the lacunar-canalicular system that help relay mechanical signals. Previous studies have shown that PLN deficiency alters pericellular space and attenuates bone anabolic response to loading. Here, we use a transgenic mouse model with reduced *Pln* expression (termed hypo) that mimics the conditions of human Schwartz-Jampel Syndrome to study PLN's role in osteocytic mechanosensing. We hypothesize that the presence of PLN in the PCM is essential for induction of a sustained bone anabolic response to load and that its absence in the lacunar-canalicular (LCS) space of hypo animals would alter their mRNA expression profile after loading when compared to wild type (WT) controls subjected to the same loading conditions. 19-week-old WT and hypo male mice were subjected to uniaxial-tibial compressive loading for either one session in one day or seven sessions on consecutive days and euthanized 24 hours post-load. RNA was extracted from the loaded and non-loaded tibiae of both WT and Hypo animals and the steady state mRNA levels of various bone cell marker genes and matrix proteins were compared using a real-time PCR approach. Among the genes studied, only *Pln,* and a couple of matrix proteinases *(Mmp2 and Mmp9)* showed significant difference at baseline (non-loaded conditions) between hypo mutant and WT control bones. One bout of loading was sufficient to induce a significant increase in the mRNA expression level of several markers of bone anabolism: prostaglandin E2 (*Ptgs2* aka as Cox-2), the alkaline

phosphatase enzyme (ALP), the membrane-associated RANK ligand (RANKL), and osteoprotegerin (OPG). This anabolic response to load was only observed in WT but not in PLN-deficient bones. Interestingly, repetitive loading (over a week period) significantly increased the transcript levels of, type I collagen (COL1A1*)* in both WT and hypo animals. In contrast, transcripts encoding for E11/podoplastin (gp38) which is the earliest osteocyte marker observed as the osteoblast differentiates into an osteoid and PLN transcripts themselves were upregulated only in WT animals. Additionally, *Mmp2* and *Mmp3* transcripts were upregulated upon loading only in hypo animals, suggestive of an increased resorptive bone phenotype in mutant animals vs. WT controls. Together, these results suggest that PLN-deficient animals are less mechanoresponsive and that PLN plays an essential function in the anabolic response that load and formation of functional osteoid in which osteocytes are embedded. The significant increase in gene expression of organic matrix components (collagen type I and PLN) with the sustained loading regimen even in the hypo mutant animals puts emphasis on the importance of loading exercises to maintain good bone health.

Chapter 1

INTRODUCTION

1.1 Bone

Bone is a highly specialized and dynamic connective tissue, which provides physical support for internal soft tissues and playing a vital role in body movements and storage of minerals. It is a complex living tissue that constantly undergoes remodeling throughout the lifetime in response to mechanical and hormonal signals [1]. The two forms of bone observed, cortical (compact) bone and cancellous (spongy) bone, respond and adapt to mechanical stimuli based on their specific structural and mechanical properties [2].

The two types of bone are trabecular or cancellous bone and cortical or compact bone. Trabecular bone is porous and spongy, accounts for 20% of the total bone mass and mainly occur in the ends of the long bones and flats bones such as the pelvis or cranial bones. Compact bone comprises 80% of the skeletal bone mass, is less porous and denser than the trabecular bone.

Bone remodeling is a process by which the integrity of the skeleton is maintained. Bone is continuously remodeled by three types of cells: osteoblasts, osteoclasts and osteocytes. Osteoblasts are fully differentiated cells derived from mesenchymal stem cells and are responsible for the formation, deposition and the mineralization of the bone tissue [3]. The development and maturation of the osteoblasts require the presence of growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factors FGFs or insulin-like growth factor IGFs,

and transcription factors such as Runx2. The post-proliferative mature osteoblasts express high levels of alkaline phosphatase, bone sialoprotein, osteopontin and osteocalcin, which also mark the mineralization phase. The mature osteoblasts are responsible for the secretion of the osteoid or bone matrix, composed of the collagen type 1, glycoproteins (osteocalcin and proteoglycans) and other non-collagenous matrix proteins. These osteoids undergo mineralization to form new bone [4, 5].

Osteoclasts are giant multinucleated cells derived from the monocyte-macrophage lineage and are responsible for bone resorption. Osteoclastogenesis is a process resulting in the activation of immature osteoclasts. Osteoclast precursors express Receptor Activator for Nuclear factor κB (RANK) in response to the Macrophage Colony Stimulating Factor (M-CSF) [6]. The membrane protein RANK ligand (RANKL) produced by the osteoblasts activates the RANK receptor and osteoclastogenesis [7].

The preosteoblasts bone lining cells are flat elongated cells that upon mechanical stimulation can induce bone formation by differentiating into osteoblasts. These cells also possess some resorptive functions and aid in digestion of the mineralized matrix by the osteoclasts [8].

Matrix metalloproteinases (MMPs), secreted by all the bone cells especially by osteoclasts, are zinc dependent proteolytic enzymes, which consists of many subtypes including collagenases (MMP1, 8, 13), gelatinases (MMP2, MMP9), and stromelysins (MMP 3,10,11). MMPs play a role in bone remodeling [9]. Bone resorptive signals or bone formation signals regulate the secretion and degradation of MMPs which either activate or sequester these enzymes to contribute to bone matrix degradation during remodeling [10].

Osteocytes, the most abundant cells in the bone tissue, are formed as osteoblasts become embedded in the the bone matrix secreted by them. These cells occupy the cavities called lacunae and project slender cell processes through the canaliculi in the matrix to connect to adjacent cells [1]. These osteocytes are involved in bone remodeling, ion- exchange between cells and serve as primary mechanosensory cells. [11]

1.2 Extracellular matrix in bone:

About 65% of the adult bone mass is comprised of inorganic components of hydroxyapatite crystals made primarily of calcium and phosphorous. These crystals are tightly packed around the collagen fibers and account for the hardness of the bone. Organic components and water make the remaining 25% of the bone mass. The organic components contribute to structure, flexibility and increased tensile strength of the bone. The adequate combination of the organic and inorganic phases provides bone strength and makes it less brittle. Collagen type 1 is the major organic part (about 90%), and therefore the major building block of the bone matrix. The remaining organic matter is non-collagenous matrix proteins and this includes the proteoglycans (perlecan and aggrecan), glycosylated proteins (alkaline phosphatase, osteopontin, sialoproteins, fibronectin) and carboxylated proteins (osteocalcin, protein S). Although the exact role of these proteins are not fully understood, they have been suggested to play a role in the regulation of mineral deposition [12]. This study in particular is focused on studying the role of perlecan in the bone and its function in mechanosensation and gene response to load.

1.3 Bone and Mechanotransduction

Mechanical stimulation is essential for the proper growth and development of the skeleton [13]. The process by which mechanical signals transmitted from an external stimulus are converted into a cellular response through gene expression is defined as mechanotransduction [14]. Exercise is a potent anabolic stimuli, increased Bone Mineral Density (BMD) has been reported in athletically trained individuals when compared to their untrained counterparts. For example, tennis player have increased BMD in their serving arms [15]. While exercise promotes bone growth, there is bone loss in the case of disuse, which is known to be associated with decreased BMD [16]. Disuse induced bone loss is studied in microgravity environments and prolonged immobilization, where a decrease in periosteal bone growth and increase in endosteal resorption is observed [17]. Hence, additional mechanical loading can be employed to externally regulate the bone remodeling cycle to prevent bone loss in suitable environments.

1.4 Osteocyte and PCM

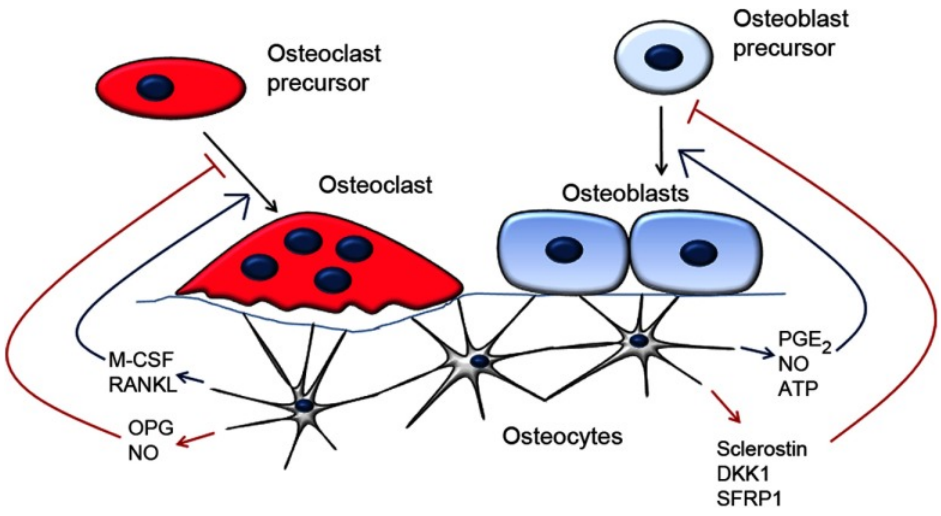

Figure 1.1: Bone cell types interaction. Osteocytes communicate with other bone cells by forming an extensive network with osteoblasts and osteoclasts that aid in cell-cell communication. Image used with permission from [6]

Osteocytes have been identified as the major mechanosensory cells in bone. They make 90-95% of the total bone cells. The location of the osteocytes in the matrix and their dendritic network makes it ideal for mechanosensation [18-20]. These osteocytes perceive the mechanical signal and transduce it to the end effector cells.

Fluid shear stress (FSS) that was typically estimated at the cell surface was found to be the most effective mechanical stimuli in inducing a cellular- level response in bone [18]. More recent *in vitro* studies on MLO-Y4 cells demonstrated that cyclic hydraulic pressure stimulation induced production of cyclooxygenase-2 (COX-2), RANK ligand (RANKL), and osteoprotegerin (OPG), a protein secreted by osteoblasts and known for its protective effect on bone tissue [21].

Osteocytes receive information through strain derived fluid flow within the canaliculi network and initiate responses, based on the magnitude of the load, that are transmitted to other bone cells like osteoblasts and osteoclasts (Figure 1.1). This fluid flow activates osteocytes, which produces ATP, nitric oxide (NO), Wnt proteins, bone morphogenetic proteins, prostaglandins, sclerostin (sost), dentin matrix protein 1 (DMP1), E11/gp38, matrix extracellular phosphoglycoprotein (MEPE) and RANKL that regulate the activity of osteoblasts and osteoclasts [20, 22-26].

The pericellular matrix (PCM) lies between the plasma membrane of the osteocytes and the mineralized extracellular matrix and houses the ectodomains of the integral membrane glycoproteins, proteoglycans and glycolipids. The PCM has

multiple roles, from mechanosensing function to cell-cell, cell-ligand and cell-ECM interactions as well as transportation of waste and nutrients to various parts of the bone tissue facilitating metabolism. This matrix forms transverse fibers, which support the cell processes in the canaliculi and and also captures drag forces from the surrounding fluid flow. The fluid drag force may act to excite osteocytes [27, 28]. Although the nature of the osteocyte PCM is not well characterized, it was identified that perlecan/Hspg2 contributes to maintenance of the lacuna-canalicular space and is contained in the PCM fibers [28, 29].

1.5 Perlecan/HSPG2

Perlecan is a large multidomain heparan sulfate proteoglycan (Figure 1.2) found in the basement membrane of all organs as well as connective tissue. PLN is extracellularly secreted and consists of a protein core of >400 kDa and has five distinct domains[30]. Domain I has three and domain V has one glycosaminoglycan (GAG) binding sites. Domain II is similar to the cholesterol-binding region of the LDL receptor. Domain III has a structure resembling the laminin A motif. Domain IV is the largest of PLN's five domains containing 14 repeats of IgG like motifs (refer Figure 1.2) [31, 32].

Perlecan supports various biological functions, including cell adhesion, growth factor binding, and modulation of apoptosis [33]. These diverse functions of PLN is due to the ability if the glycosaminoglycan (GAG) side chains and the five domains to interact with a variety of growth factors [30]. PLN is present in high quantities in developing and adult cartilage as well as bone marrow stromal cells [34]. In cartilage and bone, PLN modulates bioavailability of growth factors [34], contains binding sites for many ECM components (such as laminin, integrins, and collagen IV) [35], inhibits

mineralization of bone and regulates chondrocyte differentiation in developing bones [33], maintains the integrity of the osteocytic PCM in bone LCS [28].

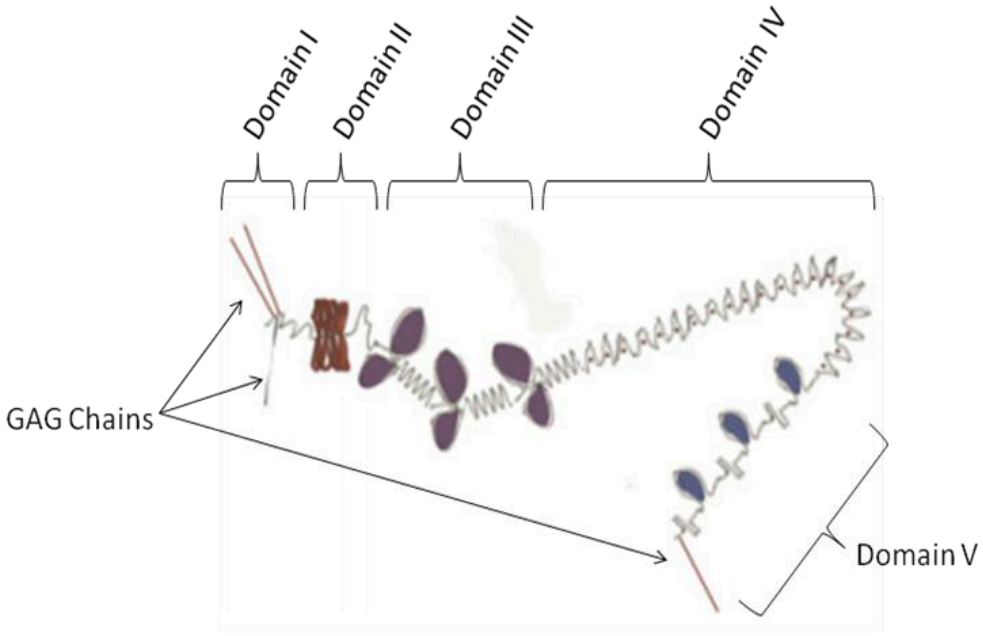

Figure 1.2: Perlecan. Structure of PLN protein indicating the five domains and the GAG attachment sites in domain I and V. Image used with permission from [28].

1.6 Perlecan-deficient mouse model

A genetic mouse model that mimics human Schwartz-Jampel Syndrome (SJS), a rare autosomal recessive disorder, was developed and characterized [34, 36]. The PLN null mutation was embryonically lethal hence a G to A point mutation, which changes the cysteine residue at 1532 to tyrosine, along with a neomycin resistance cassette inserted downstream at intron 36 to develop a hypomorphic (hence the name

'hypo' is used to refer the PLN deficient mutant animals) mutation. This hypomorphic mutation alters the transcription of the *Hspg2* gene and results in a knock-down expression of PLN. The altered transcription resulted in truncated mRNA variants and decreased levels of full-length mRNA transcripts that translated to a fully functional PLN protein. Adult mice harboring this PLN hypo mutation exhibited a phenotype characterized by dwarfism, muscle weakness, shortened and thickened bones, altered bone microarchitecture and delayed bone formation as illustrated in Figure 1.3 [28, 34, 37, 38]. The mutant mice also exhibit a flattened face and an eye phenotype, which progressively degenerates upon aging. PLN immunostaining at embryonic stage E18.5 showed that most of the PLN present in the mutant is retained intracellularly in the endoplasmic reticulum instead of being secreted in the PCM as seen in the wild type (WT). Perlecan deficiency also cause early and abnormal mineralization in the mutants compared to the WT controls [37]. Additionally, It was also found that mice with reduced expression of PLN had lower pericellular area and lesser tethering elements per canaliculus as compared to WT mice [28].

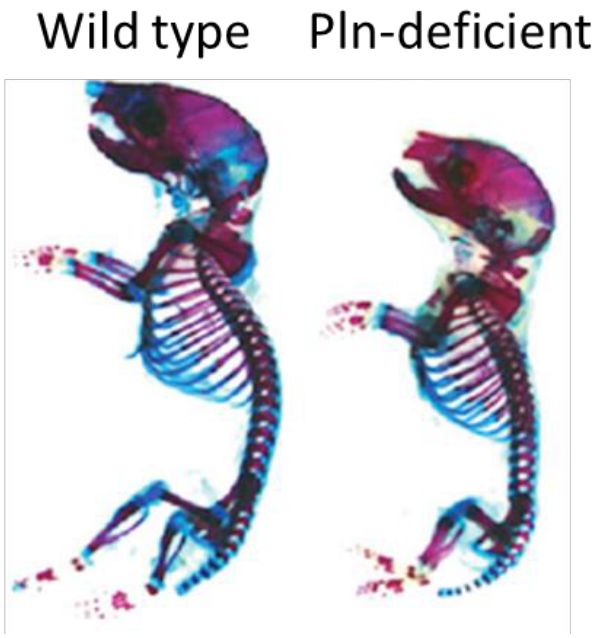

Figure 1.3 Bone phenotypic comparison of representative wild type and PLN hypo mutant animals. Whole mount stainings of a wild type (left panel) and PLN mutant (right panel) new born animals show skeletal abnormalities and developmental delay in mice harboring the PLN C1532Yneo mutation relative to wild type control. Image used with permission from [34].

A working model based on the hypothesis that PLN serves as a load-sensing antenna in the osteocytic PCM was developed. This model (Figure 1.4) depicts the differences between the PLN molecule distribution in the pericellular LCS near the osteocytic cell process of both WT control and hypo animals [27].

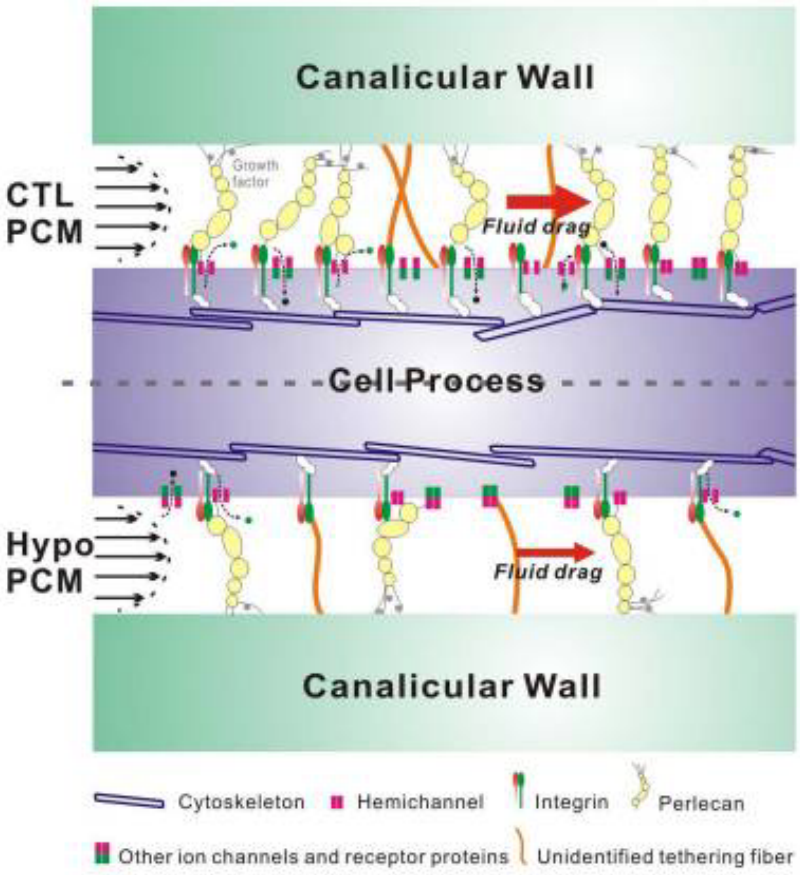

Figure 1.4: **Working model.** A model depicting the PLN fibers acting as load sensing antennae comparing the pericellular matrix (PCM) fibers and space in WT (CTL on top) and PLN mutant (hypo at the bottom) animals. Image used with permission from [27].

A recent study using the above mentioned mouse model, showed that the hypo animals were less responsive to *in vivo* compressive loading with a peak load of 8.5N at 4 Hz, with five sessions over 10 day. At the peak load of 8.5N, the strain was quantified to be 1200με, which falls in the bone-remodeling window and is anabolic to the bone, at the anterior-medial surface. A significant increase in cortical bone polar moment inertia and a reduction in the mineral apposition rate in adult (3.5 month-old)

WT bones upon loading whereas such anabolic responses were not observed in PLN-deficient mutant bones. This attenuated responsiveness to mechanical stimulation was attributed to the sparse PCM fiber density observed in the perlecan-deficient hypo animals [27].

The aim of this study was to investigate the effect of PLN deficiency on bone's mechanosensitivity and gene response to experimental load. The hypothesis of the study is that *"decreased bone sensitivity to load in PLN-deficient mice will result in reduced expression of anabolic markers of bone formation in PLN mutants vs. age-matched control animals when subjected to dynamic loading".* The above-mentioned PLN-deficient animals were used to study the changes in the gene expression profile following mechanical loading compared to WT controls.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

Perlecan-deficient hypo mice (C1532Yneo mutant) were obtained from Dr. Katherine Rodgers and were backcrossed onto the C57BL6/J background strain. The resulting homozygous C1532Yneo mutant animals were bred in the animal facility at the University of Delaware. Animal care and handling were performed in accordance with the University of Delaware Institutional Animal Care and Use Committee guidelines.

2.2 In-vivo tibial loading

19-week-old hypo male mice (n=4-6) and age-matched C57BL/6J males (WT, n=5-10) were subjected to a single session of *in vivo* loading of the left tibia (8.5N peak load, 1200 cycles, 4 Hz with 0.1s resting period) using an Electroforce LM1 TestBench loading system (Bose Corporation, Eden Prairie, MN) as described in [27] (Refer Figure 2.1, panel A). The contralateral right limbs served as non-loaded controls. Animals were euthanized 24 hours post-loading with anesthesia overdose and cervical dislocation.

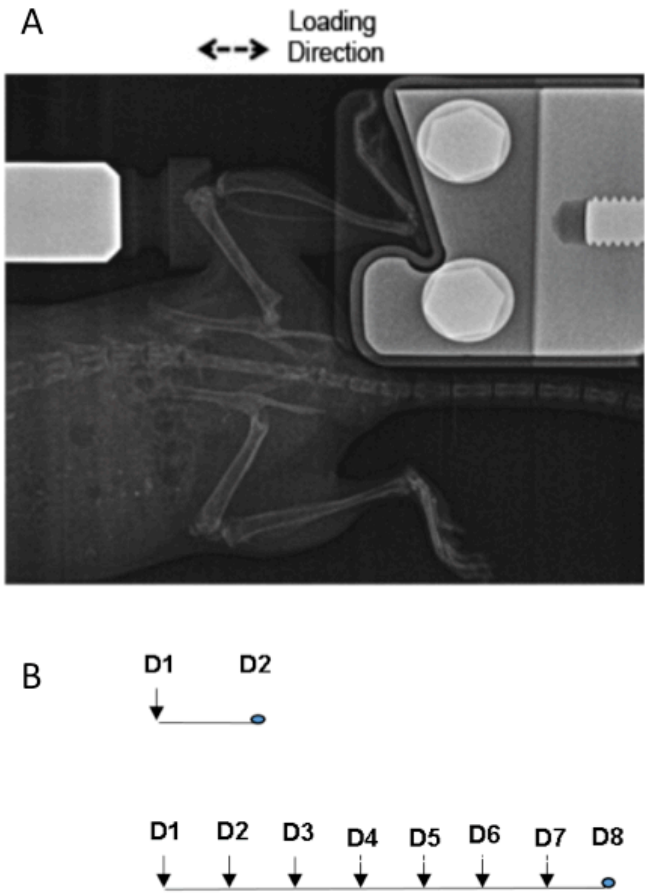

Figure 2.1: ***In vivo*** **loading mouse model and regimen used in the study**. (A) Radiograph showing the position of the mouse knee joint held for loading using the Bose ElectroForce loading apparatus. Image used with permission from [39] (B) Loading regimens used in the study: *1-day loading* -The left tibia of the animals was loaded for 1 day (arrow) and sacrificed (dot) after 24 hours (B, top; WT n=10 and hypo n= 6). *7-day loading* -The left tibia of the animals was loaded every day for 7 days (arrows) and sacrificed (dot) after 24 hours (B, bottom; WT n= 5 and hypo n= 4). The right tibia was used as the non-loaded control.

2.3 Bone loading regimen

In-vivo loading was performed for two time courses. In the one-day loadings, the animals were subjected to one session of tibial loading and sacrificed 24 hours post load. In the seven-day loadings, the animals were subjected to one session of tibial loading per day for 7 consecutive days and sacrificed 24 hours following the last session of loading (Refer Figure 2.1, panel B).

2.4 RNA isolation

Left and right tibiae were rapidly dissected out and cleansed of surrounding soft tissues. The entire tibiae were flash-frozen in liquid nitrogen. The frozen bone samples were pulverized using a mortar and pestle in liquid nitrogen. The total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific, MA, USA) and the RNeasy® Mini kit (Qiagen, CA, USA). The quality of the resulting RNA was checked using NanoDrop™ and obtaining the 260/280-absorbance ratios. The extracted RNA was treated to remove any DNA contamination using the TURBO DNA-Free™ kit (Ambion, Thermo Fisher Scientific, MA, USA). The RNA samples were stored in -80°C prior to performing cDNA synthesis and quantitative PCR analysis. An aliquot of RNA sample was reverse transcribed to 500 ng cDNA (20 µL) using iScript cDNA™ synthesis kit (Bio-Rad Laboratories, CA, USA).

2.5 Quantitative PCR analysis

16 genes were selected, including genes associated with early response to mechanical load, mature bone cell and bone cell differentiation markers (Alkaline Phosphatase (*Alp*), Prostaglandin-endoperoxide 2 (*Pge2*) synthase also known as cyclooxygenase -2 (*Ptgs2* or *Cox*-2), *E11* (also known as gp38 or podoplanin), Runt-related transcription factor 2 (*Ru*nx2), Receptor Activator of Nuclear Factor-kappaB

Ligand (*RANKL)* and Osteoprotegerin (*Opg*)), genes associated with bone matrix protein synthesis (Collagen type I alpha 1 chain (*Col1a1*), Osteocalcin (*Ocn or Bglap*), Osteopontin (*Opn*), Dentin Matrix Protein 1 (*Dmp1*), and Perlecan/Hspg2 (*Pln*)) as well as genes of matrix metalloproteinases associated with metabolic activities (MMP2, MMP3, MMP9, MMP12, MMP13). Quantitative PCR experiments were performed using Power SYBR® Green PCR mastermix (Applied Biosystems, Thermo Fisher Scientific, MA, USA) on an Applied Biosystems Quantstudio 3 machine. Primers were custom-ordered as oligonucleotides (Invitrogen/Thermo Fisher Scientific) (sequences in Table 2.1) or as pre-made mastermixes (SA Biosciences, Qiagen, CA, USA). The expression level of each gene relative to the housekeeping gene (GAPDH) was first calculated as the differential cycle threshold (Ct) values (ΔCt) for each sample. To test whether hypo/WT bones responded to loading, paired *Student's* t-tests were performed on the ΔCt values of the loaded and non-loaded (paired) samples within either hypo or WT groups. To test whether the responses to loading differed across the two genotypes (hypo and WT), fold changes (loaded/non-loaded) were calculated using the 2^-ΔΔCt method and compared for statistical differences using an unpaired *Student's* t-test. To test whether hypo and WT gene expression differed at non-loaded baseline and after loading, ΔCt values were compared (unpaired Student t-test, hypo vs. WT). Data were expressed as mean ± standard error of the mean (SE). Statistical analysis was performed in MS Excel with a significant value of $p \leq 0.05$.

Genes	Forward 5'-3'	Reverse 5'-3'
Alp	TGAATCGGAACAACCTGAC	CCACCAGCAAGAAGAAGC
Co1a1	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Cox2	Catalog number: PPM03647E (SA Biosciences)	
Dmp1	CCCAGTTGCCAGATACCACAATAC	GCTGTCCGTGTGGTCACTATTT
E11	CAGTGTTGTTCTGGGTTTTGG	TGGGGTCACAATATCATCTTCA
GAPDH	GTGCCAGCCTCGTCCCGTAGA	TGCCGTTGAATTTGCCGTGAGT
MMP2	Catalog number: PPM03642C (SA Biosciences)	
MMP3	Catalog number: PPM03673A (SA Biosciences)	
MMP9	Catalog number:PPM03661C (SA Biosciences)	
MMP12	Catalog number: PPM03619F (SA Biosciences)	
MMP 13	GCCCTATCCCTTGATGCCATT	AGAGCTCAGCCTCAACCTGCTG
Ocn(Bglap)	CTGACCTCACAGATGCCAAG	GTAGCGCCGGAGTCTGTTC
Opn	CAGCTGGATGAACCAAGTCTGGAA	ACTAGCTTGTCCTTGTGGCTGTGA
Opg	GAATGCCGAGAGTGTAGAGAGGATAA	CGCTGCTTTCACAGAGGTCAAT
Pln(Hspg2)	CCCACTCTTGGACCCTGATA	ATAGCTCCTCCTCTCCTGGGC
Rankl	ATCGGGAAGCGTACCTACAG	GTGCTCCCTCCTTTCATCAG

Table 2.1**:** Sequences and sources of the primers for all the genes used in real-time PCR studies.

Chapter 3

RESULTS

3.1 Baseline mRNA expression levels in Perlecan-Deficient mutant compared to WT animals

To investigate if there were any major changes at non-loaded conditions between the two genotypes, mRNA transcripts levels at baseline (absence of any external stimuli) were studied (Figure. 3.1). Quantification of major bone matrix proteins mRNA expression levels all showed relatively minor fold changes in mutant vs. control bones. Specifically, *Col1a1* and *Opn* transcripts showed 2-fold and 1.5-fold decrease in hypomorphs relative to WT, respectively. *Dmp1* and *Bglap* showed no change between the genotypes. *Hspg2* (*Pln*), which is transcribed into a proteoglycan secreted extracellularly in bone tissue, was down-regulated at the mRNA level ($p=0.05$) with a significant 2-fold decrease in hypo animals compared to the WT controls. In contrast, a glypoprotein known to be at the cell surface of osteocytes, *E11*, was up-regulated with a 1.6-fold increase in transcripts in hypo mutants relative to WT controls. Transcript levels of *Alp*, *Ptgs2 (Cox-2*), *Runx2*, *RANKL* and *Opg* were less than 1-fold at baseline levels in hypo vs. WT, respectively. Generally, the matrix metalloproteinases (MMPs) analyzed in this study such as MMP3, MMP9, MMP12, MMP13 transcripts were expressed to a lesser extent in hypo relative to WT as indicated by 2-, 3-, 2.5-, 1.5-fold decrease, respectively. MMP9 is the only MMP analyzed for which this decrease was found to be significantly lower in hypo ($p= 0.04$) than in WT controls. Conversely, MMP2 was slightly, but significantly ($p=0.03$), up-

regulated with an increase of around 1.25-fold in mutant vs. WT.

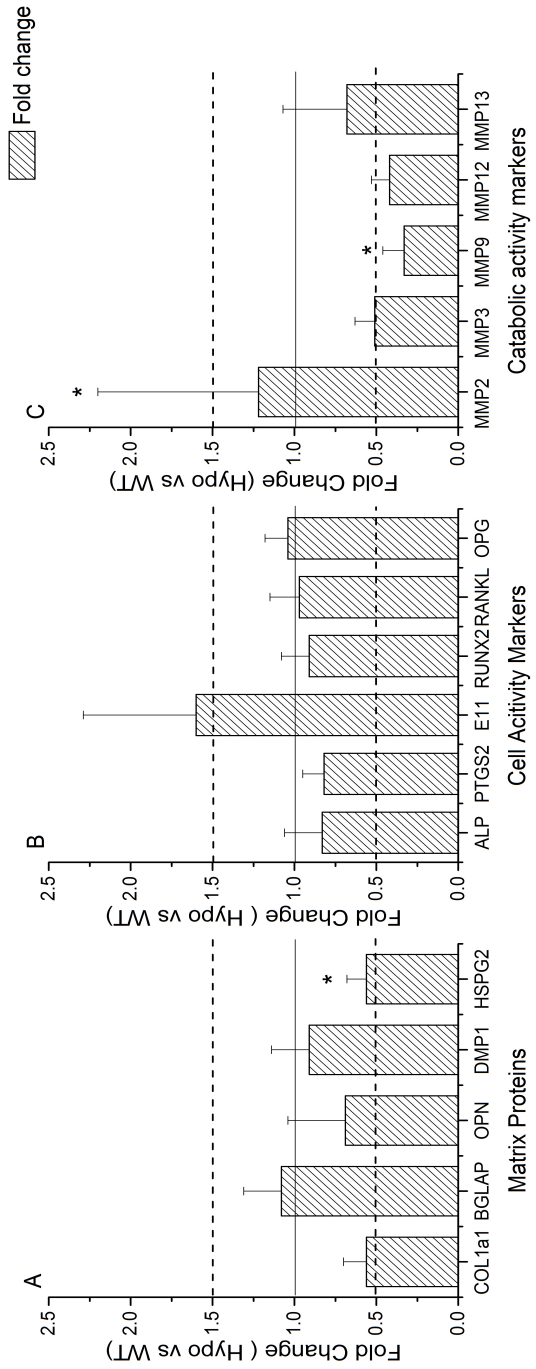
Fold change
A
B
C
Fold Change (Hypo vs WT)
Fold Change (Hypo vs WT)
2.5
2.0
1.5
1.0
0.5
0.0
*
COL1a1
BGLAP
OPN
DMP1
HSPG2
Matrix Proteins
ALP
PTGS2
E11
RUNX2
RANKL
OPG
Cell Acitivity Markers
MMP2
MMP3
MMP9
MMP12
MMP13
Catabolic activity markers

Figure 3.1: Changes observed in the baseline mRNA expression levels in adult hypo animals compared to age-matched WT controls. Transcript levels of all the genes were measured in duplicates or triplicates using real-time PCR in 19 week-old WT (n=10) and hypo (n=6). Fold changes (hypo vs. WT) were calculated using the ΔΔCt method using GAPDH as the housekeeping gene. Error bars represent standard error of the mean (SE). * indicates a $p \leq 0.05$ a significant difference in fold change between the WT and hypo bones.

3.2 Effect of mechanical loading on bone matrix genes within and between the WT and PLN mutant genotypes

To investigate the early and late responses of the genes expressing bone matrix proteins, the left tibia of the WT and hypo animals were loaded under the 1-day and 7-day regimens (Fig 3.2). The right tibia was used as the non-loaded control. We measured and compared the changes in the mRNA levels of established matrix genes known to be of expressed in bone *col1A1*, *Bglap*, *Opn*, *Dmp1* and *Hspg2(PLN)* between the WT and hypo animals. Both at day 1 and day 7, the loaded WT animals had higher mRNA levels for *Pln* with fold increase of around 2.7- fold and 3.2- fold (loaded vs. non loaded), respectively compared to the hypo animals which only showed an increase of 0.73- and 1.6- fold upon loading for 1 day and 7 day post-load, respectively. Among all the genes studied, the effect of mechanical loading across genotypes was only found to be significantly different for *Hspg2 (Pln)* transcripts. More specifically, a single session of loading showed a significant increase in relative levels of *Hspg2 (Pln)* mRNA transcripts in WT vs. hypo, and a similar trend was observed after 7 days of loading without reaching statistical significance.

In addition, there were no significant changes in the levels of other genes upon one day of loading within each of the two genotypes. In contrast, there was a significant increase following 7 days of loading in the levels of collagen type 1 mRNAs both in WT and hypo animals, and in the levels of *Hspg2 (Pln)* in WT animals.

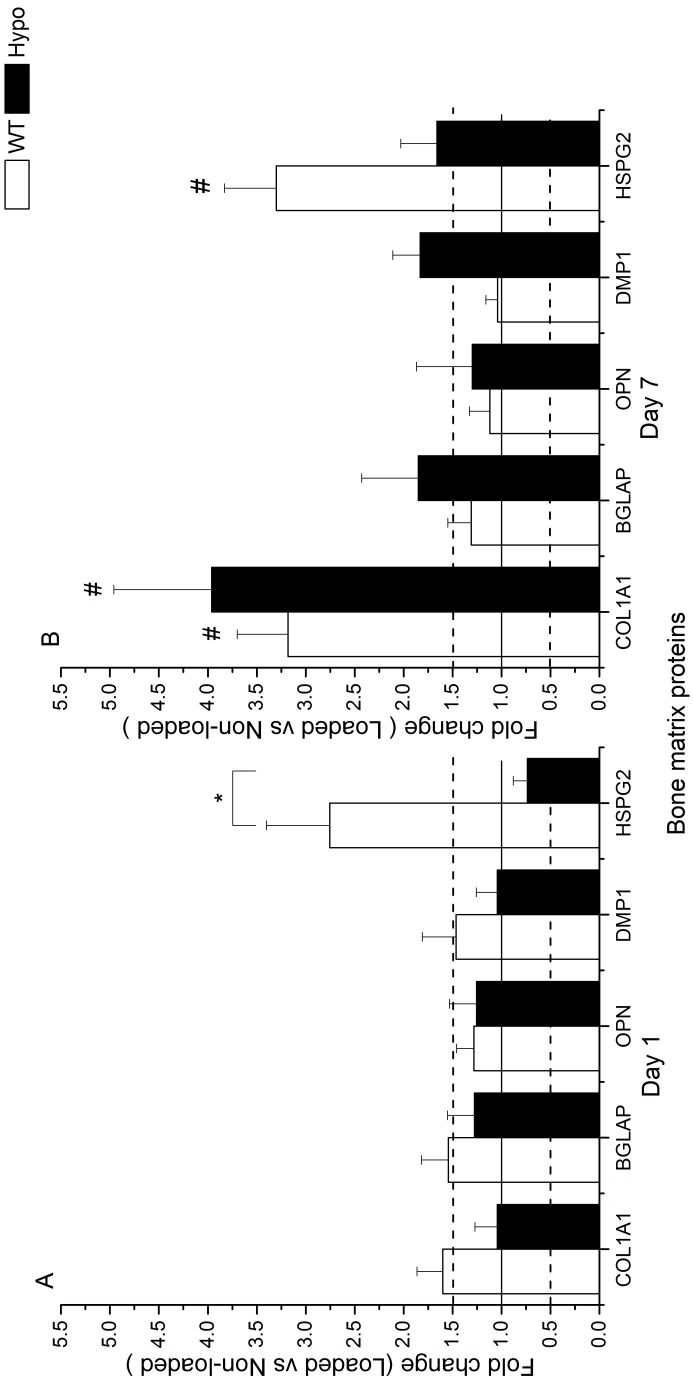
WT
Hypo
A
B
Fold change (Loaded vs Non-loaded)
Fold change (Loaded vs Non-loaded)
COL1A1
BGLAP
OPN
DMP1
HSPG2
Day 1
Day 7
Bone matrix proteins
*
#
#
#

Figure 3.2: Effect of 1-day (Panel A) and 7-day (Panel B) mechanical loading on mRNA levels of bone matrix proteins. Transcript levels of all the genes were measured in duplicates or triplicates using real-time PCR from 19 week-old WT (day 1 n=10, day 7 n=5) and hypo (day 1 n=6, day 7 n=4) male tibiae 24 hours after final session of loading. Fold changes (loaded vs. non-loaded) were calculated using the ΔΔCt method using GAPDH as the housekeeping gene. Error bars represent standard error of the mean (SE). # indicates $p \leq 0.05$ within the genotype (loaded vs non-loaded, hypo or WT); * indicates a $p \leq 0.05$ a significant difference in fold change between the WT and hypo genotypes.

3.3 Effect of mechanical loading on bone activity/formation markers within and between the WT and PLN mutant genotypes

To investigate the early and late response to mechanical loading on cell activity/formation markers, the left tibia of the WT and hypo animals were loaded under the 1-day and the 7-day regimens, respectively (Fig 3.3). The right tibia was used as the non-loaded contralateral control. The changes in the mRNA levels of the well-established mechanosensitive and bone formation marker genes *Alp*, *Ptgs2*, E11, *Runx2*, *Rankl* and *Opg* were measured and compared within each genotypes (loaded vs. non loaded) and between WT and hypo loaded animals. There was a significant increase in *Ptgs2* mRNA levels in both WT and hypo with around 5- and 7-fold increases, respectively following one day of mechanical loading. One-day loading also had the tendency to increase *E11* transcript levels up to around 5-fold in WT and 2-fold in hypo animals. *Alp, Opg* and *Rankl* transcripts were only increased in WT bones by 2.7-, 3.0-, and 1.6- while the fold changes observed in hypo animals were not significantly changed with 1.7-fold increase in *Opg* and no changes observed in *Alp* and *Rankl*.

For the day 7 experimental group, although the *Ptgs2* mRNA levels displayed a visible increase in response to loading in WT with an approximate 7.0-fold in loaded vs. non-loaded bones, the hypo animal bones showed no such dramatic response and had only a 2-fold response upon loading. This differential in response for *Ptgs2* did not reach statistical significance between WT and hypo animals. Except for an increase in E11 mRNAs in loaded vs. non-loaded WT bones, none of the other studied markers showed a significant difference in expression levels following seven days of

consecutive loading. Hypo animals showed no significant response in any of the cell activity marker genes upon seven days of loading.

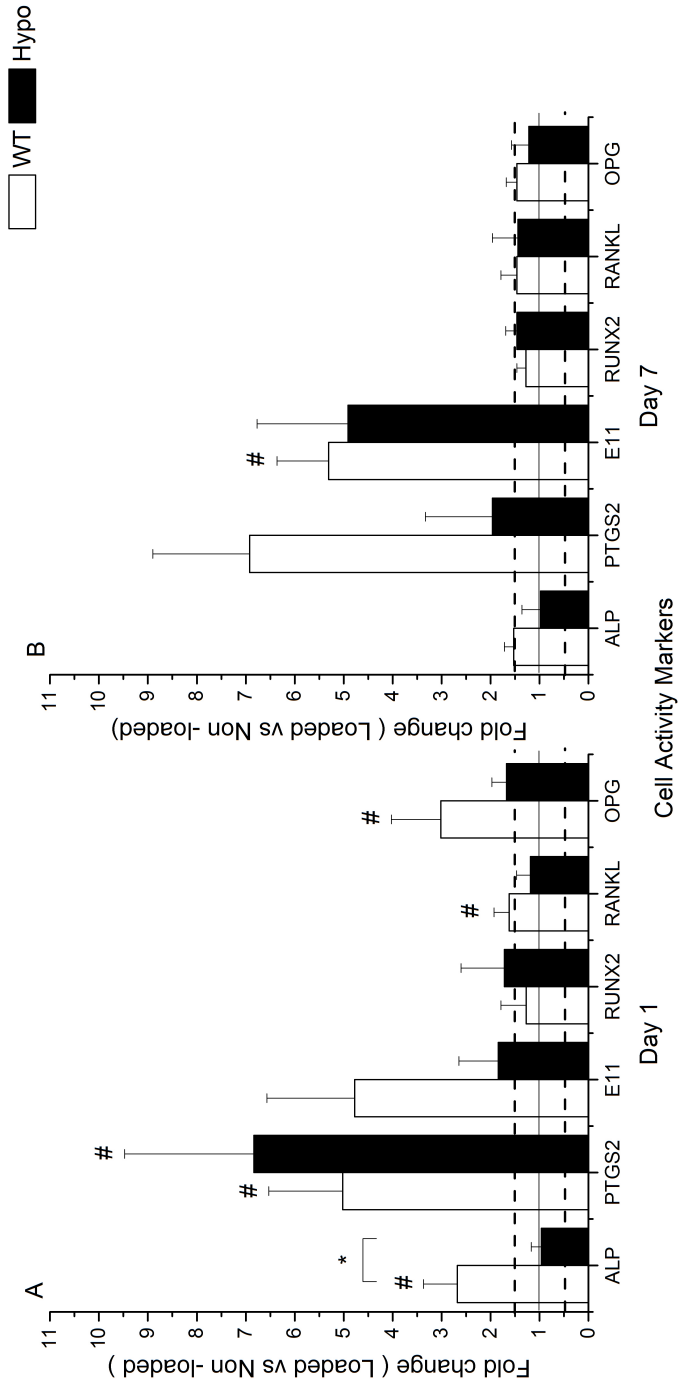
WT
Hypo
A
B
Fold change (Loaded vs Non -loaded)
Fold change (Loaded vs Non -loaded)
ALP
PTGS2
E11
RUNX2
RANKL
OPG
Day 1
Day 7
Cell Activity Markers
*
#

Figure 3.3: Effect of 1-day (Panel A) and 7-day (Panel B) mechanical loading on mRNA levels of bone cell activity markers. Transcript levels of all the genes were measured in duplicates or triplicates using real-time PCR from 19 week-old WT (day 1 n=10, day 7 n=5) and hypo (day 1 n=6, day 7 n=4) male tibiae 24 hours after final session of loading. Fold changes (loaded vs. non-loaded) were calculated using the ΔΔCt method using GAPDH as the housekeeping gene. Error bars represent standard error of the mean (SE). # indicates $p \leq 0.05$ within the genotype (loaded vs non-loaded, hypo or WT); * indicates a $p \leq 0.05$ a significant difference in fold change between the WT and hypo genotypes.

3.4 Effect of mechanical loading on bone catabolic marker genes within and between the WT and Pln mutant genotypes

To investigate the early and late catabolic response to mechanical loading, the changes in the mRNA levels of the catabolic enzymes- matrix metalloproteinases 2, 3, 9, 12 and 13 were measured and compared within each genotype (loaded vs. non loaded) and between WT and hypo animals following mechanical loading (Fig 3.4). A significant 6.5-fold increase in MMP3 mRNA transcripts observed in WT bones when subjected to short term loading (1-day) was reduced by more than half (around 3-fold increase) after long-term loading (7 days). Interestingly, whereas no transcriptional increase was found for MMP3 in mutant animals during short term loading, a significant 8-fold increase was measured in MMP3 gene expression after 7 days of loading. The effect of loading on the expression of MMP2 transcripts after one day is significantly different between the genotypes. On the other hand, 7 days of loading showed an increase in transcript levels of MMP2 in both the genotypes but no statistical significance was observed. In hypo animals, however, the effect of long-term loading (7 day) was significant when compared to the contralateral control limbs. MMP12 transcripts were up-regulated by nearly 5-fold in WT and 5.5-fold in hypo after one day of loading but remained near steady state levels in both WT and hypo animal bones after 7 days of loading. In WT animals, the transcript levels of MMP9 and MMP13 were increased by 1.7- and 2.0-fold upon one day loading while seven consecutive days of loading did not have a severe effect on the expression levels of these mRNAs (1.25-fold decrease for MMP9 and 1.2-fold increase for MMP13). Similar responses to short term and long term loading were observed for the MMP9 transcripts in hypo animals. Long term loading induced an increase in MMP13

transcript levels by 1.7-fold in loaded vs. non-loaded mutant bones whereas no noticeable change were observed after one day of loading.

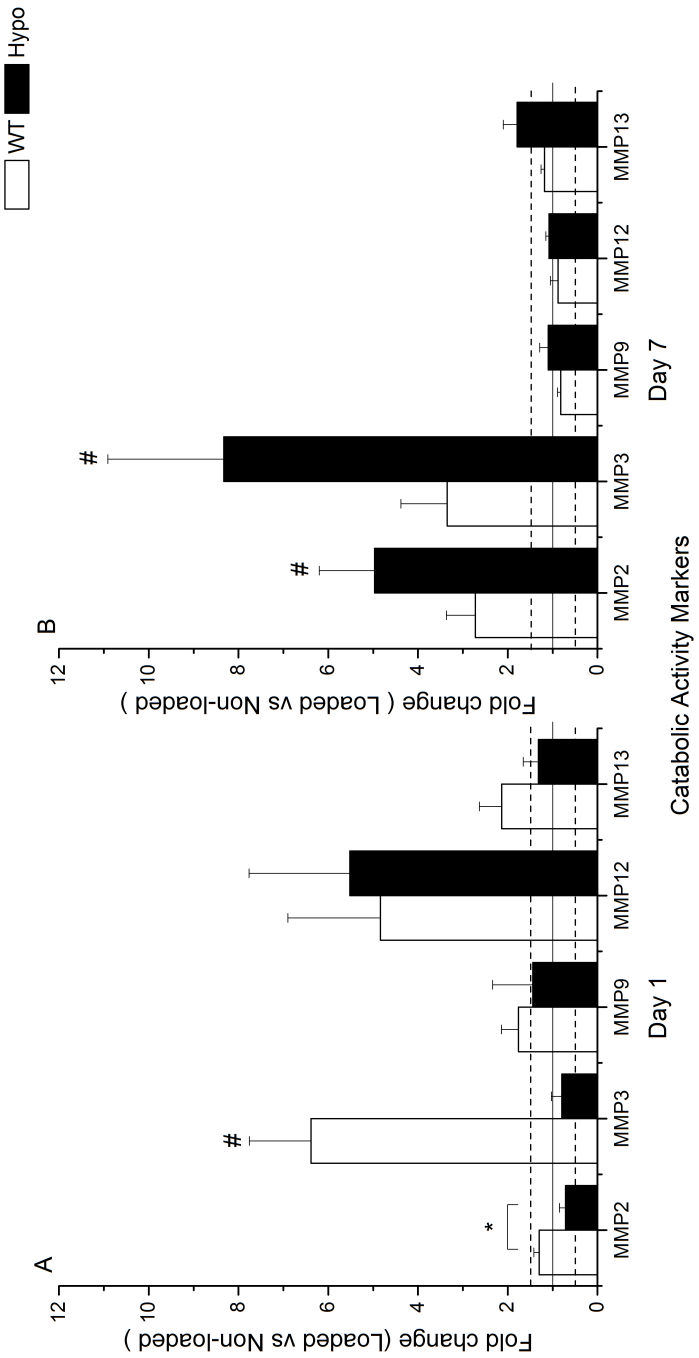
WT
Hypo
A
B
Fold change (Loaded vs Non-loaded)
Fold change (Loaded vs Non-loaded)
0
2
4
6
8
10
12
*
#
#
#
MMP2
MMP3
MMP9
MMP12
MMP13
Day 1
Day 7
Catabolic Activity Markers

Figure 3.4: Effect of 1-day (Panel A)and 7-day (Panel B) mechanical loading on mRNA levels of catabolic activity markers. Transcript levels of all the genes were measured in duplicates or triplicates using real-time PCR from 19 week-old WT (day 1 n=10, day 7 n=5) and hypo (day 1 n=6, day 7 n=4) male tibiae 24 hours after final session of loading. Fold changes (loaded vs. non-loaded) were calculated using the ΔΔCt method using GAPDH as the housekeeping gene. Error bars represent standard error of the mean (SE). # indicates $p \leq 0.05$ within the genotype (loaded vs non-loaded, hypo or WT); * indicates a $p \leq 0.05$ a significant difference in fold change between the WT and hypo genotypes.

Chapter 4

DISCUSSION

Perlecan/HSPG2 is a naturally occurring large heparan sulfate proteoglycan (HSPG) found in the ECM of cartilage, bone, most connective tissues and basal laminae. It plays an important role in maintaining normal homeostasis, cell adhesion, proliferation, embryo implantation and development and modulation of growth factors activity. Studies have shown that Perlecan is essential for proper endochondral ossification and that deficiency of perlecan in the adult mutant mice (hypo) results in short stature and altered growth of bones [34, 37]. Perlecan is secreted into the pericellular space from the osteocytes and is known to interact with the matrix proteins (Collagen Type I, fibronectin and osteopontin) and cell surface molecules (integrins and CAMs). Previous studies from our lab included the PCM fiber density measurements and dynamic histomorphometric analysis. PCM fiber density was found to be decreased with perlecan deficiency and the hypo animals experience decreased fluid drag force on loading. Histomorphometric measurements on the tibia of mechanically loaded WT animals showed a significant increase in bending stiffness, cortical polar moment intertia (Ct. pMOI) and periosteal mineral apposition rate (Ps.MAR). While the hypo animals, did not show any such anabolic responses for the same loading scheme. Hence, a working hypothesis that PCM fibers comprising of perlecan serve as load sensing antennae regulating cellular stimulation and response to external mechanical stimuli was developed [29].

Here, we examine the role of perlecan in the regulation of bone mechanosensation using the perlecan deficient mouse model and *in vivo* tibial mechanical loading model. We used the dynamic mechanical loading as it is a potent anabolic stimulus for the formation of new bone [14]. Also the gene expression patterns following mechanical loading in bone have been extensively studied and are well established in bone cell cultures and WT animal models [40, 41]. Here we compare our results with some already published research on bone mechanical loading (Table 4.1)

Gene studied	1 day - Mechanical loading		Repetitive Mechanical loading (Up to 8 days)	
	Our Results- Fold change	Trends observed in published research	Our Results- Fold change	Trends observed in published research
Collagen type I alpha 1 chain (*Col1a1*)	1.6	~0.5 fold (2 fold decrease) after one day ulna loading [40]	3.2	~3.5 fold increase upon 8 days ulna loading in rats [40], ~6 fold increase on 5 days tibial loading in mice [42]
Osteocalcin (*Bglap*)	1.5	~1.2 fold increase after one day ulna loading in rats [40]	1.3	~1.6 fold increase upon 8 days ulna loading in rats [40]
Osteopontin (*Opn*)	1.3	Fluid flow induced increase in mRNA expression observed in mineralizing 2T3 cells [26]	1.1	N/A
Dentin Matrix Protein 1 (*Dmp1*)	1.5	Increased expression upon in bone formation and resorption sites after 24 hours in tooth movement model [43]	1	Increased expression upon in bone formation and resorption sites continuous loading in tooth movement model [43]
Alkaline Phosphatase (*Alp*)	2.7	~1.2 fold increase after one day ulna loading in rats [40]	1.5	~1.6 fold increase upon 8 days ulna loading in rats [40]
Cyclo-oxygenase 2 (*Cox-2 or Ptgs2*)	5	~1.5 fold increase after 4 hours after mechanical load [44] and fluid flow induced expression of *cox-2* was observed in MC3T3	7	N/A

		cells starting from 4 hours [45]		
E11	4.8	Early responses (2-4 hours) by increase in mRNA expression in response to fluid flow in MLOY4 cells [26]	5.3	N/A
Runx2	1.3	Mechanical loading induced increased expression in MC3T3 cells. [46]	1.3	N/A
RANKL	1.6	Osteocyte induced osteoclastogenesis was observed upon mechanical loading [47]	1.5	N/A
MMP2	1.3	~1.2 fold increase after one day ulna loading in rats [40]	2.7	~1.6 fold increase upon 8 days ulna loading in rats [40]
MMP3	6.3	4 hours compressive loading increased mRNA levels in osteoblasts [48]	3.3	N/A
MMP9	1.8	~1.2 fold increase after one day ulna loading in rats [40],	0.8	~1.6 fold increase upon 8 days ulna loading in rats [40]
MMP13	2.1	Magnitude dependent upregulation observed in MC3T3 cells [49]	1.2	N/A

Table 4.1**:** Comparison of our findings with published mechanical loading studies

Before studying the effect of loading on the WT and PLN deficient mice, we analyzed the differences in the baseline gene expression profiles (non-loaded bones after 1-day loading) between the WT and hypo animals. As expected and previously reported, perlecan transcript levels were significantly lower in hypo bones compared to the age-matched WT animals at baseline [34]. Although the matrix protein and cell activity marker genes were decreased compared to the WT controls in the hypo animals, there was no significant change in these genes between the genotypes at baseline (no experimental load condition).

Cyclooxygenase-2 (*Cox-2* or *Ptgs2*), an enzyme known to be an indicator of pro-inflammation, is also well described as one of the early response gene markers expressed following mechanical loading and is believed to be an important mediator of bone anabolic response to load [50, 51]. Consistent with its previously described expression in response to load, WT (n=10) and hypo (n=6) animal bones showed increased expression of *Ptgs2* transcripts 24 hours after a single loading bout. While the response to load for this gene is preserved in WT (n=5) after 7 days of loading, hypo (n=4) animals show a milder expression of *Ptgs2* transcripts following repetitive loading. This suggests that the hypo animal bones may have desensitized and are not as mechanoresponsive than WT upon multiple loading sessions in the absence of an integral PCM that does not contain appropriate levels Pln. It is noteworthy to report that *Ptgs2* mRNA levels were found to be also increased in the non-loaded contralateral tibia after seven days of repetitive loading (data not shown). Though this may be indicative of a systemic effect from sustained tibial loading due to diffusible pro-inflammatory signals reaching the non-loaded tibia, no such conclusion can be made at this time because of the important variation in expression data observed for

this particular gene. Additional samples as a well as measurement of circulating prostaglandins in the blood stream are needed to confirm this assumption as previous studies indicate that prostaglandins mediate anabolic effects of mechanical loading on bone via induction cox-2 expression pathways [52].

Perlecan deficiency induces differentiation of bone progenitors cells *in-vitro* and also increases terminal differentiation in bone of hypo animals compared to WT, showing that Pln acts as an inhibitor of differentiation and mineralization [37]. Osteocalcin (*Bglap*) gene up-regulation in hypo animals upon seven days of loading is consistent with the observation that increased mineralization is a characteristic of muscular-skeletal tissues in which Pln is misexpressed. Supporting the above-mentioned result, Pln is known to directly interact with calcium phosphate mineral from bone extracts [53] and also increased expression of Pln was found to prevent vascular calcification in cardiac tissue [54].

Genes encoding for bone forming proteins such as matrix proteins are known to be mechanically-responsive with consecutive sessions of loading and generally show an increase in their response on multiple bouts of loading [40, 42]. Hence, the transcripts encoding for the most abundant matrix protein found in bone, *Col1a1*, are significantly increased in WT animals after seven days of loading but not after 24 hours. Interestingly, a significant increase in *Col1a1* transcripts is also observed in the Pln hypo animals after seven days of loading.

Another important marker of bone remodeling, the *Alp* enzyme, has also been reported to be increased in cells and rodent bones as an early response following load [40, 55]. The 2.5-fold increase for *Alp* transcript seen in WT after one bout of loading was attenuated, but not abolished, after multiple days of loading. Unlike WT, hypo

animals did not show any significant changes in the mRNA expression levels of *Alp* following mechanical stress at both the studied time points. This result is another indicator of reduced responsiveness of hypo animals after both short term and prolonged mechanical stimulation. Additionally, a 3-fold increase in the *Alp* transcript levels in the control (non-loaded) tibia after 7 days of loading was observed (compared to the nonleaded day 1) indicating a systemic effect caused by mechanical loading of one limb that might have resulted in an increased *Alp* levels in the other. However, the observed result could have been the result of the increased variation among the animals used in the day 7 loading study. Previous study that investigated the systemic effect following ulna loading reported an increase in bone formation response in the long bones that were not loaded. They suggest this could be the result of neuronal regulation as no such bone formation responses were observed in animals subjected to load with neuronal blocking [56].

E11, an osteocyte specific protein, increases as a result of mechanical strain and is essential for the elongation of osteocytic dendritic processes and normal bone function [26]. *In vitro* cell culture studies show that E11 mRNA transcripts are increased on fluid flow stimulation [46]. In line with previous studies, E11 transcripts are increased immediately after first session of loading and this effect is sustained up to seven consecutive days of loading. Contrastingly, hypo animals' response to load was increased only with long term loading. This is indicative of hypo bones adaptability to prolonged mechanical stimuli with an increasing number of osteoblasts being embedded in the matrix.

Effect of fluid flow induced mechanical stimulation on primary cultures and cell lines showed that an external load induces a decrease in the expression of markers

of osteoclastogenesis (RANKL/*Opg* and TRAP) [55, 57]. Ongoing TRAP staining studies (performed by Mr. Ashutosh Parajuli, University of Delaware, Department of Mechanical Engineering, data not shown) indicate that osteoclast activity is higher in hypo animals compared to WT animals of the same age when mice are experiencing normal physical daily activity and are not experimentally loaded. Gene expression patterns of RANKL and *Opg* do not reveal such a trend at non-loaded and loaded conditions in hypo animals. We suspect an alternative pathway causing an increase in osteoclast activity through TRAP and not through RANKL/*Opg*.

The regulation of matrix metalloproteinases (MMPs) is essential for bone to respond to external mechanical stress. MMPs are necessary for the normal canalicular formation and function in bone [58, 59]. MMP2, MMP3, MMP9, MMP13 were shown to be mechanosresponsive previously [40, 48, 49, 55, 58]. MMP9, a marker of bone remodeling, was significantly reduced in PLN mutants at non-stimulated conditions indicating a decreased tendency of load-induced bone remodeling. Interestingly, the significant MMP3 mRNA increase seen in WT as a result of activation of remodeling pathways is not observed in Pln-deficient bones after 24 hours but is significantly increased in Pln mutants after seven bouts of loading. The lack of initial responsiveness in Pln mutant bones appears to be counteracted after prolonged stimulation. This compensation in mutant may occur through delayed activation of matrix molecules gene expression and may be associated with increased signaling kicking in at a later time point as seen with increased transcript levels of *Col1a1* in hypo animals after 7 days of loading (but not after short term loading). This delayed activation in the mutants suggests a function of perlecan in activation of catabolic pathways upon load stimuli.

Mechanical loading based bone formation and resorption is based on the magnitude of the strain that the bone tissue is subjected to. Bone formation is known to occur at sites of high local mechanical strain and bone resorption is known to occur at sites of low local mechanical strain [60]. In our study, the induced peak strain was quantified to be at 1200με at the anterior-medial surface. This leads to an uneven loading response on the Bone Multicellular Units (BMUs) present on the anterior surface of the bone compared to BMUs on the posterior surface. This could be a possible explanation for the trend and variations observed on mechanical loading with the mRNA levels of MMPS.

An exciting novel finding is that *Pln* gene expression is upregulated upon tibial loading in the WT control animals. The increase in perlecan expression upon loading was observed after 24 hours and was sustained after multiple days of loading similar to the anabolic marker: collagen type 1. This further corroborates PLN's proposed function as an important player in bone responsiveness and as a late response gene to mechanical loading. While short-term loading had no effect on the low steady-state expression levels of PLN transcripts in Hypo animals, repetitive loading for seven days did increase the mRNA expression levels in the loaded limbs than the non-loaded limbs. Although not significant, these results indicate that repetitive loading can initiate compensatory rescue signals to increase the mRNA levels of genetically deficient genes, further emphasizing the importance of weight bearing exercises.

The present study involved *in vivo* mechanical loading experiments with animal subjects and thus had some limitations. Firstly, the inter-sample variability as observed with high standard deviations in the fold change values within the same genotype (both WT and hypo). This variability served as one of the reasons for not

observing statistical significance in most of the mRNA transcript expression data among the animals of the same genotype and also between the WT and hypo. This can be overcome by increasing the number of animals studied in each group. Secondly, the whole bone was used for mRNA analyses, transcripts from many confounding cells (mesenchymal stem cells, bone marrow cells, endothelial cells, adipocytes, etc.) were also included and studied, instead of just the bone cells. It was recently studied that the cortical and cancellous bones show different gene expression profiles in response to mechanical loading [41]. This drawback can be addressed by performing further studies on RNA extracted from bones with bone marrow cells flushed out.

An unbiased RNA sequencing (RNAseq) approach was adopted to complement the current study and perform a genome-wide comparative gene expression analysis in WT vs. hypo mutant bones after both short term and long term loading. Currently, only the 24hr post one bout of loading data were collected. The results identified by RNAseq showed similar trends as observed with our individual gene approach for *Cox2, Bglap (OCN*) and MMP3. The study also identified that WT animals were more responsive to load with a total of 55 genes being differentially expressed after one bout of loading while hypo animals expressed only 14 genes that were different with no gene overlapping between the hypo and WT groups (Fig. 4.1).

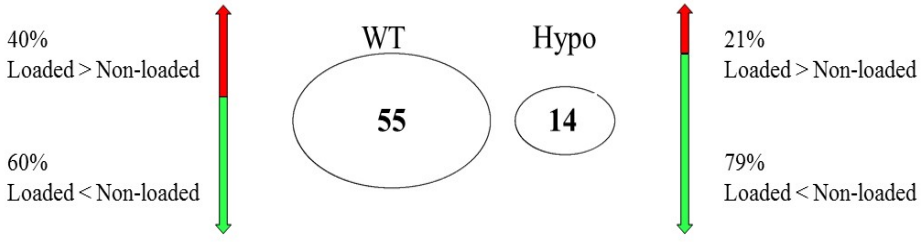

Figure 4.1: Venn diagram showing the number of differentially expressed genes between loaded and non-loaded tibiae in WT (left) and hypo (right) mice (FDR < 5%). Arrows (%) indicate proportion of genes that were more highly expressed in loaded (red) or non-loaded (green) bones. The RNA library preparation and sequencing was performed at the University of Delaware Sequencing & Genotyping Center Core Facility and the data analysis was conducted by Mr. Shaopeng Pei (University of Delaware, Department of Mechanical Engineering). Image Courtesy: Mr. Shaopeng Pei.

The RNAseq study that will identify differential gene expression pattern after seven days of loading in both the genotypes is ongoing. This information will provide a more complete assessment of the effect on long term mechanical stimuli on WT and hypo animal bones, thereby creating a full picture of how differently the hypo animals respond to mechanical stimulation relative to control animals.

4.1 Final conclusions:

Perlecan is an essential component of the ECM and plays a vital role in maintaining the tissue homeostasis and development. Here, we show that the hypo animals (harboring bone tissue deficient in perlecan) show altered mechanosensitivity compared to the WT control animals. This suggests a role for perlecan as an organizer of the PCM and also indicates an important role in cellular mechanosensation, and regulation of the downstream signaling events. Consistent with this idea, a recent study reported that knockdown of Pln in the PCM of cartilage reduced stiffness of both cartilage cells and interstitial matrix, and therefore disrupted mechanical properties of embedded cells and negatively impacted signaling in the altered tissue [61].

The significant increase in perlecan with loading similar to the other anabolic genes in the WT mice, suggests that perlecan is a mechanoresponsive gene similar to collagen 1. Although the hypo mice were less sensitive to mechanical loading compared to the WT, repetitive loading resulted in the increase of some of the anabolic genes (collagen 1 and perlecan itself). This further emphasizes that regular exercise (analogous to the sustained loading) is essential to maintain good bone health and could potentially compensate for any imbalance in the tissue by activating the synthesis machinery (even in genetically-deficient states). One of our laboratory ongoing studies is to analyze the effect of unloading (hindlimb suspension) in hypo animals vs. WT controls. It will be interesting to compare and contrast the expression profiles obtained with the dynamic mechanical loading model (described here) and the suspension study results.

4.2 Future directions:

The RNAseq data obtained at day 1 has given detailed information with the changes in gene expression in the WT and hypo with respect to loading. It has also allowed us to identify novel mechanosensitive genes. Therefore, the next step is to perform the RNAseq analysis after seven days of loading and compare the gene expression profiles with the present real-time PCR results between the WT and hypo animals. This will further allow the identification and understanding of underlying mechanisms and the variation in the results we observe in this study.

By studying the effects of age, younger animal bones were found to be more mechanoresponsive and anabolically active compared to the older animals [62]. In this study, hypo and WT animals of ages 19-20 weeks (around 5 month-old) were selected to be mechanically loaded, as the PLN hypo animals show delayed skeletal

development compared to WT animals of younger age [34]. It will be interesting to study the response to load in the hypo animals with increasing age. Also, only the male animals were used in this study. Considering that bone conditions such as osteoporosis are prevalent in aging females, it will be important to perform studies using female animals in order to take into consideration the gender based differences resulting from perlecan deficiency and mechanical loading.

In this study, we used the contralateral non-loading limb as the control for comparing with the loaded limbs. However, this could have obscured the real differences between the loaded and unloaded conditions especially because of systemic effects that likely arise after repetitive sustained loading. Therefore, future studies should focus on studying the systemic effects of tibial loading and include control non-loaded limbs from animals that were not subjected to loading to study the same. Also other physiologically relevant mouse model of loading such as treadmill running model, loading and unloading hindlimb suspension studies will further establish and demonstrate the role of perlecan in bone mechanosensation.

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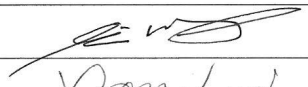
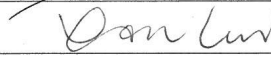
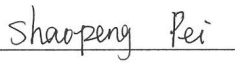
1. Liyun Wang	
2. Xiaohan Lai	
3. Shaopeng Pei	
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Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
a. Liyun Wang	lywang@udel.edu	2659	7323063026	x	Click here to enter text.

Status: Associate Professor of Mechanical Engineering

Qualifications: Wang has experience with mice, including live animal loading-9yrs, tail-vein injection-15yrs, IP and SC injections-15yrs, anesthesia-15yrs, euthanasia-15yrs, and tissue harvesting-15yrs, and microsurgery on knee-7yrs

Responsibilities: Design & oversee the experiments; train and supervise lab members; coordinate with OLAM to perform all animal related procedures. In specific, she will oversee two new techniques that will be the focus of the renewal: *dual-color Fluorescence Recovery After Photobleaching (FRAP)* velocimetry to measure pericellular matrix *in various clinical conditions; and ex vivo calcium imaging of cellular responses to mechanical loading.*

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
b. Xiaohan Lai	laixhan@udel.edu	6704	3024197960	x	Click here to enter text.

Status: Graduate student 5th year

Qualifications: Xiaohan had been working with mice for the past three years. She has experience with tail-vein injection-3yrs, anesthesia-3yrs, euthanasia-3yrs, tissue harvesting-3yrs, and FRAP experiments-3yrs.

Responsibilities: Xiaohan will perform dual-colored FRAP studies and ex vivo calcium imaging as well as other tasks required for the studies.

				Received Animal Facility

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Training	
				Yes	No
c. Shaopeng Pei	shaopeng@udel.edu	6704	7162625186	Click here to enter text.	x

Status: First year graduate student

Qualifications: Shaopeng has no previous animal experience. He will receive training from Dr. Wang and Xiaohan. He will get the training from Mr. Warren soon.

Responsibilities: Shaopeng will help Xiaohan in all her studies.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
d. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Status: Click here to enter text.

Qualifications: Click here to enter text.

Responsibilities: Click here to enter text.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
e. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

4. Non-Scientific Summary: In language understandable to a ***high-school senior, very briefly describe*** the goals and significance of this study.

a. Specific Scientific Goals: Osteocytes are very important cells in bone. They serve as sensors to detect external forces and damages in the bone matrix, so that the bone can adapt to its mechanical usage and undergo self-repair. Because these cells are buried in the concrete-like mineralized matrix, they need obtain nutrients from and dispose of waste to blood vessels through a set of interconnected pores termed "osteocyte lacunar-canalicular system (LCS). In addition for the nutrition need, the fluid flow inside the LCS occurred in a mechanically loaded bone also provides physical signals that allow the osteocytes to detect the level of mechanical loading that bone experiences during daily life. The way that osteocytes detect mechanical loading is similar to that trees detect the direction and strength of blowing winds by sensing deformations in their branches. In bone LCS, a pericellular matrix (PCM) consisting of proteoglycan-like fibers are shown to fill the annular space. These PCM serve not only as molecular sieve to filter passing solutes but also as a sensing apparatus (like branches for a tree) for osteocytes to detect the fluid flow (mechanical loading). Therefore, *solute (and fluid) transport through PCM along LCS is critical for the survival of osteocytes and their vital function of detecting mechanical loading.* In the past studies, we have successfully detected altered PCM in a transgenic fragile bone model where perlecan expression is lowered. For the next three years, we will test a better method in detecting the alterations of the PCM and to correlate the alterations of PCM with cell's intracellular calcium response to mechanical loading.

b. Significance of this Research (including the possible benefits to human and/or animal health, the advancement of scientific knowledge, or the betterment of society): The research will help gain fundamental understanding of how osteocytes detect and respond to mechanical signal. This knowledge will help us design patient-specific training program to increase bone strength.

5. Experimental Design: Explain the experimental design. This description should allow the IACUC to understand fully the experimental course of an animal or group of animals from its entry into the experiment to the endpoint of the study.

The inclusion of flow charts, diagrams, and/or tables are greatly encouraged to explain experimental design or sequential events.

Be sure to include all animal events and related details, i.e.,

- **All Procedures**-bleedings, injections, identification methods, genotyping methods, surgical procedures, euthanasia, etc.
- **Procedural details**–number of animals involved in procedure, approximate animal weight, if relevant (for injections, bleeding, etc.), route, frequency, volume, etc.
- **Pharmaceutical-grade and non-pharmaceutical grade compounds** – Identify any drugs, biologics, or reagents that will be administered to animals. If these agents are not human or veterinary pharmaceutical-grade substances, provide a scientific justification for their use and describe methods that will be used to ensure appropriate preparation.
- **Names of surgical procedures** (but reserve the surgical details for the proper Surgical Addenda)

- **Monitoring**–observations, measurements (animal weight, tumor size, etc.)
- **Monitoring details**–criteria, frequency, names of personnel monitoring, conditions for removing an animal from the study, etc.
- **Endpoints**–include endpoints for the animals/study and how will they be determined.

(Describe): Animals: perlecan-deficient homozygous mice (termed Hypo) are currently maintained in UD animal facility by Dr. Liyun Wang and Dr. Catherine Kirn-Safran. The animals will be ear punched and genotyped using tails after weaning. All the procedures except imaging will be done in OLAM and no live mice will be transferred to Spencer Lab. We will use adult Hypo mice (males and females) at 2, 4, 6, 10 months to perform two lines of studies: (1): Measuring PCM using Dual-color FRAP; and (2): Study how altered PCM impacts the intracellular calcium elevation, one of the first cellular responses under mechanical stimulation. Procedures of Study 1: Anesthesia: Although anesthesia is not mandatory for tail vein injection, we have found that isoflurane anesthesia greatly improved the successful rate of injection at the first shots, avoiding repeated piercing of the tails and excess physical constrains on the animals, and thus reducing the animals' stress. Mice will be anesthetized using isoflurane. Initial 4-5% will be used in the induction phase and then remain at 1.5-3.5% later on. The duration of the anesthesia will be approximately 5-10 min for one tail vein injection. Tail injection of dual tracers: To measure the PCM density, one small tracer and one large tracer will be injected into the same mouse. The small tracer is for tracking how fast fluid moves in the PCM. Due to the hindrance of the PCM on larger tracer, larger tracer molecules will move slower than the smaller tracer. By comparing the velocity difference between the small and large tracers, we can obtain the information of how dense the PCM fibers are spaced near the osteocytes. We call this new method "dual-color FRAP", because the velocity of tracer will be measured using FRAP technique that we pioneered (Price 2011; Wang 2014). A bolus of lactalbumin or other proteins of similar molecular weight (10-20kDa) fluorescently labeled with cy5 or other red dyes will be used as large tracer and injected first to the mice. From our experience, this sized tracer will be reach equilibrium in 3-4 hours (Li 2009). 30 min prior to the equilibrium of the large tracer, a bolus of sodium fluorescein (0.2mL, 2mg) will be injected at tail vein. Both the large red tracer and small green tracer will reach equilibrium at the same time. The animals are allowed to come out of anesthesia between the two injections and locomotion in fact promotes tracer perfusion in bone. All the tracers and fluorophores can be purchased sterile from Invitrogen or Sigma and dissolved in sterile PBS prior to injection. Sacrifice and FRAP imaging: After the required circulation time, the mouse will be sacrificed with CO_2 and cervical dislocation and tibiae will be harvested for FRAP imaging under confocal microscope and mechanical loading, following our previous publication (Wang 2014). ***Since this is mainly an ex vivo study, only tail-vein injection and tissue harvesting are involved in this animal protocol***. The outcomes of study 1 are the PCM density data at various age and gender compared with wild-type (WT) controls. The endpoints of the procedures will be freshly sacrificed bone tissues perfused with two types of tracers, which will be used for advanced imaging to probe the structure of the PCM. Procedures of Study 2: To test whether altered PCM is associated with altered cell response to mechanical loading, we will measure the intracellular calcium signaling in Hypo and WT mice. Sacrifice and tissue harvest: Mice will be sacrificed with deep anesthesia and cervical dislocation. Both tibiae will be promptly harvested in a sterile condition under a biosafety hood. Bones will be cultured in an incubator for 2-4 hours. Ex vivo intracellular

calcium imaging: we will follow our collaborator Dr. Ed Guo's protocol for imaging the intracellular calcium responses of osteocytes to mechanical loading using confocal microscopy (Jing 2014). ***Since this is an ex vivo study, only tissue harvesting is involved in this animal protocol.*** The outcomes of study 2 are the responding rates (percentage of cells showing elevated intracellular calcium within a certain time period (15 min) under physiological loading) for osteocytes in Hypo and WT bones. The endpoints of the procedures will be freshly sacrificed bone tissues with live and functional osteocytes, which will be used for advanced imaging of intracellular calcium responses to mechanical loading. Additional studies using live perfusion: To correlate the measured PCM density in the above experiments with TEM morphology, live perfusion procedure will be used. The principal reason of using live perfusion is that the bone PCM is very fragile and lacks intrinsic electron contrast. Live perfusion in deep anesthetized animals will allow fixatives to be carried by circulation into bone and preserve the PCM before autolysis and other forms of degradation occur. In addition, anions will be delivered into bone to bind to the negatively charged PCM for a better contrast for EM imaging as shown in our previous study (Thompson 2011). We will also use live perfusion techniques to explore the changes of vasculature in Hypo mice. Because perlecan is an important component of vascular base membrane, we need to make sure that our observed changes in osteocyte intracellular calcium responses are not due to changes in vasculature. We will perfuse a lead chromate-loaded silicon contrast agent (Microfil MV-117, Flow Tech Inc) at a rate of 1ml/min for ~15 min. Details seen in Terminal Surgery E form. References: Li (2009). "The dependency of solute diffusion on molecular weight and shape in intact bone." Bone **45**(5): 1017-23. Price (2011). "Real-time measurement of solute transport within the lacunar-canalicular system of mechanically loaded bone: direct evidence for load-induced fluid flow." J Bone Miner Res **26**(2): 277-85.Wang (2014) Perlecan-containing pericellular matrix regulates solute transport and mechanosensing within the osteocyte lacunar-canalicular system. J. Bone Miner Res. 29:878-91. Jing (2014): In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading. FASEB J. 28(4):1582-92. Thompson (2011). Perlecan/Hspg2 deficiency alters the pericellular space of the lacunocanalicular system surrounding osteocytic processes in cortical bone. J Bone Miner Res. 2011 Mar;26(3):618-29. doi: 10.1002/jbmr.236.

REFINEMENT, REDUCTION & REPLACEMENT

When using animals for research, it is important to consider the three Rs: reduction, refinement, and replacement to reduce both animal distress and the number of animals used in the laboratory.

Reduction: Minimizing the number of animals used
Refinement: Using techniques and procedures to reduce pain and distress
Replacement: Using non-animal methods or lower phylogenetic organisms

6. Justification for the Use of Animals (instead of *in vitro* methods)
(Check all that apply and explain):

a. ☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems: ***(Explain)***: The osteocyte lacunar-canalicular system and the pericellular matrix cannot be created in vitro. Animals have to be used.

b. ☐ There is not enough information known about the processes being studied to design non-living models: ***(Explain)***: Click here to enter text.
c. ☐ Other: ***(Explain)***: Click here to enter text.
7. Justification for Species Appropriateness: ***(Check all that apply and explain)***:
a. ☐ A large database exists, allowing comparisons with previous data: ***(Explain)***: Click here to enter text.
b. ☐ The anatomy or physiology is uniquely suited to the study proposed: ***(Explain)***: Click here to enter text.
c. ☒ This is the lowest species on the phylogenic scale suitable to the proposed study: ***(Explain)***: Mouse is the lowest species with skeletal biology similar to human.
d. ☐ Other: ***(Explain)***: Click here to enter text.
8. Justification for Number of Animals Requested: (Note: numbers should include animals used for breeding and all animals born)
a. ☐ Pilot study or preliminary project where group variances are unknown at the present time. Describe the information used to estimate how many animals will be needed: (Only a limited number of animals will be permitted.) ***(Explain)***: .

b. ☒ Group sizes are determined statistically. Describe the statistical analysis used to estimate the number (N) of animals needed: N may be estimated from a power analysis for the most important measurement in the study, usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power (e.g. $P < 0.05$). Data analysis methods should not be submitted unless directly applicable to the estimate of N.

An online calculator may be found at: http://www.math.uiowa.edu/~rlenth/Power/
or a stand-alone calculator that can be downloaded from
http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3

(Explain): Although our previous study (Wang 2014) identified that the PCM structure changed in Hypo compared with WT, the variances for the two groups are currently unknown due to the technical limitations. The dual-color FRAP method will allow us to measure the PCM density in each mouse. We will begin with a sample size of n=10 per group per gender at the age of 2 months. This sample size can provide 61% power to detect a 30% difference between the two means with an effect size (mean difference/std) of 1. We have had used this sample size and found it adequate to detect bone responses to mechanical loading in our live animal loading study (Wang 2014). Similarly, we will begin a sample size of n=20 per group per gender to test the difference of intracellular calcium signaling between the two genotypes. This larger sample size is justified based on Dr. Guo's experience and is necessary because we have to optimize the complicated calcium imaging protocol (Jing 2014). We also request a sample size of 10 per gender per genotype for live perfusion study of PCM and vasculature morphology and 5 per gender per genotype for breeding. After these initial studies on 2-month-old mice are completed in year 1, we will be in a better position to estimate how many animals are needed for other age groups and we will submit an amendment in year 2.

c. ☐ Group sizes are based on the quantity of harvested cells or the amount of tissue required for *in vitro* studies. Explain how much tissue is needed based on the number of experiments to be conducted and the amount of tissue you expect to obtain from each animal (e.g., 10g of tissues are needed: Each animal can provide 2g. 10g /2g per animal = 5 animals needed.) ***(Explain)***: Click here to enter text.

d. ☐ Teaching protocol. Specify the number of students in the class, the student to animal ratio and how that ratio was determined: Animal numbers should be minimized to the fullest extent possible without compromising the quality of the hands-on teaching experience for students or the health and welfare of the animals. ***(Explain)***: Click here to enter text.

e. ☐ Study involving feral or wild animals. Animals will be captured and released in an attempt to maximize the sample size within logistical constraints. Describe the process by which you estimate these numbers and estimate the precision needed: ***(Explain)***: Click here to enter text.

f. ☐ Observational, non-manipulative study. Animals will not be captured, their behavior will not be

interfered with, and exact animal numbers cannot be predicted: ***(Explain)***: Click here to enter text.

g. ☐ Product testing. The number of animals needed is based on FDA guidelines. Provide the citation from the regulations, the IND tracking number, or relevant FDA correspondence: ***(Explain)***: Click here to enter text.

h. ☐ Other. Elaborate, indicating the method used to determine the group size. ***(Explain)***: Click here to enter text.

9. Animals Requested:

Common Name	Genus and Species	Total Number of Animals for Three Years
1. mouse	Mus musculus	200
2. Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.

10. Where will animals be housed (or captured for wildlife)? UD OLAM

11. Where will the experiments take place?

If animals must be removed from the vivarium, please describe how they will be transported (such as hand carried in covered microisolator cages). Spencer 202 or OLAM

12. Will any animals be humanely killed, without treatment or manipulations, to be used to obtain tissue, cells, etc.? ☒ Yes ☐ No

If **Yes,** list types of tissue, etc: Bone

13. Physiological Measurements ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

14. Dietary Manipulations ☐ Yes ☒ No

If **Yes,** list and explain (Note: if food or fluid will be restricted, describe method for assessing the health and wellbeing of the animals. Body weights must be recorded at least weekly. Amount earned (if animals work for food or fluid) during testing and amount freely given must be recorded. A scientific justification must be provided for departures from the recommendations of the Guide.) Click here to enter text.

15. Environmental Stress (e.g. cold, restraint, forced exercise) ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

16. Trauma or Burn Injury ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

17. Production of Hybridoma/Monoclonal Antibodies ☐ Yes ☒ No

If **Yes,** please complete Addendum "**B**".

18. Production of Polyclonal Antibodies ☐ Yes ☒ No

If **Yes,** please complete Addendum "**C**".

19. Administration of Hazardous Chemicals, Drugs, Toxins, or Nanoparticles

☐ Yes CAS#_____ ☒ No

If **Yes,** describe hazards posed to personnel: Click here to enter text.

Methods to control exposure: Click here to enter text.

Methods of Disposal of Animals and Bedding: Click here to enter text.

20. Administration of radioactive materials ☐ Yes ☒ No

a. Type to be used. Include radioisotope(s) and chemical form(s): Click here to enter text.

b. Describe the practices and procedures to be followed for minimization of radiation exposure to workers and for the handling and disposal of contaminated materials associated with this study:

(Include the methods for management of radioactive wastes and monitoring facility for radioactive contamination, if applicable.) Click here to enter text.

c. Who will be responsible for the daily care of animals containing radioactive materials?

Click here to enter text.

d. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Click here to enter text.

Please attach a copy of any approvals or provide the approval number.

Click here to enter text.

21. Study of Irradiation *in vivo*? ☐ Yes (gamma irradiator? ☐ or x-ray irradiator? ☐)
☒ No

a. Make, model, and location of irradiator to be used:

Click here to enter text.

b. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number.Click here to enter text.

22. Administration of Biological Agents (eg microorganisms, recombinant DNA, **HUMAN** serum, tissue, cell lines, etc.) ☐ Yes ☒ No

Animal Biosafety Level ☐ 1 ☐ 2 ☐ 3 ☐ 4

Describe hazards posed to personnel:Click here to enter text.

Methods to control exposure:Click here to enter text.

Methods of Disposal of Animals and Bedding:Click here to enter text.

Approval received from UD- Institutional Biosafety Committee? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number. Click here to enter text.

23.Will tumor cells, tissue, sera, viral vectors or other biologics of RODENT origin – other than those isolated from rodents already housed in the facility – be administered to animals?

☐ Yes ☒ No

If Yes, this material must be tested for rodent pathogens and test results must be attached (Please contact the Attending Veterinarian for details). Click here to enter text.

24. Use of Genetically Engineered Animals

☒ Yes ☐ No

If Yes, please describe any anticipated phenotypes that may cause pain or distress and any special care or monitoring that the animals will require.

Our Hypo mice harbor a mutant gene resulting in reduced expression of perlecan, a large heparan sulfate proteoglycan. The mice showed bone and cartilage abnormality. We have male formation of their teeth that may need special care (trimming). Our facility staff has been doing that.

Does the proposed work involve creating new genetically modified animals, or involve crossing two genetically modified animals to produce offspring with a new genotype.

☐ Yes ☒ No

Approval received from UD- Institutional Biosafety Committee?

☐ Yes ☐ No ☐ Pending ☐ Exempt (breeding of two lines of genetically-modified rodents is exempt if 1) both parents can be housed under BL1 containment and 2) neither parent strain incorporates more than one half of the genome of an exogenous eukaryotic virus or incorporates a transgene under the control of a gammaretroviral long terminal repeat and 3)the rodent that results from the breeding is not expected to contain more than one half of an exogenous viral genome)

Please attach a copy of any approvals or provide the approval number.

_____Click here to enter text._____

25. Special Study Requirements: Please describe any special study requirements such as a requirement for single housing of the animals, exemption from environmental enrichment, or special caging:Click here to enter text.

26. Any other procedures? ☒ Yes ☐ No

If **Yes,** explain: We will run live perfusion procedures for a subset of mice to study the morphology of PCM and vasculature in lower limbs. Details will be attached in Terminal Surgery E. see below

27. Will this study involve surgery? ☒ Yes ☐ No

If **Yes,** and it is "Survival Surgery," please complete Addendum "**D**".

If **Yes,** and it is "Terminal Surgery," please complete Addendum "**E**".

28. Will any animal undergo anesthesia for any reason other than surgery? X☐ Yes ☐ No

If **Yes**,

a. List Procedures and Reason(s) for using anesthesia: Although anesthesia is not mandatory for tail vein injection, we have found from the past years that isoflurane anesthesia greatly improved the successful rate of injection at the first shots, avoiding repeated piercing of the tails and excess physical constrains on the animals, and thus reducing the animals' stress. We will inject one bolus of fluorescent tracer solution. The injection will take 1-5 min and the animal will wake up in 5 min after being removed from the anesthesia machine. In this study, we will inject two tracers in 2.5 hours apart.

b. Check the type of anesthesia to be used.

☒ Isoflurane

☐ Injectable (***For injectable, complete the following):***

Drug: Click here to enter text.
Dose: Click here to enter text.
Route: Click here to enter text.

29. Animal Use and Pain Distress. If you have indicated that **animals in your study will experience pain or distress**, even if it will be fully alleviated, please mark the appropriate check boxes below and fill in the requested information for each item marked.

You must conduct at least two (2) searches.

I have considered alternatives to the use of animals in my study. Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate. I have used the following methods and sources to search for alternatives:

Note: You may need to do more than one search per database to look for alternatives if there are multiple procedures that may cause pain and/or distress.

Database Used:

☒ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☐ **Other *(Specify)***: Click here to enter text.

Date of Search: Oct. 10, 2014
Years Covered: all years covered in Pubmed (1966-present)
Keywords Used (must include the word *alternative*): osteocyte, intracellular calcium, alternative
Number of Papers Found: 1

Discussion of the Relevancy of the Papers Found: Int Orthop. 2012 Oct;36(10):2181-7. doi: 10.1007/s00264-012-1590-x. Epub 2012 Jul 14. This paper focuses on a bone cancer cell line and is not relevant to our study.

Database Used:

☐ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☒ **Other *(Specify)***: Web of Science

Date of Search: Oct. 10, 2014

Years Covered: 1900-2014

Keywords Used (must include the word *alternative*): osteocyte, intracellular calcium, alternative

Number of Papers Found: none

Discussion of the Relevancy of the Papers Found: Click here to enter text.

30. Unnecessary Duplication of Work. Activities involving animals must not unnecessarily duplicate previous experiments performed by you or others. Provide a written narrative that assures that the activities of this project comply with this requirement and support this assurance by performing a literature search.

The search should return, at minimum, the related previous work from your laboratory.

You must conduct at least two (2) searches.

(NOT REQUIRED FOR TEACHING PROTOCOLS)

Note: You may need to do more than one search per database to look for duplication of work, especially if you are doing more than one experiment.

Database Used:

☐ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☒ **Other *(Specify)***: Web of science

Date of Search: Oct. 10, 2014

Years Covered: 1900-2014

Keywords Used: osteocyte, pericellular matrix, perlecan deficient

Number of Papers Found: 1

Discussion of the Relevancy of the Papers Found: This is the paper that Drs. Cindy Farach-Carson, Kirn-Safran and I published together showing the TEM evidence of altered PCM in perlecan deficient mice. Only one age group (12 month) was examined in the study. We propose to look at younger mice.

Database Used: ☒ **Medline** ☐ **Agricola** ☐ **Toxline** ☐ **CAB Abstracts** ☐ **Biosis** ☐ **Other *(Specify)***: Click here to enter text.
Date of Search: Oct. 10
Years Covered: all years
Keywords Used: osteocyte, pericellular matrix, perlecan deficient
Number of Papers Found: 2
Discussion of the Relevancy of the Papers Found: Both papers are from our groups. One is on TEM study of PCM and the other is on tracer velocimetry measurement of PCM density. Both studies only examined one age group (12 month).

31. What is the expected disposition of animals at the end of the experiments? ***(Check all that apply)***:
☒ Euthanized
☐ Maintained
☐ Released ***(Wildlife Only)***
☐ Other ***(Specify)***: Click here to enter text.

32. Euthanasia*
Select methods that will be used in case of emergency and/or at the end of the procedure/experiment. ***NOTE:** • Methods must be approved by the AVMA or must be scientifically justified. • A "Primary" and "Secondary" method must be selected (UD Double Kill Policy). • **If different methods will be used for different groups** of animals, indicate the group after the procedure (e.g., write "Neonates" after Decapitation, "Adults" after CO_2, "Terminal Surgery Animals" after Isoflurane Anesthesia Overdose, etc.).
☒ Animals will NOT be under anesthesia when euthanasia is performed.—For Dual-color FRAP study
☒ Animals will be under anesthesia when euthanasia is performed. ***(Check drug used below)***: For ex vivo calcium imaging study and live perfusion studies
☒ Isoflurane
☐ Injectable ***(Complete the following)***: Drug: Click here to enter text. Dose: Click here to enter text.

	Route: Click here to enter text.

PRIMARY method(s) of euthanasia

☒ CO_2 by compressed gas cylinder ***(Not for animals already under anesthesia or neonates)*** .—For Dual-color FRAP study

☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg ***(Check route below)***:

☐ IV ☐ IP ☐ IC

☐ Isoflurane Anesthesia Overdose - Inhalant

☒ Cervical Dislocation ***(only under anesthesia)*** For ex vivo calcium imaging study and live perfusion studies, where we want to maintain viability of the tissues and cells as long as possible under anesthesia without exposure to CO2.

☐ Decapitation ***(only under anesthesia or neonates)***

☐ Exsanguination or Perfusion ***(only under anesthesia)***

☐ Incision of Chest Cavity – Bilateral Pneumothorax ***(only under anesthesia)***

☐ Pithing – ***(only under anesthesia) (amphibians, reptiles only)***

☐ Removal of Vital Organ(s) ***(only under anesthesia) (Check all that apply):***

☐ Brain ☐ Kidneys

☐ Heart ☐ GI Tract

☐ Liver ☐ Lungs

☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.

☐ Other Method of Euthanasia: ***(Describe and Scientifically Justify)***:

SECONDARY method(s) of euthanasia that will be used to ensure that the animal does not survive:

☒ Cervical Dislocation
.—For Dual-color FRAP study

☐ Decapitation

☐ Exsanguination or Perfusion
☒ Incision of Chest Cavity – Bilateral Pneumothorax For ex vivo calcium imaging study and live perfusion studies
☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg ***(Check route below)***: ☐ IV ☐ IP ☐ IC
☐ Pithing – Double pithing required ***(fish, amphibians, reptiles only)***
☐ Removal of Vital Organ(s): ***(Check all that apply)***: ☐ Brain ☐ Kidneys ☐ Heart ☐ GI Tract ☐ Liver ☐ Lungs ☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.
☐ Other Method of Euthanasia: ***(Describe and Scientifically Justify)***: Click here to enter text.

University of Delaware
Institutional Animal Care and Use Committee

Application to Use Animals in Research and Teaching

ADDENDUM "E"
Terminal Surgery

AUP Number: 1149-2015-0	**← (4 digits only — if new, leave blank)**
Project: Alterations of osteocyte pericellular space and cellular responses in fragile bones	

1. Complete:

Species	Procedure	Number of Animals	Justification
a) mus musculus	Live perfusion	**40**	**10 males and 10 females of either perlecan deficient Hypo mice or WT mice will be perfused with either fixative for TEM imaging of PCM or a contrast agent for microCT imaging of vasculature trees in lower limbs**
b) Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
c) Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

2. Medications:

a. Pre-operative medications [drug; dose (mg/kg); route; frequency]:

NONE

b. Anesthesia [drug; dose (mg/kg); route; frequency]:

Inhalant isoflurane, initial 4-5% induction, 1.5-3.5% maintenance of anesthesia plane

3. Surgical Procedure: Non-survival perfusion

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

RECEIVED
OCT 5 2016
IACUC

Title of Protocol: Alterations of osteocyte pericellular space and cellular responses in fragile bones	
AUP Number: 1149-2016-2	← **(4 digits only)**
Principal Investigator: Liyun Wang	
Common Name: Mouse **Genus Species:** Mouse/Musculus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
☒ D	Procedure where pain or distress is alleviated by appropriate means (analgesics tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glenn Tallia, DVM

Date of Approval: 12/1/16

a. Briefly describe your non-survival surgical procedure:

For TEM imaging of PCM:

1. Mice will be under an adequate level of anesthesia, confirming with lack of pedal reflex
2. Open chest, insert pump needle (inlet for fixatives) into left ventricle and cut open right auricle (outlet for drainage);
3. pump fixatives of approximate 100 ml that contains blood substitutes (Perfluoro-compound FC-75), glutaraldehydes and/or paraformaldehyde, and anionic electron contrast ruthenium or terbium into heart for 30 min. Mice will be dead with fixative perfusion. The total time for the procedure including the preparation time will be approximately 45 min to 1 hr.
4. In addition to the fixative perfusion, cervical dislocation will be performed as secondary euthanasia.
5. Bone tissues (tibiae, femurs) will be harvested for EM

For Vasculature Perfusion:

The procedure is similar to the above perfusion fixative, except for replacing the fixatives with contrast agent. We will use a lead chromate-loaded silicon contrast agent (Microfil MV-117, Flow Tech Inc) at a rate of 1ml/min for ~15 min.

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Official Use Only

IACUC Approval Signature: Glenn Tallia, DVM

Date of Approval: 12/1/16

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances.  Signature of Principal Investigator 9/3/2016 Date

Rev. 10/13 #2

Aup1149 Amend

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Liyun Wang	
2. Shaopeng Pei	Shaopeng Pei
3. Shubo Wang	
4. Jerahme Martinez	
5. Suchi Parthasarathy	
6. Click here to enter text.	
7. Click here to enter text.	
8. Click here to enter text.	
9. Click here to enter text.	
10. Click here to enter text.	
11. Click here to enter text.	
12. Click here to enter text.	
13. Click here to enter text.	
14. Click here to enter text.	
15. Click here to enter text.	

IACUC approval of animal protocols must be renewed on an annual basis.

1. Previous Approval Date: 12/1/2015

Is Funding Source the same as on original, approved AUP?

☐ **Yes** **X No**

If no, please state Funding Source and Award Number: NIH MEEG322127

2. Record of Animal Use:

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Mouse	Mus musculus	200	160
2. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Protocol Status: *(Please indicate by check mark the status of project.)*

Request for Protocol Continuance:

☒ A. Active: Project ongoing

☐ B. Currently inactive: Project was initiated but is presently inactive

☐ C. Inactive: Project never initiated but anticipated starting date is:

Click here to enter text.

Request for Protocol Termination:

☐ D. Inactive: Project never initiated

☐ E. Completed: No further activities with animals will be done.

4. Project Personnel: Have there been any personnel changes since the last IACUC approval?

☐ Yes ☒No

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Victor GaoClick here to enter text.	12/1/2016
2. Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

   We have been testing ex vivo FRAP and intracellular calcium imaging using B6 controls. We have obtained successful results and are going to compare B6 and Hypo mice next year. We do not have any problems related to animal handling and the proposed procedures.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

   There are no adverse effects from the protocol.

RECEIVED
MAY 8 2015
IACUC JH

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

Title of Protocol: Transgenic Models of Skeletal and Metabolic Diseases	
AUP Number: 1204-2015-0	← (4 digits only — if new, leave blank)
Principal Investigator: Catherine B. Kirn-Safran	
Common Name: Mice **Genus Species:** Mus musculus	

Pain Category: *(please mark one)*

USDA PAIN CATEGORY: *(Note change of categories from previous form)*	
Category	**Description**
☐ B	Breeding or holding where NO research is conducted
☒ C	Procedure involving momentary or no pain or distress
☐ D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: _____

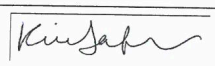
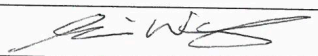
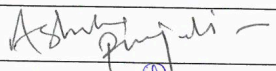
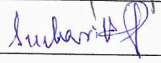
Date of Approval: 8/11/15

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. ***(Teaching Protocols Exempt)***
12. I understand that by signing, I agree to these assurances. Signature of Principal Investigator Date: 05-08-2015

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

Name	Signature	
1. Catherine Kirn-Safran		
2. Liyun Wang		
3. Ashutosh Parajuli		
4. Sucharitha Parthasarathi		
5. Click here to enter text.		
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11. Click here to enter text.		
12. Click here to enter text.		
13. Click here to enter text.		
14. Click here to enter text.		

The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked "NA" for "Not Applicable."

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

All answers are to be completed using Arial 12 size font.

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:

- Hybridoma/Monoclonal Antibodies ("B")
- Polyclonal Antibodies ("C")
- Survival Surgery ("D")
- Non-Survival Surgery ("E")
- Wildlife Research ("F")

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.

1. **Principal Investigator Information:**	
a. Name:	Catherine Kirn-Safran
b. University/Company:	University of Delaware
c. Department:	Biological Sciences
d. Building/Room:	Wolf 310
e. Office Phone:	302-831-3249
f. Lab Phone(s):	N/A
g. Home Phone:	302-836-4272
h. Mobile Phone:	302-981-8967
i. E-Mail Address:	ckirn@udel.edu

2. Protocol Status:

a. ☐ New Protocol ***OR*** ☒ Re-submission due to three (3) completed years.
If re-submission, enter Protocol Number: 1204

b. ☒ Research ***OR*** ☐ Teaching

c. ☒ Laboratory Animals ***OR*** ☐ Wildlife
If "Wildlife" please complete Addendum "F"

d. Proposed Start Date: July 1, 2015

e. Proposed Completion Date: ongoing (standard breeding protocol)

f. Funding Source: MEEG322127

g. Award Number: NIH R01 (PI: Liyun Wang; Investigator: Catherine Kirn-Safran)

3. Personnel involved in Protocol *(Include Principal Investigator):*

***Status*:** Indicate Prof, Post-Doc, Grad Student, Lab Manager, Research Assistant, Technician, etc.

***Qualifications*:** Include **procedures this person is proficient in performing** on proposed species and the time they have been doing the procedure.
Be specific (e.g. sub-mandibular bleeding on mice-2yrs, performing castrations on mice and rats-1yr, tail-vein injections on mice-2yrs, etc.) **(If no experience, list who will train.)**

***Responsibilities*:** Include **all responsibilities** this person will have with live animals on this protocol, including euthanizing animals.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
a. Catherine Kirn-Safran	ckirn@udel.edu	302-831-3249	302-981-8967	×	Click here to enter text.

Status: Investigator, faculty advisor, UD Research Assistant Professor Dept. Biological Sciences

Qualifications: Dr. Kirn-Safran has over 18 years of experience with mouse handling, breeding, and mineralized tissue collection for organ culture or histological processing; over 10 years with administration of hormones, drug, or anesthetic intraperitoneally, and 6 years with animal surgeries under anesthesia. Dr. Kirn-Safran has been studying effects of genetic mutations, growth factors, and hormones on bone/cartilage physiology and embryonic development in mouse models for over 18 years.

Responsibilities: Dr. Kirn-Safran will design, perform, and supervise personnel. Dr. Kirn-Safran will comply with any training program required by the OLAM for her staff.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
b. Liyun Wang	lywang@udel.edu	X2659	(302) 453-8372	×	Click here to enter text.

Status: Investigator, Faculty advisor, UD Associate Professor Dept. of Mechanical Engineering

Qualifications: Dr. Wang has more than 15 year experience with rodents, including live animal loading, small surgeries, IP IV injections and tissue harvesting.

Responsibilities: Dr. Wang will train her research staff for participation in these breeding

procedures and co-supervise with Dr. Kirn-Safran personnel for completion of the proposed studies.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
c. Ashutosh Parajuli	parajuli@udel.edu	831-376-4773	N/A	×	Click here to enter text.

Status: Graduate student

Qualifications: Perform research studies under the supervision of Dr. Liyun Wang. Two year experience with handling mice and collecting various tissues including bones from euthanized animals.

Responsibilities: Maintains the animal colony, cross animals for breeding and time pregnancies, sacrifice animals, and collect tissues from both adult mice and embryos.

Name	E-mail	Office Phone Number	Home/Cell Phone Number	Received Animal Facility Training	
				Yes	No
d. Sucharitha Parthasarathi	psuch@udel.edu	N/A	302-690-2331	Click here to enter text.	Click here to enter text.

Status: Graduate student

Qualifications: Perform research studies under the supervision of Dr. Catherine Kirn-Safran. Eight months experience with handling mice and collecting various tissues including bones from euthanized animals.

Responsibilities: Maintains the animal colony, cross animals for breeding and time pregnancies, sacrifice animals, and collect tissues from both adult mice and embryos. Training will be provided by animal facility personnel and Dr. Catherine Kirn-Safran

4. Non-Scientific Summary: In language understandable to a ***high-school senior, very briefly describe*** the goals and significance of this study.

a. Specific Scientific Goals: This project serves several projects in our laboratory that all look at various aspects of bone and cartilage cell function. Perlecan/Hspg2 mutant (C1532Yneo line) mice are affected by a mild short stature and skeletal bone phenotype (Rodgers et al., 2007) and were obtained in the past through a collaboration with Dr. Kathryn Rodgers. In addition, they exhibit reduced fiber density in the non-mineral compartment of their bones, a property which attenuates bone's anabolic/catabolic responses to mechanical loading/unloading. The balance between bone apposition and bone resorption will be assessed using a serological approach by comparing the levels of bone formation and degradation markers (i.e., osteocalcin, tartrate-resistant acid phosphatase) in the C1532Yneo line vs. C57BL6/J controls. Bone tissues will be harvested from different anatomical locations including the limbs, the vertebral column and the skull for comparative gene expression studies.

b. Significance of this Research (including the possible benefits to human and/or animal health, the advancement of scientific knowledge, or the betterment of society): The proposed work will provide a molecular basis for bone mechanosensation mechanisms which, in the long term, will serve to elaborate new therapeutic approaches for the treatment of patients with osteoporosis and bone lesions resulting from disuse

5. Experimental Design: Explain the experimental design. This description should allow the IACUC to understand fully the experimental course of an animal or group of animals from its entry into the experiment to the endpoint of the study.

The inclusion of flow charts, diagrams, and/or tables are greatly encouraged to explain experimental design or sequential events.

Be sure to include all animal events and related details, i.e.,

- **All Procedures**-bleedings, injections, identification methods, genotyping methods, surgical procedures, euthanasia, etc.
- **Procedural details**–number of animals involved in procedure, approximate animal weight, if relevant (for injections, bleeding, etc.), route, frequency, volume, etc.
- **Pharmaceutical-grade and non-pharmaceutical grade compounds** – Identify any drugs, biologics, or reagents that will be administered to animals. If these agents are not human or veterinary pharmaceutical-grade substances, provide a scientific justification for their use and describe methods that will be used to ensure appropriate preparation.
- **Names of surgical procedures** (but reserve the surgical details for the proper Surgical Addenda)
- **Monitoring**–observations, measurements (animal weight, tumor size, etc.)
- **Monitoring details**–criteria, frequency, names of personnel monitoring, conditions for removing an animal from the study, etc.
- **Endpoints**–include endpoints for the animals/study and how will they be determined.

(Describe): Click here to enter text.

i. **Design 1) Natural breeding** will be performed to maintain the perlecan (C1532Yneo) mutant line on the C57BL6/J background and generate age-matched controls of the same background. The C1532Yneo line was created at the University of Pennsylvania and is not commercially available.

ii. **Design 2) Tail biopsies** Genotypes of mutant mice will be determined by DNA analysis of tail tissue. Tail biopsies will be performed on mice at three weeks of age. Sampling will be done by OLAM staff according to SOP #PRO-009. If for any reason a second biopsy is required, it will be done under isoflurane anesthesia.

iii. **Design 3) Dissection of embryonic, postnatal, and adult mouse bones and control organs.** Mice will be euthanized by CO_2 asphyxiation followed by cervical dislocation to collect bone and other organs for macroscopic and microscopic analyses. Such analyses will include micro-computed tomography (microCT) for assessment of bone mineral density parameters as well as determination of molecular signature using RNA and protein extraction approaches. Bone and control organs will be mostly collected after weaning age. Isolation of primary bone and marrow cell lines will require the use of embryonic and postnatal tissues. In this case, the female if still pregnant will be sacrificed prior to pup collection from the uterine horns. Embryos or pups (age≤10 days) will be rapidly decapitated with sharp scissors in one stroke and their skull bones or long bones harvested for *in vitro* culture or specimen storage.

Design 4) Collection of blood. Blood will be collected from C1532Yneo animals after weaning age through retro-orbital route by OLAM staff (SOP #PRO-001) to investigate the presence of markers of bone formation and resorption in serum. As described in and SOP #PRO-006, no more than 10% of the mouse body weight will be collected every other week according to the NIH guidelines for survival bleeding of mice: http://oacu.od.nih.gov/ARAC/documents/Rodent_Bleeding.pdf. A maximum of four retro-orbital bleeds will be performed during the life of an animal. Because repeated retro-orbital bleeds can easily induce periorbital infection and permanent damage to the eye, sampling from the superficial temporal vein (mandibular) will be performed if more than four bleeds are needed (SOP #PRO-001). The bleeding will alternate between the right and the left eyes, and there will be at least two weeks between blood collection on the same eye.

REFINEMENT, REDUCTION & REPLACEMENT

When using animals for research, it is important to consider the three Rs: reduction, refinement, and replacement to reduce both animal distress and the number of animals used in the laboratory.

Reduction: Minimizing the number of animals used
Refinement: Using techniques and procedures to reduce pain and distress
Replacement: Using non-animal methods or lower phylogenetic organisms

6. Justification for the Use of Animals (instead of *in vitro* methods)

(Check all that apply and explain):

a. ☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems: ***(Explain)***: It is necessary to use animals because the tissue architecture of the bone and the organization of the different responsive cell types can only be found in animals not in dissociated cell cultures

b. ☒ There is not enough information known about the processes being studied to design non-living models: ***(Explain)***: The sequence of molecular events leading to osteoarthritis and osteoporosis are unknown and cannot be recapitulated in non-living systems.

c. ☐ Other: ***(Explain)***: Click here to enter text.

7. Justification for Species Appropriateness:
(Check all that apply and explain):

a. ☐ A large database exists, allowing comparisons with previous data: ***(Explain)***: Click here to enter text.

b. ☐ The anatomy or physiology is uniquely suited to the study proposed: ***(Explain)***: Click here to enter text.

c. ☒ This is the lowest species on the phylogenic scale suitable to the proposed study: ***(Explain)***: Mouse is the lowest phylogenic species that has bones similar to humans. Availability of transgenic lines in mice provides the opportunity to gain insight into the function of individual genes in bone and cartilage physiology.

d. ☐ Other: ***(Explain)***: Click here to enter text.

8. Justification for Number of Animals Requested: (Note: numbers should include animals used for breeding and all animals born)

a. ☐ Pilot study or preliminary project where group variances are unknown at the present time. Describe the information used to estimate how many animals will be needed: (Only a limited number of animals will be permitted.)
(Explain): Click here to enter text.

b. ☐ Group sizes are determined statistically. Describe the statistical analysis used to estimate the number (N) of animals needed: N may be estimated from a power analysis for the most important measurement in the study, usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power (e.g. $P < 0.05$). Data analysis methods should not be submitted unless directly applicable to the estimate of N.

An online calculator may be found at: http://www.math.uiowa.edu/~rlenth/Power/
or a stand-alone calculator that can be downloaded from
http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3

(Explain): Click here to enter text.

c. ☐ Group sizes are based on the quantity of harvested cells or the amount of tissue required for *in vitro* studies. Explain how much tissue is needed based on the number of experiments to be conducted and the amount of tissue you expect to obtain from each animal (e.g., 10g of tissues are needed: Each animal can provide 2g. 10g /2g per animal = 5 animals needed.) ***(Explain)***: Click here to enter text.

d. ☐ Teaching protocol. Specify the number of students in the class, the student to animal ratio and how that ratio was determined: Animal numbers should be minimized to the fullest extent possible without compromising the quality of the hands-on teaching experience for students or the health and welfare of the animals. ***(Explain)***: Click here to enter text.

e. ☐ Study involving feral or wild animals. Animals will be captured and released in an attempt to maximize the sample size within logistical constraints. Describe the process by which you estimate these numbers and estimate the precision needed: ***(Explain)***: Click here to enter text.

f. ☐ Observational, non-manipulative study. Animals will not be captured, their behavior will not be interfered with, and exact animal numbers cannot be predicted: ***(Explain)***: Click here to enter text.

g. ☐ Product testing. The number of animals needed is based on FDA guidelines. Provide the citation from the regulations, the IND tracking number, or relevant FDA correspondence: ***(Explain)***: Click here to enter text.

h. ☒ Other. Elaborate, indicating the method used to determine the group size. ***(Explain)***: Click here to enter text.

Mice of the C57BL6/J inbred strains will be used to generate perlecan mutant and control bone/cartilage specimens. Thus, at all time we will maintain sexually mature mutant animals of each line. The C1532Yneo line is not commercially available. Thus, this line has to be bred in-house. After six months, breeding pairs are retired and replaced by new breeders.

C1532Yneo line: This line will be used to generate bone tissue to compate the gene expression and response to load of this line relative to WT controls. The C1532Yneo line may be maintained through homozygous breeding. We observed, however, a 50% rate of perinatal death due to subfertility in C1532Yneo females as a result of short stature and small litter size. Hence, this line will require maintenance either through the breeding of mutant male to heterozygous females or through fostering of double mutant pups with C57BL6/J control females. An average of 5 stud males and 20 breeding females will be maintained year round. Since we will replace breeders after six months, we will need 10 males and 40 females/year for breeding purpose and approximately 50 mutant males for experimental osteoporosis/aging experiments. In summary, we will need 50×3 years + 50 male=**200 animals** for the 3-year period for the C1532Yneo line. Except for the initial breeders (5 males and 20 females= 25 breeders) already housed in OLAM, all the animals requested will consists of pups being born in OLAM: 175 pups.

C57BL6/J controls: An average of 2 stud males and 6 breeding females will be maintained year round. Since we will replace breeders after six months, we will need to purchase from the vendor 4 males and 12 females/year for fostering purpose. In summary, we will need 16×3 years=**48 animals** for the 3-year period for the C57BL6/J control line.

Total number of animals requested for the 3-year period is:
200+48=248 animals

9. Animals Requested:

Common Name	Genus and Species	Total Number of Animals for Three Years
1. Mouse	Mus musculus	248 (including 175 pups)
2. Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.

10. Where will animals be housed (or captured for wildlife)? OLAM

11. Where will the experiments take place?

If animals must be removed from the vivarium, please describe how they will be transported (such as hand carried in covered microisolator cages). OLAM

12. Will any animals be humanely killed, without treatment or manipulations, to be used to obtain tissue, cells, etc.? ☒ Yes ☐ No

If **Yes,** list types of tissue, etc: Calvaria and limbs for newborn and embryos. Spleen, vertebral columns (axial skeleton), and long bones for adult animals

13. Physiological Measurements ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

14. Dietary Manipulations ☐ Yes ☒ No

If **Yes,** list and explain (Note: if food or fluid will be restricted, describe method for assessing the health and wellbeing of the animals. Body weights must be recorded at least weekly. Amount earned (if animals work for food or fluid) during testing and amount freely given must be recorded. A scientific justification must be provided for departures from the recommendations of the Guide.) Click here to enter text.

15. Environmental Stress (e.g. cold, restraint, forced exercise) ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

16. Trauma or Burn Injury ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

17. Production of Hybridoma/Monoclonal Antibodies ☐ Yes ☒ No

If **Yes,** please complete Addendum "**B**".

18. Production of Polyclonal Antibodies ☐ Yes ☒ No

If **Yes,** please complete Addendum "**C**".

19. Administration of Hazardous Chemicals, Drugs, Toxins, or Nanoparticles

☐ Yes CAS#_____ ☒ No

If **Yes,** describe hazards posed to personnel: Click here to enter text.

Methods to control exposure: Click here to enter text.

Methods of Disposal of Animals and Bedding: Click here to enter text.

20. Administration of radioactive materials ☐ Yes ☒ No

a. Type to be used. Include radioisotope(s) and chemical form(s): Click here to enter text.

b. Describe the practices and procedures to be followed for minimization of radiation exposure to workers and for the handling and disposal of contaminated materials associated with this study:

(Include the methods for management of radioactive wastes and monitoring facility for radioactive contamination, if applicable.) *N/A*

c. Who will be responsible for the daily care of animals containing radioactive materials?

N/A

d. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

N/A

Please attach a copy of any approvals or provide the approval number.

Click here to enter text.

21. Study of Irradiation *in vivo*? ☐ Yes (gamma irradiator? ☐ or x-ray irradiator? ☐)
☒ No

a. Make, model, and location of irradiator to be used:
Click here to enter text.

b. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number.N/A

22. Administration of Biological Agents (eg microorganisms, recombinant DNA, **HUMAN** serum, tissue, cell lines, etc.) ☐ Yes ☒ No

Animal Biosafety Level ☐ 1 ☐ 2 ☐ 3 ☐ 4

Describe hazards posed to personnel:Click here to enter text.

Methods to control exposure:Click here to enter text.

Methods of Disposal of Animals and Bedding:Click here to enter text.

Approval received from UD- Institutional Biosafety Committee? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number. Click here to enter text.

23.Will tumor cells, tissue, sera, viral vectors or other biologics of RODENT origin – other than those isolated from rodents already housed in the facility – be administered to animals?

☐ Yes ☐ No

If Yes, this material must be tested for rodent pathogens and test results must be attached (Please contact the Attending Veterinarian for details). Click here to enter text.

24.Use of Genetically Engineered Animals

☒ Yes ☐ No

If Yes, please describe any anticipated phenotypes that may cause pain or distress and any special care or monitoring that the animals will require.

Occasional skeletal malformation but generally short-stature phenotype, some young females may have difficulties at the time of delivery

Does the proposed work involve creating new genetically modified animals, or involve crossing two genetically modified animals to produce offspring with a new genotype.

☐ Yes ☒ No

Approval received from UD- Institutional Biosafety Committee?

☐ Yes ☐ No ☐ Pending ☐ Exempt (breeding of two lines of genetically-modified rodents is exempt if 1) both parents can be housed under BL1 containment and 2) neither parent strain incorporates more than one half of the genome of an exogenous eukaryotic virus or incorporates a transgene under the control of a gammaretroviral long terminal repeat and 3)the rodent that results from the breeding is not expected to contain more than one half of an exogenous viral genome)

Please attach a copy of any approvals or provide the approval number.

_____Click here to enter text._____

25. Special Study Requirements: Please describe any special study requirements such as a requirement for single housing of the animals, exemption from environmental enrichment, or special caging:N/A

26. Any other procedures? ☐ Yes ☒ No

If **Yes,** explain: Click here to enter text.

27. Will this study involve surgery? ☐ Yes ☒ No

If **Yes,** and it is "Survival Surgery," please complete Addendum "**D**".

If **Yes,** and it is "Terminal Surgery," please complete Addendum "**E**".

28. Will any animal undergo anesthesia for any reason other than surgery? × Yes ☐ No

If **Yes**,

a. List Procedures and Reason(s) for using anesthesia: if tail biopsy has to be repeated or may be used for retroorbital bleeds.

b. Check the type of anesthesia to be used.

☒ Isoflurane

☐ Injectable ***(For injectable, complete the following):***

Drug: Click here to enter text.
Dose: Click here to enter text.
Route: Click here to enter text.

29. Animal Use and Pain Distress. If you have indicated that **animals in your study will experience pain or distress**, even if it will be fully alleviated, please mark the appropriate check boxes below and fill in the requested information for each item marked.

You must conduct at least two (2) searches.

I have considered alternatives to the use of animals in my study. Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate. I have used the following methods and sources to search for alternatives:

Note: You may need to do more than one search per database to look for alternatives if there are multiple procedures that may cause pain and/or distress.

Database Used:

☐ **Medline** ☐ **Agricola**

☐ **Toxline** ☐ **CAB Abstracts**

☐ **Biosis** ☐ **Other *(Specify)*:** Click here to enter text.

Date of Search: Click here to enter text.

Years Covered: Click here to enter text.

Keywords Used (must include the word *alternative*): Click here to enter text.

Number of Papers Found: Click here to enter text.

Discussion of the Relevancy of the Papers Found: Click here to enter text.

Database Used:

☐ **Medline** ☐ **Agricola**

☐ **Toxline** ☐ **CAB Abstracts**

☐ **Biosis** ☐ **Other *(Specify)*:** Click here to enter text.

Date of Search: Click here to enter text.

Years Covered: Click here to enter text.

Keywords Used (must include the word *alternative*): Click here to enter text.

Number of Papers Found: Click here to enter text.

Discussion of the Relevancy of the Papers Found: Click here to enter text.

30. Unnecessary Duplication of Work. Activities involving animals must not unnecessarily duplicate previous experiments performed by you or others. Provide a written narrative that assures that the activities of this project comply with this requirement and support this assurance by performing a literature search.

The search should return, at minimum, the related previous work from your laboratory.

You must conduct at least two (2) searches.

(NOT REQUIRED FOR TEACHING PROTOCOLS)

Note: You may need to do more than one search per database to look for duplication of work, especially if you are doing more than one experiment.

Database Used:

☒ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☐ **Other *(Specify)***: Click here to enter text.

Date of Search: 5/08/15

Years Covered: 2012 (last 3 year renewal)-today

Keywords Used: search: Hspg2 and bone:11 hits

Number of Papers Found: 1 and 11

Discussion of the Relevancy of the Papers Found:
Two papers are from our group. None of the other papers represent an overlap with our current research.Two papers are review articles including one from one of our collaborator in Texas, Dr. Farach-Carson. One paper reports the bone phenotype of human patients carrying a rare variant of HSPG2. Two papers are on neovascularization and cancer research studies and use HSPG2 as a marker for cancer metastasis to bone. One paper studies the effect of prolactin (a protein produced during pregnancy) on the expression of Hspg2 in an established clonal cell line (ATDC5) different from the primary cell culture system proposed in our studies. Two other papers focus on other musculoskeletal pathologies: knee arthritis and tendinopathy.

Database Used:

☐ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☒ **Biosis**	☐ **Other *(Specify)***: Click here to enter text.

Date of Search: 5/08/15

Years Covered: 2012 (last 3 year renewal)-today

Keywords Used: Search: Hspg2 and bone: 13 hits

Number of Papers Found: 8 + 13

Discussion of the Relevancy of the Papers Found: Click here to enter text.
There is no overlap between the 13 papers found and the studies proposed in our R01 grant and covered under this animal protocol. Out of the 13 papers, 3 are our reports on the biology of perlecan in mouse skeletal tissues, 2 are cell studies, 2 are reviews, 1 is on idiopathic scoliosis in a human

variant of HSPG2, 1 is on HSPG2 and prostate cancer in men, and two are on other musculoskeletal disorders including tendinopathy.

31. What is the expected disposition of animals at the end of the experiments?
(Check all that apply):

☒ Euthanized

☐ Maintained

☐ Released ***(Wildlife Only)***

☐ Other ***(Specify)***: Click here to enter text.

32. Euthanasia*

Select methods that will be used in case of emergency and/or at the end of the procedure/experiment.

***NOTE:**

- Methods must be approved by the AVMA or must be scientifically justified.
- A "Primary" and "Secondary" method must be selected (UD Double Kill Policy).
- **If different methods will be used for different groups** of animals, indicate the group after the procedure (e.g., write "Neonates" after Decapitation, "Adults" after CO_2, "Terminal Surgery Animals" after Isoflurane Anesthesia Overdose, etc.).

☒ Animals will NOT be under anesthesia when euthanasia is performed.

☐ Animals will be under anesthesia when euthanasia is performed. ***(Check drug used below)***:

☐ Isoflurane

☐ Injectable ***(Complete the following)***:

Drug: Click here to enter text.

Dose: Click here to enter text.

Route: Click here to enter text.

PRIMARY method(s) of euthanasia

☒ CO_2 by compressed gas cylinder ***(Not for animals already under anesthesia or neonates)***

☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg ***(Check route below)***:

☐ IV ☐ IP ☐ IC

☐ Isoflurane Anesthesia Overdose - Inhalant

☐ Cervical Dislocation ***(only under anesthesia)***

☐ Decapitation ***(only under anesthesia or neonates)***

☐ Exsanguination or Perfusion ***(only under anesthesia)***

☐ Incision of Chest Cavity – Bilateral Pneumothorax ***(only under anesthesia)***

☐ Pithing – ***(only under anesthesia) (amphibians, reptiles only)***

☐ Removal of Vital Organ(s) ***(only under anesthesia) (Check all that apply):***

☐ Brain ☐ Kidneys

☐ Heart ☐ GI Tract

☐ Liver ☐ Lungs

☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.

☐ Other Method of Euthanasia: ***(Describe and Scientifically Justify)***:

SECONDARY method(s) of euthanasia that will be used to ensure that the animal does not survive:

☒ Cervical Dislocation

☐ Decapitation

☐ Exsanguination or Perfusion

☐ Incision of Chest Cavity – Bilateral Pneumothorax

☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg ***(Check route below)***:
☐ IV ☐ IP ☐ IC

☐ Pithing – Double pithing required ***(fish, amphibians, reptiles only)***

☐ Removal of Vital Organ(s): ***(Check all that apply)***:

☐ Brain ☐ Kidneys

☐ Heart ☐ GI Tract

☐ Liver ☐ Lungs

☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.

☐ Other Method of Euthanasia: ***(Describe and Scientifically Justify)***: Neonates will be euthanized by decapitation