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Animal Models for the Study of Bone Derived Pain

Austen L. Thompson, Tally M. Largent-Milnes, Todd W. Vanderah

University of Arizona, Department of Pharmacology College of Medicine USA.

Abstract

Bone pain is a prevalent issue in society today and also is one of the hardest types of pain to control. Pain originating in the bone can be caused by many different entities including metastatic and primary neoplasm, fracture, osteoarthritis as well as numerous other metabolic disorders. In this chapter we describe the methods and protocols that currently are accepted and validated for the study of bone pain in models of metastatic cancer, bicortical fracture and osteoarthritis. These animal models provide invaluable information as to the nature of bone pain and give rise to potential new targets for its treatment and management.

Keywords

Bone pain; Cancer pain; Osteoarthritis; Fracture; Metastatic disease

1. Introduction

Bone derived pain is a common complaint amongst patients with a variety of conditions ranging from simple fractures to more serious conditions like metastatic cancer, which affect a large number of people around the world and across age groups.[1–3] Bone pain is also notoriously difficult to control. Mild pain from disorders like osteoarthritis can be relatively controlled with pharmacological interventions such as nonsteroidal anti-inflammatory agents (NSAIDs) or acetaminophen. Chronic use of NSAIDs can have severe gastrointestinal, cardiovascular, renal, hemostatic and hepatic side effects, especially in patients with other co-morbidities such as diabetes, cardiovascular disease or hematological disorders. More severe, persistent pain, as well as breakthrough pain, from primary and metastatic cancers are often treated with potent opiate narcotics. Opiate narcotics have many negative side effects, especially long term, such as constipation, pruritus, nausea, sedation, respiratory depression and opioid-induced hypersensitivity. Additionally, the potential for abuse, which has become of extreme concern in recent years, is great.[4] Moreover, there are very few studies on how chronic opioids influence bone integrity [5, 6]; preclinical studies have demonstrated that chronic opiates may negatively affect bone. Thus, the ability to generate appropriate models for bone pain is paramount to understanding how to properly treat these vast arrays of disorders. Over the last 20 years, many independent and collaborative groups

have worked to develop well-defined and highly translational animal models for bone pain disorders such as metastatic cancer, osteoarthritis, and fracture.

Pain originating from cancer metastasizing to the bone is excruciating and a difficult problem to manage through standard clinical and pharmacological methods. Although any cancer can theoretically metastasize to the bone, carcinomas and sarcomas of the breast, lung, prostate, thyroid and kidney are the most common clinically seen.[7] Metastatic animal cancer models originally utilized an intra-cardiac injection of cancerous cells that would disseminate hematogenously to the bone.[8] This strategy of cancer induction was positive in the fact that it mimicked the route of metastasis that normally occurs during natural disease progression. However, this model was not ideal for studying cancer pain due to the high variability of the tumor burden and location between mice, as well as resulting in a very ill animal that could not clearly display pain behavior due to metastatic bone cancer. Models improved when direct, intramedullary injection of cancer cells were performed in a mouse model as this allowed for consistent delivery of a defined tumor burden into a specific location in the animal.[9, 10] This model proved superior to the intra-cardiac model due to the fact that it localized the tumor in a reproducible fashion and it limited the confounding of pain data by limiting the involvement of potential secondary metastasis and soft tissue invasion and destruction. This model resulted in clear behavioral signs of pain of the inoculated limb and allowed for comparison to the non-inoculated limb. The pain behaviors that typically were seen and tested in this model included spontaneous pain (flinching and guarding) as well as tactile/mechanical allodynia. This model was expanded to a rat model, which was beneficial due to the larger species size for tissue collection and imaging compared to the mouse.[11] A limitation of the rat model from an experimental design standpoint is the significant lack of techniques for genetic manipulation when compared to mouse models, for mechanistic investigations. The intra-bone cancer metastasis model has been performed in both syngeneic and non-syngeneic models of cancers. The benefits and limitations of a syngeneic versus a non-syngeneic model are discussed at length in a review by Slosky et al.[12] In short, the non-syngeneic model is one in which tumor cells from another species were implanted in an immune-deficient animal; allowing for the study of human neoplasms within the bone microenvironment. However, this non-syngeneic model limited the study of how the tumor-immune system interacted in the development of pain, tumor burden and bone integrity. In the syngeneic models, tumor cells were chosen and implanted based on the species and strain of the animal model being used. This proved superior since this allowed for the study of how the immune system interacted with the tumor in regulating its proliferation, local inflammation, osteoclasts and osteoblasts and all their contribution to cancer-induced bone pain. Using these rodent models of metastatic bone cancer pain, mechanisms of cancer induced bone pain (CIBP) have been more elucidated and novel potential therapies for the management of CIBP have been identified.

Fracture is one of the more common causes of acute bone pain. The severity of fracture as well as improper fixation leading to non-union or mal-union can cause severe, chronic pain. Orthopedic surgeries for fracture fixation affect patients of all ages that experience severe trauma, and these procedures disproportionately affect the ever-increasing aging population. [13, 14] The study of fracture pain is of utmost importance since pain control is the major factor that allows for loading of the bone, which is necessary for participation in physical

rehabilitation. If this pain is improperly controlled, especially in the elderly, rehabilitation is hindered, which can lead to altered healing or non-union of fracture. This can lead to atrophy of the affected limb and eventual development of chronic musculoskeletal pain further reducing patient quality of life.[15, 16] A model of a controlled, bicortical fracture was developed that allows for the study of fracture pain during the healing process. This model has also proven useful to study the biology of fracture healing. The pain behaviors that are typically tested in this model are similar to those in the metastatic cancer model (see above).

Osteoarthritis is a chronic disorder that arises from the progressive loss of articular cartilage within the joint leading to bone articulating with bone causing pain. Clinically, it is associated with new bone formations creating intraarticular osteophyte formation, loss of joint function and immense pain.[17] The pain associated with osteoarthritis is typically described as dull and aching chronically with intermittent breakthrough bouts of intense pain. Osteoarthritis is one of the major causes of disability, especially in the aging population, as well as one of the most common musculoskeletal disorders.[18]

The monosodium iodoacetate (MIA) model was first described by Kalbhen in 1987, a single, intraarticular injection of monosodium iodoacetate is placed into the joint. [19] MIA is an inhibitor of glycolysis, which disrupts the normal metabolism of chondrocytes and this leads to the degeneration of chondrocytes. The degeneration of chondrocytes leads to articular cartilage degeneration that inevitably leads to similar histopathology that is seen within the joints of osteoarthritic patients. Intra-articular injections of MIA have been shown to decrease weight bearing on the affected limb, movement-evoked pain, allodynia and hyperalgesia.[20, 21] Although this model does lead to a transient inflammatory response within the joint that isn't typically characteristic of osteoarthritis, the inflammation is typically resolved by day 7 and is not thought to play a role in the pain behavior that is observed. This model has the advantage that it is rather easily induced and reproducible as compared to the ligamentous transection and meniscal disruption models. The critique of this model as compared to the traumatic models is an artificial destruction of the articular cartilage that is not typical of the clinical disease, while the ligamentous disruption models are more typical of the clinical history of patients with osteoarthritis.

There have been multiple different models that have been outlined for induction of osteoarthritis through ligamentous disruption in rodent models. In humans, anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) injuries are common causes of osteoarthritis.[3] Therefore, models have been developed in which the ACL and PCL are surgically disrupted, which leads to the development of osteoarthritic pain symptoms as well as histopathology in a murine model.[22] This model demonstrates that we can induce osteoarthritis in a manner that follows the natural course of disease progression similar to one of the most common causes of human knee osteoarthritis.

2. Materials

2.1 Metastatic Model of Bone Cancer Pain

1. First, one must determine the cell lines and whether the model is a syngeneic or non-syngeneic model. In order to study human cancer cell lines a non-syngeneic model must be used. These models consist of utilizing immunodeficient mice (e.g. SCID mice) that will allow the tumor cells to survive and proliferate within the intramedullary space.

Hereon will describe methods based on a syngeneic model of metastatic breast cancer using BALB/c mice (Envigo) and 66.1 breast adenocarcinoma cells (American Type Culture Collection, representing breast cancer metastasis to bone). For other cell culture conditions, please see Slosky et al. for the complete list of metastatic cancer models in mouse and rat and see the specific references cited within the review for each model.[12]

2. Cultured 66.1 cells between passage 10–20
 - a. Media requirements: Minimum Essential Medium (MEM) enriched with 10% Fetal Bovine Serum (v/v) and 1% Penicillin-Streptomycin (v/v) (see Note 1)
3. BALB/c mice (3 months, 18–20g)
4. Faxitron (UltraFocus DXA) used for acquiring plain x-ray films as well as for DXA images for analysis of bone quality.
5. Vision DXA (UltraFocus) image acquiring software
6. 7mm or 9mm stainless steel wound clips
7. 10–25µL Microinjector (Hamilton Company Inc.) (see Note 2)
8. 28-gauge micro-injector needle (PlasticsOne)
9. MicroLab 350/450 Dental Drill (RAM Products, Inc.)
10. 0.45 – 0.6 mm drill bit for femoral reaming (Stoelting Co). Smaller size used for mouse femur, larger sized used for rat.
11. Gentamycin Sulfate (0.8mg/mL) used for infection prophylaxis. (see Note 3)

¹-Cell Culture: 66.1 cells have media replaced every 3–4 days and are passaged every 7–8 days. When placed into a new flask, the cells are diluted 1:10 and incubated at 37°C with 5% CO₂. During passaging, trypsin EDTA is allowed to rise to room temperature without being placed into incubator or warm water bath and cells are washed briefly with 4 mL of trypsin EDTA. Then, 2 mL trypsin EDTA is placed onto the cells and the flask is placed into the incubator for 10–15 minutes or until cells fully detach from the culture flask bottom. On day of harvest for injection, cells are released from the flask bottom using trypsin as above. The cells are then collected in 10 mL of media and centrifuged for 4 minutes at 2000 RPM. The media is then removed and the pellet is washed with OPTI-MEM, then centrifuged again for 4 minutes at 2000 RPM. Cells are then suspended 10 mL of media and counted by hemocytometer. The cells are then diluted to the desired concentration for implantation into the mouse.

²-The microinjector is attached to a piece of plastic tubing to which the 28 gauge injection needle is then attached. This is done to prevent the cells or media from getting into the microinjector (28g needle will not shear stress the cells, smaller gages or larger cells may need adaption of needle up to 26 g). The tubing is color coded to denote whether it was used for media or the cancer cells and the same tube is used for each surgery. Before every surgery, test the microinjector and tubing for its ability to properly draw and expel liquid to ensure that there are no issues with the needle or tubing as they are prone to blockage and tearing, respectively.

³-Gentamycin injection is done following anesthesia induction as a 10 mL/kg subcutaneous bolus.

12. Veterinary grade sterile saline used for the solubilizing of ketamine/xylazine as well as the dilution of ketamine/xylazine and gentamycin sulfate from stock solutions. Also, used for pre-operative fluid bolus. (see Note 4)
13. Bone cement or dental amalgam
14. Acrylic used for the activation of the dry powdered bone cement/dental amalgam to be used as a plug for the injection site of the cancer cells.
15. Ketamine/xylazine (8.0 mg/kg and 1.2mg/kg, respectively) for anesthesia. Store at 4°C. Must not be kept active for longer than 6 months. Store the anesthetic in an opaque vial as the ketamine is UV sensitive. Xylazine is temperature sensitive and mix must be kept on ice when out of the refrigerator.

2.2 Fracture Induced Pain

1. 0.011-inch-diameter and 11-mm-length stainless steel wire (Small Parts Inc.)
2. Dental amalgam
3. Bone Cement (Stryker)
4. 3-point bending device (BbC Specialty Automotive Center)

2.3 MIA Induced Osteoarthritis

1. Monosodium acetate (Sigma-Aldrich)
2. 26-gauge needle for injection [23]

2.4 Cruciate Ligament Transection

1. Bvi Beaver EdgeAhead Safety knife Sideport MVR 0.90 mm 20G microsurgical scalpel (Beaver-Visitec International, Inc.)
2. Surgical microscope (Leica LZ-6, Leica Microsystems Inc.)

3. Methods

3.1 Behavioral Testing in mice

1. Flinching and Guarding (Spontaneous Pain)
 - a. Animals are placed into small, raised plexiglass cages with wire mesh bottom and allowed to acclimate to the cage for at least 30 minutes before the observation/testing begins
 - b. Each mouse is watched individually for 2 minutes and the number of flinches of the inoculated femur that are spontaneously observed are counted as well as the amount of time the animal guards the ipsilateral foot. Guarding is defined as the amount of time the paw is held in a fully retracted position next to the animal's side without touching the

⁴Saline injection is done following anesthesia induction as a 3 mL subcutaneous bolus for hydration support.

ground. A flinch is defined as a rapid rising of the inoculated leg from the mesh floor.

2. Von Frey Filament Test (Mechanical Allodynia)
 - a. Animals are placed into small, raised Plexiglas cages with wire mesh bottom and allowed to acclimate to the cage for at least 30 minutes on test days: prior to cancer or media inoculation, and on post-inoculation days 7, 10 and 14.
 - b. Tactile allodynia was assessed as previously described[24]. Briefly, withdrawal threshold of the paw ipsilateral to the site of tumor cell inoculation was assessed in response to the application of calibrated von Frey filaments (0.002–4.56g) to the plantar surface using the Chaplan up-down method[25].
 - c. The 50% paw withdrawal threshold was determined by the nonparametric method of Dixon [26] and reported in grams.
3. Palpation-Evoked Pain
 - a. Pain with non-noxious palpation is a common clinical test for assessing the healing of bone fracture.
 - b. Palpations induced behavior are assessed at baseline on the day of the surgery, the day of fracture and the day of euthanasia.
 - c. Nocifensive behaviors were provoked by palpating the distal femur of the animals over a period of two minutes. Nocifensive behaviors are recorded and include: flinching, vocalizations, biting and guarding.[10] [27]

3.2 Behavioral Testing in rat

1. Von Frey Filament Test (Mechanical Allodynia)
 - a. Mechanical stimulation was performed with von Frey filaments with logarithmically increasing stiffness (range: 0.400–15.0g).
 - b. Animals are placed into Plexiglas boxes with a wire mesh floor and allowed to acclimate for at least 30 minutes before testing.
 - c. Each von Frey filament is applied in ascending order of stiffness to the plantar surface of the hindpaw that will be/was operated upon.
 - d. A single trial consisted of 10 applications of each filament for 2–3s. At least 2 minutes was allowed between each trial and 5 trials were performed for each filament. A trial was suspended if there was a hindpaw withdrawal noted.
 - e. The amount of paw withdrawals in the five trials was then expressed as a percent response frequency.
2. Thermal Hyperalgesia

- a. Rats placed in a clear plastic chamber (18cm x 29 cm x 12.5 cm) with a glass floor and allowed to acclimate for 5 minutes prior to testing [28].
 - i. Animals are considered acclimated when they no longer explore the cage and have minimal grooming behavior.
- b. A radiant heat source is placed beneath the cage 40 mm and it consists of a high intensity projector lamp (Osram, 58–8007, 8 V, 50 W) that projects through a 5mm x 10 mm aperture.
- c. The light beam was focused onto the affected paw and a photoelectric cell that detects the light reflected from the paw shut off the light beam with movement of the paw.
- d. Withdrawal latency measured to the nearest 0.1 second was determined.
- e. Data are expressed as mean paw withdrawal latency

3.3 Cancer Induced Bone Pain – A Mouse Model of Metastatic Breast Cancer

1. Female BALB/c mice are utilized for this protocol but can be replaced with other mouse strains/sex depending on the study and end-points desired.
2. On day of surgery, baseline behavioral testing should be performed.
 - a. Animals must be placed into small, raised Plexiglas cages with a mesh wire bottom and allowed to acclimate for at least 30 minutes before the behavioral testing is begun. (see Note 5)
3. Following behavioral testing, 66.1 cells are harvested and diluted to appropriate concentration for implantation and kept on ice for use. (see Note 1)
4. Following the behavioral testing, animals are anesthetized under ketamine/xylazine (8 mg/kg and 1.2 mg/kg, respectively) anesthesia via an intraperitoneal injection (10mL/kg body weight). (see Note 6)
5. Faxitron images are take of the caudal end of the animal with special attention made to be sure that both the ipsilateral and contralateral femurs and proximal tibias are in the field of view on x-ray. (see Note 7)
6. Following baseline images, the mice have their right hind limb shaved and the skin is cleaned with betadine scrub then 70% ethanol 3 times. A final application of betadine solution (10%) is performed immediately before incision.
7. Pre-operative subcutaneous bolus of saline and prophylactic antibiotics should be administered. Eye lubricant should be applied as well to prevent corneal drying while under anesthesia. Surgical field should be equipped with a heating pad

⁵Any of the behavioral tests to measure pain, whether spontaneous or evoked, can be used based on the study design. Most commonly, we test flinching, guarding and von Frey, but additional tests can be added depending on the study outcome measures.

⁶Animals should be monitored for deep anesthesia. This is done by looking for a pinch reflex on the hindpaw. Animals under full anesthesia should have no withdrawal reaction when their hindpaw is compressed. If the reflex persists for over 6 minutes after induction, it can be appropriate to add a booster of ketamine/xylazine that doesn't exceed half of the original induction dose.

⁷Faxitron images are then contrast adjusted with the range set to be between 9000 on the upper end and 1507 on the lower end.

underneath the station to maintain body temperature as ketamine/xylazine mixture drops core body temperature $\sim 3^{\circ}\text{C}$.

8. A lateral approach to the distal femur is performed and an incision is made over the skin of the ipsilateral femur and the thigh is exposed near the distal femur (see Note 8)
9. Using forceps, the fascial compartment is dissected and vastus lateralis muscle is separated to allow access to the underlying femur. (see Note 9) It is at this step that neurovascular compromise can occur most commonly – be careful not to disrupt the sciatic nerve or femoral vessels. (see Note 10)
10. The knee is accessed by medially translating the patella and putting the leg into full flexion to allow for maximum exposure of the distal femur.
11. An arthrotomy of the knee joint is performed and using the dental drill, the distal femur is reamed in a retrograde fashion to enter the medullary canal. (see Note 11)
12. Following the opening of the medullary canal, the cannulation injection needle is inserted into the reamed femur and 5 μL of 66.1 cells are injected (40,000–80,000 cells in total volume – see Note 12). Figure 1 demonstrates the position of the injection needle within the medullary canal.
13. Before removing the injection needle, the bone cement should be mixed with acrylic to begin the activation process. Once the proper consistency is attained and the injection needle is removed, a small amount is placed into the hole of the femur to create a plug that prevents the cancer cells from escaping the medullary canal and into the surrounding soft tissue. (see Note 13)
14. Once the femur is properly sealed, the knee is rearticulated and the patella repositioned. (see Note 14)

⁸. Skin incision can be performed with surgical scissors or using a scalpel. For our methods, typically surgical scissors are used to open the skin as it allows for the most control and cleanest incision. Also, the scissors are used to blunt dissect the subcutaneous tissue to remove the skin from the underlying muscular compartment.

⁹. As a landmark, we use the iliotibial band that connects at the proximal tibia and the fascial compartment is dissected around the knee. The dissection is extended proximally in order to adequately mobilize the quadriceps muscles. Once adequately dissected, the curved forceps are used to hook femur from lateral to medial near the knee. With the leg in full flexion, the patella and quadriceps tendon and distal portions of the muscle are medially translated in order to expose the distal femur and intercondylar space.

¹⁰. If excessive bleeding occurs at any point during the surgery, which can happen due to transection of the femoral artery/veins, compression of the site a sterilized cotton swab should be performed immediately. This should be done until bleeding has ceased. Special attention should be paid while doing the muscular compartment dissection to avoid the femoral artery. Similarly, the sciatic nerve runs on the posterior aspect of the femur and can be accidentally damaged during the muscular dissection. Following the recovery from anesthesia, the animal should be assessed for viability of the limb. If there is disuse or paralysis, the animal may need to be euthanized.

¹¹. Caution needs to be taken when performing this step as the femur is very fragile. It is also easy to ream through the cortex. If either of these events occur, the animal must be euthanized.

¹². Concentration of cells that is injected ranges from 40,000 to 100,000 in our experiments and exact amount depends on the severity of bone loss that is desired. The increased number of cells placed in the medullary canal, the faster the rate of bone loss and the greater the tumor burden. Our typical experiments use 80,000 cells/5 μL . Unicortical/bicortical fracture of the femur in a given animal is an exclusion criterion which requires removal of subject from experiment and human euthanasia (AVMA).

¹³. The bone cement needs to reach a proper consistency before it is able to be successfully applied to the femur. Small amount of powder is applied and 1 drop of acrylic onto the powder and mixed using the end of a cotton swab.

¹⁴. The relocation of the patella is easily done by extending the leg and pushing the patella laterally. Use the curved forceps to help realign the patella. Faxitron can be taken after the closure of the leg to ensure the proper alignment of the patella. Also, functional

15. Finally, the muscular layer should be closed using 5–0 Vicryl or PDS suture. The skin can be closed using 7mm or 9mm stainless steel wound clips.
16. Animals should be placed into a recovery chamber where they can passively come out of anesthesia. Body temperature must be maintained while recovering so chamber should be warmed by an insulated heating pad set to a low temperature.
17. Animals should be checked daily for 3 days post-operatively to ensure functional use of the limb and for signs of infection.

In response to the development of a mouse model of metastatic cancer to bone, a rat model was developed. This was done because rat bones are larger and this allows for easier access to the medullary canal, which makes the implantation of the cancer cells much easier.

3.4 Cancer Induced Bone Pain – A Rat Model of Metastatic Cancer to the Tibia

Based on the original description of the protocol [11] and our experience with a murine model of intramedullary metastatic disease, we have modified a procedure for producing a model of metastatic cancer to the tibia in rats. Female Sprague-Dawley rats (Charles River, 150–180 g) are utilized in this model and are implanted with MRMT-1 rat mammary gland carcinoma cells (Novartis Oncology Research)(see Note 15)

1. On the day of surgery, baseline behavioral testing is performed including mechanical allodynia, weight bearing and mechanical hyperalgesia as well as baseline Faxitron images.
2. The animals are deeply anesthetized using isoflurane inhalation anesthetic or ketamine/xylazine (80mg/kg and 12mg/kg, respectively)
3. The animal is then placed supine with the abdomen facing up.
4. The left leg of the rat is shaved and the skin is disinfected with 70% ethanol solution.
5. A 1 cm incision is made in the skin over the proximal tibia and the tibia is exposed. Special attention should be paid to prevent damage to the surrounding musculature and popliteal and tibial blood vessels.
6. A 23-gauge needle is inserted through the tibial cortex 5 mm distal to the epiphyseal plate. The needle must be inserted at an angle in order to access the medullary canal without fracturing through the opposite cortex.
7. Needle is removed and replaced with a blunt tipped injection needle connected to a 10µL Hamilton microinjector.

assessment should be made of the limb after the mouse recovers from anesthesia. If there is improper positioning of the patella, rupture of the quadriceps tendon or other functional compromise, the animal will need to be euthanized.

¹⁵MRMT-1 cells are cultured and harvested according to conditions set by Medhurst et al.[11] In short, cells were cultured in RPMI 1640 enriched with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin. When being harvested, cells were released from the culture flask by incubation with 0.1% trypsin (w/v). The cells were collected by centrifugation in 10mL of media for 3 minutes at 1200 RPM. The pellet was then washed with 10mL of Hank's Balanced Salt Solution (HBSS) lacking calcium, magnesium or phenol red. The suspension was then centrifuged a second time at 1200 RPM for 3 minutes and resuspended in 1 mL of HBSS. Cells were then counted by hemocytometer and diluted to the final desired concentration for injection into the tibia.

8. A volume of 3 μ L of cells (3000–30,000 cells/injection) or vehicle are then slowly injected into the medullary canal while slowly withdrawing the injection needle to ensure filling of the canal. Care is taken to ensure cells do not spill out of the medullary canal.
9. After the injection needle is removed, the injection site is sealed with bone wax, dental amalgam or bone cement.
10. Skin is then closed with metal skin clips or suture.
11. Animals are placed into a recovery chamber with a heating pad until consciousness is regained.

3.5 Fracture Induced Pain:

A procedure for inducing a controlled, full thickness, bicortical fracture was developed based on our collaboration with the Mantyh lab at the University of Arizona and their previously published works. [13, 27]

3.5.1 Pin Implantation

1. On day of surgery, baseline behavioral testing should be performed. Spontaneous pain behaviors should be performed first, followed by the palpation evoked behaviors.
 - a. Animals must be placed into small, raised Plexiglas cages with a mesh wire bottom and allowed to acclimate for at least 30 minutes before the behavioral testing is begun.
2. Following behavioral testing, the animals are anesthetized using a mixture of ketamine and xylazine (8 mg/kg and 1.2 mg/kg, respectively). (see Note 6)
3. Following anesthesia, the mice are given a bolus of saline (3mL) and gentamycin (0.8mg/mL, 1 mL) subcutaneously.
4. Faxitron images are taken of the caudal end of the animal with special attention made to be sure that both the ipsilateral and contralateral femurs and proximal tibias are in the field of view on x-ray. (see Note 7)
5. Following baseline images, the animals have their ipsilateral hind limb shaven and the skin is cleaned with betadine scrub then 70% ethanol 3 times. A final application of betadine solutions (10%) is performed prior to incision.
6. Pre-operative subcutaneous bolus of saline and prophylactic antibiotics should be administered. Eye lubricant should be applied as well to prevent corneal drying while under anesthesia. Surgical field should be equipped with a heating pad underneath the station to maintain body temperature as ketamine/xylazine mixture drops core body temperature $\sim 3^{\circ}\text{C}$.
7. The skin over the lateral femur is incised roughly 10mm and blunt dissection is performed to separate the skin from the underlying muscular compartment.

8. The fascial compartment is dissected and vastus lateralis is split with forceps. The quadriceps tendon, patella and patellar ligament are then translated medially.
9. The translation of the patella allows for exposure of the distal femur.
10. Access to the medullary canal is gained by coring through the bone using a 30 gauge needle in the intracondylar space of the distal femur.[27]
11. Faxitron images were taken after gaining access to the medullar canal with the needle still in place to ensure that the reaming of the femur was done appropriately without compromising the cortices and causing fracture. (see Note 11)
12. A pre-cut 0.011-inch diameter and 11-mm length stainless steel K-wire was inserted into the medullary canal in order to stabilize the fracture. Insertion of the wire was done carefully so to not induce a fracture upon placement, if fracture occurred at this step the animal was euthanized.
13. Dental amalgam was used to secure the implanted wire in place to close the hole in the distal femur. (see Note 16)
14. Area was irrigated quickly with sterile saline and muscular compartment was closed using absorbable 5–0 PDS suture.
15. Skin was closed using 6–0 silk suture (see Note 17)
16. Animals should be placed into a recovery chamber where they can passively come out of anesthesia. Body temperature must be maintained while recovering so chamber should be warmed by an insulated heating pad set to a low temperature.
17. Animals should be checked on every day for 3 days post-operatively to ensure functional use of the limb and for signs of infection. Wound clips are removed 7 days post-surgery.

3.5.2 Fracture Procedure

1. Prior to the fracture procedure, the animals are tested for behavioral measures of pain (see behavioral testing above)
2. On day 21 following the placement of the femoral pin, a closed, mid-diaphyseal fracture was induced under ketamine/xylazine anesthesia.
3. A 3-point bending device (BbC Specialty Automotive Center, Linden, NJ) is utilized to support the mouse femur.
4. The anesthetized mouse is placed supine with the femur in the 3 point device with the medial side faced up directly over the support anvil of the bending device.

¹⁶Bone cement could be substituted for dental amalgam. Bone cement is radiolucent and will appear the same density as bone on x-ray, while dental amalgam will appear radiodense on x-ray.

¹⁷Skin could also be closed using 7mm or 9mm wound clips.

5. The blunt guillotine blade is then lowered onto the hindlimb equidistant between the hip joint and knee joint.
6. A 168-g weight is dropped onto the guillotine blade from a height of 19.8 cm, which resulted in a close fracture of the femoral diaphysis.
7. Immediately following the fracture procedure, the animals were radiographed to ensure the proper placement of the fracture. If the animal following the fracture met at least 1 of the exclusion criteria, they were removed from the study. (see Note 18)

3.6 Monosodium Iodoacetate (MIA) Model of Osteoarthritis

Our proposed protocol has been modified based on our knowledge of using arthrotomy models and the expertise of osteoarthritis groups who have previously published in the field. [29–31]

1. Male Sprague-Dawley Rats (Harlan, 275–300g) were utilized for the procedure.
2. Baseline behavioral testing for nocifensive behavior was performed on the day of the procedure. Procedures included von Frey testing and thermal hyperalgesia.
3. Animals were placed under light anesthesia with 3% isoflurane.
4. Following induction of anesthesia, a single, intraarticular injection of monosodium acetate (1–3 mg in 0.02–0.05 mL sterile saline) is introduced into the right knee joint. The needle is inserted through the patellar ligament, just below its origin on the inferior pole of the patella.
5. Animals are allowed to waken from anesthesia in a recovery chamber with a heating pad before being placed into home cages.
6. Animals are allowed to 14–21 days post-injection before behavior testing is begun again to allow for arthritis to develop.

3.7 Surgical Destabilization of the Cruciate Ligaments to Induce Osteoarthritis – A Model of Severe Traumatic Arthritis in Mice

This surgical protocol has been modified from orthopedics groups studying traumatic ligamentous disruption leading to severe osteoarthritis. [22]

1. 8 week old male FVB/N mice utilized in the described surgery (other strains could be supplemented, if needed)
2. Behavioral testing is performed at baseline before the surgical procedure.
3. Mice are anesthetized using ketamine/xylazine (8mg/kg and 1.2mg/kg, respectively).

¹⁸Exclusion Criteria for Fracture Model: fracture not at the mid-diaphysis of the femur, the pin becomes displaced by the impact, fracture isn't observed following impact, and excessive comminution of bone.[13]

4. The ipsilateral knee is prepared for aseptic surgery by shaving the area and the skin was prepared with betadine and 70% ethanol. This cleaning procedure was performed three times. (see Note 19)
5. An arthrotomy was performed from the medial side of the joint by making a skin deep 5mm incision parallel to the patellar tendon using a No. 11 scalpel blade.
6. A Bvi Beaver EdgeAhead Safety knife Sideport MVR 0.90 mm 20G microsurgical scalpel is then inserted into the joint space and the anterior cruciate and posterior cruciate ligaments are transected. (see Note 20)
7. Skin is then closed with 5–0 monofilament suture. Suture will be removed 2 weeks post-surgery.

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¹⁹. This procedure has been described in the literature as a unilateral and bilateral procedure. Therefore, either can be performed.[32]

²⁰. Success of transection is determined by the valgus and varus laxity of the knee as well as unrestricted anterior and posterior translation of the tibia relative to the femur.

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Figure 1.
Representative Faxitron image of the caudal end of a mouse following femur cannulation with a 28 gauge blunt injection needle. Note that the needle is placed into the medullary canal without disrupting the cortex of the femur.