**Supplemental Material**

**Animal Treatments**

**Cells:** NCTC 2472 cells were maintained at 37oC in NCTC-135 media (Sigma-Aldrich; St. Louis, MO; N3262) containing 12% horse serum (Sigma-Aldrich; St. Louis, MO; H1270), 26.8 mM sodium bicarbonate (Sigma-Aldrich; St. Louis, MO; S5761), 100 U/ml penicillin, 100 g/ml streptomycin (Sigma-Aldrich; St. Louis, MO; P4333) and 100 g/ml Geneticin (Life Technologies Corp; Grand Island, NY; 10131) in BioLite tissue culture flasks (Fisher Scientific; Fairlawn, NJ; 130190) in a 5% CO2-humidified chamber. In preparation for surgery, cells were harvested, counted, and suspended in HBSS (1X) (Life Technologies Corp; Grand Island, NY; 14170-112). The time between the harvest of NCTC 2472 cells for injection and the actual injection of these cells into the mouse femur was never greater than 3 hr. NCTC 2472 cell suspensions were kept on ice following cell harvest and, immediately prior to being placed in the injector device, were gently mixed to ensure an evenly distributed cellular suspension.

**Surgery:** Injection of NCTC 2472 cells directly into the intramedullary space of the mouse femur was as previously described([1-13](#_ENREF_1)). Briefly, following induction of deep anesthesia with ketamine/xylazine (0.01 ml/g, 100 mg/10 kg, s.c.; Western Medical Supply; Arcadia, CA), mice were prepared for surgery by shaving and swabbing the surgical area with betadine then 70% ethanol (repeated twice). Mice were then placed on their left side on heating pads (Braintree Scientific; Braintree, MA), and using small iris scissors, a one cm incision was made in the skin of the right hind limb (lateral side parallel to the femur) to expose the muscle. The skin was separated from the underlying muscle, and using a #11 blade, an incision was made between the rectus femoris and vastus medialis muscles using the line of connective tissue as a guide. With curved forceps, the rectus femoris muscle and patella were moved to the medial side of the knee to expose the condyles of the femur. The connective tissue between the femur and the patella was cut, but the patellar tendon was not.

Using a foot-activated dremel (fitted with a 0.5 mm bit), a hole was made at the top to the middle of the patellar groove (aligned parallel with the femur). Spinning a 29G needle through the bone and into the intramedullary space completed the hole. Following the insertion of a dummy injector into the femur (until it reached the proximal end of the medullary space and then backed out until the tip is halfway into the femur), an X-ray was taken to confirm injector placement. Using a cell injection system comprised of a 10-L Hamilton syringe, C313I injector (Plastics One, Inc.; Roanoke, VA), and tygon tubing, a 5-L aliquot of cancer cells (105 cells) was injected. To prevent leakage of cancer cells outside the bone, the injection site was sealed with bone cement. The leg was then straightened, and the patella gently returned to its correct orientation using thumb and forefinger. To avoid possible patella displacement (see **Supplementary Fig. 1**), muscles were secured back into position using a horizontal mattress suture technique and absorbable sutures. The injection site was well irrigated with sterile saline and wound closure was achieved with two 7-mm auto wound clips (Becton Dickinson; Sparks, MD). The same surgical procedure was used for sham animals except that HBSS was injected instead of the NCTC 2472 cells. Animals recovered from anesthesia on heating pads, and received injections of antibiotic (Amikacin, 10mg/kg, i.m.; Western Medical Supply; Arcadia, CA) and sterile saline (1mL, s.c.). Following surgery, mice were individually housed. Wound clips were removed at Day 7 following surgery. Surgeries were performed by the same surgeon, and pre-op, X-ray, and post-op tasks were carried out by assistants so that at all times sterile operating conditions could be maintained.

**Dynamic weight bearing:** The mouse was placed in a small Plexiglas cage (11 x 11 x 22 cm) and a camera was placed on top of the enclosure. The animal was allowed to move freely within the apparatus for 5 min while the pressure data and live recording were transmitted to a laptop computer via a USB interface. Following completion of the test, mice were removed, and the test chamber was cleaned with alcohol wipes to reduce the potential for stress-induced analgesia as a result of any stress odor from the previous animal, and data was stored on the computer for subsequent analysis. For data analysis, the raw pressure data was automatically synchronized with images from the video camera and the averaged values were encrypted and re-recorded on a computer. Using the BioSeb software v1.3, the operator then manually validated each test period, ensuring each print corresponded to the appropriate paw using the synchronized video feed as a reference. A zone was considered valid when the following parameters were detected: ≥4 g on one captor with a minimum of 2 adjacent captors recording ≥1 g. For each time segment where the weight distribution was stable for more than 0.5 sec, zones that met the minimal criteria were then validated and assigned as either right or left hind paw or front paw by the experimenter according to the video and the scaled map of activated captors.

**Supplementary Figure 1. Patella displacement is a confounding factor in bone cancer pain.** To deliver cancer cells directly within the intramedullary space of the mouse femur, it is necessary to temporarily displace the patella (see **Methods**). To prevent the patella from becoming displaced post-arthrotomy, surgical steps are taken which include making the 1cm skin incision on the hip instead of over the knee and using a modified mattress suture technique to secure the patella in place. In addition, after surgery, animals are individually housed and allowed to recover for one week before being handled for behavioral and radiological assessment. Representative high-resolution radiographs of a femur with an intact patella (**A,C**), and a femur with a displaced patella (**B,D**). (**E**) The time spent in spontaneous nocifensive behavior for animals with patella displacement (closed circles, n=5) is significantly greater than for animals without patella displacement (open circles, n=8). Error bars represent SEM; \*p<0.05, one-way ANOVA.

**Videos of spontaneous nocifensive behavior**

Assessment of spontaneous and movement-evoked nocifensive behaviors was performed by analysis of videos taken of mice placed in small raised Plexiglas chambers (see **Methods**). Time spent in “nocifensive” behavior was assessed during a 5-min observation period (between minutes 15-20 of the filmed behavior). Nocifensive behavior was defined as: (a) full guarding (lifting the affected limb and holding it against its body), (b) reduced weight-bearing (affected limb is not completely held up against its body and some weight is borne on it, possibly with toes poking through the wire), (c) tending to the affected limb (abnormal grooming behavior directed solely to affected limb), and (d) sporadic hopping (intermittent jumps without utilizing affected limb). Rearing is not considered an example of nocifensive behavior. During the five-minute period of observation, the researcher used a stopwatch to precisely record the number of seconds spent in each of the above-mentioned nocifensive behaviors.

**Video 1: Spontaneous Nocifensive Behavior in the Naïve Mouse.** This film depicts the nocifensive behavior of a naïve mouse. During the film, when the mouse is stationary,the toes of the left foot are spread out and a range of brightness appears on the bottom of the foot because it is bearing weight on the wire floor. Therefore it is distributing its weight evenly across its hind limbs. Time spent in this position would not be counted as nocifensive behavior. When the mouse moves about the enclosure, weight bearing is evident for the left hind limb since the foot has variation in its brightness and spread out toes that firmly rest on the wire cage floor during each step. Time spent in this behavior would not be counted as nocifensive behavior.

**Video 2: Spontaneous Nocifensive Behavior in the Sarcoma-injected Mouse**. This film depicts the spontaneous nocifensive behavior of a mouse at Day 17 post-cancer cell injection. Throughout the film there are several visual cues that are indicative of nocifensive behavior. The pronounced changes in behavior and movements of the hind limbs when compared to naïve animals allow the observer to precisely measure the time spent in nocifensive behavior. ***Seconds 1-6***: Full guarding behavior is present in the left hind limb: it is fully lifted above the wire mesh with the toes held together, and the foot pad appears darkened as the mouse is holding the limb close to its body. In contrast, the non-tumor-bearing limb bears weight on the wire floor. The right foot pad skin has variation in its brightness and spread out toes that firmly rest on the wire cage floor during each step. ***Seconds 9-11***: Reduced weight-bearing behavior is seen: the toes of the left foot poke through the wire floor, indicating that the foot pad is not resting on the floor and the mouse is not bearing its full weight on this limb. ***Seconds 11-14***: Additional display of reduced weight-bearing behavior: the left leg and foot are cupped and turned down to the wire with toes held together. ***Seconds 15-17***: Sporadic hopping: the left leg is lifted and held at an angle above the wire with the foot pad cupped and turned/rotated to face the midline of the body during each step as it hops. ***Seconds 17-21***: Reduced weight-bearing behavior is displayed: the left foot is held above the wire and it appears dark with toes held together. ***Seconds 21-31:*** The toes of the left foot are spread out and a range of brightness appears on the bottom of the foot because it is bearing weight on the wire floor; time spent in this position would not be counted as nocifensive behavior. ***Seconds 33-49:*** Full guarding behavior is displayed. ***Seconds 49-53:*** Reduced weight-bearing behavior is displayed. ***Seconds 55-60:*** Sporadic hopping and reduced weight-bearing. **Note:** This video does not include footage of a mouse tending to the affected limb or grooming its haunches. Tending to the affected limb is abnormal grooming/licking behavior directed solely to the affected foot and limb. Grooming of the haunches of the affected limb is not counted as nocifensive behavior.

In present day orthopedic clinical settings, appointments are brief because of high patient volume. It is necessary to select measures that effectively investigate the variability of locomotion, gait, and breakthrough pain during the short time that the patient is present. Health providers utilize questionnaires and assessments of walking and weight bearing of the lower extremities to measure the disease progression ([14-20](#_ENREF_14)). Observational analysis techniques, such as the Edinburgh Visual Gait Scale, have been upheld as standard tools for evaluating multiple parameters of a patient’s gait ([14](#_ENREF_14), [21](#_ENREF_21)). In our pre-clinical mouse model of bone cancer, in lieu of questionnaires, videos of spontaneous behavior are used to mirror the observational clinical measures of gait and pain. The videos document mice experiencing movement-related pain. Guarding, partial weight-bearing, and limping are visual cues that closely mirror clinical observations of patients adopting a new gait pattern in an attempt to change the proportion of weight distributed on the affected limb during ground level walking while experiencing spontaneous breakthrough pain. All of the videos are archived, which allows for detailed analysis and public inspection ([22](#_ENREF_22)).

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