Fig. E-1

Image of surgical dissection of L4-L5 posterolateral intertransverse process fusion in the rat. The transverse processes of L3, L4, and L5 are visible at the top of the image.

Fig. E-2

Schemata of intertransverse process fusion contrasting two implant materials. The upper diagram represents an implant of growth factors, growth factors with cells (bone marrow or stem cells), or cells alone on ACS placed in the L4-L5 intertransverse process space. The lumbar spine of a rat has six vertebral bodies, L1 o L6. The lower diagram represents the placement of bone-grafting material such as autograft or demineralized bone matrix-based products.
Fig. E-3

Example of manual palpation of a lumbar segment (after explantation) in neutral, flexion, and extension from a rat spine treated with a target bone-grafting material or implant. A fused L4-L5 segment does not yield to manual force, whereas a nonfused segment easily moves.

Fig. E-4

High-resolution ex vivo radiographs (after rats were killed) demonstrating a continuous region of density of the fusion mass with slightly increased density at the border of an L4-L5 fused segment (Fig. E-4A) compared with no density in the L4-L5 space of a nonfused lumbar segment (Fig. E-4B).
Appendix

Detailed Description of the Posterolateral Intertransverse Process Fusion Procedure Performed in a Rat Model

This research employed the posterolateral intertransverse process fusion procedure performed in the Lewis rat.

Introduction and Purpose of the Model

The posterolateral intertransverse process fusion procedure performed in a rat is typically employed to assess the induction and formation of new bone after the implantation of experimental growth factors or grafting materials. The posterolateral gutter is a site where bone does not exist anatomically in the native spine. Growing bone in this site, between the transverse processes, where it does not normally exist, may be a good test to determine the processes of bone formation osteoinduction, osteogenesis, osteopromotion, and/or osteoconduction and the potency of the experimentally implanted and targeted grafting materials.

The posterolateral intertransverse process fusion procedure was modified and translated from larger animal models (sheep, pigs, dogs) to a rat model by our research team around 1995 (Kanim et al., 1998, animal models conference)33. It was specifically designed to test materials or growth factors that were expected to accelerate spinal fusion. Since that time, experimental modifications have been made to adapt this procedure to test (1) different types of grafting materials sometimes less potent than rhBMP-2, (2) various host conditions (inhibitors to fusion such as osteoporotic rat, aged rat, or nutritionally deficient rat), (3) surgical procedures (such as greater or lesser decortication of the transverse processes and laminar decortication), (4) removal of facets versus the intact condition, and (5) grafting methods (such as greater versus lesser amount of grafting materials, particle size, and/or placement of the graft).

Experiments involving this surgical procedure and model are designed to screen the potency of novel growth factors, carrier matrix materials, and other osteoinductive or conductive materials at an early stage of investigation.

Compared with the requirements for fracture-healing, forming bone between transverse processes after a posterolateral surgical procedure offers the increased challenge that potential bone formation is in a place where it does not exist natively: between the transverse processes at the lateral fusion gutter.

Therefore, the osteoinductive properties of a grafting material may be experimentally controlled by adding or eliminating factors related to the host environment, such as release of bone marrow via decortication of the transverse processes or the facets.

Compared with the osteoinductivity requirements of muscle pouch implantation testing, the posterolateral fusion procedure requires a remodeling of the newly formed
bone and connectivity to the transverse processes. Since the adoption of this model in 1998 for the testing of growth factors, many studies have employed a version of the posterolateral procedure in the rat for screening bone-grafting substitutes and gene delivery for fusion applications. The specific advantages are that the rat provides possibly one of the smallest models employable at a low cost, is challenging (i.e., fusion does not spontaneously occur), and somewhat clinically relevant. However, it is limited in that its biological and mechanical loading factors are very different from the human spine. A detailed chapter on animal models used for the evaluation of bone-grafting materials in spinal fusion was published in 1999. That review included the use of a few small animal models. In several early studies, investigators performed a posterior spinous process fusion procedure in rats (employed by Guizzardi et al. in 1992). Gould et al. used mice in 2000. Thomas et al. used guinea pigs in 1975 and placed bone grafts between the spinous processes, a variation of the procedure used herein. A more recent update to this earlier literature is provided as references in the present study. Increasingly, the rat is used as a model because it is potentially the smallest animal model wherein the surgical procedure for spinal fusion can be adequately performed and easily controlled. Also, depending on the subspecies selected, isografts may be tested with use of Lewis rats, whereas xenografts (human stem cells, grafts derived from human bone [i.e., demineralized bone matrix-based products]) may be directly tested by using the available subspecies of Athymic rNu rNu.

**Description of the Lewis/Crl Rat Model Used in This Study**

The Lewis/Crl rat subspecies was employed (Charles River, www.criver.com; LEW/Crl [inbred]). Lewis/Crl is an Albino rat and MHC haplotype RT1 (Rt1A1). Lewis/Crl is fully inbred, homozygous for all 107 microsatellite markers, and no variation has been found within 32 single nucleotide polymorphisms (SNPs).

Bone marrow, an isograft, was harvested from one rat (Lewis/Crl donor rat) and implanted into another rat (Lewis/Crl host rat) without host disease during the two-month observation interval as these rats are fully inbred.

**Posterolateral Intertransverse Process Spinal Fusion—Survival Surgery, Lumbar Levels (L4-L5)**

The rat is anesthetized with inhaled isoflurane administered at 4% to 5% via the chamber of a rat anesthesia apparatus that is then maintained at 2% via an anesthesia machine with a connected scavenging system. A cone at the end of a silicone hose attached to the anesthesia machine is placed over the rat’s nose and mouth to allow placement of the rat and connecting nose cone in the prep area (typically a “blue chux” underpad placed in a fume hood). The rat is placed on the prepping field, and the entire posterior side (thoracic and lumbar area) of the rat is then clipped free of hair and
prepared with betadine scrub followed by a 70% alcohol scrub wipedown, which is repeated three times.

For surgery, the rat is placed on a separate sterile field. The field is created by a sterilized, absorbent disposable pad (blue chux) that is laid out on a water-circulating heating pad and placed on a surgical table. The rat is then draped. Prior to surgery, a sterile ophthalmic ointment may be placed on the eyes to protect them from the dry environment for longer-length surgery. Rectal temperature may be monitored via a thermometer during the procedure.

A scalpel (#10 to #15 blade) is used to make a posterior midline longitudinal incision (approximately 3 to 4 cm) in the skin/fascia over the lower lumbar spine. The L6, L5, L4, and L3 spinous processes of the vertebrae and the sacrum are identified. The transverse processes of the lumbar vertebrae, L4 and L5, are surgically exposed (Fig. E-1) on the right and left (bilaterally); alternatively, bilateral muscle-splitting incisions may be used. The transverse processes are scraped of soft tissues. Once exposed, a high-speed drill with cutting burr attached is used to gently remove the outer cortical surface of the transverse processes (only) until punctate bleeding (decortication) in order to prepare the site for implantation. On completion of surgical dissection and site preparation, the site is well irrigated with warm, lactated Ringer solution containing antibiotic. The site is dried with sterile gauze pads or swabs, ensuring adequate hemostasis.

Graft materials, implants, and/or growth factors—all test materials—are placed in the posterolateral gutter bilaterally so that the implanted material spans the transverse processes of L4 and L5 (Fig. E-2) according to a randomly preassigned schema of grafting conditions. Typically, one treatment condition is placed bilaterally per L4-L5 site and per animal.

Surrounding muscle and tissue are gently lifted over the implant. Closure of the incision is completed in two layers. The fascia is closed with use of absorbable sutures in a continuous locking manner (interrupted if desired), and the skin is closed with use of 4-0 Vicryl suture in a subcuticular closure. No postoperative dressings are used. Warm subcutaneous fluids (99°F, about 1 mL) and buprenorphine (0.05 to 0.075 mg/kg) are administered at the end of the procedure once the rat exhibits some spontaneous movement and every twenty-four hours, if needed for pain control, during the first two to three days.

All procedures are performed without spinal instrumentation, postoperative immobilization, or restriction. Once a rat recovers mobility and is upright (sternal), the rat is placed in a cage. Standard rat chow is soaked in water and is placed in a small tray on the cage floor immediately after surgery. Cages are returned to the vivarium.
The rats are individually housed in separate cages after surgery in order to prevent any possible rat-to-rat mutilation of the fresh wound. They are allowed to eat and drink ad libitum. At about two weeks postoperatively, rats are cohabitated (two rats per cage).

High Resolution Radiographs—in Vivo

Immediately after surgery, and monthly thereafter, rats are transported in their cages to the room where the cabinet-type radiograph machine (LX-60; Faxitron X-Ray LLC, Lincolnshire, Illinois) is located. Rats are anesthetized with use of a rodent anesthesia machine with an attached waste-gas scavenging system that is attached to the cabinet Faxitron machine. Rats are placed on a shelf in the prone position in the Faxitron, the automatic exposure adjusts the kV and time according to the mass of the rat (in vivo), and the radiograph is made while anesthesia is being administered for about thirty-seconds. Rats immediately awake when they are removed from the cabinet, and are placed back in their same cage. The actual imaging process takes a few minutes per rat. Rats are returned to their housing facility after, or up to an hour after, the radiograph.

Killing of Rats

Rats were killed at eight weeks in this main study, either by means of isoflurane gas inhalation anesthesia at 2% to 5% via a rodent anesthesia machine with an attached waste-gas scavenging system to effect, followed by vital tissue harvest or by means of carbon dioxide asphyxiation in a chamber, followed by vital tissue harvest. The entire lumbar spine segment was removed en bloc.

Manual Palpation

Immediately after the rat is killed, the T12 to L6 spinal segment is harvested, cleaned of soft tissues, and tested by means of manual palpation. Using the investigator’s right and left hands (thumb and index finger), a manual bending force is applied to a lumbar segment by simultaneously first pressing upward with the thumb on the anterior spine and downward with the index finger on the posterior spine then pressing in the opposite directions (Fig. E-3). This is repeated several times, and each lumbar segment of L1-L2, L2-L3, L3-L4, L4-L5, L5-L6, and L6-S1 is systematically tested. Motion (not fused) versus no motion (fused) is recorded at each level; all levels are tested as fusion may extend beyond the treated level after treatment with some growth factors at very high doses. Three investigators who are blinded to the grafting treatment conditions perform these evaluations.

High-Resolution Radiographs—ex Vivo

The cleaned rat spine segment is then placed on the platform (shelf) in the Faxitron cabinet and the radiograph is made as per the procedure above. Examples of high-resolution radiographs showing “radiographically fused” and “radiographically not
fused’ specimens are shown in Figures E-4A and E4-B. Segments are then either sent for histological or biomechanical analysis.

High-Resolution Radiographs: Grading

Radiographic fusion was classified as Grade 1 (no bone or density between the transverse processes of L4-L5), Grade 2 (osseous nodules or density between the transverse processes of L4-L5 but no attachment to the transverse processes, with <50% fill between the transverse processes), Grade 3 (>50% density or bridging bone and <75% fill between the transverse processes of L4-L5), or Grade 4 (continuous density or bridging bone extending and connecting to the L4-L5 transverse processes, with >75% of the interspace filled with dense bone mass).