A preclinical large-animal model for the assessment of critical-size load-bearing bone defect reconstruction

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Critical-size bone defects, which require large-volume tissue reconstruction, remain a clinical challenge. Bone engineering has the potential to provide new treatment concepts, yet clinical translation requires anatomically and physiologically relevant preclinical models. The ovine critical-size long-bone defect model has been validated in numerous studies as a preclinical tool for evaluating both conventional and novel bone-engineering concepts. With sufficient training and experience in large-animal studies, it is a technically feasible procedure with a high level of reproducibility when appropriate preoperative and postoperative management protocols are followed. The model can be established by following a procedure that includes the following stages: (i) preoperative planning and preparation, (ii) the surgical approach, (iii) postoperative management, and (iv) postmortem analysis. Using this model, full results for peer-reviewed publication can be attained within 2 years. In this protocol, we comprehensively describe how to establish proficiency using the preclinical model for the evaluation of a range of bone defect reconstruction options.

Introduction

Development and application of the protocol

Critical-size segmental bone defect reconstruction has remained a surgical challenge for centuries1. In the setting of long-bone defects and fractures, the tibial diaphysis is the most common site of synchronous segmental bone loss2, with a non-union rate as high as 21%3. An inability to effectively replace the skeletal support for a limb renders it useless, with amputation being the only treatment option in certain cases4. The current management paradigm for these defects in humans is broad and includes limb shortening, non-vascularized autograft alone, delayed non-vascularized autograft placed into an induced membrane, distraction osteogenesis and vascularized bone with or without allograft1. Alongside advances in the understanding of bone blood supply5, novel tissue-engineering methods for addressing these defects are an emerging frontier in reconstructive surgery6. As such, reproducible preclinical animals that closely reflect the clinical situation are mandated for the successful exploration and translation of new research in this field.

Critical-size bone defects are considered to be those that will not heal spontaneously during the lifetime of the animal in the absence of intervention7. Currently, many animal models exist for...
critical-size bone defect research, although large-animal models are the most useful preclinical models for assessing the clinical suitability of novel bone reconstructive approaches. In particular, sheep older than 6–7 years provide a robust surrogate for the human clinical situation, having very similar bone mineral composition, macro- and micro-architecture and remodeling capacity and biomechanics similar to those of humans. In addition, the use of human osteosyntheses in sheep makes the ovine model attractive from a practical and cost-effective point of view. As such, the ovine long-bone defect model has remarkable relevance to clinical application because of the high capacity for the translation of study findings. In this protocol, we describe how to establish and use this model. Particularly novel to this protocol is the extensive description of preoperative and postoperative management aspects of this large-animal model. Our extensive experience with the performance of >10 studies and >300 surgeries provides evidence that these aspects are critical to the overall success and the ability to assess bone defect healing at lengthened time points. Furthermore, we discuss previously unreported insights into critical steps involved during all preoperative, operative and postoperative stages of this animal model to limit complications and ensure satisfactory defect creation and stabilization by interested research groups. This model is of particular relevance to those involved in the biomedical sciences, including orthopedic, reconstructive and plastic surgeons; surgical oncologists; veterinary surgeons; medical scientists; and tissue engineers.

Animal models in segmental bone defect research
Surgical reconstruction of the tibial segmental defect is one of the most challenging procedures in orthopedic trauma. Current approaches to these defects are multiple and include limb shortening, non-vascularized autograft alone, delayed non-vascularized autograft placed into an induced membrane, distraction osteogenesis and vascularized bone with or without allograft. However, in a smaller yet still considerable patient population, these conventional reconstructive techniques are not sufficient, and historically these patients have had to have limbs amputated. This is a result of insufficient donor sites available for such extensive tissue reconstruction and the associated pragmatic limitations seen with non-flap-based options such as distraction osteogenesis (e.g., lengthy healing phase, discomfort) and bone grafting (e.g., high rate of resorption, non-union). In a bid to address the limitations associated with existing surgical approaches to segmental defects of the tibia, a variety of polymer-based tissue-engineered constructs have been developed for partial replacement of extensive bone defects. To assess the effectiveness and refinement of tissue-engineering methods and also for the safety testing required for regulatory approval, in vivo preclinical testing is a critical step. The main key benchmarks in the selection of models for preclinical testing are (i) an animal model that mirrors the human physiological response as much as possible, (ii) target sites that are anatomically matched to the clinical setting, and (iii) invasive/noninvasive interventions and treatment conditions that mimic the real clinical situation.

Small-animal models
Small-animal models (mice, rats, guinea pigs and rabbits) are frequently used as an initial step for in vivo evaluation of a hypothesis to determine if it is worthwhile to proceed to more clinically translatable animal models. These animals are particularly useful for this because of their accessibility, low cost and ethical acceptance. Despite a high level of genetic similarity to humans, in the setting of critical-size segmental bone defect analysis there are substantial disadvantages that limit the use of such animals. Such limitations of small-animal models include limited or rapid cortical remodeling and secondary osteon formation, thinner femoral condyle cartilage and a cortical bone composition (e.g., hydroxyproline and protein content) differing from that of humans. This has been discussed extensively in the literature. Thus, when aiming to directly introduce a particular technique or implant into the clinical realm, especially with regard to the critical-size bone defect problem, a large-animal model is required for demonstrating sufficient translational capacity.

Large-animal models
Large, skeletally mature animals, such as sheep, goats, pigs, and dogs, are the most clinically relevant models used for preclinical testing. Osseous microarchitecture, physiology and biomechanical properties in large animals are similar to those of humans. Larger skeletal surfaces in the large animals also provide the opportunity to mimic internal and external fixation techniques and implants commonly used in humans. These properties encompass a histological osseous microstructure similar to that of humans, with well-developed Haversian, trabecular bone remodeling and highly localized bone fragility.
associated with stress shielding\(^7\). The use of large animals also enables the use of fracture, osteotomy, and defect sites that more closely match those seen in clinical settings\(^20\). Large-animal models typically used in bone tissue-engineering research include dog, swine, goat and sheep models; several reviews are available on this topic\(^9,17,21\). The pros and cons of each model are outlined in Table 1.

Dogs (Canis lupus familiaris). Dogs have been used in bone-regenerative research for several decades. Despite displaying some differences from human bone tissue in regard to microstructure and remodeling\(^19\), canine bone tissue has organic and inorganic bone material characteristics, as well as weight and density, that are similar to those of human bone\(^18\). Considered a characteristic associated with rapidly growing animals\(^22\), the differences in bone microstructure for dogs primarily relate to the osteonal bone architecture existing in the central component of the cortical bone, with laminar bone featured in endosteal and periosteal areas\(^19\). Although bone remodeling and turnover occurs at a higher rate in dogs as compared with humans, there is much biomechanical crossover, owing to the Haversian-type microstructure of the cortical bone\(^19\). Pragmatically, dogs are relatively easy to maintain and house during a lengthy study period, and, although human implants are generally too large for use in dogs, there are plenty of commercially available osteosyntheses specifically designed for dogs.

Historically, the aforementioned biological similarities and practical ease in cost as well as husbandry has helped garner favor for canines as a representative model of load-bearing human skeletal tissue\(^22\). As such, a number of research groups evaluating segmental bone defects have based their preclinical assessments around the canine large-animal model for a range of load-bearing bones (radius\(^24\), ulna\(^25\), tibia\(^26\), femur\(^27\) and fibula\(^28\)). Nevertheless, contemporary use of companion animals has been markedly decreasing because of the emotional impact and the legal aspects of their experimental use. Moreover, there is now general agreement that there is no specific reason for their use as experimental subjects when goats and sheep are also available for most orthopedic animal studies.

| Table 1 | Pros and cons of various large-animal species as preclinical models of tibial segmental defects |
|---------|---------------------------------------------------------------|
| Species | Pros                                                                 | Cons                                                                 |
| Dog (canine)\(^9,22,21\) | Highly tractable nature:  
Easy handling and husbandry  
Uneventful recovery period where properly acclimatized and conditioned  
Availability of commercial osteosynthesis  
Similarities in bone composition and biology to those of humans | Quadruped locomotion  
Enormous genetic diversity  
Major ethical concerns and public scrutiny |
| Pig (swine)\(^9,122,123\) | Bone structure and physiology:  
Lamellar bone structure similar to that of humans  
Bone regeneration rate is comparable to that of humans  
Public acceptance | Quadruped locomotion  
May exhibit aggression:  
Hard to handle and house  
Higher cost of husbandry and agistment  
Denser bone trabecular network  
Fast growth rates; not suitable for long-haul survival studies (may gain high body weight) |
| Goat (caprine)\(^17,121,124,125\) | Bone macro- and microstructures similar to those of humans  
Thinner coat and less muscular bulk  
More tolerant of stress and isolation | Quadruped locomotion  
Have horns: occupational health and safety concerns for staff  
Naturally independent, curious, and active animals; easily startled; hard to establish postoperative partial-weight bearing using sling or leg plaster  
Peak contact pressures in the caprine tibiofemoral joint at peak flexion are higher than normal human tibiofemoral contact pressure |
| Sheep (ovine)\(^9,12,37,50,124\) | Cooperation and compliance (docile):  
Easy handling and husbandry  
Uncomplicated postoperative limb coaptation and support (period of partial-weight bearing) using sling or coaptation device  
Suitable long bones of dimensions for testing human implants and prostheses  
Bone macrostructure similar to human bones  
Body weight similar to that of adult humans  
Low cost  
Readily available  
Public acceptance | Quadruped locomotion  
Bone metabolism maybe affected by environmental conditions (season, photoperiod cycle, geographical location, and diet)  
Only in sheep \(\geq\) 6 years old is secondary Haversian bone tissue remodeling similar to that of humans |
Swine (Sus scrofa domesticus). Pigs represent another commonly used large-animal model for segmental bone-defect assessment, with anatomy and bone mineral composition that closely resemble those seen in human long bones. Although the micro-architecture of porcine long bones has a denser trabecular arrangement, remodeling capacity and bone turnover rates remain nearly identical to those of humans. Pigs present some physiologic analogies to humans, including similar juvenile and adult skeletal phases. Pigs are relatively cost effective from an operative point of view because human implants can be used and use in experimentation is generally not viewed unfavorably, in contrast to the use of other animals such as dogs. Groups involved in porcine-based segmental bone defect research have explored the tibia for a variety of bone-regenerative applications. However, practical issues inherently limit their broader usability, mainly the husbandry requirements relating to the size of the animals and the complexity of handling them during the postoperative period. Such factors must be taken seriously when considering what husbandry infrastructure is available for experimentation and its associated costs.

Goats (Capra aegagrus hircus). Goats represent medium-sized ruminants and tend to have weight and limb dimensions similar to those of humans, making them suitable for human implants. Another advantage is their tolerance to fluctuations in temperature (including higher temperatures), which is an advantage compared to sheep. Segmental bone defect models in goats have included the femur, tibia and radius. Limitations of this animal choice include a lack of homogeneity in bone microstructure in the tibia in contrasted to that of humans, as well as difficulties in handling and restricting the animals’ mobility during the postoperative phase. Goats tend to be easily startled and, as such, can pose a substantial risk to themselves for postoperative complications (osteosynthesis failure). Although they represent a highly useful animal group for the study of segmental bone defects, issues with their postoperative handling, alongside a perceived elevated risk for adverse events, make them less than ideal, particularly as compared to the more docile sheep model.

Sheep (Ovis aries). Sheep, similar to goats, have a body morphology (weight, size) comparable to that of adult humans—with similar long-bone dimensions—which permits the use of implants designed for human surgeries. Sheep, in particular, have a well-described biomechanical loading profile with loading across the hind limb that is roughly half of that encountered in humans during the gait cycle. Despite differences in some histological characteristics (such as delayed osteonal remodeling), there are substantial analogies in bone mineral composition as well as similar bone turnover and remodeling capacity, in sheep as compared with humans. Sheep, in general, are particularly docile creatures and carry many other pragmatic advantages not observed with other preclinical models (e.g., pigs or dogs), such as ease of husbandry, use of human (non-customized) implants and acceptability to society. For the above-mentioned reasons, we established our long-bone segmental defect model in this animal. Skeletally mature, castrated male sheep (wethers), aged 60 kg, were used for our preclinical model to ensure a stable rate of bone turnover but also that secondary bone remodeling has occurred. The species (Ovis aries) has a predictable bone defect models in goats have included the femur, tibia and radius. Limitations of this animal choice include a lack of homogeneity in bone microstructure in the tibia in contrasted to that of humans, as well as difficulties in handling and restricting the animals’ mobility during the postoperative phase. Goats tend to be easily startled and, as such, can pose a substantial risk to themselves for postoperative complications (osteosynthesis failure). For the current model, leg length was not an issue when procuring the sheep from the farm. However, investigators should be aware that expanding on this model for diaphyseal defects of size, there is a minimum tibial length (22–23 cm) required for adequate bony fixation. These sheep can be difficult to find, and this was somewhat problematic for our group when we recently expanded the defect size.

The choice of long bone used for creation of a preclinical segmental defect model in the sheep has been varied. The femur, metatarsus, and tibia have all been studied. The ovine femur is located deeply in the thigh muscular mass, rendering surgical access and exposure of the femur challenging. However, this provides a favorable biological environment for bone healing. The contrast, the tibia has much less soft tissue coverage than the femur, and therefore makes it a better model for trying to identify clinically relevant differences between different treatment modalities. In addition, the femoral cortex is relatively thinner than the tibia, which can potentially lead to a higher morbidity following surgical creation of a diaphyseal defect and fixation placement. Although the metatarsus is classified as a “long bone”, in sheep it is much shorter than the tibia. This leads to some limitations in the choice of fixation device as compared with those used in humans. Anatomical location of the metatarsal bones can also be a potential risk factor for acquiring postoperative infections because of its immediate proximity to the animal’s soiled hooves. Furthermore,
the metatarsal shaft in the sheep is slightly four-sided, which can impose a biofabrication burden on
the bioengineers in customizing a lopsided tissue-engineering construct for segmental defect studies.

The tibia appears to be the most commonly used bone in sheep segmental defect models (Table 1). This reflects the clinical experience, in which the tibia is the most commonly fractured long bone and also has a high incidence of segmental defects requiring reconstruction. Segmental tibial defects can occur as a result of a large tumor or bone infection removal, trauma, or blast injuries in global conflicts. Impaired functionality is a major clinical problem because the tibia is a weight-bearing bone and tibial shaft fractures, especially compound injuries, carry a lengthy postoperative phase for recovery.

Limitations of the sheep model in segmental defect research
To date, no ideal animal model for the creation of long bone segmental defects with the same characteristics as those found in human beings has been established. A simple literature search of bibliographic databases shows that ‘sheep’ has been the most commonly used animal species for segmental bone defects. Nevertheless, the distinctiveness of the sheep musculoskeletal system as a limiting factor in translation of experimental results from orthopedic research to clinical practice should be strictly taken into consideration before planning a segmental defect study, especially if the biomechanical aspect is predominant. Limitations encountered in our model include, but are not limited to, the factors listed below:

Postoperative kinematics
Taylor et al. studied peak knee (stifle joint) contact forces in sheep and showed a considerable difference in magnitude compared to the corresponding forces reported in the humans. Quadrupedal stance is certainly responsible for lower forces in the sheep knee. These findings indicate that in our preclinical segmental defect studies, the mechanical conditions that certain surgical treatments in the clinic are required to withstand can be underestimated. The sheep knee also has a higher ratio of shear to axial loading, which most likely is due to the higher knee flexion angles in sheep compared to humans. The larger flexion angle of the sheep knee and the difference in force vector may affect the local forces seen in the surrounding soft-tissue structures, such as the ligaments. In our study, slight postoperative changes in the sheep kinematics were expected as a result of full-leg cast application for 6 weeks after the operation and sling support in a customized sheep sling. Although changes in external limb loading may alter diaphyseal internal limb loads, joint contact loading is more strongly affected by the action of the muscles, all of which are carefully preserved during surgical creation of segmental defects. Slinging the sheep after surgery also allows some degree of choice as to the external loading (unloading of the operated limb), which is to some extent reproduced in the clinic when patients rest or use crutches during motion activities.

Age and breed of the sheep
Reichert et al. advised the use of sheep >6 years for studying long-bone segmental defects, because of secondary osteonal remodeling, which would make the defects more similar in structure to those of humans. Sourcing sheep within the aforementioned age range can be a tedious task; it requires pre-planning and establishing contacts with private livestock sale yards and may cause unreasonable lengthy delays, affecting project feasibility. Selection of a suitable breed of sheep was another obstacle we encountered while our tibial segmental defect was in the developmental stages. The predominant breed of sheep in Australia is the Merino, which is a superb forager and an absolutely adaptable sheep. Because the Merino is predominantly used for wool, these sheep are raised for several years for wool production, which can make sourcing older sheep (≥6 years old) more feasible for our preclinical model.

Postoperative limb coaptation and support
One of the confounders in our segmental defect preclinical model was the uncontrolled, high-bending moments that the defect site and fixation hardware were subjected to during movements of sitting down and getting up. To minimize that, sheep body weight was supported during the early stages of bone healing by placing an external coaptation (a modified partial full-leg cylindrical fiberglass cast) on the operated limb and also cradling the sheep in a customized sling support for 4 weeks. Heavy padding was used under the cast, and a window was cut from the cast over the point of the hock joint to further reduce risks of potential complications (e.g., pressure sores). Furthermore, wound checks
Table 2 | Fixation approaches described for the sheep tibia segmental bone defect model

| Fixation approach | Advantages | Disadvantages |
|-------------------|------------|---------------|
| External fixation device | Versatile, Simplified fixation approach, Minimal soft-tissue trauma, Easy introduction of tissue-engineered constructs/materials into the defect | Pin screw loosening, Pin track infection (and osteomyelitis), Frame can be cumbersome and cause local injury to sheep (pressure sores), Delayed bone healing time compared to other methods |
| Intramedullary nail/rod | Standard approach for diaphyseal bone injury in humans, Unreamed nail available to avoid reaming complications, Central load carrier, High tolerance to applied forces, Reamed debris can be source of stem cells | Reaming-related complications (thermal necrosis, altered endosteal blood supply, air/fat embolism, unintentional bone erosion and fracture risk), Risk of fracture when inserting an unreamed nail, Difficult to attain absolute reduction of fracture/defect (if desired), Limited defect space available for tissue-engineered constructs/materials, More technical application than external fixation, Obstruction of intramedullary space, limiting the regenerative potential of bone marrow |
| Plate fixation | Common alternative fixation approach for diaphyseal bone injury in humans, Anatomical reduction attainable if required, Easy introduction of tissue-engineered constructs/materials into the defect | Eccentric load carrier, Prone to frontal-plane deviation and associated implant failure, Can impair periosteal blood flow at bone-plate contact site, More technical application than external fixation |
| Internal fixation | Same advantages as plate fixation, Designed for bridging construct fixation; therefore, construct stability is not dependent on ongoing compression of the plate onto the bone surface, More defined mechanical environment | No commercial locking plates available to suit specific application in this model (i.e., strong enough to sustain limb loading without bending, but slim enough not to protrude through thin, soft tissue layers on top of the tibia), Construct may be too stiff to stimulate bone regeneration |

were performed weekly during the first 4 weeks after surgery. Our customized sling enabled the operated sheep to fully load their limbs and walk around within the constraints of the sling support, but prevented them from sitting. The proportion of body weight that was supported was adjusted as required by fine-tuning eight suspension tension ropes attaching the sling and the sheep to four overhead points. To further reduce risks associated with lengthy limb immobilization and sling support, such as myopathies, pressure sores and skin maceration, use of sheep >60 kg (presurgical, pre-fasting weight) for this model was avoided.

Implant fixation approaches in segmental bone defect research
As seen in the clinic, a variety of fixation techniques are used in segmental bone defect research, each associated with its own advantages and limitations (Table 2). In mid-diaphyseal defects of the tibia encountered clinically, intramedullary nailing is the approach typically used. Although this approach is effective in animal models, it serves to obstruct the placement of a solid, single-piece load-bearing scaffold. Similar to plate fixation, external fixation systems avoid this issue but have their own limitations relating to pin track infection and delayed healing times. Here we discuss the different fixation approaches described in the literature specific to the sheep tibia segmental defect model and justify our choice of plate fixation.

External fixation
From a clinical perspective, external fixation is routinely applied as a temporary fixation measure, especially in lower-limb trauma in the setting of synchronous soft-tissue defects. In segmental bone defect research, external fixation is a commonly used method, owing particularly to a lesser degree of soft-tissue injury and the relative ease of surgical application.

A major recognized issue for this mode of fixation relates to pin track infections, which result in Schanz screw loosening. By contrast, in plate fixation, a plate or intramedullary nail is internally fixated and effectively ‘buried’ away from the external environment, which is a major advantage over
external fixation models. In our experience using an external fixator for the sheep segmental tibial defect, we restricted free mobilization of the sheep with the use of a sling harness to reduce the risk of complications such as pin site infections and soft-tissue injuries related to the external fixation constructs. Another recognized limitation associated with the use of external fixation is that healing generally takes longer than when internal fixation methods are used. Such limitations must be taken into account when determining the study design and associated time line.

**Intramedullary nail fixation**

For tibial fractures affecting the mid-diaphysis, intramedullary nailing is the most common procedure. Although it has widespread clinical use for bone injury in this area of the tibia, advantages and disadvantages are associated with its use. To introduce the nail into the bone, reaming is usually performed to provide enough space and a clear pathway within the intramedullary canal. Reaming also increases the possible nail diameter. If reaming is not performed carefully, unintentional path formation can occur and lead to iatrogenic bony erosion from the reaming device, with or without associated fractures. The process itself markedly increases the intramedullary temperature, which alongside impairments in cortical bone circulation, can induce bone-tissue necrosis and alter the endosteal architecture. Damage through these mechanisms can take several weeks to resolve and may influence bone healing for studies at earlier time points. The use of intramedullary nail systems without reaming has a high rate of osteosynthesis failure, and if smaller-diameter nails are used, these are prone to inadequate bony fragment stability. An ample rise in intramedullary pressure occurs during the reaming process; this can lead to air/fat embolism and associated tissue infarction with physiological impairments. Despite these disadvantages, intramedullary nails are central load carriers, and, unlike plate fixation, these systems are less susceptible to tilting in the frontal plane and have a higher tolerance to applied forces. As a result of this, many research groups have evaluated segmental defects in the sheep tibia using this approach. Unfortunately, this approach cannot be used without compromising the potential internal space of the defect under evaluation for scaffold placement or filling with another biomaterial.

**Plate fixation**

Plate fixation is the main alternative definitive procedure used in the management of mid-diaphyseal segmental bone defects in humans. With respect to our study design, a variety of reasons justify our use of plate fixation. Defect stabilization with a human-sized 10- to 12-hole dynamic compression plate (DCP) using 6–8 bicortical self-tapping screws has advantages for defect bridging and stabilization: First, using a plate enables synchronous stabilization while permitting filling of the entirety of the defect, which is essential when aiming to evaluate different options for segmental bone defect reconstruction. Second, the plate can be accurately bent for congruity with the tibial contour, permitting rigid fixation of the defect. For our sheep, this is performed by an experienced operator manually (by eye) using a plate-bending device specifically designed for DCP plates, just as is done in the surgical treatment of human bone fractures. This accuracy is adequate and sufficient for this application in a mid-diaphyseal defect, where the plate serves as a template for the general alignment of the segments after defect creation. Third, the predominant blood supply to the sheep tibial diaphysis is through the nutrient vessels that lie proximal and lateral. These are not interrupted by placement of the plate along the anteromedial tibial surface. This approach preserves blood supply in the residual tibia and helps to produce reproducible bone healing. Finally, owing to limb loading and resulting plate bending in a plate-fixation construct, the defect site is subjected to compressive loads, which have relevance for osseointegration of the defect filler under evaluation (e.g., a rigid biodegradable scaffold, such as medical-grade polycaprolactone–tricalcium phosphate (mPCL-TCP)).

A number of other groups have used plate fixation in sheep segmental defect research. Recently, Pobloth et al. compared two different titanium-mesh scaffolds (soft and stiff) combined with autologous bone graft and then stabilized with either a locking compression plate (LCP) or a rigid, customized shielding plate. The study was undertaken in 27 adult Merino mix sheep with a 4-cm diaphyseal segmental bone defect. Incorporating finite element techniques, the scaffolds were designed to minimize stress shielding while simultaneously ensuring resistance to mechanical failure. Over 24 weeks, they found that lower stress shielding led to earlier defect bridging and enhanced bone regeneration within the defect. The choice of a 4-cm rather than a 3-cm diaphyseal segmental defect further highlights the versatility of this animal model. We have recently expanded our defect size to 6 cm, which has a clinical relevance to more extensive bone tissue loss (as seen following oncological ablation and severe osteomyelitis). Other groups report a combination fixation approach to the sheep
tibia in the segmental defect model, using a dual-plate fixation technique\textsuperscript{24,74,75,77}, a plate alongside an external fixator\textsuperscript{79} or a reamed intramedullary nail technique with a medial plate\textsuperscript{70}. Other than to reduce stress at the screw–bone interface\textsuperscript{70}, justification for these approaches by the authors appears unclear. For mid-diaphyseal segmental bone defects, combination-fixation approaches have no real clinical equivalent and may represent stronger rigidity in fixation than what is seen in our model and also in the clinical context, which further limits the translational capacity of these alternative models.

We recognize the inherent limitations of plate fixation in our animal model. Although the 3-cm defect size does represent a critical-size bone defect as compared with an appropriate control group\textsuperscript{7}, this is only the case if meticulous removal of the periosteum is performed during defect creation, because residual periosteum, especially on the muscle, may allow for spontaneous defect regeneration if overlooked. Furthermore, in humans, diaphyseal segmental defects of the lower extremity are typically managed with an intramedullary nail, which is a central load carrier with a high tolerance for applied forces. A fixation plate is considered an eccentric load carrier and frequently carries low to moderate rates of implant axial deviation with associated risk for implant failure. There is also the potential for periosteal compromise secondary to direct bone surface compression, as well as bone resorption or impaired healing as a result of stress protection from the absolute rigidity of the DCP fixation. However, the latter may represent advantages in experimental methods because they impair bone regeneration. Recognizing these limitations, our major justification for using the DCP fixation approach relates to its role in preserving the space under evaluation in the defect for tissue-engineered construct placement.

**Experimental design**

Our methodology includes four key stages, each as important as the others for the overall success of the procedure: (i) preoperative planning and preparation, (ii) the operative procedure, (iii) post-operative management, and (iv) post hoc analysis.

**Critical-size bone defect**

We define the critical-size bone defect per the accepted definition in the literature, that is, “the smallest size intra-osseous wound in a particular bone and species that will not heal spontaneously in the lifetime of that animal”\textsuperscript{77}. Although this definition was created in relation to craniomandibular discontinuity defects, there is no accepted definition for size and volume in load-bearing bones. Applying this definition to load-bearing bone research, there is a requirement for a control group to confirm that the defect size under evaluation will not heal during the study period. Our 3-cm defect was established in the \textit{Ovis aries} large-animal model with a control group without intervention, aside from a bridging plate across a 3-cm defect in the sheep tibia. The periosteum in the posterior muscular septum is excised, in addition to 1 cm of tibial periosteum on either side of the defect, because this eliminates spontaneous healing of the defect\textsuperscript{70}. After 3 months, the absence of bone healing was confirmed radiographically via plain X-ray, micro-computed tomography (micro-CT) and histology\textsuperscript{6}. Intervention groups for this particular defect size include autologous bone graft (current gold standard), mPCL-TCP scaffold, bone graft and scaffold, mesenchymal stem/stromal cells (MSCs) and scaffold, and bone morphogenetic protein-7 (BMP-7) with scaffold (Fig. 1; ref. \textsuperscript{6}).

**Analysis and evaluation methods**

Determination of successful restoration of function through viable bone regenerate in load-bearing bone defects should be made through a combination of modalities, the most important of which is the testing of the mechanical integrity. Our team routinely uses mechanical assessment through torsional loading of the bone regenerate to assess successful bone healing. Further methods, such as serial radiographic assessment, micro-CT, and histological and immunohistochemical techniques, serve to complement the findings of the mechanical testing. By performing sequential analysis, all of the aforementioned forms of bone healing assessment can be undertaken. First, sheep limbs are X-rayed during the postoperative assessment period at sequential time intervals until euthanasia. Following euthanasia, the limb is assessed biomechanically with torsional load after plate removal. A fracture through the bone regenerate induced by biomechanical assessment does not impair the ability to evaluate key features found through micro-CT, such as bone volume and architecture. We then embed the bone samples into paraffin and resin medium to assess bone regeneration histologically, in addition to assessment of neovascularization and new bone growth by immunohistochemical techniques.
Biomechanical analysis and mechanical testing

Our large-animal model is validated through a robust evaluation of the mechanical integrity of the healed bone. Historically, various mechanical testing methods have been applied to long bone specimens to assess their mechanical properties after fracture or defect healing. The methods include bending tests (three-point bending, four-point bending and cantilever bending), axial compression/tensile tests and torsional testing (constrained torsion test and unconstrained torsion test). Some mechanical properties, such as stiffness, can be assessed via any of these tests non-destructively, as long as the loads remain within the linear-elastic range of the tested bones. However, the stiffness of a healing bone presents only an incomplete picture of the restoration of the mechanical function of the bone, because it typically does not correlate with the yield strength, which is a more relevant measure of its mechanical integrity. Yield strength, on the other hand, can obtained only when the load is over the failure point, resulting in destructive testing. Fortunately, in carefully controlled...
loading this does not typically result in catastrophic failure of the tested bone into individual fragments, but rather results in the formation of single-fracture cracks (while maintaining overall sample integrity), which can be easily identified as artifacts of the mechanical testing, and subsequent analysis using other experimental methods such as micro-CT imaging or histomorphology remains possible.

Although three-point bending is the most physiologically relevant test, it has the disadvantages of high dependency on the direction of the applied load\(^81,82\) and compression of newly regenerated tissue in the critical region of the specimens. Axial compression is error prone (up to 80% of such tests result in long-bone stiffness, according to Steiner et al.\(^83\)), making it unsuitable for the determination of long-bone mechanical properties, and axial tensile tests do not replicate a critical, physiological load. Ultrasound elastography, specifically compression elastography, is a non-destructive method of biomechanical assessment that allows direct visualization of strain (Young’s elastic modulus can be obtained by calculation). However, the method has substantial drawbacks\(^84\): strong artifacts and a lack of quantitative measurement and standardization; in addition, the pressure is limited to a certain range for the linear elastic properties of tissues. These limitations have rendered this technique inapplicable to the mechanical testing of sheep long bones.

Torsional testing of the bone along its long axis appears to be the most robust method for our experimental setup, because it is direction independent. Torsional testing also has the advantage of providing the characterization of bone specimens as a whole, with failure occurring at its weakest point, typically in the area of the regenerating defect\(^85\). It is for these reasons that we prefer this method over alternative mechanical measures for the assessment of bone defect healing in our model.

In addition to methods that assess the mechanical integrity of the healed bone after the experimental endpoint, our team has also developed an indirect method to measure strain within the healing bone. This indirect method involves instrumenting the DCP plate within the segmental bone defect model. Others have described sensors built into newly designed fixation devices\(^86,87\); however, our biomechanical monitoring device is a removable attachment that can be fixed to the existing DCP and does not interfere with the plate’s mechanical integrity\(^88\). The sensitivity of the sensor was validated first in ex vivo bone and was found to be sufficient to monitor bone healing in a temporal manner (Fig. 2a–d). In addition to recording information about the progression of bone defect healing, the sensor provides data about the animal’s activity, including the number of loading cycles and intensity ranges (Fig. 2e,f).

**Statistical analysis**

We carry out analysis using SPSS v.22.0 (IBM: https://www.ibm.com/products/spss-statistics). Comparison between the treatment limb and a control dataset(s) can be undertaken with a two-tailed Mann–Whitney U test and \(P\) values adjusted according to Bonferroni–Holm using a 95% confidence interval. Data can be presented in graphic and tabular formats.

Sample size for experimental work carried out should be determined with a power analysis performed on the torsional strength data reported for a comparable critical-size defect study in sheep tibiae\(^73\). To determine a 25% difference in torsional strength with a power of 80% on a significance level of \(P \leq 0.05\), we found a minimum of eight animals is generally required for treatment group comparison.

**Time points in experimental protocols**

The time points chosen for analysis should reflect the hypothesis in addition to the goals of the research work. Standardized guidelines for the evaluation of regeneration in ovine critical-size bone defects state that study periods of \(\geq 3\) months are necessary to comprehensively characterize bone repair and regeneration\(^89\). Previous studies over 3-month\(^6,52\), 6-month\(^6,51,90\), and 12-month\(^6,53\) periods resulted in effective bone bridging (as reviewed in ref. 21). In the clinic, internal fixation devices are unlikely to be removed within 12 months after initial surgery for segmental bone defects, and thus a 12-month time point is, from a clinical point of view, a condition sine qua non. Furthermore, the 12-month time point is particularly clinically relevant for studying functional outcomes of bone remodeling and maturation, such as torsional stiffness and polar moment of inertia\(^6,53\). The choice of a 6-month time point has practical advantages through a reduced study length while still enabling a robust assessment of bone regeneration with a large number of study groups and early evaluation of biomechanical strength\(^50\).
Record keeping

Standard record-keeping processes throughout the preoperative, intraoperative and postoperative periods should be adhered to, in conjunction with guidelines issued by committees issuing animal ethical guidance. Features to be taken into consideration include animal identification information (species, age, sex, weight, identification record number, sheep micro-chip identifier); protocol information (study coordinator, ethics approval number); the names of those involved where applicable (e.g., anesthetist, primary surgeon); preoperative information (vital signs, drugs/ fluids administered for anesthesia); anesthetic operative data (type/administration of anesthesia, vital signs throughout the procedure); surgical operative data, including how the defect was reconstructed and any difficulties/issues encountered (to assist post hoc analysis correlation); postoperative data (method/timing of sheep recovery, vital signs, timing of mobilization, ongoing weight-bearing status of the operated limb and feeding); and routine postoperative monitoring, which should be performed three times each day by an animal technician and remotely on weekends/holidays via live video feed.

Materials

Biological materials

- Skeletally mature sheep (Merino, >6 years old, <60 kg) Age determination in our sheep was based on the number, condition, and order of eruption of their permanent incisor teeth\(^{39,91}\) CAUTION Experiments involving animals must conform to all relevant standards of ethical treatment of animals and must be approved by the local institutional animal ethics committee. All procedures discussed here were approved by the QUT University Animal Ethics Committee and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Reagents

- Sterile normal saline (IV fluid, 0.9% sodium chloride, Baxter, cat. no. AHB1324) or Hartmann’s solution (Baxter, cat. no. AHB2324), to be administered at 2–5ml/kg/h, i.v.
• Midazolam (Alphapharm; Patterson Veterinary, cat. no. 07-888-9994), to be administered at 0.2 mg/kg body weight, i.v.
• Propofol (Prorive, Claris; Clifford Hallam Healthcare, cat. no. 1782382), to be administered at 5 mg/kg body weight, i.v.92
• Isoflurane (IsoFlo, Zoetis; Patterson Veterinary, cat. no. 07-806-3204), to be administered at 2–2.5% in 40% oxygen, by inhalation3! CAUTION Isoflurane is a volatile halogenated anesthetic and can cause irritation to the eyes, skin and respiratory system. It is recommended to work with isoflurane under ventilated conditions and with a carbon dioxide absorber canister. Isoflurane is suspected to be capable of damage to fertility or the unborn child. Pregnant or lactating personnel should avoid exposure to this drug.
• Genteal eye gel (Patterson Veterinary, cat. no. 07-893-0475)
• Buprenorphine, injectable (Temgesic; Clifford Hallam Healthcare, cat. no. 1238366), to be administered at 0.005–0.1 mg/kg body weight, i.v.93
• Fentanyl citrate injection, 500 µg/10 ml (Generic Health; Clifford Hallan Healthcare, cat. no. 9002802), to be administered at 10 µg/kg/h, i.v. constant-rate infusion (CRI)94,95
• Fentanyl transdermal patch, 50 µg/h (Sandoz; Clifford Hallan Healthcare, cat. no. 1993841), to be administered at 2 µg/kg/h, transdermally96
• Flunixon injection (Norbrook Laboratories; Clifford Hallam Healthcare, cat. no. 1953611), to be administered at 1.1 mg/kg body weight, i.m.96
• VAM injection, injectable supplements (Ceva Healthcare, cat. no. APVMA 50147), to be administered at 1ml/45 kg body weight17
• Depredil (methylprednisolone; Troy Laboratories; Clifford Hallam Healthcare, cat. no. 1905082), to be administered at 1.3 mg/kg body weight, i.m.97

**Antibiotics**
▲ CRITICAL Prophylactic and postoperative antibiotics should be administered once 30 min before initial incision and then daily during the first 3 d postoperatively.
• Gentamicin injection (Pfizer; Clifford Hallam Healthcare, cat. no. 08060080), to be administered at 3 mg/kg body weight, i.v.98
• Cefazolin-AFT (AFT Pharmaceuticals; Clifford Hallam Healthcare, cat. no. 2016874), to be administered at 20 mg/kg body weight, i.v.96
• Sodium pentobarbitone (Lethabarb, Virbac; Clifford Hallam Healthcare, cat. no. 1905213), to be administered at 100 mg/kg body weight, i.v.99
• Short-acting broad-spectrum antibiotic (Trisoprim-480; Troy Laboratories; Clifford Hallam Healthcare, cat. no. 180635; to be administered at 1.5 ml/kg body weight, i.m.)

**Mechanical testing**
• Palapress vario dental acrylic powder and monomer (Kulzer)
• Triton X-100 (Sigma-Aldrich Pty Ltd, cat. no. T8787)

**Additional medications**
▲ CRITICAL The additional medications below should also be available as required for use in respiratory/cardiovascular emergencies.
• Calcium gluconate injection (Phebra, cat. no. INJ022)100
• Adrenaline MYX (Mayne Pharma; Clifford Hallam Healthcare, cat. no. 2220058), to be administered at 0.03 mg/kg body weight, i.v.101
• Sodium bicarbonate (8.4%; Phebra, cat. no. INJ127)102
• Metaraminol (Clifford Hallam Healthcare, cat. no. 2349724), to be administered at 0.2–0.6 mg/kg body weight, infusion at a pump flow of 80ml/kg/min103
• Noradrenaline MYX (Mayne Pharma; Clifford Hallam Healthcare, cat. no. 2220058, to be administered at 60 µg/ml in 5% dextrose, i.v.104
• Cordarone X (Amiodarone HCL, i.v.; Clifford Hallam Healthcare, cat. no. 2035055), to be administered at a 100-mg i.v. loading dose105
• Betaloc, (Metoprolol, AstraZeneca; Clifford Hallam Healthcare, cat. no. 1015914), to be administered at 30 µg/kg, i.v.106
• Povidone–iodine (7.5% (wt/wt) surgical scrub; Pfizer; Clifford Hallam Healthcare, cat. no. 1726303)107
• Ethanol 100 high grade (HG) (80% (vol/vol); Recochem, cat. no. 15100); dilute 800 ml of ethanol 100 HG in 200 ml of ddH2O and store at room temperature (RT; 20–22 °C) for up to 24 months107
• Bone wax (Ethicon; Henry Schein Halas, cat. no. JJ-W810T)108

**PROTOCOL NATURE PROTOCOLS**
**Specimen fixation**
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. 158127) ! CAUTION PFA is toxic upon inhalation, upon skin contact and if swallowed. Use only in a laboratory fume cabinet and with appropriate PPE.
- Sodium hydroxide (NaOH)

**Embedding (paraffin)**
- Histoplast paraffin wax (Thermo Fisher Scientific, cat. no. 8330)

**Embedding (resin)**
- Technovit 9100 PMMA powder (Heraeus Kulzer, cat. no. 66010251) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Technovit 9100 basic solution (Heraeus Kulzer, cat. no. 66006735) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Technovit 9100 regulator (Heraeus Kulzer, cat. no. 66039184) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Aluminum oxide 90 active neutral (Merck, cat. no. 101077100)
- Technovit 7100/9100 Hardener 1, (Heraeus Kulzer, cat. no. 64709022) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Technovit 9100 Hardener 2, (Heraeus Kulzer, cat. no. 66039185) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.

**Mounting (resin)**
- Technovit 4000 powder (Heraeus Kulzer, cat. no. 66032003) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Technovit 4000 syrup I (Heraeus Kulzer, cat. no. 66032002) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Technovit 4000 syrup II (Heraeus Kulzer, cat. no. 64712092) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
- Primer RC (adhesion primer to increase the bond between resin and ceramic surfaces; Heraeus Kulzer, cat. no. 66015461/00) ▲ CRITICAL Degrease glass slide and make sure that the surface is free of dust.
- One-component precision adhesive, light-curing (Technovit 7210 VLC; EXAKT Advanced Technologies, cat. no. 64709017)
- Alumina slurry (ProSciTech, cat. no. 10400)

**Mounting (thin section)**
- Paladur (Kulzer; Emgrid, cat. no. 64707938)
- Poly-L-lysine solution (Sigma-Aldrich, cat. no. P8290)
- Gelatin, type A (Thermo Fisher Scientific, cat. no. G8500)
- Glycerol (Sigma-Aldrich, cat. no. G7757)
- Phenol (Sigma-Aldrich, cat. no. P1037)
- Technovit 3040 powder (Kulzer, cat. no. 64708806)

**Additional reagents**
- Ammonia (25%, Sigma-Aldrich, cat. no. 1054321011)
- Hematoxylin monohydrate (Merck, cat. no. 1159380025)
- Hydrogen peroxide (30%; Sigma-Aldrich, cat. no. H3410)
- Aqueous eosin (Amber Scientific, cat. no. YGR-EOA1-1L)
• Harris hematoxylin (POCD, cat. no. HHXIMP2.5)
• Fuchsin acid (Merck, cat. no. 1052310025)
• 2-Methoxyl ethylacetate (2-MEA) (Merck, cat. no. 8060612500)
• 1% Alcian Blue 8GX solution (Sigma-Aldrich, cat. no. B8438)
• Saffron (Sigma-Aldrich, cat. no. S8381-5G)
• Liquid DAB + substrate chromogen system (DAKO/Agilent, cat. no. K3468)
• Dako wash buffer (Dako, cat. no. DM831)
• Phosphate-buffed saline (PBS) (Thermo Fisher Scientific, cat. no. BR0014G)
• Ammonium hydroxide (28.0–30.0%, Sigma-Aldrich, cat. no. 4101042466)
• Weigert’s iron hematoxylin (Sigma-Aldrich, cat. no. HT1079)
• Ferric chloride iron (III) chloride (Merck, cat. no. 8039450500)
• Crocein scarlet (Sigma-Aldrich, cat. no. C8822)
• Acid fuchsin (Chroma–Waldeck, cat. no. 1B 525)
• Phosphotungstic acid (Sigma-Aldrich, cat. no. P4006)
• Glacial acetic acid (Chem-Supply, cat. no. 64197)
• Xylene (Ajax Finechem; Thermo Fisher Scientific, cat. no. 576/2.5L/P) !CAUTION Xylene is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate personal protective equipment (PPE).
• Acetone (Ajax Finechem; Thermo Fisher Scientific, cat. no. 67641) !CAUTION Acetone is a flammable and harmful substance. Use only in a downdraft fume hood and with appropriate PPE.
• Eukitt quick hardening mounting medium (Sigma-Aldrich, cat. no. 03989)
• Formaldehyde (37%; Thermo Fisher, cat. no. 9311) !CAUTION Formaldehyde is toxic upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate personal protective equipment.
• Potassium ferricyanide (Thermo Fisher Scientific, cat. no. P232-500)
• Silver nitrate (Thermo Fisher Scientific, cat. no. S181-100)
• Sodium carbonate (anhydrous; Fisher Scientific, cat. no. S263-500)
• Sodium thiosulfate (Thermo Fisher Scientific, cat. no. S445-500)
• Safranin O (Sigma-Aldrich, cat. no. S2255)
• Hydrochloric acid (32%; Ajax Finechem; Thermo Fisher, cat. no. AJA256)
• Light green SF yellowish (Merck, cat. no. 1159410025)
• Orange G (Merck, cat. no. 1159250025)
• Ponceau xylidine (Sigma-Aldrich, cat. no. 16150)
• Hydrogen peroxide (Sigma-Aldrich, cat. no. H3410) Store at 4 °C for up to 24 months
• Tris base (Roche Applied Science, cat. no. 11814273001) Store at RT for up to 36 months
• Dako wash buffer (Dako, cat. no. DM831) Store at 4 °C for up to 36 months
• Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A7906). Store at 4 °C for up to 36 months
• Secondary antibody: EnVision+ dual-link system (HRP rabbit/mouse kit; Dako, cat. no. K4061) Store at 4 °C for up to 24 months
• Mayer’s hematoxylin (Sigma-Aldrich, cat. no. MHS32-1L) Store at RT for up to 24 months
• EDTA

Equipment
• Bag valve mask apparatus (Mayo manual resuscitators; Mayo health care, cat. no. 150117)
• Instron Material Testing System (Instron, model no. 8874) ▲CRITICAL See Fig. 3 for photographs of some selected items.
• Drape clamps (Henry Schein, cat. no. 1013568)
• Artery forceps (Aesculap, cat. no. BH339R)
• Fine tissue scissors (Henry Schein, cat. no. AE-BC061)
• Metzenbaum dissecting scissors, curved tip (18 cm long; Aesculap, cat. no. BC263R)
• Dressing scissors, straight tip (14.5 cm long; Henry Schein, cat. no. AO-0325-1)
• Right-angle forceps, 400 mm long (Aesculap, cat. no. MB924R)
• Right-angle forceps, 200 mm long (Aesculap, cat. no. BJ057R)
• Adson non-toothed tissue forceps (Henry Schein, cat. no. AE-BD151)
• Debakey atraumatic tissue forceps (Aesculap, cat. no. FB402R)
• Adson toothed tissue forceps (12 cm long; Aesculap, cat. no. BD511R)
• Toothed tissue forceps (Aesculap, cat. no. BD500R)
Fig. 3 | Instruments required for the operative procedure. (1) Ten drape clamps; (2) four artery forceps; (3) two pairs of fine tissue scissors; (4) two pairs of Metzenbaum dissection scissors with curved tips; (5) dressing scissors; (6) small right-angle forceps; (7) large right-angle forceps; (8) Adson non-toothed tissue forceps and Debakey atraumatic tissue forceps; (9) Adson toothed tissue forceps and Gilles toothed tissue forceps; (10) large and small Hohman retractors; (11) large and small needle holders; (12) two no. 3 scalpels handles; (13) periosteal elevator; (14) skin preparation dressing holder; (15) hexagonal screwdriver; (16) 10-hole DCP; (17) self-retaining fragment clamp; (18) large metal ruler; (19) Verbrugge bone-holding forceps; (20) bone tap; (21) small metal ruler; (22) Coban wrap; (23) depth gauge; (24) drill guide; (25) sagittal saw blade; (26) disposable Yankauer suction tip; (27) three 2-mm drill bits; (28) lockable System 6 drill chuck; (29) key for drill chuck; (30) sagittal saw; (31) drill; (32) three small plastic bowls; (33) light handle; (34) two kidney dishes.

- Hohman retractor, 28 cm long (Teleflex, cat. no. KM46848)
- Hohman retractor, 27 cm long (Medline, cat. no. MDS3288024)
- Needle holder, 20 cm (Aesculap, cat. no. BM387T)
- Needle holder, 15 cm (Aesculap, cat. no. BM385T)
- Sterile disposable scalpel blades, no. 10 (Clifford Hallam Healthcare, cat. no. 1117668)
- Sterile disposable scalpel blades, no. 15 (Clifford Hallam Healthcare, cat. no. 1117756)
- Scalpel handle, stainless steel, no. 3 (Clifford Hallam Healthcare, cat. no. 1840861)
- Periosteal elevator (Aesculap, cat. no. MG024R)
- Skin preparation dressing holder (Henry Schein, cat. no. AE-BF118)
- Hexagonal screwdriver (DePuy Synthes, cat. no. 314.27)
- Cortex screws, self-tapping, 45-mm diameter, 28-mm length (DePuy Synthes, cat. no. 214.828S)
- 14-hole condylar dynamic compression plate (DCP), 95° (DePuy Synthes, cat. no. 237.420), cut down to a 10-hole plate length
- Self-retaining fragment clamps (Buxton Biomedical, cat. no. 56-1010)
- Steel ruler, 300 mm (Aesculap, cat. no. AA805R)
- Verbrugge bone-holding forceps (Zimmer Surgical Orthopedic, cat. no. 2309-48)
- Bone tap (Aesculap, cat. no. LS059R)
- Steel ruler, 150 mm (Aesculap, cat. no. AA804R)
- Depth gauge (DePuy Synthes, cat. no. 319.100)
- DCP drill guide (DePuy Synthes, cat. no. 322.440)
- Sagittal saw blades (Stryker, cat. no. 6118-89-90)
- Yankauer suction device with vent (Convatec, cat. no. 309.01.000)
- Drill bit (3.2 mm; Stryker, cat. no. 5120-108-32)
- Lockable drill chuck (System 6; Stryker, cat. no. 6203-131)
- System 6 sagittal saw (Stryker, cat. no. 6208)
- System 6 rotary handpiece (Stryker, cat. no. 6205)
- Small plastic bowls (Clifford Hallam Healthcare, cat. no. 1408920)
- Light handle cover (Clifford Hallam Healthcare, cat. no. 2298173)
• Kidney dishes (Clifford Hallam Healthcare, cat. no. 1844117)
• Bone plate press (plate-bending device; Synthes, cat. no. 329.30)
• REM PolyHesive patient return electrodes (Covidien; Patterson Veterinary, cat. no. 07-858-0779).
• Electrical clipper (Aesculap, cat. no. GT300) with no. 40 blade (Aesculap, cat. no. GT310).
• Fiber optic laryngoscope set (Welch Allyn Miller; Patterson Veterinary, cat. no. 07-813-0243) or 4 (Patterson Veterinary, cat. no. 07-820-0638), depending on the size of the sheep’s oral cavity
• Discofix three-way stopcock (B Braun; Patterson Veterinary, cat. no. 07-890-7587)
• Gravity blood infusion set (Becton Dickinson, cat. no. 901-020E)
• Extension tubing set, 150 cm (McFarlane Medical, cat. no. 19289TE)
• Portex tracheal tube, 9.0 mm (Smiths Medical, cat. no. 100/199/090)
• HotDog patient warming system (HotDog multi-functional controller (Augustine Temperature Management, cat. no. WC52-VET) and full-body warming blanket (Augustine Temperature Management, cat. no. V104)
• Bionet BM5 patient monitor (Mediquip, cat. no. BTHBM5)
• Ultiport 100 breathing system filter (Pall, cat. no. BB100E)
• DAR catheter mount, extendable (Covidien; Clifford Hallam Healthcare, cat. no. 2051274)
• Multi-lumen central venous catheterization set (Teleflex Medical; Clifford Hallam Healthcare, cat. no. 1612164)
• Diathermy rocker switch pencil (Covidien; Clifford Hallam Healthcare, cat. no. E2515)
• Surgical skin marker (Patterson Veterinary, cat. no. 07-849-0214)
• Syringe pump (UniVet; DLC Australia, cat. no. 210321)
• Closed-circuit large-animal anesthesia machine (Signet 615; Ulco medical, SN 4028) equipped with a mechanical respirator (EV 500; Ulco medical, SN 4062)

**Post-operative analysis equipment**

• Instron Materials Testing System (Instron, model 8874)
• Aperio Image Scope image analysis software (Leica Biosystems, ScanScope v. 12.3)
• Slide press holder (Kulzer; Emgrid, cat. no. 64712819)
• Laboratory fume cabinet
• Micro-computed tomography (micro-CT) scanner (Scanco Medical, model no. μCT40)
• Biaxial materials testing machine (Instron, cat. no. 8874)
• Kos Rapid multifunctional microwave tissue processor (Milestone, Abacus DX, Brisbane, model no. 67051/W)
• Excelsior ES tissue processor (Thermo Fisher Scientific, cat. no. ASHA78410023).
• Shandon Histocentre 3 embedding station (Thermo Fisher Scientific, cat. no. B64110040)
• Rotary microtome (Leica Biosystems, model no. RM2265, cat. no. 050338780)
• Embedding molds (33 ×24 ×12 mm; Bio-Optica, cat. no. 07-MBM6)
• Slide file storage system (Hurst Scientific, cat. no. M700-100G)
• Heavy-duty sledge microtome (Polycut-S; Reichert-Jung; International Medical Equipment, cat. no. SM2500)
• Upright microscope (Carl Zeiss Microscopy, model no. Axio Imager 2)
• Water bath (Labec)
• Autostainer XL (Leica Biosystems, model no. ST5010)
• Diamond band saw (EXAKT Advanced Technologies, model no. EXAKT 310)
• Micro-grinding system (EXAKT Advanced Technologies, model no. Exakt 400 CS)
• Electronic measuring and control system (EXAKT Advanced Technologies, model no. AW 110)
• Variable-speed grinder–polisher (EcoMet 4000; Buehler Industrial Technologies, cat. no. 49-1780); Coolant-proof digital micrometer (0–0.25 mm; 0.001 mm precision; Mitutoyo)
• Scanner (Leica, model no. 400SCN)

**Surgical supplies**

• U-drape, 193 cm × 305 cm (Clifford Hallam Healthcare, cat. no. 2228148)
• Drape, universal pack, containing reinforced table cover; 2 smart gowns, large; Mayo stand cover, reinforced (59 ×141 cm); 2 trilaminate side sheets with adhesive (97 ×196 cm); trilaminate foot sheet with adhesive (136 ×253 cm); trilaminate head sheet with adhesive (184 ×192 cm); outer wrap/table cover, reinforced (125 ×230 cm) (Clifford Hallam Healthcare, cat. no. 2224171)
Surgical drape, 120 cm × 140 cm (McFarlane Medical, cat. no. 31016MU)
Ioban 2 antimicrobial incise drape, 60 cm × 45 cm (3M; Clifford Hallam Healthcare, cat. no. 1050182)
Sterile suction tubing, flexible (AIMS Medical Group, cat. no. AN050003)
Surgicel Nu-Knit absorbable hemostat (Ethicon, cat. no. 1943GB)
ABdetex X-ray-detectable abdominal sponge, 30 cm × 30 cm (Multigate, cat. no. 88-888)
Detex X-ray-detectable pudding sponge (Multigate, cat. no. 15-002)
Syringes, 2, 5, 10, 20 and 50 ml (Becton Dickinson, cat. nos. 302204, 302130, 302149, 300613 and 300144)
Drawing-up needle, 18 gauge (Becton Dickinson, cat. no. 300204)
Needles, 18, 21 and 23 gauge (Becton Dickinson, cat. nos. 302032, 302017 and 302008)
Gauze swab, 10 cm × 10 cm (Multigate, cat. no. 17-888)
Vicryl sutures (0-0, 2-0, 3-0 and 4-0; Ethicon, cat. nos. J518, J317, J316 and J304)
Electrode tip cleaner (Covidien; Clifford Hallam Healthcare, cat. no. 1614346)
Soffban synthetic bandage, 10 cm × 2.7 m (BSN Medical; Clifford Hallam Healthcare, cat. no. 07-807-2245)
Coban wrap, 100 mm × 2 m (3M; Clifford Hallam Healthcare, cat. no. 1243234)
Personal protective equipment (PPE), including fluid-shield surgical mask (Clifford Hallam Healthcare, cat. no. 1403832) and gloves (Clifford Hallam Healthcare, cat. no. 2222332)

Post-operative analysis supplies
Microtome blade histo cutter, plasma LH35 stainless steel (Hurst Scientific, cat. no. LH35BOX)
Uniset tissue-processing cassette (Instrumec, cat. no. M405-4)
Exakt 800, 1000, 1200, 2500M and 4000 grit sandpaper (EXAKT Advanced Technologies)

Embedding (resin)
Quickfit Chromatography Column (Thermo Fisher Scientific, cat. no. 15537770) ! CAUTION Use only in a laboratory fume cabinet and with appropriate PPE.
Spark-free refrigerator

Mounting (paraffin)
Slide super frost polysine-coated white (25 × 75 × 1.0 mm) (Thermo Fisher Scientific, cat. no. MENSF41296PL)

Mounting (resin)
Microscope slides, 50 × 100 mm, 2 mm thick (EXAKT Advanced Technologies)

Mounting (thin section)
Microscope slides, 76 × 50 mm, 1.0–1.2 mm thick, single frosted (Hurst Scientific, cat. no. 7107)
Liner Brush Set (Mont Marte 6pc)

Reagent setup
Palapress embedding cement
To 40 ml of palapress dental acrylic monomer add 80 g of palapress dental acrylic powder. Leave it to polymerize for 10 min. ▲ CRITICAL Coat inner surface of embedding molds with Triton X-100 for easier cement removal after mechanical testing. Use it only in a laboratory fume cabinet and with appropriate PPE.

4% (wt/vol) PFA (pH 7.4)
To 40 g of PFA, add 1 liter of PBS and heat to 60 °C. Adjust the pH to 7.4 by adding NaOH. ! CAUTION PFA is toxic upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate PPE. ▲ CRITICAL Use the solution fresh or store it at −20 °C for up to 5 years.

3% (vol/vol) hydrogen peroxide
Dilute hydrogen peroxide 1:10 in ddH2O. ▲ CRITICAL Prepare fresh before use and discard remaining solution.
2% (wt/vol) BSA
Add 1 g of BSA to 50 ml of PBS. Prepare fresh before use and discard remaining solution.

0.1% (vol/vol) ammonium hydroxide
Dilute 2.89 ml of ammonium hydroxide in 500 ml of ddH₂O; store the solution at RT for up to 3 months.

Dako wash buffer
Dilute 100 ml of 20× concentrated Dako wash buffer stock in 1,900 ml of ddH₂O; store the solution at RT for up to 3 months.

Filtered Technovit 9100 basic solution
Filter the desired amount of basic solution using aluminum oxide 90 active neutral. Keep destabilized (filtered) basic solution at 4 °C in a spark-free refrigerator and use within 2 months. CRITICAL Use a chromatography column half filled with aluminum oxide 90 active neutral. Each column may take up to 40 min to filter 75 ml of basic solution. Ensure that the aluminum oxide 90 active neutral has filtered <45–50 column volumes. If the log shows that 45–50 column volumes have been filtered, change the sand.

Technovit 9100 pre-infiltration solution
To 200 ml of filtered Technovit 9100 basic solution, add 1 g of Technovit 9100 hardener 1. Keep the pre-infiltration solution at 4 °C in a spark-free refrigerator and use within 2 months. CAUTION Pre-infiltration solution is flammable and harmful upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate PPE. CRITICAL Ensure that all glassware and stirring flasks are cleaned with 100% ethanol and allowed to dry completely before use. Any water present in the resin solutions will interfere with the polymerization process. Full solution mixing may take up to 30 min.

Technovit 9100 infiltration solution
To 250 ml of filtered Technovit 9100 basic solution, add 20 g of Technovit 9100 PMMA powder and 1 g of Technovit 9100 hardener 1. Keep the infiltration solution at 4 °C in a spark-free refrigerator and use within 2 months. CAUTION Infiltration solution is flammable and harmful upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate PPE. CRITICAL Avoid clumping by adding Technovit 9100 PMMA powder a little at a time over ~5 min. Full solution mixing may take up to 2 h. Ensure that all glassware and stirring flasks are cleaned with 100% ethanol and allowed to dry completely before use. Any water present in the resin solutions will interfere with the polymerization process.

Technovit 9100 embedding stock solution A
To 500 ml of filtered Technovit 9100 basic solution, add 3 g of Technovit 9100 hardener 1 and 80 g of Technovit 9100 PMMA powder. Keep Technovit 9100 embedding stock solution A at 4 °C in a spark-free refrigerator and use within 2 months. CAUTION Technovit 9100 embedding stock solution A is flammable and harmful upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate PPE. CRITICAL Add the required amount of Technovit 9100 hardener 1 and stir for 15 min. Slowly add the Technovit 9100 PMMA powder a little at a time to the Technovit 9100 filtered basic solution while mixing. Note: 4× more Technovit 9100 PMMA powder is added to solution A (compared to fresh infiltration solution); hence, a longer time for addition of Technovit 9100 PMMA powder is required to prevent clumping. Ensure that all glassware and stirring flasks are cleaned with 100% ethanol and allowed to dry completely before use. Any water present in the resin solutions will interfere with the polymerization process.

Technovit 9100 embedding stock solution B
To 50 ml of filtered Technovit 9100 basic solution, add 4 ml of Technovit 9100 hardener 2 and 2 ml of Technovit 9100 regulator. Mix the solutions for at least 15 min. Keep Technovit 9100 embedding stock solution B at 4 °C in a spark-free refrigerator and use within 2 months. CAUTION Technovit 9100 embedding stock solution B is flammable and harmful upon inhalation, upon skin contact and if swallowed. Use it only in a laboratory fume cabinet and with appropriate PPE. CRITICAL Ensure that
all glassware and stirring fleas are cleaned with 100% ethanol and allowed to dry completely before use. Any water present in the resin solutions will interfere with the polymerization process.

**Technovit 9100 embedding working solution**  
To 9 parts (vol/vol) of Technovit 9100 embedding stock solution A add 1 part (vol/vol) Technovit 9100 embedding stock solution B. ▲CRITICAL Mix Technovit 9100 embedding stock solution A and Technovit 9100 embedding stock solution B immediately before use.

**Mounting resin base**  
Add 25 g Technovit 3040 powder to 15 ml Paladur. Leave it polymerizing for 10 min. ▲CRITICAL Place masking tape around the resin block and fill it with the mounting resin base cement. Use it only in a laboratory fume cabinet and with appropriate PPE.

**Gelatin coating**  
In 100 ml of water, dissolve 1 g of gelatin at 30 °C. Add 2 g of phenol crystals and 15 ml of glycerol. Stir and filter the solution. Store at 4 °C and use within 6 months.

**1% Alcian Blue**  
Dissolve 5 g of Alcian Blue in 500 ml DI water. Filter solution and add 5 ml 1% acetic acid solution. ▲CRITICAL Prepare fresh before use and discard remaining solution.

**Alkaline alcohol**  
To 90 ml of 96% ethanol, add 10 ml of 25% ammonia; store at RT for up to 2 months.

**2% (wt/vol) aqueous ferric chloride**  
Add 2 g of ferric chloride to 100 ml of distilled water; store at RT for up to 1 month.

**Crocein scarlet stock solution**  
Add 0.1 g of crocein scarlet to 99.5 ml of distilled water and 0.5 ml of glacial acetic acid; store the solution at RT for up to 1 month.

**Acid fuchsin stock solution**  
Add 0.1 g of acid fuchsin to 99.5 ml of distilled water and 0.5 ml of glacial acetic acid; store the solution at RT for up to 1 month.

**Crocein scarlet–acid fuchsin staining solution**  
The crocein scarlet–acid fuchsin staining solution is a 4:1 ratio of crocein scarlet stock solution to acid fuchsin stock solution. Add 80 ml of crocein scarlet stock solution to 20 ml of acid fuchsin stock solution. ▲CRITICAL Prepare fresh before use and discard remaining solution.

**5% (wt/vol) aqueous phosphotungstic acid**  
Add 10 g of phosphotungstic acid to 200 ml of distilled water; store the solution at RT for up to 1 month.

**6% (wt/vol) alcoholic saffron**  
Add 6 g of saffron to 100 ml of 100% alcohol at 58 °C for 48 h. Store at RT for up to 6 months.

**0.5% (vol/vol) acetic acid water**  
Add 0.5 ml of acetic acid to 100 ml of distilled water. ▲CRITICAL Prepare fresh before use and discard remaining solution.

**70% (vol/vol) ethanol**  
To 2,210 ml of 100% ethanol, add 790 ml of DI water.

**Silver nitrate solution**  
To 400 ml of DI water, add 20 g of silver nitrate; store the solution at RT for up to 6 months. ▲CRITICAL Protect the solution from light to prevent precipitation.
Sodium carbonate–formaldehyde solution
To 337.5 ml of DI water, add 112.5 ml of formaldehyde and 22.5 g of sodium carbonate; store at RT up to 6 months. ▲CRITICAL The solution should be filtered before each use.

Farmer’s diminisher solution
To 420 ml of DI water, add 40 g of sodium thiosulfate and 2 g of potassium ferricyanide ▲CRITICAL The solution is stable for <1 h after the addition of potassium ferricyanide; thus, make immediately before use.

0.1% (wt/vol) Safranin O
To 100 ml of DI water, add 0.1 g of safranin O. Store the solution at RT up to 6 months.

MacNeal’s tetrachrome
Add 12 g of tetrachrome stain, certified (MacNeal) to 600 ml DI water and stir solution at 60 °C for 48 h. Filter solution. Store at RT for up to 6 months.

Wiegert’s iron hematoxylin solution A
Dissolve 10 g of hematoxylin monohydrate in 1,000 ml of 95% ethanol. Store at RT for up to 6 months.

Wiegert’s iron hematoxylin Solution B
Dissolve 5.8 g of ferric chloride and 5 ml of hydrochloric acid in 500 ml of distilled water. Store at RT for up to 6 months.

Wiegert’s iron hematoxylin working solution
Mix Wiegert’s iron hematoxylin solutions A and B in a 1:1 ratio. ▲CRITICAL Filter the solution before use. The solution is stable for only 1 d. Discard unused solution.

Acid fuchsin–Ponceau solution A
Dissolve 6 g of Ponceau xylidine in 600 ml of distilled water. Store at RT for up to 6 months.

Acid fuchsin–Ponceau solution B
Dissolve 2 g of Fuchsin acid in 200 ml of distilled water. Store at RT for up to 6 months.

Acid fuchsin–Ponceau stock solution
Mix acid fuchsin–Ponceau solutions A and B in a ratio of 3:1. ▲CRITICAL Prepare stock solution just before use.

Acid fuchsin–Ponceau working solution
Dilute the stock solution in a ratio of 1:5 with 0.2% acetic acid. ▲CRITICAL The solution is stable for only 1 d. Discard unused solution.

Phosphotungstic acid–Orange G solution
To 500 ml of distilled water, add 10 g of Orange G and 20 g of phosphotungstic acid; the solution can be collected and reused. Store at RT for up to 6 months. ▲CRITICAL Filter the solution before use to avoid Orange G precipitation.

Light green SF yellowish solution
To 500 ml of distilled water, add 1 g of light green and 1 ml of glacial acetic acid; the solution can be collected and reused. Store at RT for up to 6 months. ▲CRITICAL Filter the solution before use.

1% (vol/vol) glacial acetic acid
To 1,000 ml of distilled water, add 10 ml of glacial acetic acid. ▲CRITICAL Prepare 1% glacial acetic acid fresh just before use. Do not store.

0.5% (vol/vol) glacial acetic acid
To 1,000 ml of distilled water, add 0.5 ml of glacial acetic acid. ▲CRITICAL Prepare 1% glacial acetic acid fresh just before use. Do not store.
Equipment setup

Facilities
Facility requirements are fairly standard. We undertake all of our large-animal studies at the Medical Engineering Research Facility (MERF) in Brisbane (QUT, Queensland, Australia). As stated earlier, all procedures are approved by the QUT University Animal Ethics Committee and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

An operating theater with standard gas/pressure components (oxygen/nitrogen/suction), lighting (two surgical lights) and storage space is required. We use a state-of-the-art pedestal operating table that permits automated control of height and position, although all that is required is a standard operating table with a manual vertical positioning capability. A standard adult ventilator is required during the anesthesia.

Personnel
Personnel requirements to undertake this type of work include a veterinarian for the induction, maintenance and weaning of anesthesia; a primary operating surgeon to undertake the procedure, alongside an assistant surgeon (operative technical expertise not necessarily required); and an animal technician to assist with preoperative and postoperative measures, as well as to help source any additional utensils/consumables required intraoperatively.

Husbandry
The husbandry capabilities required to facilitate the preoperative and postoperative care of the animals are important. The animals should be brought in the week before the operation to allow a preoperative check to be undertaken. The sheep health check should be performed by a veterinarian and the following parameters should be noted: body weight, age, visual body condition, core body temperature, heart rate, respiratory rate, chest auscultation results, eye condition (e.g., blindness, lacerations, infection), ear condition, dentition (broken, chipped or missing teeth), state of mucous membranes (laceration, ulceration and color), superficial lymph nodes (normal versus enlarged), coat condition (dirty, unkempt or presence of lice), skin condition (presence of lacerations or inflammation), fecal or urine soiling, fecal output and consistency, movement (e.g., signs of stiffness, lameness), and general behavior. Any sheep that do not meet suitable criteria must be excluded from the study, or remedial action must be taken. All sheep should also be tested for Q-fever and should be excluded from the study if a positive result is obtained. All sheep should be vaccinated, drenched for internal parasites, given cobalt and grinder, sprayed for external parasites and shorn.

Preoperative checks over the duration of the 1-week period also permit a period for the animals to become acclimatized to the facility and to the weight-bearing slings. Sling acclimatization should be performed for a total period of 7 d in interrupted phases (e.g., 12 h overnight) during the preoperative week. We use three separate staged areas for the sheep. During the immediate postoperative phase, a separate chamber with six slings is used to help the animal control its own weight-bearing status on the operated limb. Control of weight-bearing status at this point is useful for pain control and allows the animal a graduated period in which to adjust to the operated limb. The way the sling is constructed also facilitates the ability of the animal to choose when to bear weight and which limbs to bear weight on.

Procedure

▲ CRITICAL An overview of the procedure is shown in the flow diagram (Fig. 4).

Preoperative steps (day before surgery)  ● Timing 24 h

1. The day before surgery, check the sheep’s general health and blood profile in order to ensure the animal’s suitability for the procedure. Weigh the sheep for accurate dosing of the medications. Clip the sheep’s neck, forelegs and right hind leg using a clipper with a no. 40 blade.
2. Apply a fentanyl transdermal patch to the skin of the sheep’s foreleg. Place a few sheets of cotton gauze dressing on the patch and protect it with a Coban wrap.

▲ CRITICAL STEP Opioid patches alleviate postoperative pain in the research sheep for up to 3 d (72 h). The patch can be replaced once or twice every 72 h for longer pain management, where required\(^{10}\).
3 Remove food from the sheep for 12 h before the general anesthesia and surgery. Ad libitum access to water can be provided until 2 h before general anesthesia. **CRITICAL STEP** Fasting reduces the risks associated with recumbency and general anesthesia, for example, tympany, regurgitation, and aspiration pneumonia.\(^{113}\)

**Preoperative steps (day of surgery) • Timing 1 h**

4 On the day of surgery, cradle the sheep in a custom-made sheep lifter/trolley in an upright position and establish a peripheral i.v. line. Establish venous access via the left jugular vein with a multi-lumen central venous catheter using a Seldinger technique\(^{114}\) (Fig. 5a). **CRITICAL STEP** We use a custom-made sheep lifter/trolley for the purpose of physical restraint, which considerably reduces anxiety/stress and makes the sheep more tractable research subjects. The sheep lifter/trolley enables the animal to be confined and then, using a winch and a stretcher, safely lifted in an upright position to a suitable working height. Experimental procedures (e.g., establishing venous access) can be safely performed in this position, and then the stretcher can be lowered onto a trolley and the animal safely transported to the surgical theater.

5 Administer 0.2 mg/kg midazolam and 0.005 mg/kg buprenorphine i.v. via the jugular venous catheter for sedation and breakthrough analgesia, respectively.

6 Once a reasonable level of sedation has been achieved (this should occur ~5–10 min post-injection), palpate the tracheal outer diameter in order to select the appropriate size of endotracheal...
tube (ETT). A transparent PVC disposable cuffed ETT (Portex tracheal tube) is recommended for small ruminants.

**CRITICAL STEP** Endotracheal intubation is highly recommended in small ruminants to provide a secure airway and prevent aspiration of saliva and ruminal contents (passive regurgitation)\(^\text{113}\).

7 Pre-oxygenate the sheep before intubation with bag–valve–mask ventilation (BVM) to extend the safe apnea time\(^\text{117}\). Induce anesthesia with a single i.v. dose of 4–6 mg/kg 1% Propive\(^\text{113}\). Normally, it takes ~10 s for the sheep to fall asleep (become unconscious).

8 Hold the sheep’s head and neck in a hyperextended position and open its mouth (this is generally done by an assistant). Introduce a laryngoscope with a straight blade size 3 or 4 (depending on the size of the sheep’s oral cavity) intraorally. Advance the blade toward the oropharynx until tip of the blade comes into contact with the tongue base (torus linguae).

9 Depress the tongue–epiglottis complex ventrally so the larynx (rima glottidis) is visible. Lubricate the tip and cuff of the ETT with 2% lignocaine gel and tie a loop of linen tracheostomy tape immediately below the tube’s connector.

10 Hold the tube convex side up and gently introduce the lubricated tube above and alongside the laryngoscope blade into the sheep’s rima glottidis. Inflate the tube’s cuff with 10 ml of air, using a syringe connected to its spring-loaded one-way valve.

11 Secure the tube by passing and tying the tracheostomy tape behind the head under the ears. Depth (length) of insertion depends on the size of the sheep jaws, but in no circumstances should the connector be placed past the labial commissure. A deep ETT insertion may result in cumbersome placement of the catheter mount and breathing tube.

**CRITICAL STEP** Apnea is a common finding following propofol induction. To ensure that the sheep is adequately oxygenated post-intubation, manually ventilate with BVM for 20–30 s until the oral mucous membranes are pink and bright.

12 Confirm correct placement of the ETT by direct visualization of the tube while it is advancing between the vocal cords into the larynx (rima glottidis), visualization of bilateral chest rise when the sheep is bagged (BVM ventilation), presence of fogging (condensation) of the tube, and the presence or odor of ruminal fluid in the ETT.

**CRITICAL STEP** A misplaced ETT can lead to hypoxemia and aspiration pneumonia.

13 Place a warming blanket on the operating table above the sheep’s body. Transfer the sheep to the operating table from the trolley, and position the sheep in right lateral recumbency. Connect the sheep to a closed-circuit large-animal anesthesia machine equipped with a mechanical respirator. Maintain anesthesia with 2–2.5% isoflurane in a mixture of oxygen/air (40:60).

**CRITICAL STEP** Use a warming blanket during the procedure to keep the animal’s body temperature stable and prevent hypothermia associated with general anesthesia.

**CRITICAL STEP** Follow the warming blanket’s instruction manual to ensure proper positioning of the blanket.

**CRITICAL STEP** Assess depth of anesthesia using cardinal vital signs, eye reflexes, jaw tone, and inter-digital pinch reflex every 15 min throughout the surgery. Also continuously monitor cardiac electrical activity (ECG) using the base apex lead, heart rate, capillary refill time and oxygen saturation level (SpO\(_2\)) with a portable patient monitor. Record observations during the surgery in an intra-operative anesthesia log.
14 Apply enough Genteal eye gel to cover the sheep’s eyes.
   ▲ CRITICAL STEP Sterile eye lubricant is needed to protect the sheep’s eyes from desiccation during the procedure.
15 Administer a maintenance fluid (e.g., Hartmann’s solution) i.v. via the jugular vein access at a rate of 2.5–5 ml/kg/h.
16 To ensure a steady state of analgesia during the procedure, i.v. administer fentanyl at a rate of 10 µg/kg/h\(^{95}\) using a syringe pump (continuous-rate infusion). In addition, administer a nonsteroidal anti-inflammatory (Flunixin, 1.1 mg/kg, i.m.).
17 Administer a first-generation cephalosporin (Cefazolin, 15–20 mg/kg, i.v.) as a bolus to ensure adequate tissue concentrations at the time of surgical incision.
   ▲ CRITICAL STEP Surgical antibiotic prophylaxis is recommended because artificial devices (mPCL scaffold and internal fixation device) are implanted into the tibial segmental defect.

Operative procedure ● Timing 2.5–3 h
18 Position the sheep in the right lateral decubitus position, with the left hind limb in a flexed position cranially, supported by a cushion pad, and the metatarsus retracted with a lace tie knot under tension. The medial side of the right hind limb should be fully exposed to at least 4 cm above the tibiofemoral joint line (Fig. 5b).
19 Prepare the limb for surgical access (Fig. 5b,c) by clipping the medial and anterior aspects of the tibia to the skin with a fresh shearing clipper and then cleaning the limb with a povidone-iodine scrub.
20 Wrap a self-adherent wrap (Coban) around the hoof and suspend the limb in the air, using an i.v. pole for circumferential access to the limb.
21 Spray the limb with an aqueous ethanol solution (70–75% ethanol) and let it dry for 30 s.
22 Prepare the skin with a povidine-iodine solution (Betadine).
23 Carefully detach the limb from the fluid pole while wrapping a sterile Coban wrap around the limb distally and over the nonsterile Coban wrap.
   ▲ CRITICAL Coban wrap only comes non-sterile. Sterilize Coban wrap before use.
24 Place sterile drapes around the limb and then place an antimicrobial incise drape cover (Ioban) over the operative site.
25 Palpate and mark two major landmarks: (i) the proximal tibial plateau and (ii) the summit of the medial malleolus. Measure the length of the tibia and mark its midpoint. Place the DCP over the middle of the tibia longitudinally, with the midpoint of the plate and the midpoint of the tibia matched. Use this as a guide for the skin incision.
26 Make a skin incision with a size 10 scalpel blade down to the panniculus carnosus. Dissect down to the periosteum carefully with Metzenbaum dissecting scissors and perform diathermy cauterization as required (Fig. 6a).
   ▲ CAUTION It is important to keep the soft tissues wet with saline-soaked gauze to limit periosteal desiccation while measuring out the defect and bending the plate.
27 Mark the proximal end of the DCP with a 3-0 Vicryl suture and place it over the medial to anteromedial surface of the tibia, abutting the periosteum. Use the plate-bending device to bend the plate to match the tibial contour. Having the suture proximally on the plate ensures no confusion after plate bending has occurred.
   ▲ CRITICAL STEP It is imperative to ensure that the midpoint of the plate is matched with the midpoint of the tibia during this process. The plate can be marked with a pen at its midpoint and the periosteum of the tibial midpoint can be marked with a no. 15 scalpel blade.
28 Release the tibia from the soft tissues in preparation for osteotomy. This is performed by releasing the musculature at the anterior, lateral and posterior surfaces of tibia in a plane that is superficial to the periosteum. Release should be performed over the middle 3 cm, with an additional 1 cm proximal and distal to the defect. Start at the anterior and posterior edges of the tibia with a size 15 scalpel blade, followed by blunt dissection. For the lateral aspect, use blunt dissection only. Use 90° artery forceps and a large Hohman retractor to dissect between the neurovascular bundle (NVB) and the periosteum (Fig. 6b).
   ▲ CAUTION Be careful not to damage the anterior tibial NVB during this process. This can be avoided by carefully identifying the NVB during the anterior approach and using a Hohman retractor to bluntly dissect between the NVB and the periosteum.
29 Temporarily fix the plate to the medial surface of the tibia with a Verbrugge bone clamp.
   ▲ CRITICAL STEP Ensure that the midpoint of the plate matches the midpoint of the defect.
Perform diathermy on the periosteal surface of the tibia at each hole of the DCP in the proximal and distal tibial segments.

**CAUTION** Be careful not to place the diathermy pencil on the plate because this will serve as a conduction device and cause a low-grade burn to the periosteum.

**CRITICAL STEP** This will ensure that no periosteal tissue becomes caught in the drill while drilling the tibia.

Counting from proximal to distal, drill hole no. 7, measure with the depth gauge and then tap the screw hole. If the plate does not seem to rotate well around this hole in the first instance, consider drilling hole no. 9 in the first instance rather than no. 7. This enables rotation of the plate around this hole. Measure and place a screw.

**CAUTION** It is important to tap the screw holes closest to the segmental defect, because this reduces the potential for fracture from the screw hole into the osteotomized bone surface when inserting the screw later in the procedure. We use this approach even with self-tapping screws.

**TROUBLESHOOTING**

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**Fig. 6 | Illustration of the various key stages in the operative approach.**

- **a.** The soft tissue and skin of the medial leg are incised to facilitate exposure of the tibia.
- **b.** The 3-cm defect periosteum is scored with a marking for the midpoint of the defect, and the anterior musculature of the limb is retracted to expose the neurovasculature bundle.
- **c.** The periosteum is stripped proximal and distal to the defect for at least 5 mm to reduce spontaneous healing.
- **d.** The proximal osteotomy. Note placement of the Hohman retractor to ensure that the neurovascular bundle is protected sufficiently.
- **e.** Final fixation of the defect with the DCP, with the scaffold placed centrally and tied to the plate with a single 0-0 Vicryl suture to reduce scaffold migration during the early postoperative phase.
- **f.** The soft tissue is closed in layers with 3-0 Vicryl sutures.
- **g.** Postoperative image showing the application of the Delta-Lite Plus fiberglass cast tape to the sheep’s hind limb. All animal experiments received Queensland University of Technology animal ethics approval.
32 Drill hole no. 3, measure, tap and insert a screw.
33 Drill a second hole for each of the proximal and distal fragments. This is necessary to ensure the alignment of the fragments following osteotomy. There is no need to place screws into these holes at this point.
34 Remove the screws from holes 3 and 7, and remove the plate from the defect.
35 Measure the distance between drill holes 4 and 7. This should be 5 cm, and the midpoint should correspond to the previously scored periosteum in Step 5. Use a fresh size 15 scalpel blade to score the periosteum and bone. Mark 1.5 cm proximal and 1.5 cm distal of the midpoint perioseal marking and ensure that the marking lines are perpendicular to the ruler. This will mark the orientation of the osteotomies. Use a scalpel to score the two lines transversely and score the whole anterior–posterior width of the tibia on the medial surface.
36 Use a periosteal elevator or dissection scissors to denude the periosteum ~5 mm from the medial surface of the tibia at the lines of the scored periosteum in the direction toward the bone segment that is to be resected (Fig. 6c).
37 To assist the safe passage of the Hohman retractor, use the 90° artery forceps from the anterior aspect initially. Then place a Hohman retractor from posterior to anterior, between the periosteum and the NVB. Slide the Hohman retractor to the level of the proximal osteotomy to protect the soft tissues and NVB while performing the osteotomy.
38 Perform the proximal osteotomy with the sagittal saw, cutting from medial to lateral.
  ! CAUTION Irrigation with normal saline during the osteotomy is essential to prevent thermal necrosis of the bone.
  ! CAUTION Ensure that the assistant holds the distal part of the limb throughout the osteotomies, as well as holding the Homan retractor and performing irrigation. An additional assistant can make this part of the procedure easier but is not essential.
  ▲ CRITICAL STEP Ensure that only the medial half of the tibial width is osteotomized at this point.
39 Remeasure from the proximal osteotomy line to 3 cm distal to ensure the correct position for the distal osteotomy. Place a small ruler in the proximal partial osteotomy to help with alignment while performing the distal osteotomy.
40 Perform a complete osteotomy distally. Introduce the Hohman retractor from the posterior aspect to protect soft tissues and the NVB.
  ▲ CRITICAL STEP It is essential that the assistant hold the distal segment of the tibia in a neutral position during cutting. This will help to prevent irregular split of tibia at the distal osteotomy site.
41 Secure the 3-cm bone fragment with Verbrugge bone-holding forceps and finalize the proximal osteotomy (Fig. 6d).
  ? TROUBLESHOOTING
42 Remove the segmental bone fragment. Detach the remaining insertion of the gastrocnemius and other muscles from the lateral tibial surface.
  ! CAUTION Be sure to protect the anterior tibial NVB during this process.
43 Place the DCP back over the proximal and distal segments in alignment with the screw positions. Replace the screws in hole positions 4 and 7. This will ensure that the tibia is stable during the remaining fixation process.
  ! CAUTION The hind limb musculature tends to contract and reduce defect length following creation of the bone defect. Ensure that the defect is still 3 cm before tightening the screws.
  ▲ CRITICAL STEP Place saline-soaked gauze on the defect and wound to keep the soft tissues wet.
44 The defect should be placed under compression through the use of screw positioning in the distal aspect of the DCP holes in the distal fragment. This moves the distal fragment toward the proximal fragment, effectively loading a compression force on the defect or its content. Retighten the screws in the compressed position.
  ▲ CRITICAL STEP For a rigid scaffold, it is essential that when the device is placed within the defect, it is under a slight compression load to facilitate satisfactory osseointegration during the healing process.
45 Drill remaining holes through the DCP, measure and then place the screws.
46 Further fix the scaffold with a 0-0 Vicryl suture through the scaffold and plate holes 6 and 7 (Fig. 6e).
47 Close the wound in layers. First, close the released fascial tissue with 3-0 Vicryl to ensure that the plate is completely covered. Next close the skin continuously with 3-0 Vicryl (Fig. 6f).
48 Cover the wound with sterile gauze. Wrap the tibia with Soffban synthetic bandage, followed by wrapping it around the whole length of hind limb.
49 Take X-ray images to evaluate screw length and tibia alignment.

50 Fashion a cast for the whole leg using Delta-Lite Plus fiberglass cast tape (Fig. 6g).

**CAUTION** It is recommended to fenestrate the cast over the tuber calcanei to reduce risk of pressure sores while the cast remains on.

**Immediate postoperative management**

**Timing 2–3 h**

51 Upon completion of the surgical procedure, switch the vaporizer off. Provide the sheep with pure oxygen via the ventilator while continually monitoring the cardinal vital signs. Give a prophylactic dose of cortisone (methylprednisolone, 40–60 mg, i.v.) to prevent airway spasm due to extubation.

52 After about 5 min, gradually wean the sheep from the active ventilator, while still supporting spontaneous breathing using CPAP (continuous positive airway pressure). Closely monitor the sheep until it regains its ability to maintain its own ventilation and oxygenation requirements (spontaneous breathing recovers).

53 Disconnect the sheep from the ventilator as soon as its reflexes recover. Move and place the sheep into the custom-made large-animal trolley in an upright position. Monitor the sheep’s cardinal vital signs (particularly its SpO2) for another 5–10 min before moving it back to the recovery room in the animal house. Here, place the sheep in a sling with access to food and water and carefully monitor vital signs until features of normal behavior are observed (e.g., healthy appetite, normal demeanor) as outlined in Fig. 7. Generally, we keep our animals in the slings for 2 weeks after the operation and for up to 4 weeks if the sheep avoids bearing weight on the limb.

**Early postoperative monitoring**

**Timing 2 weeks**

54 For the first 14 d, monitor sheep by observing the sheep’s demeanor, looking for signs of pain or distress, while keeping the sheep in a supportive sling. Particular note should be made of the sheep’s appetite and drinking behavior, walking behavior (mobility), weight-bearing pattern of its operated leg, urination and defecation, and interest in surroundings. Monitor the sheep three times a day during this period (morning, midday, evening) and appropriately document in observations in the sheep’s patient file. This enables researchers to identify any potential postoperative issues (osteosynthesis screw loosening, periprosthetic fracture) that may have arisen before a major

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**Fig. 7** | The custom sling used for sheep limb support during the postoperative period. **a, b.** Sling harnesses are fixed to the ceiling using multiple adjustable fixation points (**a**), which permit ‘as tolerated’ weight-bearing on the limb in a dynamic fashion (**b**). All animal experiments received Queensland University of Technology animal ethics approval.
complication occurs in the sheep limb. If required, intervention or early euthanasia can be undertaken to spare the animal any considerable pain.

▲ CRITICAL STEP Provide the sheep with suitable antibiotics postoperatively, such as a short-acting broad-spectrum antibiotic (e.g., Trisoprim) once daily for 3 d postoperatively and administer further doses as required.

▲ CRITICAL STEP Provide bimodal postoperative analgesia with slow-release transdermal fentanyl patches (2–3 μg/kg/h) and parenteral administration of a suitable nonsteroidal anti-inflammatory (e.g., flunixin meglumine). Use a total of three patches for each sheep to ensure that 10 d of postoperative analgesia is provided. Give nonsteroidal anti-inflammatories once daily for 5 d postoperatively. Because there are variations between individual animals in the rate at which opioids are absorbed via the skin, additional doses of buprenorphine can be used on the basis of signs of pain or discomfort during the postoperative period.

Further postoperative monitoring ● Timing 3–12 months

55 From postoperative week 3 onward, observe the animals only once per day. As before, document the demeanor, appetite, drinking, weight-bearing pattern, mobility and any signs of pain or distress in the sheep. For the first 6 weeks following surgery, house the animals inside in a larger communal indoor pen with the remaining sheep. This restricts their mobility and keeps their leg cast away from the ravages of climate and the outdoor environment while providing ample room for the sheep to move, eat/drink and interact with each other. House the sheep in this larger pen for up to 3 months before placing them in an outside non-enclosed pen.

56 4–6 weeks after the operation, X-ray the sheep to rule out any implant-mediated complications (implant failure or other pathologies). If there are no complications, remove the cast (4–6 weeks postoperative) using a sagittal saw under sedation. To do this, firmly press the cast saw blade against the cast at a 90° angle until it completely passes through the cast shell. Then lift the blade out, move it to an adjacent spot, and repeat the process (vertical ‘in-and-out’ sawing motion). The cast should be cut down both sides. Once the cast shell on both sides is cut down, use a cast spreader to further widen the cuts until the two cast shells can be separated and removed. Then use bandage scissors to carefully cut off and remove the underlying cast padding.

57 Once the cast is removed, house the sheep indoors for another week and monitor them closely. Return the sheep to the agistment facility at 3–6 months after surgery if no complications are recorded. Check the animals daily. Remote monitoring via video is also possible.

58 Perform additional X-ray analysis at required intervals for up to a year following surgery. Generally, we perform this at the following intervals: time of surgery, 6 weeks postoperative, 12 weeks postoperative, 6 months postoperative, and 12 months postoperative (before euthanasia). But other X-rays can be performed as clinically indicated during the postoperative phase.

59 At 12 months, transport the sheep back to the research facility for euthanasia and limb explantation.

▲ CRITICAL STEP The social requirement for these animals is particularly high, and if a sheep is left alone for an extended period, this can produce anxiety in the animal.

Euthanasia ● Timing 30 min

60 Carry out euthanasia at the designated time point after the surgery. Typically, we perform this with an i.v. combination of pentobarbital and potassium chloride (sodium pentobarbitone 325 mg/ml, 0.5 ml/kg, Lethabarb) administered to the animal in a controlled fashion by a veterinarian.

61 Confirm death by a combination of the following signs: absence of heartbeat/pulse/respiratory movement for at least 3 min, fixed and dilated pupils, or loss of color in mucous membranes.

▲ CRITICAL STEP Post hoc analysis must be performed only after death is confirmed.

Post-mortem analysis

62 Examples of postmortem analyses that can be performed are plain radiography (Fig. 8a–e, option A), bone volume evaluation (Fig. 8f,g); mechanical testing (Fig. 8h,i, option B); micro-CT (Fig. 9, option C) and histology of explanted tibial defects (Figs. 10–16, option D, Boxes 1–4 and Table 4). Resin and paraffin samples can be processed from the same tissue explant for histology, as indicated in Fig. 11. Resin sample processing does not require decalcification and therefore is generally performed before paraffin sample processing.
(A) **Plain radiography** ● **Timing 30 min each time**

(i) Perform medial–lateral and anterior–posterior views using plain radiography (3.2 mA·s, 65 kV) to progressively assess the extent of bone regeneration across the defect following
surgery (t = 0 d) at 1 month, 3 months, 6 months, 9 months and 12 months to determine radiographic features of regenerate bone formation and the presence radiographic union. (B) Mechanical testing

(i) After limb explantation, disarticulate the operated tibiae from the hind limbs and assess.

(ii) Remove the soft tissues from the tibiae, embed proximal and distal tibial ends in Palapress ('Reagent setup') dental acrylic and place samples into a biaxial materials testing machine.

▲ CRITICAL Coat inner surface of the embedding cups with Triton X-100 or detergent for easier cement removal after mechanical testing.

(iii) Perform torsion under angular displacement at a targeted velocity of 0.5/s and a constant compression load of 0.05 kN until the first signs of fracture are observed, at which time instantly stop the process.

(iv) Use the contralateral tibia as a paired reference point for each animal.

(v) Calculate the maximum torsional moment and torsional stiffness values from the slope of the torque–angular displacement curves and normalize against the values of the contralateral tibiae.

(vi) After mechanical testing, excise the 3-cm defect and 1 cm proximal and distal to the defect from the tibia and place into 4% PFA for 72–96 h at RT (Fig. 10).

Fig. 8 | Mechanical testing results after 3 months. (a–e) Representative X-ray images from 8 animals studied, showing an empty control defect (a), a defect reconstructed with a cancellous bone graft from the iliac crest (b), a defect treated with the mPCL-TCP scaffold only (c), and defects augmented with scaffold + rhBMP-7 (d) or scaffold + MSCs (e). Scale bar, 1 cm. f Median BVs were determined by CT. g BV distribution along the z axis. The total length of the defect was divided into three parts of equal length (proximal, middle, and distal thirds). h,i Torsional moment (h) and torsional stiffness (i) measurements. Empty control defects (n = 8) were excluded because they were filled with soft tissue only. *P < 0.05, Bonferroni–Holm test. Data are presented in box plots (f–i) that depict the mean (horizontal lines), the upper and lower quartiles (boxes), and the minimum and maximum (whiskers; n = 8). Circles indicate the outliers. ABG, autologous bone graft; BV, bone volume. Images adapted from ref. 6. All animal experiments received Queensland University of Technology animal ethics approval.

Fig. 9 | Histological and imaging analyses of bone defects after 3 and 12 months. Images are representative of n = 3–8 samples. The empty defect was not studied at 12 months to avoid implant failures. The scaffold + MSCs group was not studied at 12 months because it did not regenerate bone as well as the ABGs or the scaffold + rhBMP-7 combination. 3D renderings of µCT reconstructions and a frontal section of one representative sample per group (left) and safranin O–von Kossa–stained histology sections (right; orientation: top, proximal; left, medial). Images adapted from ref. 6. All animal experiments received Queensland University of Technology animal ethics approval.
(C) Micro-CT ● Timing 4.5 h per limb, including preparation time

The newly formed mineralized tissue can be imaged and quantified after animal euthanasia at 12 months post-operation, as previously described.

(i) Place specimens into a sample tube and scan using a desktop micro-CT scanner at a power of 70 kV (peak, kVp), an intensity current of 114 µA, and a voxel size of 19 µm.

(ii) Analyze the defect region only as the volume of interest. Segment the reconstructed scans with a Gaussian filter of a width of 0.8 and support of 1.0. Determine thresholds by visual evaluation of 20 random tomograms/specimen for four samples per group.

(iii) Select a global threshold that best depicts the morphology of the mineralized bony tissue of interest.

(D) Histology ● Timing 4–5 months

(i) Resin sample processing. Cut tibial sample defects at 5-cm length (3-cm defect plus 1 cm each on the proximal and distal sides of the host bone) using the EXAKT 310 diamond band saw.

Fig. 10 | Example specimens. a, Specimen after removal from residual tibia, following biomechanical testing. b, Specimen freshly isolated from the sheep, demonstrating excellent biocompatibility of the mPCL scaffold, with bone marrow extruding from the DCP holes. All animal experiments received Queensland University of Technology animal ethics approval.

Fig. 11 | Schematic overview of the histological cutting plane methodology applied to the tibial defects, paraffin and resin samples format. P, paraffin plane section. All animal experiments received Queensland University of Technology animal ethics approval.
(ii) First, section the samples into two resin sagittal planes at 2 mm thick and retain the entire length (50 mm) as illustrated in the schematic sample cutting plane in Fig. 11 (resin sagittal plane section). Remove tissue excess and allocate it for resin processing and staining (Boxes 1–3).

**CRITICAL STEP** After sectioning the sample into two sagittal planes, allocate the two halves remaining for paraffin processing (see step 62D(xix))

(iii) Dehydrate the samples allocated for resin processing through one change of 70%, 80% and 90% ethanol and two changes of 100% ethanol for 1 week for each solution.

(iv) Following dehydration, degrease the samples in two changes of xylene for 4 h each.

**CAUTION** Xylene is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.

(v) After clearing the samples, prepare the required amounts of Technovit 9100 solutions for infiltration and embedding tissue samples. An example of Technovit 9100 solutions for infiltration and embedding is provided in Table 3116.

(vi) Incubate the samples in fresh Technovit 9100 pre-infiltration solution for 7 d at 4 °C. Store samples at 4 °C.

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Fig. 12 | Morphology of newly formed bone in scaffold-rhBMP-7-treated animals. **a,b.** Movat’s pentachrome stain. The histology sections show the interface of cortical bone and newly formed bone (**a**) and a higher-magnification image of the new bone (**b**). **c.** New bone could also be visualized around a scaffold strut with unmineralized osteoid (red). **d-f.** Osteoblasts, osteoclasts, and mature osteocytes were embedded in lacunae (**d**), and blood vessels were embedded in soft tissue (light blue; **e,f**). **g-i.** BSE microscopy images of a transversal section of a contralateral tibia. These images illustrate the plexiform bone morphology characteristic of ovine bone, comprising a combination of woven and lamellar bones within which vascular plexuses are sandwiched (**g**). Secondary osteon formation (yellow arrowheads) is seen in the vicinity of the marrow cavity (**h,i**). Backscattered electron (BSE) microscopy images. Images reproduced from ref. 6. All animal experiments received Queensland University of Technology animal ethics approval.
Technovit 9100 solutions are flammable substances and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.

**Critical Step** Fresh Technovit 9100 pre-infiltration solution can be reused once. If reusing solution, incubate the samples in used Technovit 9100 pre-infiltration solution for 4 d at 4 °C, followed by incubation in fresh Technovit 9100 pre-infiltration solution for 7 d at 4 °C. Discard used Technovit 9100 pre-infiltration solution in appropriate disposal bottles.

(vii) Incubate samples in fresh Technovit 9100 infiltration solution for 7 d. Store samples at 4 °C.

Technovit 9100 infiltration solution is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.

**Critical Step** Use a vacuum chamber to remove air from the solution containers. Fresh Technovit 9100 infiltration solution can be reused once. If reusing solution, incubate samples in used Technovit 9100 infiltration solution for 4 d, followed by incubation in fresh Technovit 9100 infiltration solution under vacuum for 7 d. Discard used Technovit 9100 infiltration solution in appropriate disposal bottles. Containers used for embedding.
samples need to have a proper lid, which seals the container well. If the containers are not sealed properly, the embedding solution will not achieve polymerization.

(viii) Embed the samples in the low-temperature Technovit 9100 embedding working solution ('Reagent setup'), using a vacuum chamber to remove air bubbles. Store the samples at −17 °C for 7 d or until polymerization of the Technovit 9100 embedding working solution is achieved.
**Critical Step** Use a vacuum chamber to remove air from solution containers. Containers used for embedding samples need to have a lid that seals the container well. If the containers are not sealed properly, the embedding working solution will not achieve polymerization. Containers should be filled with embedding solution leaving enough space for the lid to cover the container only. Discard any excess Technovit 9100 embedding working solution in appropriate disposal bottles.

(ix) After polymerization of samples is achieved, trim the resin blocks with the EXAKT 310 diamond band saw, leaving 1 mm of resin around the tissue samples. Use the trimmed blocks for thin and ground sectioning.

**Critical Step** Ground sectioning utilizes a 300-µm thickness of tissue per cut. To be safe and to avoid using too much sample, first perform thin sectioning and then perform ground sectioning.

(x) Section resin blocks at 8- to 10-µm thickness using a microtome. Section a minimum of five slices.

**Critical Step** Before thin sectioning, coat the resin slides with gelatin and allow them to dry overnight. Mount resin base (‘Reagent setup’) for the resin blocks prior sectioning.
Apply 30% ethanol to the surface of the block before slicing. This will enable the blade to cut the block smoothly.

**CRITICAL STEP** Never use 100% ethanol on the resin blocks. 100% ethanol will crack and damage the resin blocks.

(xi) Flatten the resin slices with a few drops of 100% ethanol on the glass slide. Use a liner brush to flatten the resin slices.

(xii) Place a plastic sheet on top of the section and place the resin slide into the resin press holder.

(xiii) Dry the slides at 60 °C for 48 h before staining.

(xiv) Stain the thin sections with Movat pentachrome (Box 1) and von Kossa (Box 2) techniques (Figs. 12 and 13).

(xv) After performing thin sectioning, mount the resin blocks and section longitudinally at 200-µm thickness, using the EXAKT 310 diamond band saw according to the technique described previously.¹¹⁷

(xvi) Grind the resin slides at 50 µm, using the EXAKT 400 CS microgrinder according to the technique described previously.¹¹⁷

(xvii) Histologically assess the sections using Goldner’s trichrome staining (Box 3; Fig. 14).

(xviii) Image thin and ground section slides using the ZEN 2.6 software for the Zeiss microscope (CZI images) with a 2.5× objective at 2.58 µm/pixel resolution via the brightfield tile acquisition mode. Using the ZEN 2.6 software in the ‘post processing’ tab, adjust the parameters for exposure (focused on tissue), white balance (focused on slide) and shade correction as desired. In the ‘advanced tiling’ tab, select the tissue region of interest to be tiled and start the experiment. In the ‘post processing’ tab, stitch the tiles into a whole image. In the ‘Graphics’ tab, insert a scale bar. In the ‘processing tab’, export the image as a Tiff-format file.

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**Box 1 | Imaging newly formed bone in thin sections with Movat’s pentachrome stain**

**Timing** 1 d (preparation); 4 h 30 min (procedure)

Movat’s pentachrome stain is a histological staining procedure that enables differentiation between collagen, elastic tissue, muscle, mucin and fibrin in thin resin sections.

**Additional reagents**
- 1% Alcian Blue 8GX solution (Sigma-Aldrich, cat. no. B8438)
- Saffron (Sigma-Aldrich, cat. no. 58381-5G)

**Procedure**
1. Deplasticize thin sections with three changes of 2-MEA (20 min each) at RT.
2. Hydrate samples with a descending ethanol series (100%, 90%, 80% and 70%) for 2 min each.
3. Rinse slides in DI water for 2 min.
4. Stain slides with 1% (wt/vol) Alcian Blue (‘Reagent setup’) for 10 min. Rinse with running tap water for 5 min.
5. Incubate the slides with alkaline alcohol (‘Reagent setup’) for 1 h. Wash slides in running tap water for 10 min, followed by a rinse in distilled water.
6. Stain the slides with Weigert’s iron hematoxylin working solution (‘Reagent setup’) for 10 min. Rinse with running tap water for 10 min.
7. Stain with crocein scarlet-acid fuchsin (‘Reagent setup’) for 15 min. Rinse with 0.5% (vol/vol) acetic acid water solution.
8. Incubate the slides into 5% (wt/vol) aqueous phosphotungstic acid (‘Reagent setup’) for 20 min each. Rinse with 0.5% (vol/vol) acetic acid water solution.
9. Rinse the slides in 100% ethanol for 2 min three times. Stain with 6% (wt/vol) alcoholic (‘Reagent setup’) saffron for 60 min. Rinse with 100% ethanol for 2 min three times.
10. Dehydrate, clear and mount the slides with Eukitt quick-hardening mounting medium.

**Expected stain results**
Examples of typical results can be seen in Reichert et al.⁶ and also in Fig. 12. The expected stain results should show the following:
- Bright yellow: mineralized woven bone/collagen
- Reddish to yellow: cartilage
- Reddish: cytoplasm
- Red: elastic fibers, muscle
- Dark red: osteoid
- Blue-green: mineralized cartilage
- Bright pale blue: glycosaminoglycan
- Blue-black: nuclei
Paraffin sample processing. With the two halves resulting from the previous cut (Step 62D (ii)), transversally cut the samples allocated for paraffin embedding into six paraffin (P) transverse sections (P1–P6) using the EXAKT 310 diamond band saw. A schematic sample cutting overview is provided in Fig. 11.

After removing tissue excess, decalcify the samples in 10% EDTA, using a Kos microwave HistoStation rapid decalci fier at 37 °C for about 8 weeks.

EDTA solution must be changed weekly.

Ensure that the tissue decalcification endpoint is reached by using temporal micro-CT reconstructions, as described previously118.

Process the decalcified samples overnight in a tissue processor (Excelsior ES tissue processor).

Embed the tissue samples using the Shandon Histocentre 3 embedding station with molten paraffin at 60 °C.

Cut 5-µm tissue sections and flatten the paraffin ribbons in a 40 °C water bath for subsequent collection onto Super Frost polysine microscope slides.

Dry the microscope slides at 60 °C for 16 h before staining.

Evaluate morphological tissue structure preservation via hematoxylin and eosin (H&E) staining using a Leica Autostainer XL.

Additional reagents

- Safranin O (Sigma-Aldrich, cat. no. S2255)
- Tetrachrome stain, certified (MacNeal; Polysciences, cat. no. 02783-5)
- Deionized (DI) water
- Tap water

Additional equipment

- Whatman filter paper

Procedure

1. Deplasticize thin sections with three changes of acetone (20 min each) at RT.

2. Hydrate the sections with a descending ethanol series (100%, 90% and 70%) for 5 min each.

3. Rinse the samples three times in DI water for 1 min each before staining.

4. Stain slides in silver nitrate solution (‘Reagent setup’) for 5 min. Wash the slides in DI water three times for 1 min each wash.

5. Immerse the slides in sodium carbonate-formaldehyde solution (‘Reagent setup’) for 2 min.

6. Wash the slides in DI water two times for 1 min each wash.

CRITICAL STEP This is a time-critical step. Do not discard this solution. It can be stored at RT and reused for future staining.

7. Submerge the slides in fresh Farmer’s diminisher solution (‘Reagent setup’) for 30 s.

CRITICAL STEP This solution is stable for only ~30 min; thus, it must fresh before use.

8. Wash the slides under running tap water for 20 min. Rinse the slides in DI water for 1 min.

9. Counterstain sections for 10 min with 0.1% (wt/vol) safranin O solution or counterstain sections for 5 min with MacNeal’s tetrachrome solution.

CRITICAL STEP Slides can be counterstained with 0.1% (wt/vol) Safranin O (Fig. 9) or MacNeal’s tetrachrome (Fig. 13) solution.

10. Rinse the sections with DI water (4 × 30 s).

11. Dehydrate samples with ascending grades of ethanol (70%, 90% and 100%, 30 s each) and coverslip with Eukitt mounting medium.

CAUTION The mounting medium contains xylene, which is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.

Expected results

Typical results are shown in Fig. 9 (Safranin O) and Fig. 13 (MacNeal’s tetrachrome), and the colors indicate the following:

- Black: mineralized bone
- Pink or light blue: soft tissue
- Blue/black: nuclei

(xvii) Safranin O staining. With the two halves resulting from the previous cut (Step 62D (ii)), transversally cut the samples allocated for paraffin embedding into six paraffin (P) transverse sections (P1–P6) using the EXAKT 310 diamond band saw. A schematic sample cutting overview is provided in Fig. 11.

After removing tissue excess, decalcify the samples in 10% EDTA, using a Kos microwave HistoStation rapid decalci fier at 37 °C for about 8 weeks.

EDTA solution must be changed weekly.

Ensure that the tissue decalcification endpoint is reached by using temporal micro-CT reconstructions, as described previously118.

Process the decalcified samples overnight in a tissue processor (Excelsior ES tissue processor).

Embed the tissue samples using the Shandon Histocentre 3 embedding station with molten paraffin at 60 °C.

Cut 5-µm tissue sections and flatten the paraffin ribbons in a 40 °C water bath for subsequent collection onto Super Frost polysine microscope slides.

Dry the microscope slides at 60 °C for 16 h before staining.

Evaluate morphological tissue structure preservation via hematoxylin and eosin (H&E) staining using a Leica Autostainer XL.
Box 3 | Imaging newly formed bone in ground nondemineralized bone sections with Goldner’s modified trichrome stain

● **Timing** 2 d (preparation); 3 h (procedure)

This stain is used for the microscopic identification and evaluation of osteoid, mineralized bone, soft tissue, osteoblasts, osteoclasts and cartilage.

### Additional reagents
- Fuchsin acid (Merck, cat. no. 1052310025)
- Tap water
- Deionized (DI) water

### Additional equipment
- Plasticine wells

### Procedure ● **Timing** 60 min

1. **Glass or plastic surface etching and hydration.** Etch the surface of the sections in xylene and absolute ethanol for 20 min each.
   - **CAUTION** Xylene is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.
   - **CRITICAL STEP** Save all solvent changes for future etching and filter through a paper towel if cloudy or dirty.

2. Hydrate the sections with two changes of ethanol (90%, 70%) and one change of DI water for 5 min each.
   - **PAUSE POINT** At this point you can leave the slides in DI water for up to a few hours if necessary.

3. Place proper-size plasticine wells on the slides containing the sections while the slides are in the DI water.
   - **Timing** 90 min

4. **Goldner’s modified trichrome method.** Stain the slides in Wiegert’s iron hematoxylin working solution for 25 min.

5. Rinse the slides with DI water for 1 min.

6. Wash the slides gently in running tap water for 10 min.

7. Rinse the slides with DI water for 1 min.

8. Stain the slides in acid fuchsin-Ponceau working solution for 10 min.

9. Rinse the slides in two changes of fresh 1% (vol/vol) acetic acid solution for 30 s each.

10. Stain the slides in freshly filtered phosphotungstic acid orange G solution for 20 min.

11. Rinse the slides in two changes of fresh 1% (vol/vol) acetic acid solution for 30 s each.

12. Stain the slides in freshly filtered light-green SF yellowish solution for 15 min.

13. Check whether the slides have been sufficiently stained; if not, repeat step 12.

14. Remove the plasticine wells and rinse the slides in two changes of fresh 1% (vol/vol) acetic acid solution for 30 s each.

15. **Dehydration and coverslipping.** Air-dry the slides for 2 h in darkness.

16. Briefly clear the slides in xylene.
   - **CAUTION** Xylene is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.

17. Coverslip the slides with extra caution to avoid any air bubbles, using Eukitt (xylene-based) mounting medium (diluted with xylene at a ratio of 1:1).
   - **CAUTION** Xylene is a flammable substance and harmful upon inhalation or skin contact. Use only in a downdraft fume hood and with appropriate PPE.
   - **CRITICAL STEP** Let the slides dry overnight. Before imaging the samples, the slides must be polished to remove scratches.

18. **Plastic slide polishing.** Turn on the Buehler grinder and adjust the speed of the grinding wheel to 100–200 r.p.m. on the basis of personal preference.
   - **CRITICAL** Steps 18–21 are to be performed the day after coverslipping the sample slides.

19. Apply alumina slurry (polish solution) to the grinding wheel.

20. Place the slide on the grinding wheel with its back facing down.

21. Hold the slide securely on the wheel until the back of it is polished.

### Expected stain results

Examples of typical results can be seen in Kirby et al.48 and Yong et al.127, as well as in Fig. 14. The expected stain results should show the following:

- Mineralized bone: green or blue
- Connective tissue: orange
- Osteoid: orange-red
- Nuclei: blue-gray
- Cartilage: purple
- Osteoblasts: red with pink Golgi complex
- Osteoclasts: ruffle border—light red; sealing zones—dark red

(xxvii) Image two H&E slides from each treatment tested (Fig. 15) using a Leica 400SCN scanner with a 20× objective at 0.25 μm/pixel. The images will be hosted in the Digital Hub (DIH) platform. Export the images as TIFF files, via the Leica’s Aperio ImageScope software (ScanScope v.12.3).

(xxviii) If desired, stain the slides for markers of bone extracellular matrix (ECM) or vasculature using immunohistochemistry as described in Box 4.

(xxix) If desired, image the slides as described in Step 62D(xxvii).
**Box 4 | Immunohistochemistry to evaluate cellular morphology**  ●  **Timing**  1 d (preparation); 3 h 30 min (procedure)

To demonstrate the presence of bone ECM proteins and vasculature in the newly formed bone, immunohistochemistry analysis with a range of antibodies can be used (Fig. 16). Blood vessels are indicated by vWF staining (Fig. 16a). CD31 is used to evaluate evidence of new blood vessel formation and the angiogenesis process (Fig. 16b). VEGF detects endothelial cells and injury-site revascularization (Fig. 16c). Markers for bone ECM deposition include Col-I to evaluate osteogenic activity (Fig. 16d) and ALP to detect early bone mineralization (Fig. 16e). To determine evidence of mineralized bone regeneration, osteoblast marker OC is used (Fig. 16k) and to evaluate early bone formation, BMP2/4 is used (Fig. 16l).

**Additional reagents**
- Dako wash buffer (Dako, cat. no. DM831)
- Protease K (Dako, cat. no. S3020) Store at 4 °C for up to 24 months
- PBS (Thermo Fisher Scientific, cat. no. BR0014G)
- Osteocalcin (OC) antibody (Abcam, cat. no. ab34710, RRID: AB_731684) Store at −20 °C for up to 24 months
- von Willebrand factor (vWF) antibody, ready to use, rabbit polyclonal (Dako/Agilent, cat. no. IR52761-2, RRID: AB_2810304) Store at 4 °C for up to 24 months
- Cluster of differentiation 31 (CD31) antibody (Santa Cruz Biotechnology, cat. no. sc-1505R, RRID: AB_632173) Store at 4 °C for up to 24 months
- Vascular endothelial growth factor (VEGF) antibody (Santa Cruz Biotechnology, cat. no. sc-152, RRID: AB_2212984) Store at 4 °C for up to 24 months
- Collagen type I (Col-I) antibody (Abcam, cat. no. ab125212, RRID: AB_10975465) Store at 4 °C for up to 24 months
- Bone morphogenetic protein 2/4 (BMP-2/4) antibody (Santa Cruz Biotechnology, cat. no. sc-137087, RRID: AB_2258985) Store at 4 °C for up to 24 months
- Liquid DAB + substrate chromogen system (DAKO/Agilent, cat. no. K3468)

**Additional equipment**
- Dako pen (Dako, Cat. No. S2002) Store at RT for up to 24 months

**Procedure**
1. Dewax paraffin slides in two changes of xylene for 5 min each.
2. Dehydrate the slides in two changes of 100% ethanol for 3 min each, followed by 1 min in each of 90%, 70% and 50% ethanol changes.
3. Delineate sections on the slides with a Dako pen.
4. Perform enzyme-mediated antigen retrieval with protease K for 5 min at RT. Wash the slides in Dako wash buffer.
5. Block endogenous peroxidase activity with 3% (vol/vol) hydrogen peroxide (Reagent setup) e for 10 min. Wash the slides in Dako wash buffer.
6. Block nonspecific binding sites with 2% (wt/vol) BSA (Reagent setup) at RT for 60 min.
7. Incubate the sections with primary antibody at the required dilution in 2% (wt/vol) BSA at RT for 60 min (see Table 4). Wash the slides in Dako wash buffer.
   ▲ CRITICAL STEP  For negative-control samples, incubate tissue sections with a non-immune isotype control from the same species as the primary antibody to exclude false-positive staining as a result of nonspecific IgG binding. 2% BSA can also be used as a negative control.
   ▲ CRITICAL STEP  Incubation must be done in a humid dark chamber.
8. Incubate the sections with the secondary antibody (EnVision+ dual-link system (HRP rabbit/mouse kit)) at RT for 30 min. Wash the slides in Dako wash buffer.
9. Detect immunoreactivity via color development with liquid diaminobenzidine (DAB) chromogen according to the specific primary antibody (Table 4).
10. Counterstain with Mayer’s hematoxylin for 30 s at RT, followed by bluing in 0.1% ammonium hydroxide for 30 s.
11. Dehydrate, clear and mount the slides with Eukitt quick-hardening mounting medium.
12. Image the stained slides (Figs. 15 and 16) using a Leica 400SCN scanner with a 20× objective at 0.25 µm/pixel. The images will be hosted in the Digital Image Hubs (DIH) platform (https://histology-dihi.ihib.qut.edu.au/dih/login.php). Export the images as TIFF files via the Aperio ImageScope software (ScanScope v12.3).

**Troubleshooting**

**Difficulties encountered to date**
As our experience has grown with this particular animal model, so has our ability to troubleshoot issues that have arisen along the way.

**Osteosynthesis failure**
Osteosynthesis failure (i.e., fixation plate bending/breakage or screw failure) occurred in <5% of cases during the postoperative period (Fig. 17). Notably, plates and screws that have been used for a period of load bearing in one animal will probably not have the biomechanical integrity required for load bearing over a 12-month study period in another animal. In our early experience, osteosyntheses were reused in ~50 animals, and in 2 of these animals, device failure occurred. We believe that device reuse...
in these cases may have contributed to failure. Despite cost issues, it is important to consider using exclusively new commercially available plates and screws (with manufacturer confirmation regarding the biomechanical properties of the implant) for each individual animal. Another consideration applies to limiting the selection of sheep to those that weight <60 kg. Osteosynthesis failure was also seen in sheep that weighed >60 kg. In our opinion, controlling device quality and sheep weight collectively reduces the likelihood of osteosynthesis failure and ensures consistency between study groups.

**Tendon irritation**

This complication has occurred in <1% of our animals. The anatomical approach is medial, and the gliding tendons are positioned laterally in the distal limb. Placement of screws that over-compensate for the length across both bone cortices can potentially cause pain and irritation from tenosynovitis as well as soft-tissue irritation. To avoid this, after placement of each screw we apply intraoperative palpation and also confirm our screw lengths at the end of the case with X-ray. If necessary, the wound can be reopened before recovering the animal post-X-ray and the screw length adjusted accordingly.

**Nerve injury**

This complication has occurred in <1% of our animals. A careful dissection approach with anatomical familiarity with the sheep hind limb is necessary, not only to undertake defect creation and fixation, but also to prevent nerve injury. The saphenous nerve and tibial nerves travel in close proximity to

| Table 3 | Solution recipes for resin samples (Heraeus Kulzer, user instruction Technovit 9100) |
|---------|--------------------------------------------------------------------------------------|
| Solution | Destabilized Technovit 9100 basic solution (A) | Technovit 9100 PMMA powder (B) | Technovit 9100 hardener 1 | Technovit 9100 hardener 2 | Technovit 9100 regulator | Storage |
| Pre-infiltration | 200 ml | 1 g | 4 ml | 4 °C |
| Infiltration | 250 ml | 20 g | 1 g | 4 °C |
| Embedding stock solution A | 500 ml | 80 g | 3 g | 4 °C |
| Embedding stock solution B | 50 ml | | | |

| Table 4 | Verified antibody staining to detect bone ECM proteins and blood vessel markers in the preclinical critical-size bone defect model |
|---------|---------------------------------------------------------------------------------------------------------------|
| Purpose | Marker | Source | RRID | Host species | Antibody dilution | DAB incubation time |
| Blood vessel marker | vWF | Dako/Agilent (IR52761-2) | AB 2810304 | Rabbit polyclonal | Ready to use | 1 min 30 s |
| Evaluating evidence of new blood vessel formation and angiogenesis | CD31 | Santa Cruz Biotechnology (sc-1505R) | AB 632173 | Rabbit polyclonal | 1:1,000 | 1 min 30 s |
| Endothelial cells and injury site revascularization | VEGF | Santa Cruz Biotechnology (sc-152) | AB 2212984 | Rabbit polyclonal | 1:500 | 1 min 30 s |
| Macrophage marker to determine macrophage infiltration into the new bone site | CD68 | Abcam (ab125212) | AB 10975465 | Rabbit polyclonal | 1:300 | 2 min |
| Early bone ECM protein to determine early osteogenic activity | Col-I | Abcam (ab34710) | AB 731684 | Rabbit polyclonal | 1:100 | 2 min |
| Depicts early bone mineralization | ALP | Abcam (ab108337) | AB 10862036 | Rabbit monoclonal | 1:500 | 5 min |
| Osteoblast marker; to determine evidence of mineralized bone regeneration | OCN | Abcam (ab13418) | AB 300332 | Mouse monoclonal | 1:1,000 | 1 min 30 s |
| Evaluate bone formation | BMP2/4 | Santa Cruz Biotechnology (sc-137087) | AB 2258985 | Mouse monoclonal | 1:50 | 1 min 30 s |

DAB, diaminobenzidine.
the bone defect and should be identified before performing any osteotomies. Iatrogenic nerve injury can lead to chronic pain in the sheep hind limb and lead to reduced weight bearing on the limb during the postoperative study period, which will inherently affect results. Skilled surgeons with demonstrated experience in the animal model and approach to dissection are paramount to avoiding this issue.

Infection
Infection rates in our experimental model are <1%. We use a strategy consistent with standard operative technique in human operations in which foreign body implants are used. This includes preoperative wash, scrub and shearing of the wool; spray of the skin with 70% alcohol solution; sterile wound site preparation and the use of sterile draping/utensils; primary closure of wounds in layers with a fascial closure around the implant before superficial skin closure; and application of broad-spectrum antibiotic spray to the sutured wound edge. We have encountered a single infection of the implant and only a few wound dehiscences, which were managed with a short, conservative course of antibiotics in >300 sheep operations.
Pressure sores
Pressure sores can be seen in animals where uninterrupted pressure on the soft tissue is sustained over lengthy time periods. Early experience with our cast immobilization of the sheep hind limb involved some cases of pressure sores (<1%), particularly over the tuber calcanei. Subsequent to this, we modified our technique with additional padding and the creation of windows in the cast to ensure that no pressure was exerted against the soft tissue (Fig. 18). Furthermore, a sling was used during the postoperative period that bears the risk of pressure sores. In our studies, pressure sore development was prevented using a breathable sling fabric, regular sling change (weekly) and the full weight-bearing behavior of all the operated sheep. Further troubleshooting advice can be found in Table 5.

Timing

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 31   | Unable to view lateral aspect of the tibia where screw tip will sit once screwed in place | Incorrect screw size used | Correct probing technique with depth gauge: if depth gauge indicates exactly 28 mm, use the 30-mm screw size. If depth gauge measures 27 mm, use the 28-mm screw size. If it measures >27 mm but <28 mm, then use the 28-mm screw size |
| 41   | Additional jagged bone at proximal osteotomized bone surface | When performing the osteotomy with the sagittal saw, a lateral bone spur can be sometimes left behind on the proximal segment due to partial fracturing of the bone defect from the residual proximal tibia | Carefully remove the excess bone using a burr rather than a bone nibbler or another simple device. The bone nibbler carries a risk of fracture of the residual proximal bone fragment, which can be sufficient reason to abort the operation entirely |
|      | Bleeding from the proximal tibial fragment after osteotomy | The perforating nutrient artery enters into the lateral surface of the tibial cortex and will frequently bleed immediately after the osteotomy is performed | Simple cauter of the arterial bleed will not suffice. We advocate the application of bone wax to the site of the bleeding. With sustained compression and occlusion by the bone wax, the bleed will eventually cease |

Anticipated results
Over the past 10 years we have trained six surgeons and performed >300 procedures to evaluate segmental bone defect healing using this technique. All surgeons were either fully qualified orthopedic, trauma or veterinary surgeons or were undertaking accredited surgical training when performing the procedures. Clinical familiarity is a requirement for this animal model, because identical surgical equipment and techniques, including fixation methods, are applied. Generally, for
each surgeon, experience with at least two mock surgeries using ovine cadaveric material and assisting in a minimum of four live surgeries has been required before being considered to have baseline proficiency to act as the lead surgeon for a study.

The results to date using this technique have been widely published, and a number of studies have been undertaken. In particular, our team has investigated a variety of scaffold-based bone-regeneration concepts, alongside several different mediators to enhance the regenerative process (Fig. 1).

Most studies by our group used a 3D-printed and good manufacturing practice (GMP)-fabricated mPCL-TCP scaffold (Osteopore International, Singapore) that has been used with morcelized autologous bone graft (current gold standard model), autologous and allogenic mesenchymal bone marrow precursor cells, platelet-rich plasma and BMP-7 and BMP-2. In the study by Reichert et al. in 2012, 12 months after implantation of an mPCL-TCP scaffold with BMP-7, we reported 100% bridging of the 3-cm defect with enhanced bone volume and mechanical strength in contrast to an autologous bone graft control group (Figs. 8–10, 13).

Given the reproducibility of this large-animal model, we have also modified the defect length to 6 cm, which represents >30% of the total tibial length in these sheep. From a translational research point of view, this defect represents, to our knowledge, the most challenging large preclinical animal model with other scaffold designs and materials for bone regeneration, such as a baghdadite bioceramic and silk scaffolds. Furthermore, the model has also been used to investigate the role of soft-tissue (ligamentous/tendinous) integration into the scaffold interface, as well as the effect of BMP-7 in guiding neo-ostegenic micro-architecture of the scaffold during the bone regenerative process, as compared with standard scaffold-loading conditions.

Given the reproducibility of this large-animal model, we have also modified the defect length to 6 cm, which represents >30% of the total tibial length in these sheep. From a translational research point of view, this defect represents, to our knowledge, the most challenging large preclinical animal model with which to assess bone regeneration. Studies using the 6-cm model have important implications for orthopedic tumor surgery and large-volume non-unions in the clinical arena.

**Reporting Summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The datasets that support this study are available from the corresponding author upon request.

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Author contributions
D.S.S. wrote the manuscript with the assistance of S.S., F.M.S., J.R., I.A.M. and D.W.H. S.S. and J.C.R. designed and performed the experiments, and S.S. provided veterinary expertise. D.S.S., F.M.S., A.C., C.E.D., A.B., J.H., J.C.R., M. Wullschleger and J.R. performed the experiments and prepared and analyzed the data. S.S. designed, performed and analyzed the biomechanical testing. S.S., M. Wagels, M.A.W., M.A.S. and D.W.H. supervised the project. M.A.S. and M. Wagels provided clinical input into the design of the model. D.W.H. led the design of the experiments and supervised the project. All authors read and critiqued the manuscript extensively and agreed on the final version of the manuscript.

Competing interests
D.W.H. is a cofounder and shareholder of Osteopore International Pty Ltd, a company specializing in 3D bioreabsorbable implants to assist with bone healing.

Additional information
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**Sample size**
Torsional strength data from a comparable study was used to determine sample size. To determine a 25% difference in torsional strength with a power of 80% on a significance level of \( p<0.05 \), a minimum of eight animals was generally required for treatment group comparison.

**Data exclusions**
No data were excluded from the analysis

**Replication**
No repeated measurements were performed for this study

**Randomization**
Sheep were allocated at random into experimental groups.

**Blinding**
No blinding was used in these experiments

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| ☑   | Palaeontology          |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data          |

### Methods

| n/a | Involved in the study |
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| ☑   | ChIP-seq               |
| ☑   | Flow cytometry         |
| ☑   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**
Primary antibodies: Anti-Osteocalcin (1:1000, Abcam, cat. no. ab13418), Anti-CD31 (1:1000, Santa Cruz Biotechnology, Cat. no. sc-1505R), Anti-von Willebrand Factor (ready-to-use, Dako, Agilent, cat. no. IR52761-2), Anti-VEGF (1:500, Santa Cruz Biotechnology, cat. no. sc-152), Anti-CD68 (1:1000, Abcam, cat. no. ab125212), Anti-Collagen Type I (1:100, Abcam, cat. no. ab34710), Anti-Alkaline Phosphatase (1:500, Abcam, cat. no. ab108337), Anti-BMP2/4 (1:150, Santa Cruz Biotechnology, cat. no. sc-137087). Secondary antibody: HRP-labelled secondary antibody kit (Dako, Cat. no. K4061).

**Validation**
Antibody specificity and cross reactivity was evaluated using positive and negative controls. The tests were carried out using Standard Operating Procedures at the Queensland University of Technology.

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**Laboratory animals**
Skeletally mature sheep (Merino Sheep, >6 years of age, <60kg bodyweight).

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The study did not involve wild animals.

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The study did not involve samples collected from the field.

**Ethics oversight**
Ethics approval was obtained from the Queensland University of Technology University Animal Ethics Committee.

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