A novel mouse model to study fracture healing of the proximal femur

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Abstract
The majority of fractures, especially in elderly and osteoporotic patients, occurs in metaphyseal bone. However, only a few experimental models exist to study metaphyseal bone healing in mice. Currently used mouse models of metaphyseal fracture healing are either based on drill hole defects, lacking adequate biomechanical stimulation at the site of fracture and therefore endochondral ossification in the fracture callus, or are introduced into the distal part of the mouse femur stabilized by a locking plate, which is challenging due to the small specimen size. Therefore, the aim of the current study was to develop a new mouse model to study metaphyseal fracture healing of the proximal femur. We chose a combination between an open osteotomy and a closed intramedullary stabilization. A 24 G needle was inserted into the femur in a closed manner, then an osteotomy was made with a 0.4-mm Gigli wire saw between the third and the lesser trochanter of the femur using an open approach. Fractured femurs were analyzed using microcomputed tomography and histology at days 14 and 21 after surgery. No animals were lost due to surgery or anesthesia. All animals displayed normal limb loading and a physiological gait pattern within the first three days after fracture. We found robust endochondral ossification during the fracture healing process with high expression of late chondrocyte and early osteogenic markers at day 14 (d14). By day 21 (d21), all fractures had a bony bridging score of 3 or more, indicating successful healing. Callus volume significantly decreased from d14 to d21, whereas high numbers of osteoclasts appeared at the fracture callus until d21, indicating that callus remodeling had already started at d21. In conclusion, we successfully developed a novel mouse model to study endochondral fracture healing of the proximal femur. This model might be useful for future studies using transgenic animals to unravel molecular mechanisms of osteoporotic metaphyseal fracture healing.

KEYWORDS
fracture, osteoporosis, repair
1 | INTRODUCTION

The majority of fractures, especially in elderly and osteoporotic patients, occurs in metaphyseal bone due to the susceptibility of trabecular bone to microstructural damage. These fractures are challenging to treat because the weak bone quality limits proper osteosynthesis fixation and there is evidence for reduced healing capacity of osteoporotic bone. Furthermore, recent research has demonstrated that there are significant differences between the fracture healing process in metaphyseal and diaphyseal bone. Tätting et al. revealed increased numbers of lymphocytes and monocytes and decreased numbers of granulocytes when comparing the inflammatory response after fracture in diaphyseal vs metaphyseal bone. Further studies demonstrate different effects of both antiresorptive and osteoanabolic treatment strategies including Wnt/β-catenin activation, parathyroid hormone application, and bisphosphonate injections on metaphyseal and diaphyseal bone healing. Therefore, metaphyseal osteoporotic fractures require more complex strategies to facilitate optimal bone healing. This is especially true for osteoporotic hip fractures, which occur mainly in the region of the femoral neck and the femoral trochanter and are therefore classified as metaphyseal fractures.

While these injuries are important from a clinical standpoint, adequate small animal models to study them are lacking. In fact, only a few animal models exist to study metaphyseal bone healing, and the majority of these studies have been performed in sheep, dogs, and rats, limiting the possible use of genetically modified animals to unravel molecular mechanisms involved in bone regeneration. For this purpose, mouse models to study metaphyseal fracture healing are desirable because of the large number of available knock-out, knock-in, and transgenic mouse strains. Indeed, due to the small specimen size and challenging orthopedic surgical procedures, most fracture healing studies using mice have been performed on the diaphysis of the femur or tibia. Currently used mouse models of metaphyseal fracture healing are either based on drill hole defects, lacking adequate biomechanical stimulation at the site of fracture and therefore endochondral ossification in the fracture callus, or are introduced into the distal part of the mouse femur stabilized by a locking plate, which is challenging due to the small specimen size. Therefore, to begin to understand the molecular mechanisms of fracture healing and investigate possible treatment options in a more clinically relevant manner, a robust and reliable mouse model of metaphyseal fracture healing would be useful. Therefore, the aim of the current study was to develop a novel mouse model to study metaphyseal fracture healing of the proximal femur.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

All animal experiments were approved by the local animal welfare committee (IACUC UCSF AN143402-03B) and were performed in compliance with international regulations for laboratory animal welfare and handling (ARRIVE guidelines). Twelve-week-old female C57BL/6J mice were purchased from Jackson laboratories. Young mice were chosen for this study to determine the feasibility of the new surgery technique and to investigate the physiological healing process of proximal femur fractures in mice. Mice were housed in groups of up to five animals with free access to standard mouse diet and water. The surgical procedure was conducted under general anesthesia using ketamine hydrochloride (100 mg/mL; 50 mg/kg bodyweight; Henry Schein Animal Health) at a ratio of 1:1 with dexmedetomidine hydrochloride (0.5 mg/mL; Orion Pharma) and analgesia using buprenex (0.03 mg/mL; 0.05 mg/kg bodyweight). Buprenex was injected subcutaneously every 6 hours for the first 24 hours. Mice were allowed to move freely directly after surgery. Animals were monitored daily to investigate nonphysiological or physiological limb loading and gait pattern. After 14 and 21 days, mice were euthanized using carbon dioxide and cervical dislocation. Fractured femurs were harvested and fixed in 4% paraformaldehyde for 48 hours. Afterward, implant material was removed and fractured femurs were subjected to microcomputed tomography (µCT) scans and decalcified histology. The sample size was 6 per time point.

2.2 | Surgical procedure

A 24 G needle was introduced retrograde into the right femur according to previously established procedures (Figure 1). For femur osteotomy, a medial 0.5-cm skin incision was made longitudinally along the femur. The femoral muscles were separated bluntly and the tendon insertion at the third trochanter was cut to allow free access to the proximal part of
the femur. The osteotomy was sawed between the third and the lesser trochanter using a 0.44-mm Gigli wire saw (Figure 1). The bone was sawed from the bottom to the top until the saw hit the needle, then the sawing direction was changed to top-bottom until the osteotomy was complete. After flushing the osteotomy gap with sterile sodium chloride, the muscles were sutured together using a continuous U-suture with absorbable Vicryl 5-0. The skin was sutured using an interrupted stitch with nonabsorbable Resolon 5-0.

### 2.3 µCT analysis

Volume of interest was defined as the periosteal fracture callus in a 6-mm region spanning from the proximal to the distal part of the osteotomy. This region was chosen to analyze the whole fracture callus in all animals. The original cortical bone was excluded from the analysis by contouring. The fractured femurs were analyzed using µCT (Skyscan 1172) operating at a resolution of 8 µm and a voltage of 50 kV and 200 mA. For determination of the apparent bone mineral density (BMD), two phantoms with a defined density of hydroxyapatite (250 and 750 mg/cm³) were scanned within each scan. For the determination of total volume (TV), bone volume (BV), and bone volume to total volume fraction (BV/TV) a global threshold of 642 mg hydroxyapatite/cm³ according to Morgan et al.10 was used to distinguish between mineralized and nonmineralized tissue. Moment of inertia (MMIx) in the anterior-posterior direction of the femur was determined without thresholding the tissue. Therefore, the data shown account for MMIx for the whole callus tissue. µCT analysis was done with the 3D analysis software from Skyscan (NRecon, DataViewer, CTAn, CTVox). Bony bridging was analyzed in a blinded fashion in two perpendicular planes of the µCT data set. A maximum score of four bridged cortices could be achieved.

### 2.4 Histological analysis

After µCT scans, right femurs were subjected to decalcified histology as described previously.11 Sections of 7 µm were stained with safranin O. The amounts of bone, cartilage, and fibrous tissue at days 14 and 21 after fracture were determined using image analysis software (Leica MM AF 1.4.0 Imaging System). The region of interest was whole fracture callus. The number and surface of osteoclasts (NOc/BPm, OcS/BS) were determined using tartrate-resistant alkaline phosphatase (TRAP) staining. Osteoclasts were defined as multinucleated, TRAP-positive cells residing on the bone surface. Bone cells and bone surface were evaluated using OsteoMeasure system (OsteoMetrics). Staining procedures are described previously.12

### 2.5 Immunohistochemical stainings

Paraffin-embedded 7-µm longitudinal sections were prepared for immunohistochemical staining. The following antibodies were used: rabbit anti-mouse collagen X (ABIN1077945, dilution 1:200; Antibodies Online), rabbit anti-mouse Runx2 (8486, 1:50; Cell Signal ing), rabbit anti-mouse osteocalcin (orb77248, 1:200; Biorbyt), goat-anti rabbit IgG-biotin (sc-3840, 1:200, Santa Cruz) and horse-radish peroxidase-conjugated streptavidin (Zytomed Systems). Anti- gen retrieval was done by incubating the sections in 10 mM citrate buffer at 95°C for 20 minutes. Blocking was done by incubating the sections in 5% goat serum at room temperature for 1 hour. 3-Amino-9-ethylcarbazole (Zytomed Systems) was used as the chromogen. The sections were counterstained using hematoxylin (Waldeck). Species-specific nontargeting immunoglobulins were used as isotype controls. Quantification of the positively stained regions was performed using the image analysis software Adobe Photoshop CS4 (Adobe, Dublin, Ireland). The color gamut of positive staining was determined with the color picker tool and a tolerance of 40. The positively stained pixels were counted in the histogram and calculated against all pixels of the image to determine the percentage of positively stained area. The region of interest was whole fracture callus.

### 2.6 Statistical analysis

Data were tested for normal distribution using Shapiro-Wilk normality test. Most data sets were normally distributed. Therefore, results are presented as dot plots with mean ± standard deviation. Statistical analysis was done by Student’s t test (GraphPad Prism 9). The level of significance was set at *P* < .05. The group size was six, and was calculated based on the findings of a previous fracture healing study with the main outcome parameters flexural rigidity and BV/TV in the fracture callus.13

### 3 RESULTS

No animals were lost due to surgery or anesthesia. All animals displayed normal limb loading and a physiological gait pattern within the first 3 days after fracture.

µCT analysis revealed successful osteotomy between the lesser and the third trochanter in all animals (Figure 2A,B). Bony bridging score increased significantly between day 14 (d14) and day 21 (d21) from values 0 or 1 at d14 to values 3 or 4 at d21 (Figure 2C). BV ratio also increased significantly between d14 and d21 (Figure 2D). Total BV did not differ between the different time points, however, total callus volume decreased significantly between d14 and d21 (Figure 2E,F). Furthermore, whole callus MMIx decreased significantly, whereas BMD of the bony callus increased significantly between d14 and d21 (Figure 2G,H).

Histomorphometric analysis of safranin O-stained sections from the fractured femurs revealed that all fractured healed via endochondral ossification (Figure 3A). Whole callus area decreased significantly between d14 and d21 (Figure 3B). The relative amount of fibrous tissue did not differ between the two time points, whereas the relative amount of cartilage decreased.
and the relative amount of bone increased between d14 and d21 (Figure 3C-E). All fracture calluses at d21 displayed less than 10% of cartilage tissue, indicating a successful cartilage-to-bone transition between d14 and d21. TRAP staining showed high osteoclast abundance and activity at the outer edges of the fracture callus at d14 and throughout the whole fracture callus at d21 after fracture (Figure 3F), indicating that fracture callus remodeling has already started at d21 after fracture. The number of osteoclasts per bone perimeter (Figure 3G) and osteoclast surface per bone surface (Figure 3H) increased significantly from d14 to d21.

Immunohistochemical staining for the hypertrophic chondrocyte marker collagen X (Figure 4A) revealed high expression at the cartilage-to-bone transition zone of the fracture callus and in the trabecular bone in the peripheral callus at d14. At d21, collagen X was present only sparsely in the trabecular bone in the fracture callus and the collagen X-positive area in the whole fracture callus
decreased significantly from d14 to d21. Immunohistochemical staining for the early osteogenic marker runx2 (Figure 4B) revealed high expression in hypertrophic chondrocytes at the cartilage-to-bone transition zone and in osteoblasts throughout the bony callus at d14. At d21, runx2 was expressed in some cells at the outer edges of the fracture callus and in some bone marrow cells and osteoblasts in the bony callus. Runx2-positive area in the whole fracture callus decreased significantly from d14 to d21. The late osteogenic marker osteocalcin was stained in hypertrophic chondrocytes, osteoblasts, osteocytes, and bone matrix both at d14 and d21 (Figure 4C). Osteocalcin-positive area in the whole fracture callus increased significantly from d14 to d21.
The aim of this study was to develop a new model to study metaphyseal fracture healing in the proximal femur of mice because the majority of human osteoporotic hip fractures occur in proximal, metaphyseal bone. For this purpose, we successfully combined an open osteotomy approach to the proximal femur with an approach for intramedullary stabilization. All fractures were located between the third and the lesser trochanter of the mice. No animals were lost during the procedure. All animals appeared to have normal limb loading and a physiological gait pattern within 3 days of fracture. We found robust endochondral ossification during the fracture healing process with high expression of late chondrocyte and early osteogenic markers at d14. All fractures displayed a bony bridging score of 3 or more on d21, indicating successful healing of the fractured bones within 21 days. Callus volume significantly decreased from d14 to d21, whereas high numbers of osteoclasts appeared at the fracture callus until d21, indicating that callus remodeling had begun. The advantages of our new model are that the surgical approach and the location of the fracture are highly standardized due to the combination of the open osteotomy and the closed intramedullary stabilization. Furthermore, the location between the third and the lesser trochanter represents an area where many osteoporotic fractures occur in humans, although the anatomy of the proximal

**FIGURE 4** Immunohistochemical staining of fractured femurs at day 14 (d14) and day 21 (d21) after fracture. A, Representative images of fractured femurs stained for collagen X and quantification of the positively stained area in the whole fracture callus. B, Representative images of fractured femurs stained for runx2 and quantification of the positively stained area in the whole fracture callus. C, Representative images of fractured femurs stained for osteocalcin and quantification of the positively stained area in the whole fracture callus. Scale bar = 200 µm. C, cortex; G, fracture gap. *0.05 > P > 0.01; ***0.01 > P > 0.0001 [Color figure can be viewed at wileyonlinelibrary.com]
femur of mice is not the same as in humans and therefore the biomechanical environment might differ. Furthermore, the stabilization by a cannula is inexpensive and relatively easy to place into the intramedullary canal, and importantly, it did not appear to cause sustained pain. In conclusion, we successfully developed a novel mouse model to study endochondral fracture healing of the proximal femur.

The limitations of our model are that stabilization by a cannula did not provide rotational stability as is achieved when proximal femur anterotational nails or plates are used. Furthermore, many osteoporotic human fractures occur at the femoral neck and it is not possible to replicate this in mice due to a lack of adequate fracture implant devices for the mouse hip. Another limitation is that there are only a few trabecular bone structures in the area between the third and the lesser trochanter, therefore the real “metaphyseal” area in the mouse proximal femur is very small.

To date, there are only a few published studies of metaphyseal fracture models in mice. Most of these studies used drill hole defects in the mouse femur or tibia. However, drill hole defects lack biomechanical stimulation at the fracture site and furthermore, no fracture implants are used for this approach. This might critically influence the type of healing. Furthermore, the inflammatory response is likely to be different after osteotomy or fracture due to the different amounts of tissue injury. As it was shown in diaphyseal fracture models that the inflammatory and angiogenic response might be altered during osteoporotic fracture healing, it may be of critical importance to use a metaphyseal healing model with a certain amount of tissue injury to study this in more molecular detail. An alternative mouse model to study metaphyseal fracture healing used a plate at the distal mouse femur. However, positioning this plate in the trabecular metaphyseal region could be challenging due to the small specimen size.

Comparing the data from our novel mouse model to published data from a diaphyseal fracture healing study using the same age, sex, and strain of mice (female, 12-week-old C57BL/6J mice), it is obvious that total callus volume and BV in the fracture callus are significantly higher at d21 with our novel approach. This might be due to a different time course of healing or to different biomechanical conditions caused by different fracture implants used. BV/TV seems to be comparable between the study from Wehrle et al and the present study. Both models showed a significant amount of cartilage in the intermediate phase of healing, indicating endochondral ossification during callus maturation. Comparing our data with other studies investigating metaphyseal fracture healing in mice, it is obvious that stabilizing a distal femur fracture with a plate led to intramembranous ossification in the fracture callus in contrast to endochondral ossification seen in our model. Furthermore, callus size was significantly smaller. This might be due to the different locations of the fracture and/or due to the different biomechanical conditions caused by different fracture implants used. Also, the study by Histing et al used CD-1 mice, therefore the results are not directly comparable with our data from the present study.

In conclusion, our novel model provides a fast, reliable, and inexpensive way to study endochondral metaphyseal fracture healing in mice. Future studies using osteoporotic mice in combination with our model might help unravel molecular mechanisms of delayed osteoporotic fracture healing. We would expect differences in the healing process and healing time in aged and/or ovariectomized mice compared with nonosteoporotic animal, therefore this model might be used to determine the cellular and molecular mechanism of delayed osteoporotic fracture healing in metaphyseal bone.

ACKNOWLEDGMENTS

We thank Iris Baum for excellent technical assistance. This study was supported by the Hertha-Nathorff program (travel grant to MHL and MK), the DAAD (travel grant to BW), the Deutsche Forschungsgemeinschaft (CRC1149), and the Orthopaedic Trauma Institute at UCSF.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

MHL, BW, CL, VF, IL, MK: conducted experiments. MHL, BW, MK, AI, RSM, TM: substantial contributions to research design. MHL, AI, RSM, TM: interpretation of data. MHL: drafted the paper. All authors: revised the paper critically and approved the submitted version.

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How to cite this article: Haffner-Luntzer M, Weber B, Lam C, et al. A novel mouse model to study fracture healing of the proximal femur. J Orthop. 2020;38:2131-2138. https://doi.org/10.1002/jor.24677