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BONE HEALING IN MICE: DOES IT FOLLOW GENERIC MECHANO-REGULATION RULES?

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Abstract. *Mechanical signals are known to influence bone healing progression. Previous studies have postulated inter-species differences in the mechanical regulation of the bone healing process. The aim of this study is to investigate whether mechanical "rules" explaining tissue formation patterns during bone healing in rat can be translated to a mouse model of bone regeneration. We have used an established mechano-biological computer model that uses finite element techniques to determine the mechanical conditions within the healing region and an agent-based approach to simulate cellular activity. The computer model is set up to simulate the course of bone healing in a femoral osteotomy model stabilized with an external fixator. Computer model predictions are compared to corresponding histological data. Generic mechano-regulation "rules" able to explain bone healing progression in the rat are not able to describe tissue formation over the course of healing in the mouse. According to the differentiation theory proposed by Prendergast, mechanical stimuli within the healing region immediately post-surgery are determined to be favorable for cartilage and fibrous tissue formation. In contrast, in vivo histological data showed initial intramembraneous bone formation at the periosteal side. These results suggest that in mice, bone does not require as much stability as is required in rat to reach timely healing. This finding emphasizes the need to further investigate the species-specific mechano-biological regulation of bone regeneration.*

Key Words: *Mechano-biology, Mouse Bone Healing, Agent-based Model, Finite Element Analysis, Tissue Differentiation*

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1. INTRODUCTION

Although bone is able to self-repair, in many situations its regeneration capacity is impaired, leading to delayed or non-unions. The healing process is known to be influenced by many factors; among them mechanical signals have been shown to play a fundamental role [1, 2]. It is well known that the course of bone healing is related to mechanical stability, which in turn influences the local mechanical conditions within the callus. Mechanical instability has been shown to prolong the endochondral healing phase [3, 4], while a lack of mechanical stimulation may inhibit the bone healing response [5].

Elucidating mechanical "rules" driving bone regeneration has been the focus of many studies in the last 30 years, since such knowledge has a great relevance in the design of clinical strategies for the treatment of bone fractures. Although local mechanical strains/stresses within the healing region cannot be measured experimentally, they can be determined using computer modeling techniques, such as finite element (FE). Using FE models to quantify the distribution of biophysical stimuli in a fracture gap, Claes and Heigele [6], determined a relationship between the magnitude of these stimuli and the distribution of the tissues present in histological sections. They observed intramembranous bone formation for strains smaller than +/- 5% and hydrostatic pressures smaller than +/- 0.15 MPa. Endochondral ossification was associated with compressive pressures larger than about -0.15 MPa and strains smaller than +/- 15%. All other conditions were related to the formation of connective tissue or fibrous cartilage. Although a globally accepted theory explaining the mechanical regulation of tissue repair does not exist [7, 8], it has been shown that callus tissue volume and shape changes due to mechanical loading are a good indicator of further differentiation processes [9]. Over the last several years, these theories have been successfully implemented in computer models to simulate the mechanical regulation of tissue repair and differentiation in fracture healing [10, 11]. The role played by the local mechanical conditions during the healing process has been investigated by simulating the influence of fixation stiffness [12], gap size [13], fracture type [14] and external loading conditions on the healing outcome [15, 16].

So far, the majority of these studies have used the sheep as an animal model to compare computer model predictions to experimental data in order to formulate hypotheses about how mechanical signals drive bone healing responses [17]. This is due to the notion that the process of bone healing in sheep is thought to closely mimic that in humans. However, the rat [18, 19, 20] and mouse [21, 22, 23, 24, 25] have become increasingly popular as animal models in experimental bone healing studies due to ease of handling, low costs, and the availability of molecular biological tools. Using a computer modeling approach, Checa et al. [26] showed inter-species differences in the mechanical regulation of the bone healing process between sheep and rat, where different levels of mechanical stimuli were determined as favorable for the bone formation response.

Mice allow an additional advantage of relatively easy genetic modification, thus permitting the study of molecular mechanisms controlling fracture healing [27]. Unfortunately, few experimental studies exist which have examined how mechanical factors influence bone healing in mice. Holstein et al. [22] compared bone healing under rigid and flexible conditions in a closed fracture model using a conventional or a locking nail with higher stiffness. The initial phase of fracture healing was delayed under flexible conditions. Gröngröft et al. [25] showed that a rigid internal plate induced solely intramembranous ossification, whereas a semi-rigid plate led to a mixture of endochondral

and intramembranous bone formation. Röntgen et al. [24] compared the healing outcome using a rigid versus flexible external fixators to stabilize an osteotomy in the mouse femur. They showed delayed fracture healing with a larger callus formation and prolonged endochondral ossification in the flexible compared to the rigid case. Steck et al. [28], characterized the time course of strength recovery and callus development of mouse femoral osteotomies stabilized with internal fixation plates that allowed either low or high flexibility (in bending and torsion). They observed earlier bridging of the mineralized callus under less flexible conditions.

Few computational models have been developed to investigate bone healing progression in mice. Geris et al. [29] developed a mathematical model to investigate a murine tibia fracture semi-stabilized by an intramedullary fixating pin. Although they were able to show a qualitative agreement between the experimentally measured and numerically simulated cartilage and bone formation, they did not consider the effect of fracture fixation stability on the healing outcome. Isaksson et al. [30] investigated the emergence of a double cortex in the remodeling phase of healing in mice using an established remodeling algorithm. They showed that this peculiarity might be a consequence of different mechanical loading in mice, which may result from differences in skeletal structure or posture during gait. However, they did not investigate the influence of these loading conditions in the earlier healing phases.

Therefore, it remains largely unknown how the local mechanical strains within the healing region regulate intramembranous and endochondral ossification in mice, particularly during the early phases of healing. Whether mechanical "rules" able to explain bone and cartilage formation in other species, such as in rat and sheep, can be translated to mice remain to be determined. Therefore, the aim of this study is to investigate the mechanical regulation of bone healing in a mouse femoral osteotomy, stabilized with a rigid external unilateral fixator. Using an established mechano-biological computer model, we have determined how local mechanical strains within the healing region relate to tissue formation responses over the course of healing. We hypothesize that, due to anatomical similarities between rat and mouse, bone healing in mice can be explained using the same mechanical rules as those earlier derived in rat.

2. MATERIAL AND METHODS

To investigate the mechanical regulation of bone healing in mice, we used a previously established mechano-biological computer model which uses FE techniques to determine the mechanical conditions within the healing region and an agent-based modeling approach to simulate cellular activity [26]. Computer model predictions were compared to histological data of an externally stabilized mouse femoral osteotomy model.

2.1. Animal model

A 0.5 mm osteotomy was performed on femurs from 26 week old (adult) C57BL/6 female mice under general anaesthesia (75 mg/kg ketamin and 1 mg/kg medetomidin intraperitoneal). The fracture was stabilized with an external fixator that was mounted onto the femur in a cranio-lateral direction by four pins (0.45 mm, RISystem, Switzerland) (Fig. 1). After mounting the fixator in the correct position, a 0.5 mm osteotomy gap was created using a gigli saw (0.44 mm, RISystem, Switzerland). After 7,14 and 21 days of healing, the mice were sacrificed and the femora were fixated with paraformaldehyde,

decalcified in EDTA for 2 weeks, dehydrated with alcohol and xylol, and embedded in paraffin. Sections (4 μm-thick) were cut in a longitudinal direction and stained with Movat Pentachrome. Healing proceeded via a combination of intramebranous ossification and endochondral ossification. Intramembranous ossification was evident at all three time points (7, 14, and 21 days post-osteotomy) in the periosteal region and also at day 14 and 21 in the endosteal region. Endochondral ossification was visible at 14 and 21 days of healing and was located primarily within the intracortical region of the bones. These islands of cartilage centered within the intracortical region extended into the periosteal and endosteal callus region (Fig. 3C).

2.2. Finite element model

A FE model of the stabilized fracture was developed to determine the mechanical conditions within the healing region (Fig. 1). The bone was modeled as a hollow cylinder where the inner region represents the medullary cavity (Fig. 1). The osteotomy was simulated, creating a 0.5 mm gap between the bone ends. The model was developed in Abaqus 6.12 and meshed with C3D8 elements, with an average element size of 0.15 mm (Fig. 1B).

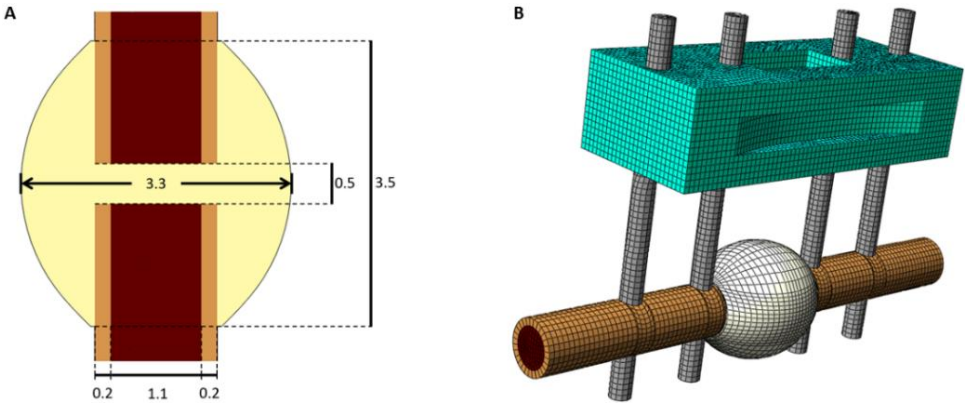

Fig. 1 A) Dimensions of the finite element model obtained from histological images (values are reported in mm). B) Finite element model developed to determine the local mechanical conditions within the healing region in a 0.5 mm femoral osteotomy in mouse stabilized with an external fixator [31]. Different colors represent different material properties

Material properties were assigned following Checa et al. [26] (Table 1). PolyEtherEtherKetone (PEEK) material properties (E= 3800 MPa, ν=0.38) were assigned to the external fixator, while titanium properties (E=17000 MPa, ν=0.33) were used for the four nails.

Table 1 Material mechanical properties

	Granulation tissue	Fibrous tissue	Cartilage	Immature bone	Mature bone	Cortical bone	Marrow
Young's Modulus (MPa)	0.2	2	10	1000	5000	5000	2
Permeability ($m^4/N\ s * 10^{-14}$)	1	1	0.5	10	37	0.001	1
Poisson's ratio (-)	0.167	0.167	0.3	0.3	0.3	0.3	0.167
Bulk modulus grain (MPa)	2300	2300	3700	13940	13940	13920	2300
Bulk modulus fluid (MPa)	2300	2300	2300	2300	2300	2300	2300

Loading conditions in mice are largely unknown. Based on anatomical similarities, we assumed that mice experience similar loading conditions than rats. We simulated two loading cases: a compression load and a combined compression and bending load. The compression load was equivalent to six times body weight (BW) (F= 1.5 N) and the bending load was such that it would result in the intact bone in a maximum bending moment of 10.7 BWmm (2.7 Nmm) at the femoral mid-shaft, as reported by Wehner et al. [32]. Over the course of healing, a certain percentage of limb loading was simulated [26]. Loads were applied in the proximal bone surface, while the distal end was restrained to move in all directions.

2.3. Bone healing simulation

To simulate the bone healing process inside the callus, a discrete 3D lattice mechano-regulation model was created following Checa et al. [26]. Briefly, the callus region was divided into a regular 3D grid, where each position represented a possible location for a cell and its extracellular matrix. The distance between two lattice points was considered 10 μm. The healing response was simulated as an iterative process. Initially mesenchymal stem cells (MSCs) originated from the periosteum and the marrow cavity (30% of volume fraction [32]) and were allowed to migrate and proliferate at a certain rate (Table 2), following a random walk model. After cell maturation, considered to be 6 days, 30% of the mature MSCs [30] were allowed to differentiate based on the local mechanical stimulus at their location, following Prendergast et al. [8]. Differentiated cells

Table 2 Cell activity rates according to Checa et al. [26]

Cell type	Proliferation rate (day^{-1})	Apoptosis rate (day^{-1})	Migration rate (μm/h)
MSC	0.6	0.05	30
Fibroblasts	0.55	0.05	30
Chondrocytes	0.2	0.1	-
Osteoblasts	0.3	0.16	-

were then assumed to synthesize a new extracellular matrix, changing the material properties of the tissue within the callus. New material properties were then updated in the finite element model and a new iteration started.

3. RESULTS

3.1. Mechanical conditions within the healing region immediately post-surgery

Under both loading cases, strains and fluid velocities were the highest within the fracture gap, while lower magnitudes were predicted to occur at the periosteal side. Under compression loading, strains up to 20 % were determined at the osteotomy gap. When combined with bending, strains increased up to 50 %, with the maximum located opposite to the fixator (Fig. 2). The external load influenced fluid velocity, where higher velocities were predicted for the combined loading case. As for strains, maximum fluid velocities were found within the gap with values up to 0.005 and 0.01 mm/s in the compression and combined loads, respectively.

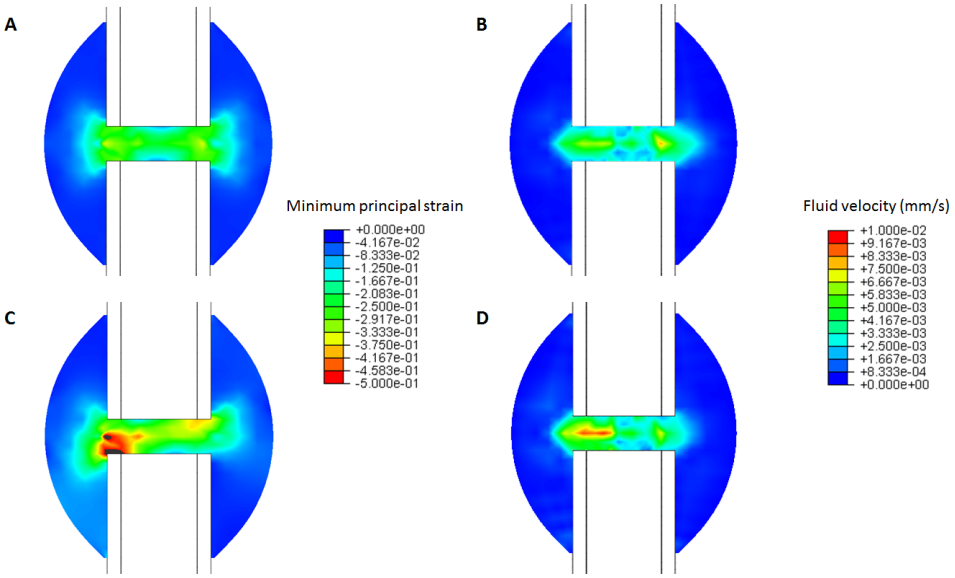

Fig. 2 Mechanical environment inside the callus determined using finite element analysis. Figure shows the predicted minimal principal strain distribution (A, C) and fluid velocity (B, D) in the situation immediately after surgery. Two loading cases are shown: only compression (A, B) and combined compression and bending load (C, D)

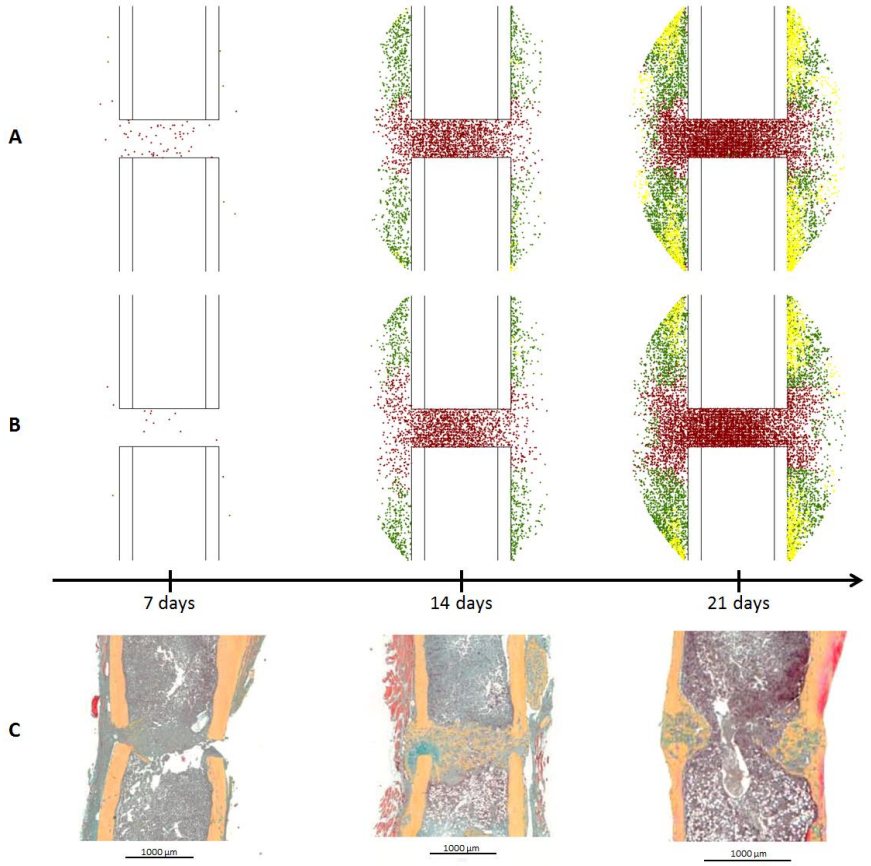

Fig. 3 Prediction of fibrous tissue (brown), cartilage (green) and bone (yellow) at 7, 14 and 21 days post-fracture under compression loading (A) and combined compression and bending load (B). Histology sections stained with Movat Pentachrome showing in vivo the formation of bone (yellow), cartilage (green), as well as fibrous connective tissue and bone marrow (reddish brown) over the course of healing (C)

Both loading conditions (compression and combined loading) led to similar tissue formation patterns during the first three weeks of healing (Fig. 3). After 14 days, the computer model predicted fibrous tissue and cartilage formation in the fracture gap and in the periosteal region, respectively. No intramembranous ossification was predicted. After 21 days, bone formed through endochondral ossification was predicted in the periosteal region and the external callus partially joining the cortical ends (Fig. 3). After 21 days, high amounts of fibrous tissue were predicted to be still present in the osteotomy gap.

Loading had an influence on tissue formation patterns at later time points. Under compression loading, complete healing was predicted after 14 weeks, while combined compression and bending led to a non-union situation (Fig. 4).

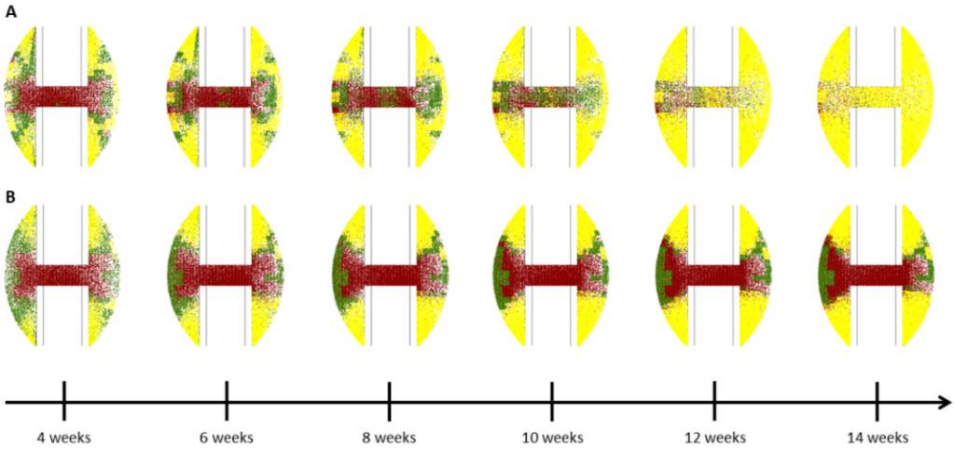

Fig. 4 Predicted bone (yellow), cartilage (green) and fibrous tissue (brown) formation between 4 and 14 weeks under compression (A) and combined compression and bending load (B)

The percentage of the different tissues formed within the callus area clearly showed that both loading conditions led to similar healing at the early stages, and continued following two different healing paths in the later healing phases (Fig. 5). Under compression loading, at the later stages of healing, the amount of fibrous tissue and cartilage decreased while the amount of bone increased. In contrast, under combined compression and bending load, the amount of fibrous tissue and cartilage remained relatively constant, indicating a non-union situation.

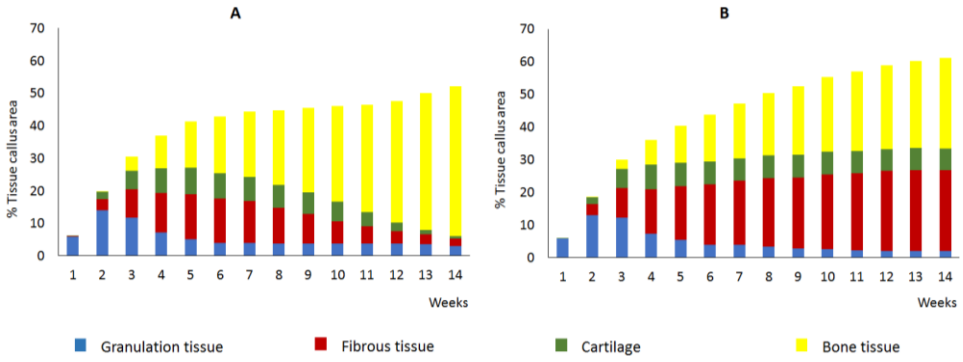

Fig. 5 Evolution of the healing response, described as temporal variation of the amount of different tissues predicted within the callus area for both loading cases: only compression (A) and compression in combination with bending (B)

4. DISCUSSION

Understanding how mechanical signals influence bone regeneration processes has great relevance in the design of clinical strategies for bone fracture treatment. Although different species have been shown to respond to different levels of mechanical stimuli, little is known about how fixation stability and therefore mechanical signals influence callus tissue formation processes over time in mice. Mice are a popular animal model due to the availability of a broad spectrum of molecular biological tools and ease of genetic modification. Therefore, the aim of this study was to investigate the mechanical regulation of bone healing in a mouse femoral osteotomy model. An established mechano-regulation computer model was used to predict tissue formation patterns over the course of healing, which were compared to experimental histological data.

Generic mechano-regulation rules, which were able to describe bone healing in rat [26], were not able to explain experimentally the observed tissue formation patterns in a femoral osteotomy model in mouse [31]. Computer model predictions showed periosteal cartilage formation at the early healing phases, which were not observed *in vivo*. Finite element analyses showed that mechanical strains within the callus immediately post-surgery were significantly higher than those reported in a rat osteotomy model [26]. The mechanical stimuli created within the callus by the external fixation were in the range of those postulated to promote cartilage and fibrous tissue [8]. Experimental studies have suggested that mice bone does not require as much stability for timely healing as the one in humans [25]. This could explain higher mechanical strains within the healing region immediately post-surgery in bone osteotomy models leading to uneventful secondary bone healing in mice [31].

Experimentally, bony bridging in the mouse was reached after three weeks. In contrast, computer model predictions showed large amounts of fibrous tissue in the gap and no bony bridging after three weeks of healing. Bony bridging was predicted at much later time points, approximately after 12 weeks under compression load. One reason for the difference results between the experimental and computational models is the absence of bone formation at the initial healing phases in the computational model. Computer models predicted initial bone formation to occur through endochondral ossification, which requires a longer time period than intramembranous ossification. Experimentally a combination of endochondral and intramembranous ossification was observed. Additionally, in this study we assumed that cellular activity in the mouse occur at the same rate as in rat. Faster cellular activity in the mouse compared to the rat could explain the slower bone healing response predicted by the computational model. However, this needs to be further investigated.

We investigated two different loading conditions, compression and combined compression and bending. Loading mode had an influence on tissue formation pattern predictions, especially at the later stages of healing. We observed that combined bending and compression loads led to a non-union situation. Loading conditions in mice are not well understood. Here, we assumed that due to the anatomical similarity, loading conditions in rat and mouse are comparable. Our loading conditions were therefore based on values reported for the rat [31], scaled to take into account differences in animal weight. Isaksson et al. [30] used a computer model to investigate the development of a second cortex during the remodeling phase of healing in mice and showed that its appearance could be explained when external bending loads were considered. They used

a compression load of 0.75 N and a load that resulted in a bending moment at the fracture site of 1.8 Nmm. Following Wehner et al. [32], we applied a compression force of 6 times the mouse body weight, which resulted in 1.5 N and a load which resulted in a maximum bending moment of 2.7 Nmm at the femoral midshaft. Our loads are approximately twice as those reported by Isaksson. They estimated the loads based on a remodeling algorithm, to best describe bone shape. We decided to adapt rat derived loading conditions, from a musculoskeletal model, since they should better take into account anatomical and gait patterns. Further studies will further investigate the influence of the loading conditions on mechano-biological predictions of bone healing progression.

In summary, we have shown that mechano-regulation "rules" able to explain bone healing in rat are not able to explain tissue formation patterns over the course of healing in a mouse osteotomy model. It appears that bone healing in mice occurs under significantly higher magnitudes of mechanical strain compared to rat. These results are relevant if experimental observations of mechano-transduction responses in mouse are to be translated to humans.

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