Osteoporotic krm2-Transgenic Mice Show Impaired Diaphyseal Fracture Healing

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Introduction:
Canonical wnt-signaling in bone is now recognized as a crucial pathway in maintaining bone homeostasis. Impairment of this pathway could contribute to osteoporosis, as shown by the loss of function mutation of Lrp5 in osteoporosis pseudoglioma patients [1]. Osteoporotic patients in general show a disposition for delayed fracture healing, often leading to dramatic clinical problems. The molecular reasons for such deficient fracture healing in osteoporotic patients remain unknown. We recently generated a mouse model, in which an osteoporotic phenotype was developed due to an impairment of the canonical wnt-pathway via overexpression of kremen-2 (krm2-tg), a ligand of Lrp5/6 at the cell surface [2]. The aim of the present study was to investigate diaphyseal fracture healing in this osteoporotic mouse model compared to strain-matched wild type mice. As interfragmentary movement has a great influence on the quality, progress and final success of the fracture healing process, and as wnt-signaling is known to be involved in mechanotransduction [3], we investigated fracture healing under well defined rigid and flexible fixation. We hypothesized that we would find a delay of the fracture healing process in krm2-tg compared to wild-type mice and that ideal fixation conditions would differ between krm2 transgenic mice and wild-type mice.

Material and Methods:

Animals: This study was conducted following national regulations for the care and use of laboratory animals and was approved by the national Ethical Committee. Female 6 months old krm2-tg mice (krm2-tg) and strain matched female C57BL/6 mice (wt) were raised under identical conditions (average weight approximately 25g). A rigidly and a flexibly fixated wild-type group and a rigidly and a flexibly fixated krm2-tg group were operated (each subgroup n=5-8).

Surgery and fixation: Two alternatives of a mechanically characterized unilateral external fixator for femoral mouse osteotomies were used. The unilateral external fixation optimized rotation stability of the osteotomy site and could be reproducibly varied from rigid to flexible fixation in the axial direction. The chosen axial in vitro stiffness for fixators in the rigid groups was E*I≈18.1 N/mm, and for the flexible groups E*I≈1.3 N/mm. The femoral osteotomy (0.5 mm gap size) was performed using a gig saw wire. Four titanium pins (0.5 mm diameter) placed into predrilled holes held the external fixator in position, no dislocation of the bone fragments having occurred (Fig.1). The weight of the whole device was only 0.22g, which allowed the animals to move freely.

Fig. 1: Rigid external fixator and x-ray of the osteotomy site

Evaluation: Using a motion detection system (Actimot-System, TSE, Germany) during the 12 hours dark cycle, activity and ground reaction forces of the animals were monitored prior to operation and during the healing period of 21 days. Animals were sacrificed on day 21 and femora were collected. Flexural rigidity E*I of each bone was evaluated by continuously recording the bending load F versus sample deflection during a non-destructive 3-point-bending in a material testing machine (Zwick, Germany). Micro computed tommography (resolution 30 µm) was used for quantitative measurements. 3D reconstructions of the samples were visualized using VG StudioMax 1.0 3D software (Volume Graphics, Germany). Two volumes of interest (VOIs), the whole callus and the osteotomy gap, were created by segmentation, for which the total volume (TV) and the bone volume (BV) were obtained, and the bone volume fraction (BV/TV) was calculated. Afterwards these femora

were dehydrated and embedded in methyl methacrylate to obtain longitudinal sections for histological evaluation.

Statistical analysis: Student’s t-test, level of significance: p < 0.05.

Results:
Activity measurements: All mice recovered quickly after the operation. They tolerated the external fixator well and showed little impairment of their activity during the initial days after surgery, reaching the pre-surgery activity level during the second week of healing. Recovery started in tendency earlier in the rigidly fixated groups. The ground reaction force of the right hind limb reached approximately 80% of the pre-surgery values by day 4 and was then constant until the end of the healing period for all groups.

Biomechanical testing: Non-operated intact femora of osteoporotic krm2-tg mice revealed a significant loss of flexural rigidity in 3-point-bending (Table 1).

Table 1: Flexural rigidity E*I in Nmm

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<tr>
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<th>E*I conrtols</th>
<th>E*I rigid</th>
<th>E*I flexible</th>
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<tbody>
<tr>
<td>wt</td>
<td>2932.2±542.8*</td>
<td>2245.6±569.9*</td>
<td>1636.5±448.9*</td>
</tr>
<tr>
<td>krm2-tg</td>
<td>2214.9±606.0*</td>
<td>950.4±433.3*</td>
<td>457.8±301.6*</td>
</tr>
</tbody>
</table>

(* genotyp-dependent significance; † fixation-dependent significance)

Testing of the operated bones showed that healing of rigidly fixated osteoporotic mice was impaired compared to wild-type mice. Both genotypes showed significantly impaired healing on flexible fixation, the observed loss of stiffness being even more apparent in the krm2-tg mice (E*I decreased by 27% in wt-mice and by 52% in krm2-tg). Micro computed tomography: Analysis of the segmental VOIs from µct-scans revealed a more than doubled total periosteal callus volume and a significant increase in callus diameter from rigid to flexible fixation in both genotypes (Table 2).

Table 2: Callus volume (CV [mm³]) and callus diameter (CD [mm])

<table>
<thead>
<tr>
<th></th>
<th>CV rigid</th>
<th>CV flexible</th>
<th>CD rigid</th>
<th>CD flexible</th>
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<tbody>
<tr>
<td>wt</td>
<td>4.85±0.81*</td>
<td>9.67±3.83*</td>
<td>2.17±0.17*</td>
<td>2.95±0.51*</td>
</tr>
<tr>
<td>krm2-tg</td>
<td>3.50±1.57*</td>
<td>9.68±3.79*</td>
<td>1.80±0.39*</td>
<td>2.70±0.41*</td>
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(† fixation-dependent significance)

The evaluated bone volume fraction (BV/TV) for the osteotomy gap was significantly decreased in flexibly fixated krm2-tg mice (Fig. 2). This result was confirmed by histological evaluation.

Discussion:
The present study demonstrated that female, middle-aged, transgenic krm2 mice, afflicted with a malfunction of wnt-signaling in bone, suffer from severe osteoporosis. For the first time it was shown that the osteoporotic bone of these animals show delayed fracture healing. Compared to wild-type mice, the healing process of krm2-tg mice was stronger impaired under flexible conditions. Furthermore, it was demonstrated that impairment of fracture healing in krm2-tg mice was not due to inadequate development of callus-volume, but to poor calcification of the tissue in the bridging area.

References:

Fig. 2: Fraction of calcified tissue in the osteotomy gap (BV/TV)