Full Length Article

Healing of fractures in osteoporotic bones in mice treated with bisphosphonates – A transcriptome analysis

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ABSTRACT

Bisphosphonates (BP) are inhibitors of bone resorption and are used to treat postmenopausal osteoporosis. Long-term treatment with BP attenuates bone remodeling, possibly leading to detrimental consequences for the bones’ ability to repair defects. To test this hypothesis, an animal model was established. Twelve week old mice were ovariectomized (OVX). Following confirmation of bone loss 8 weeks after OVX, the animals were treated with Alendronate (ALN) until sacrifice. After 5 weeks of ALN injections, the femoral bones were osteotomized and the osteotomies were either rigidly or non-rigidly stabilized. In rigidly fixed defects, no callus developed between 1 and 5 weeks after osteotomy, whereas after non-rigid fixation, callus development occurred. The administration of ALN resulted in an increase in newly formed bone at the defect site 5 weeks after osteotomy, irrespective of the estrogen status or fixation system. Transcriptome analysis demonstrated that both rigid and non-rigid fixation affected gene expression primarily during the middle phase of bone repair. Furthermore, the number of differentially expressed genes in tissues from non-rigidly fixed defect sites increased in animals treated with ALN over the course of bone repair. This indicates that ALN-dependent repair processes become increasingly dominant in the late phases of the healing process. Ranking of the factors affecting the composition of the transcriptome and their impact on the healing process revealed fixation at the defect site to be the strongest causative factor, followed by bisphosphonate treatment and estrogen deficiency. The present study suggests that the continuous administration of ALN is detrimental to bone repair, eventually causing a delay in healing in mechanically compromised situations. Consequently, rigid fixation may prove essential for a successful intervention.

1. Introduction

Bisphosphonates (BP) are widely used for the treatment of postmenopausal osteoporosis [1,2]. Treatment of osteoporotic patients with alendronate (ALN), the most frequently prescribed BP, results in a reduction of fractures of the hip, wrist and spine [3,4]. The beneficial effects are achieved by the suppression of bone resorption, followed, through a coupling mechanism, by a reduction in bone formation [3–9]. Due to this mechanism of action, the widespread use of BP and the fact that elderly osteoporotic patients are prone to fractures, there exists a vivid interest in understanding the effects of BP on bone repair.

The process of bone repair is characterized by a sequence of distinct phases that, to a large extent, parallel wound healing. An initial phase of inflammation and removal of debris and blood clots is followed by stabilization of the defect site through membranous or endochondral bone formation. The repair process is concluded by removal of the primary woven bone, which is replaced by lamellar bone. The pathway of repair depends on the mechanical stability at the defect site. Non-rigid fixation of a defect, allowing for inter-fragmentary motion, favors endochondral bone formation and is characterized by the formation of a cartilaginous callus to enhance primary stability. This process is followed by subsequent remodeling and restoration of the original shape [10,11]. In rigidly fixed defect sites and in the absence of inter-fragmentary motion, intramembranous bone formation will occur. The healing process is characterized by the absence of a callus and a direct remodeling of the Haversian canals [12]. Elucidation of the molecular events in bone repair is essential for the prediction and treatment of the aberrant healing of bone defects.

The potentially adverse effects of BP on fracture healing have been investigated in animal models. Most notably, studies have demonstrated that rats [13–15] and mice [16,17] under treatment with BP experienced a delay in the remodeling of the cartilaginous callus in non-
vertebral fractures. The elucidation of signaling pathways activated during defect repair has revealed crucial involvements of the TGF-β [18,19], Wnt [20] and TNF-α pathways [21,22]. Transcriptome analyses have been performed on different animal models of fracture repair [23,24]. Detailed knowledge on the molecular events involved in normal and impaired bone repair processes, however, remains scarce. Moreover, the interplay between the mechanical environment, estrogen depletion and BP therapy and their respective effects on bone repair are relatively unexplored. The aim of the present study was to elucidate gene expression during fracture healing in a femoral defect model in dependence of rigidity of fixation, treatment with BP and estrogen depletion and to combine the data with histology and MicroCT analysis. The data provides evidence as to whether treatment with BP exerts detrimental effects on the repair of osteoporotic bone and also demonstrates which of the three parameters, has the strongest modulatory effects on bone healing.

2. Methods

2.1. Reagents

Alendronate was purchased from Sigma-Aldrich (Buchs, Switzerland). The anesthetic mix for surgery and peripheral Quantitative Computer Tomography (pQCT) measurements contained fentanyl dihydrogen citrate (Sintetica, Switzerland), Dormone* (Dr. E. Graeub AG, Bern, Switzerland) and Dormicum* (Roche, Basel, Switzerland). The antidote mixture was composed of Alzane (Dr. E. Graeub AG, Bern, Switzerland), Anexate (Roche, Basel, Switzerland) and Temgesic (Reckitt Benckiser Healthcare Ltd., Berkshire, UK). The antidote mix to end anesthesia after pQCT measurements was the same, except for Temgesic, which was replaced by Naloxon (Orpha Swiss, Küsnacht, Switzerland).

2.2. Experimental design

This study was approved by the Local Committee for Animal Experimentation (Bern Committee for the Control of Animal Experimentation, Bern, Switzerland, permit number BE10/14 to WH). According to local regulations, animals were kept in the specific pathogen free (SPF) facility of the Medical Faculty of the University of Bern following FELASA regulations [25]. In this study, 12 week old female C57BL/6J mice (Charles River, Sulzfeld, Germany) were assigned to one of the 8 experimental groups representing the possible combinations of surgical and treatment protocols including the following parameters: sham/ovariectomy (OVX), vehicle/ALN, and rigid/non-rigid fixation of the osteotomy (MouseFix™ rigid/MouseFix™ non-rigid). The experimental design and the time course of the in vivo study are depicted in Fig. 1. Briefly, at the start of the experiment, animals underwent either OVX or sham surgery. Vehicle (control)/ALN treatment was initiated 8 weeks after OVX/sham, and continued until sacrifice of the animals. A femoral defect was introduced 5 weeks after the onset of vehicle/ALN treatment and was stabilized either with a rigid (MouseFix™ rigid; RISystem AG, Davos, Switzerland) or non-rigid (MouseFix™ flexible; RISystem AG, Davos, Switzerland) osteosynthesis system. For surgical procedures, the mice were anaesthetized by subcutaneous injections (2 ml/kg body weight) of fentanyl dihydrogen citrate (0.05 mg/kg body weight; 0.02 mg/ml)/medetomidine hydrochloride (0.5 mg/kg body weight; 0.2 mg/ml)/climazolame (5 mg/kg body weight; 2 mg/ml). Post-operatively, an antidote of Alzane (1.1 mg/kg; 0.22 mg/ml)/Anexate (0.45 mg/kg; 0.09 mg/ml)/Temgesic (0.075 mg/kg; 0.015 mg/ml) was injected s.c. (2.5 ml/kg body weight). The antidote used after pQCT measurements was composed of Alzane (1.02 mg/kg; 0.17 mg/ml)/Anexate (0.42 mg/kg; 0.07 mg/ml)/Naloxon (0.6 mg/kg; 0.1 mg/ml) and was injected s.c. (3 ml/kg body weight). Group sizes were n = 6 for histological and MicroCT analyses and n = 3 for RNA sequencing. The mice were sacrificed at 3, 7, 14 and 28 days after osteotomy for the purpose of RNA sequencing. All animals (192) were subjected to pQCT analysis.

2.3. Ovariectomy

For OVX, ovaries were approached through two 0.5 cm flank incisions at the mid-dorsum. The ovaries were located, clamped and removed (gently pulled through the incisions and a hemostat was placed between the oviduct and the ovaries). The oviducts were ligated and a cut was made between the hemostat and the ovaries. Hemostasis was controlled before replacing the ligated oviducts into the abdomen. The muscle layer was closed with absorbable sutures, and the skin was sutured with a non-absorbable thread. Sham operated animals underwent the identical surgical procedure, but without ligation of the oviducts and removal of the ovaries.

2.4. Treatment with alendronate

ALN in 0.9% NaCl (1.61 μmol/kg body weight; 2 ml/kg bodyweight) or vehicle (0.9% NaCl; 2 ml/kg bodyweight) was administered 8 weeks after OVX by s.c. injection twice weekly until sacrifice of the animals [28].

2.5. Femoral osteotomy

At 13 weeks after OVX, and 5 weeks after start of the ALN treatment, the left femora were surgically osteotomized. Briefly, a longitudinal incision in line with the left femur was made into the lateral thigh. The interval between the vastus lateralis and the biceps femoris was developed to expose the bone, and the gluteus superficialis tendon was detached from the trochanter tertius. Either a MouseFix™ rigid or MouseFix™ flexible system, manufactured from pure Titanium, was mounted onto the intact femora. Subsequently, by using a Gigli saw, a mid-femur osteotomy of 0.22 mm was created. Care was taken to avoid

![Fig. 1. Experimental design of the ALN-treated osteoporotic femoral defect model. At 13 weeks before the femoral osteotomy, animals were OVX- or sham-operated. Vehicle or ALN was applied twice/week s.c. for 5 weeks prior to osteotomy until sacrifice of the animals. A 0.22 mm femoral mid-diaphysis osteotomy was stabilized with a rigid or non-rigid fixation plate. RNA sequencing was performed on the total RNA isolated from tissues collected at the defect site 3, 7, 14 and 28 days post-osteotomy. MicroCT and histological evaluations were performed at 7 and 35 days post-osteotomy.](image-url)
harming the periosteum. Debris was removed from the defect site by rinsing with a sterile physiological saline solution, followed by wound closure. After recovery from anesthesia, analgesia with Temgesic (0.0075 mg/kg body weight; 0.3 mg/ml) was provided for 3 days and full load bearing and unrestricted cage activity were allowed.

2.6. Radiography

After surgery, the integrity of the surgical site was documented through high-resolution radiography (MX-20, Faxitron X-Ray Corporation, Edimex, Le Plessis, France). Post-operatively, the defects looked identical, independently whether a rigid or the non-rigid fixation system was mounted. No animals needed to be excluded from the experiment post-operatively due to incorrect localization of the osteotomy and/or plate positioning, and no animals were lost prematurely.

2.7. pQCT

The effects of OVX on bone mass and structure were evaluated by in vivo pQCT (XCT research SA, Stratec Medizintechnik GmbH, Birkenfeld, DE) measurements at the left distal femur and proximal tibia, at 4 and 7 weeks after OVX.

2.8. MicroCT

The tissues assigned to histological analysis were fixed in 4% paraformaldehyde in phosphate-buffered saline 24 h and subsequently transferred to 70% ethanol for microCT analysis (MicroCT40, SCANCO Medical AG, Brüttisellen, CH), using the built-in software from Scanco (Scanco Module 64-bit; V5.15). The region of interest (ROI) in the vertebral bodies of L3, L4, and L5 was manually delineated and the measurements were recorded perpendicular to the longitudinal axis of the vertebrae. The long axis of the femur was oriented orthogonally to the axis of the X-ray beam. The X-ray tube was operated at 70 kVp and 114 mA, and the integration time was set at 200 ms. Bone repair was evaluated in the area between the two central screws of the fixation systems. To distinguish between woven and lamellar bone, the tissue was segmented into 3 tissue types based on their greyscale, i.e. < 200 Hounsfield unit (HU) for soft tissues, between 200 HU and 360 HU for low density mineralized tissues (LDMT) and > 360 for high density mineralized tissues (HDMT) [29]. The analysis was performed at the highest resolution with a voxel size of 6 μm [30].

2.9. Histological analysis and histomorphometry

For histological evaluation, the tissues were embedded in MMA as described previously [31]. Thereafter, ground sections of approx. 200 μm, were prepared (Leica SP1600, Leica Microsystems, Glattbrugg, CH). The sections were polished and stained with MacNeal’s tetra-chrome [32,33]. Microphotographs were taken using a Nikon Eclipse E800 microscopy system (Nikon Inc., Switzerland, Egg, CH). The area of cortical lamellar bone at the defect site was quantified using ImageJ (1.50i) after manual delineation of lamellar bone from woven bone based on color gradients [34]. For in vivo labeling of the mineralizing surfaces, calcine (10 mg/kg body weight) and xylene orange (90 mg/ kg body weight) were dissolved in 2% NaHCO₃ at pH 7.4, and the solutions were administered i.p. at 12 days and 5 days before sacrifice of the animals. The contralateral femora were embedded in MAA and 16-μm sections were prepared (n = 3). For microscopy, filter settings for FITC (Excitation max: 490 nm; Emission max: 525 nm) and TRITC (Excitation max: 557 nm; Emission max: 576 nm) were used for calcine and xylene orange imaging, respectively.

2.10. Uterus dry weight and bone metabolic markers

The uteri of the animals were collected post-mortem and their dry weights were measured 24 h later. Serum levels of TRAP5b levels were determined 3 and 5 weeks after the onset of the treatment with ALN, using a commercially available kit according to the manufacturer’s instructions (MouseTRAP™, IDS, Boldon Colliery, UK).

3. RNA sequencing

3.1. Isolation of RNA, preparation of TruSeq library, and sequencing

To collect the tissues from the repair sites, the mice were sacrificed at 3, 7, 14 and 28 days post-osteotomy. Within 5 min of sacrifice, the femora were extracted and placed in RNALater® (Sigma-Aldrich, Buchs, Switzerland). The repair tissues were collected by removing the two central screws and positioning a custom built guide on the fixation plate. Two Gigli saws were used to cut and detach the tissues. Particular attention was paid to maintain the integrity of the marrow and bone tissues. The total RNA was isolated by homogenizing the isolated tissues in microtubes containing metal beads and 300 μl Lysis Buffer/30 μl β-Mercaptoethanol, using a benchtop tissue homogenizer (TissuLyser 2, Qiagen, Hilden, DE), followed by a digestion with DNase, using the RNA easy kit (Macherey-Nagel, Düren, DE) according to the manufacturer’s instructions. The quality of the RNA (eluted in 30 μl RNase-free water) was assessed (Agilent Technologies, Santa Clara, CA, USA). The concentrations of RNA were measured with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The RNA was stored at − 70 °C. RNA sequencing libraries were prepared with the TruSeq Stranded mRNA Sample Preparation Kit, v2 (Illumina, San Diego, CA, USA) and were sequenced for 150 bp paired-reads on an Illumina HiSeq3000 with the Next Generation Sequencing (NGS) Platform of the University of Bern.

3.1.2. Mapping reads to the reference genome

The quality of the reads was assessed using fastqc v.0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were mapped to the mouse reference genome (mm38, ensembl release 75) with TopHat v.2.0.13 [35]. The htseq-count v.0.6.1 [36] was used to count the number of reads overlapping with each gene, as specified in the ensembl annotation (release 82).

3.1.3. Identification of differentially expressed genes and outliers, and analysis of temporal profile patterns of genes and gene ontology enrichment analysis

Transcriptome analyses were carried out using the Bioconductor package DESeq2 v. 1.12.3 [37]. Differentially expressed genes (DEG) were identified for the following comparisons at each time point: OVX vs. sham, controls vs. ALN and rigid vs. non-rigid fixation. Differences in gene expression with a false discovery rate (FDR) < 0.05 were considered significant. Hierarchical clustering was performed in R with the function “hclust” using the method “complete”. This particular clustering method defines the cluster distance between two clusters to be the maximum distance between their individual components. The identification of outliers was based on total mapped reads correlation studies and cluster identification with principal component analysis. Bayesian hierarchical modeling using the R package EBSeq-HMM (version 1.4.0) [38] was applied to identify dynamically expressed genes over time. Three patterns were identified at each temporal transition: up-regulated, down-regulated and no change. A total of 27 patterns were considered along 3 transitions (D3-D7; D7-D14; D14-D28). Temporal profiles of gene expression for each of the following conditions were identified: vehicle; ALN; rigid fixation and non-rigid fixation. Thereafter, dynamically expressed genes were grouped into subsets of genes exhibiting either identical or distinct temporal profiles.
3.1.4. Statistical analysis

The statistical analyses were performed with GraphPad Prism 7 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com): The unpaired t-test was used for the analysis of bodyweight and uterus weights and two-way analysis of variance (ANOVA) with Tukey post-hoc was applied to pQCT, MicroCT and histomorphometry analyses.

4. Results

4.1. Effects of OVX and treatment with BP in C57BL/6J mice

The suitability of the OVX/ALN mouse model was assessed by analyzing body weight and bone architecture in response to OVX and treatment with ALN. Body weight was determined at time of OVX and 2, 4 and 12 weeks thereafter. Uterine dry weights were measured post-mortem (Suppl. Fig. 1B). Body weight was significantly increased in OVX animals by 8%, 14%, and 14%, at 2, 4, and 7 weeks after surgery when compared to sham animals (Suppl. Fig. 1A). Uterine dry weight was reduced by 70% (Suppl. Fig. 1B). Between the time of osteotomy (13 weeks after OVX) and analysis of the repair tissues (up to 18 weeks after OVX) the weight of the animals no longer increased (data not shown).

The effect of OVX on bone mass was determined in vivo by pQCT in the distal tibiae and proximal femora at time of OVX and 4 and 7 weeks thereafter (Fig. 2A–B, Suppl. Table 1). Cortical and trabecular densities did not vary between the OVX and sham groups at the time of OVX, but were significantly decreased in OVX animals after 4 and 7 weeks.

4.1.1. Treatment with ALN inhibits vertebral bone loss in OVX animals

To assess the effects of ALN on bone mass and architecture, vertebrae L3–L5 were measured by MicroCT (Fig. 2C–D; Suppl. Table 2) at 1 week after osteotomy (14 weeks after OVX and 6 weeks treatment with ALN). OVX caused a significant decrease in vertebral BV/TV as well as in trabecular number ( Tb.N), trabecular thickness ( Tb.Th) and an increase in trabecular spacing ( Tb.Sp) (Suppl. Fig. 2). Treatment of the animals with ALN caused an increase in BV/TV by a factor of 1.85 in OVX and sham animals, resulting in a significant increase in BV/TV in the sham/ALN group and no significant change in BV/TV in the OVX/ALN group compared with sham/vehicle controls (Fig. 2D). Changes in BV/TV were accompanied by an increase in Tb.N and Tb.Th, and a decrease in Tb.Sp (Suppl. Fig. 2) and in the levels of serum TRAP levels (Suppl. Fig. 3).

Using fluorochrome labeling with calcine and xylene orange 12 days and 5 days before sacrifice of the animals, mineral apposition rates of 1.8 ± 0.5 μm/day (sham/vehicle); 1.6 ± 0.1 μm/day (OVX/vehicle); 0.8 ± 0.1 μm/day (sham/ALN); 0.6 ± 0.2 μm/day (OVX/ALN) were measured (Suppl. Fig. 4).

4.2. Development of repair tissues in femoral defects

4.2.1. Histological analysis of the healing process

The healing progress of the osteotomies was visualized histologically after 1 and 5 weeks (Fig. 3). After 1 week with rigid fixation, bone and cartilage formation were observed in all 4 experimental groups (Fig. 3A–D, Suppl. Fig. 5A–D). Each of the groups showed both endosteal and periosteal reactions with bone formation. Endosteal and periosteal bone formation was also observed in femora with non-rigidly fixed osteotomies (Fig. 3E–H, Suppl. Fig. 5E–H). In addition, prominent formation of cartilage-like tissue was visible (Suppl. Fig. 5E–H). At 5 weeks after osteotomy, the defects were closed in the experimental groups with rigid repair fixation (Fig. 3I–L). After non-rigid defect fixation, bridging of the gap was not as advanced as in rigidly stabilized osteotomies (Fig. 3M–P) and a callus had formed in each of the groups. The callus mainly comprised a mineralized cortex and bone marrow in the control sham and OVX animals (Fig. 3M, O), while the callus was filled with cancellous bone in the ALN-treated groups (Fig. 3N, P).

4.2.2. MicroCT analysis of the defect site

At 1 week after osteotomy, the bone volumes did not differ among the experimental groups (Fig. 4B), whereas after 5 weeks, the total bone volumes were increased by 20% in cases of rigidly stabilized defects, with a further increase of 15% noted in the sham/ALN group (Fig. 4B). Upon non-rigid fixation, the TV of bone at the defect sites was increased 3- to 5-fold at 5 weeks after osteotomy. Treatment with ALN resulted consistently in higher bone volumes as compared to controls (Fig. 4B).

MicroCT analysis revealed that by 1 week after osteotomy the high density mineralized tissues (HDMT)/TV did not differ significantly between fixation systems (Fig. 4C), independent of sham/OVX surgery and ALN/vehicle treatment, respectively. Notably, measurement of the low density mineralized tissues (LDMT)/TV revealed a significant increase of LDMT in non-rigidly stabilized defects as compared with rigidly fixed osteotomies (Fig. 4D), while LDMT/TV was not affected by sham/OVX surgery and ALN/vehicle treatments. At 5 weeks after osteotomy, the LDMT/TV at the defect site was the same in sham/OVX and ALN/vehicle animals upon rigid stabilization, while upon non-rigid stabilization, LDMT/TV was increased by a factor of 2 in animals treated with ALN compared with controls, irrespective of their estrogen status. At 5 weeks after osteotomy, HDMT/TV in rigidly fixed osteotomies was significantly increased with ALN. At the same time, HDMT/TV in non-rigidly stabilized defects was reduced by a factor of 2 to 3 in comparison to 1 week after surgery, independently of sham/OVX surgery and ALN/vehicle treatment, respectively. Bone density was significantly decreased in non-rigidly fixed osteotomies from ALN-treated animals compared with those from controls, irrespective of their sham/OVX status (Fig. 4A).

4.3. Transcriptome in developing repair tissues

4.3.1. RNA sequencing

To evaluate the molecular mechanisms regulating bone repair, RNA was isolated from tissues in the defect site at 3, 7, 14, and 28 days after surgery, and a global RNA sequencing analysis was performed. On average, 21 million reads/library with an overlap of 83% (72% lowest mapping) with the annotated genome (genome m38, build 75, ENSEMBL) were obtained.

4.3.2. Data clustering

A plot of the first two axes from a principal component analysis (Fig. 5A) revealed that the samples primarily clustered depending on the time of collection. In the top left quadrant, the samples collected 3 day post-osteotomy formed one cluster, independent of the applied treatments. The samples collected 28 day post-osteotomy were grouped within the bottom left quadrant, again irrespective of the applied treatment. In the central area of the map, clustering of day 7 and day 14 post-osteotomy samples depended on rigid and non-rigid fixation. Samples from non-rigidly stabilized defects clustered on the right part of the map while samples obtained from rigidly stabilized defects were located on the left side. To further evaluate the data, a complete linkage hierarchical clustering analysis was performed (Fig. 5B). On the dendrogram, 5 top clusters were identified. Cluster 1 is composed exclusively of samples that were collected 3 days post-osteotomy. Non-rigidly stabilized samples collected 7 days post-osteotomy formed cluster 2. In cluster 3, samples from non-rigidly stabilized osteotomies collected 14 days post-surgery were grouped together. In cluster 4 the rigidly stabilized samples collected at day 7, 14 and 28 days were grouped with the vehicle-treated non-rigidly stabilized samples collected at day 28. Finally, cluster 5 included all ALN-treated non-rigidly...
stabilized samples collected at day 28.

Within each of the 5 top clusters, samples clustered by time points and/or by fixation system, irrespective of sham/OVX or ALN/vehicle treatment. Exceptions are those samples obtained from tissues isolated 28 days post-osteotomy, where the samples from non-rigidly fixed defects clustered depending on the treatment with ALN/vehicle. The branching of the clusters revealed that samples at day 3 did not cluster depending on rigid or non-rigid fixation. Similarly, non-rigidly stabilized osteotomy samples from vehicle-treated animals at day 28 did not cluster with samples from ALN-treated animals but clustered with rigidly stabilized osteotomy samples collected at days 7, 14 and 28.

4.3.3. Identification of outliers
A principal component analysis and a correlation analysis were used to identify outliers. Four inadequately clustering, rigidly stabilized osteotomy samples, expressing similar transcript patterns as did same time/treatment non-rigidly stabilized repair tissues, were considered as outliers. Further evaluations were performed without these outliers (Suppl. Fig. 6).

4.3.4. Differentially expressed genes
To further explore gene expression of the developing repair tissues, the numbers of DEG among the different treatments applied during bone repair, were assessed for each time point (rigid vs. non-rigid fixation; ALN vs. vehicle; OVX vs. sham; Fig. 5C). Stability at the defect site (between 9405 and 16,989 DEG) followed by ALN/vehicle treatment (between 0 and 1987 DEG) and OVX/sham (between 15 and 151 DEG) were found to affect the number of DEG in decreasing order. Over the course of healing, the number of DEG was high when tissues from rigidly fixed and non-rigidly fixed defects were compared. Treatment with ALN and OVX did not affect DEG during the first 2 weeks of the healing process, but the number of DEG increased to ca. 2000 in the ALN/vehicle-treated animals and to ca. 150 in OVX animals after 28 days.

4.3.5. Biological process ontology during bone healing
Several biological processes essential for skeletal healing were investigated by analyzing the GO terms characterizing repair and bone turnover. As combined MicroCT, histological, DEG analyses and data clustering showed no effects of OVX on the development and composition of the repair tissues, further analyses were performed irrespective of sham and OVX conditions.

4.3.5.1. Temporal mRNA expression profiles. Dynamically expressed genes, the expressions of which were upregulated or downregulated during each of the temporal segments of the study (D3–D7; D7–D14; D14–D28) were identified using EBSeq-HMM and were clustered according to their temporal profiles. Temporal pattern analysis revealed that the number of dynamically expressed genes was 1.7-fold higher in transcriptomes from the non-rigidly stabilized
osteotomies than in those from the rigidly stabilized osteotomies (8130 vs. 4829; Fig. 6A). Similarly, treatment with ALN led to an increase in the number of dynamically expressed genes both with the use of rigid (1.1-fold increase; 8740 vs. 8192) and non-rigid fixation system (1.2-fold increase; 8538 vs. 7079). As a first step in the evaluation process, the dynamically expressed genes common to two treatment conditions were identified. Subsequently, these genes were divided into two groups that contained genes exhibiting either identical or divergent temporal profiles. Comparison of mRNA temporal profiles revealed that transcriptomes from rigidly and non-rigidly stabilized osteotomies shared 69% and 40% of the dynamically expressed genes over the entire healing course, respectively. Notably, 50% of these commonly expressed genes were grouped into identical temporal profile clusters.

While an enrichment analysis of commonly expressed genes by using PANTHER revealed GO terms related to skeletal biological processes, genes uniquely expressed in transcriptomes from either rigidly and non-rigidly stabilized tissues did not reveal GO terms related to skeletal healing. Further enrichment analysis with PANTHER as to the commonly expressed genes clustered in identical temporal profiles revealed the top GO terms related to skeletal healing. The GO terms related to skeletal healing were less enriched with commonly expressed transcripts clustered in different temporal patterns (Fig. 6A). We applied an identical approach to identify genes that were expressed in rigidly or non-rigidly stabilized osteotomy tissues treated with either ALN or vehicle. Evaluation of the transcriptomes from rigidly stabilized defects revealed that 37% of the dynamically expressed genes in tissues from vehicle-treated animals and 35% of the dynamically expressed genes in tissues from ALN-treated animals are shared among the two treatment groups. 56% of these commonly expressed genes share identical temporal profile over the entire healing course (Fig. 6B).

Analysis of the transcriptomes from non-rigidly stabilized osteotomies revealed that 77% of dynamically expressed genes in tissues from vehicle-treated animals and 64% of dynamically expressed genes in tissues from ALN-treated animals are shared. Of the commonly expressed genes, 86% were grouped into identical temporal profile clusters (Fig. 6C).

4.3.5.2. Angiogenesis. Reestablishment of the vasculature during bone repair is essential for bone healing. Analysis of GO terms revealed the GO term GO: 0001525 “angiogenesis” to be significantly enriched (adjusted p value < 0.05) in transcriptomes from rigidly or non-rigidly stabilized osteotomies for each time point (181 DEG at day 3;
290 DEG at day 7; 333 DEG at day 14; 267 DEG at day 28). Further functional analysis of the temporal profiles (EBSeq-HMM) of the 1647 genes, which were dynamically expressed in transcriptomes prepared from tissues derived from rigidly and non-rigidly stabilized osteotomies, showed grouping into different temporal profile clusters (hereafter 1647 dynamically distinct genes) and revealed that the GO term "GO: 1904018 "positive regulation of vasculature development" (fold enrichment = 2.99, p = 1.78E−02) was significantly enriched. Further analysis of the 25 DEG included in this GO term revealed 7 genes to be significantly up-regulated, and 18 genes to be significantly down-regulated in the rigidly stabilized osteotomy transcriptomes, compared with transcriptomes from non-rigidly stabilized defects (Table 1).

4.3.5.3. Cartilage. Functional analysis of the RNA temporal profile analysis (EBSeq-HMM) of the 1647 dynamically distinct genes demonstrated the GO term "cartilage development" (fold enrichment = 2.97, p = 1.07E−10) to be significantly enriched. Levels of transcripts encoding markers characterizing chondrocyte lineage cells, such as ACAN, COL2A1, COMP, LOXL4, IHH, PTH1R, PTHLH, and SOX9, were significantly increased and peaked at day 14 in transcriptomes from non-rigidly stabilized osteotomies, compared with transcriptomes from rigidly stabilized osteotomies (Table 2). RNA temporal profile analysis (EBSeq-HMM) of the 760 genes that were dynamically expressed in transcriptomes from non-rigidly stabilized osteotomies upon ALN and vehicle treatment, but grouped into different temporal profile clusters, revealed significantly enriched GO terms "cartilage development" (fold enrichment: 4.23; p = 1.70E−02) and "connective tissue development" (fold enrichment: 3.55;
4.3.5.4 Osteoblast differentiation and activity. Functional analysis of the 1647 dynamically distinct genes (EBSeq-HMM) revealed the significantly enriched GO terms “regulation of biomineral tissue development” (fold enrichment: 3.99, \( p = 1.06 \times 10^{-02} \)), “regulation of bone mineralization” (fold enrichment: 3.94, \( p = 4.72 \times 10^{-02} \)), and “positive regulation of ossification” (fold enrichment: 3.58, \( p = 2.54 \times 10^{-02} \)). Increased expression levels of transcripts encoding markers of osteoblast lineage cells (APLP, COL1A2, IBSP, POSTN, RUNX2, SPARC, SPP1 SP7 and VDR) were observed from day 3 to day 14 followed by decreased levels between day 14 and day 28 in the transcriptomes derived from both rigidly and non-rigidly fixed tissues (Table 3). While kinetics of mRNA expression over the course of healing are similar between tissues from non-rigidly and rigidly stabilized osteotomies, transcripts from non-rigidly stabilized osteotomies were significantly upregulated at days 7 and 14 (Table 3) compared with those from rigidly fixed osteotomies. Expression of sclerostin and osteocalcin followed distinct expression profiles. Levels of transcripts encoding sclerostin, an inhibitor of osteoblast formation and activation,
were increased in the rigidly stabilized osteotomies compared with the non-rigidly stabilized osteotomies at days 14 and 28, respectively. Levels of transcripts encoding osteocalcin were increased in transcriptomes from rigidly stabilized osteotomies as early as day 3, reaching max. levels at day 14, and then decreasing at day 28. The effects of treatment with ALN on bone formation-related GO terms were observed only in transcriptomes from non-rigidly stabilized tissues collected at day 14 post-osteotomy. The significantly enriched GO terms included: "endochondral ossification" (fold enrichment: 9.62, p = 2.20E-02), "replacement ossification" (fold enrichment: 9.62, p = 2.20E-02), "regulation of biomineral tissue development" (fold enrichment: 6.31, p = 2.71E-04) and "positive regulation of osteoblast differentiation" (fold enrichment: 6.22, p = 7.42E-03).

4.3.5.5. Osteoclast differentiation and activity. The GO terms “osteoclast development” and “osteoclast differentiation” were significantly enriched solely at 28 days after osteotomy in samples from non-rigidly fixed compared with those from rigidly fixed defects. Further analysis revealed significant upregulation of osteoclasts markers (ACP5, CALCR, CTSL, DCSTAMP, ITGAV, OCSTAMP, and OSCAR) at late healing stages in transcriptomes from both rigidly and non-rigidly stabilized osteotomies (Table 4).

5. Discussion

Annually, > 8.9 million osteoporotic fractures are registered worldwide [42]. The characteristic reduction in bone mass and...
BP is associated with impaired bone healing in humans [44]. Presently, there is no consensus as to whether treatment with ALN on bone repair.

Table 1
Expression of angiogenic marker genes during bone healing in non-rigidly and rigidly stabilized femoral defects. Differentially expressed genes were computed with DESeq2: n = 4-6.

<table>
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<th>Non-rigid vs. rigid fixation</th>
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<tbody>
<tr>
<td>Log 2 fold increase</td>
<td></td>
<td>D3</td>
<td>D7</td>
<td>D14</td>
<td>D28</td>
<td></td>
<td></td>
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<td>4.14E−08</td>
<td>1.25E−10</td>
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<td>9.17E−08</td>
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<td>0.71</td>
<td>0.0128</td>
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<tr>
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Table 2
Expression of chondrogenic marker genes during bone healing in non-rigidly and rigidly stabilized femoral defects. The expression of arbitrarily selected genes related to chondrogenesis were significantly upregulated at each time point (peaking at day 14) in non-rigidly stabilized osteotomies compared with rigidly stabilized osteotomies. Differentially expressed genes were computed with DESeq2: n = 4-6.

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<tr>
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<th>Non-rigid vs. rigid fixation</th>
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<td>0.0003</td>
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<td>3.63</td>
<td>1.17</td>
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<td>1.38E−155</td>
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</table>
transcriptome analyses revealed extensive changes that could be attributed to genes related to osteoclasts were increased late during the healing process (day 14 and day 28) in ALN-treated animals as compared to controls. Diurnal expression of osteoclast marker genes during bone healing in non-rigidly and rigidly stabilized femoral defects. The levels of transcripts encoding arbitrarily selected genes related to bone formation were significantly increased at each time point in non-rigidly stabilized defects as compared to rigidly stabilized defects. SOST and BGLAP transcript levels were found to be decreased at days 14 and 28 (SOST) and days 7 and 14 (BGLAP) in non-rigidly stabilized defects compared with rigidly stabilized defects. Differentially expressed genes were computed with DESeq2: n = 4–6.

Table 3
Expression of bone formation marker genes during bone healing in rigidly and non-rigidly stabilized femoral defects. The expression levels of arbitrarily selected genes related to bone formation were significantly increased at each time point in non-rigidly stabilized defects as compared to rigidly stabilized defects. The stability at the defect site was the experimental variable with the strongest effect on the composition of the transcriptomes during the middle phase of healing. The later stages of healing are largely dependent on the mechanical environment exerted minimal effects on the initial and late healing stages, the differences in stability exerted the strongest effects on the composition of the transcriptomes during the middle phase of bone repair. Furthermore, GO analyses revealed that the biological processes related to skeletal healing, such as angiogenesis, and bone and cartilage formation, were significantly increased in tissues from non-rigidly stabilized defects. The analysis of the dynamically expressed genes of the repair tissues from rigidly and non-rigidly stabilized defects at the most enriched GO terms and the clustering pattern. This suggests that the ALN effect on bone repair is largely dependent on the mechanical condition applied at the defect site. In non-rigid fixation, remodeling of the mineralized callus requires extensive osteoclastic resorption activity, which is blocked by the ALN that is incorporated during mineralization. Impaired remodeling results in inferior material properties at the fracture site due to a failure in replacing woven bone by lamellar bone [14,15]. The present data using whole mRNA transcriptomics and histological and MicroCT analyses, revealed that estrogen depletion has minor effects on bone repair, compared to those of the mechanical conditions at the defect site and to treatment with ALN. It can be safely assumed that growth of the experimental animals assumed that growth of the experimental animals, a factor assumed that growth of the experimental animals, a factor that may be necessary to allow for the description of the final composition and quality of the repair tissues. These data may contribute to a better understanding of the effects of BP on bone repair, in particular in either the cause, or the consequence, of the observed differences in the healing processes between rigidly and non-rigidly stabilized defects. A major finding of this study is seen in the formation of a cluster based on transcriptomes from ALN treated, non-rigidly stabilized osteotomies at day 28. For this particular group, the significantly enriched GO terms related to skeletal healing confirmed the strong effect of ALN on bone repair at the transcriptome level. In contrast, transcriptomes from tissues collected at rigidly stabilized defect sites do not reveal any significant effects of ALN on the healing process, with respect to both the enriched GO terms and the clustering pattern. This suggests that the ALN effect on bone repair is largely dependent on the mechanical condition applied at the defect site. In non-rigid fixation, remodeling of the mineralized callus requires extensive osteoclastic resorption activity, which is blocked by the ALN that is incorporated during mineralization. Impaired remodeling results in inferior material properties at the fracture site due to a failure in replacing woven bone by lamellar bone [14,15]. The present data using whole mRNA transcriptomics and histological and MicroCT analyses, revealed that estrogen depletion has minor effects on bone repair, compared to those of the mechanical conditions at the defect site and to treatment with ALN. 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In contrast, transcriptomes from tissues collected at rigidly stabilized defect sites do not reveal any significant effects of ALN on the healing process, with respect to both the enriched GO terms and the clustering pattern. This suggests that the ALN effect on bone repair is largely dependent on the mechanical condition applied at the defect site. In non-rigid fixation, remodeling of the mineralized callus requires extensive osteoclastic resorption activity, which is blocked by the ALN that is incorporated during mineralization. Impaired remodeling results in inferior material properties at the fracture site due to a failure in replacing woven bone by lamellar bone [14,15].

Table 4
Expression of osteoclast marker genes during bone healing in non-rigidly and rigidly stabilized femoral defects. The levels of transcripts encoding arbitrarily selected genes related to osteoclasts were increased late during the healing process (day 14 and day 28) in ALN-treated animals as compared to controls. Differentially expressed genes were computed with DESeq2: n = 4–6.
situations, where the fixation of the bone is critical. In conclusion, the continuous administration of ALN will be detrimental to bone repair in mechanically compromised situation, eventually causing a delay of healing. In addition, rigid fixation may prove essential for a successful intervention.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.04.017.

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Authors’ roles: Study design: MH and WH. Study conduct: MH, MS, and WH. Data collection: MH, MS, SD, and IA. Data analysis: MH, MS, IK, and WH. Data interpretation: MH, MS, IK, and WH. Drafting manuscript: MH and WH. Revising manuscript content: MH and WH. Approving final version of manuscript: MH, MS, SD, and IK. MH and WH take responsibility for the integrity of the data analysis.

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References


