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Expression of antagonists of WNT and BMP signaling after non-rigid fixation of osteotomies

Marc-Olivier Montjovent [a,1], Mark Siegrist [a], Frank Klenke [a,b], Antoinette Wetterwald [a], Silvia Dolder [a], Willy Hofstetter [a,*]

[a] Group for Bone Biology & Orthopaedic Research, Department Clinical Research, University of Bern, Bern, Switzerland
[b] Department of Orthopaedic Surgery, Inselspital, Bern University Hospital, Bern, Switzerland

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ABSTRACT

Delayed fracture healing and non-unions represent rare but severe complications in orthopedic surgery. Further knowledge on the mechanisms of the bone repair process and of the development of a pseudoarthrosis is essential to predict and prevent impaired healing of fractures. The present study aimed at elucidating differences in gene expression during the repair of rigidly and non-rigidly fixed osteotomies. For this purpose, the *MouseFix*™ and the *FlexiPlate*™ systems (AO Development Institute, Davos, CH), allowing the creation of well defined osteotomies in mouse femora, were employed. A time course following the healing process of the osteotomy was performed and bones and periimplant tissues were analyzed by high-resolution X-ray, MicroCT and by histology. For the assessment of gene expression, Low Density Arrays (LDA) were done. In animals with rigid fixation, X-ray and MicroCT revealed healing of the osteotomy within 3 weeks. Using the *FlexiPlate*™ system, the osteotomy was still visible by X-ray after 3 weeks and a stabilizing cartilaginous callus was formed. After 4.5 weeks, the callus was remodeled and the osteotomy was, on a histological level, healed. Gene expression studies revealed levels of transcripts encoding proteins associated with inflammatory processes not to be altered in tissues from bones with rigid and non-rigid fixation, respectively. Levels of transcripts encoding proteins of the extracellular matrix and essential for bone cell functions were not increased in the rigidly fixed group when compared to controls without osteotomy. In the *FlexiPlate*™ group, levels of transcripts encoding the same set of genes were significantly increased 3 weeks after surgery. Expression of transcripts encoding BMPs and BMP antagonists was increased after 3 weeks in repair tissues from bones fixed with *FlexiPlate*™, as were inhibitors of the WNT signaling pathways. Little changes only were detected in transcript levels of tissues from rigidly fixed bones.

The data of the present study suggest that rigid fixation enables accelerated healing of an experimental osteotomy as compared to non-rigid fixation. The changes in the healing process after non-rigid fixation are accompanied by an increase in the levels of transcripts encoding inhibitors of osteogenic pathways and, probably as a consequence, by temporal changes in bone matrix synthesis.

Introduction

The regeneration of bone during fracture healing is a process in which pre- and post-natal developmental programs take place in a highly coordinated manner [1]. Despite the complexity of the process, delayed healing or the formation of cartilaginous pseudarthroses is rare at 2.5% for non-unions and 4.4% for delayed unions in tibial fractures [2]. Though low in number, these complications present severe cases in orthopedic practice due to the debilitating effects on the affected patients and the required extensive restorative surgery.

The formation of a pseudo-joint can be brought about mechanically, i.e. by unstable fixation of an otherwise inconspicuous fracture or by reduced physiological healing capacity due to diseases such as diabetes, inflammatory conditions, and age [3–5]. To improve prediction and treatment of the course of fracture repair, detailed knowledge of the underlying cellular and molecular processes is essential. In the past, four parameters have been defined to be critical for functional bone repair, (i) the local and temporal availability as well as the concentrations of the signaling molecules present at the repair site, (ii) adequate progenitor cells, (iii) the scaffold/extracellular matrix, and (iv) the mechanical stability [6]. When these preconditions are met, the process, mirroring embryonic developmental steps, will proceed to the successful repair of the fractured bone [1,7–9]. Several

* Corresponding author at: Group for Bone Biology & Orthopaedic Research, Department Clinical Research, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland. Fax: +41 31 632 3297.
E-mail address: hofstetter@dkf.unibe.ch (W. Hofstetter).
[1] Present address: Celerion (Switzerland), Fehraltorf, Switzerland.

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clinical and pre-clinical studies focused on the conditions leading either to normal or delayed healing [10,11]. Clinical studies, however, usually lack sufficient numbers of samples and are hampered by a heterogeneous collective. Animal studies, on the other side, frequently use fracture models with fractures that differ within and between the groups [12].

Investigations on the cellular processes during fracture healing revealed a fixed order of distinct developmental processes to take place [3]. The first step in the healing cascade is characterized by an inflammatory response, formation of a blood clot and attraction of leucocytes. Subsequently, angiogenesis is initiated and mesenchymal progenitor cells are attracted to the site of injury [13,14]. Depending on the stability of fracture fixation, either endochondral or membranous bone formation, both followed by remodeling of the immature woven bone and replacement by lamellar bone are initiated [15,16]. Each of these stages of the repair process is characterized by a specific microenvironment generated by the participating tissues and cell lineages. Inflammatory growth factors dominate during the initial phase of fracture repair. These are subsequently replaced by vasculogenic factors, by factors acting on chemotaxis and differentiation of mesenchymal progenitor cells and lastly, to initiate remodeling of the primary bone, by factors coupling bone formation and resorption [3].

Recently, the AO Development Institute developed an internal fixation system optimized for mouse femora [17]. The system allows the creation of highly reproducible femoral osteotomies with defined edges and an accurate realignment of the bone ends. Furthermore, by using the two different fixation systems, *MouseFix*™ and *FlexiPlate*™, the latter with only one fourth of the mechanical top/bottom stiffness as compared to the former, one component of the key parameter mentioned before, the mechanical stability, could be modified. With the *FlexiPlate*™ System, a delay in bone repair and formation of a stabilizing cartilaginous callus was observed previously [18].

Using these internal fixation systems, we aimed at elucidating the gene expression during healing of osteotomies in mouse femora. The data suggests that an overshooting induction of inhibitors of WNT and BMP signaling pathways may contribute to the observed callus formation and the differences in the healing process of bone defects after rigid and non-rigid fixation.

Methods

Surgical procedures

Female 12 week old *C57Bl/6J* mice (Charles River, Sulzfeld, Germany) were used in this study, which was approved by the local Committee for Animal Experimentation (Bernese Committee for the Control of Animal Experimentation, Bern, Switzerland, permit number 103/07 to MOM) and conducted in accordance with its regulations. The mice were anaesthetized by subcutaneous injections of a mixture of fentanyldihydrogencitrate (50 μg/kg body weight), medetomidine hydrochloridum (500 μg/kg body weight) and climazolame (5 mg/kg body weight). During the surgical procedure, the animals were placed on a heating pad to prevent hypothermia. After shaving and disinfection, a longitudinal incision in line with the femur was made on the lateral thigh. The interval between the *vastus lateralis* and the *biceps femoris* was developed to expose the bone. Subsequently, the *gluteus superficialis* tendon was detached from the *trochanter tertius*. The *MouseFix*™ or *FlexiPlate*™ Systems (AO Development Institute, Davos, CH) were then fixed to the femur with 4 interlocking screws. A mid-femur osteotomy, width 0.22 mm, was created between the 2 central screws using a Gigli saw. Care was taken not to harm the periosteum. Thereafter, the wound was closed.

The experimental groups, each with an $n=3$, were defined as *MouseFix*™ ± osteotomy, and *FlexiPlate*™ with osteotomy. Animals were sacrificed 3 days and 1, 3, and 4.5 weeks after surgery.

X-ray and MicroCT analysis

After surgery and after excision of the femora at the end of the experiment, the integrity of the surgical site was documented by high resolution X-ray (MX-20, Faxitron X-Ray Corporation, Edimex, Le Plessis, France). The tissues assigned to histological analysis were fixed in paraformaldehyde (PFA, 4% in PBS) for 24 h and subsequently transferred into 70% ethanol for MicroCT analysis (MicroCT40, Scanco, Bruettisellen, CH), using the software that was provided by Scanco with the purchase of the MicroCT40 system. For measurements, 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, the integration time was set at 200 ms. Bone repair was evaluated in the area between the two central screws close to the osteotomy. To distinguish between woven and lamellar bone, the tissue was segmented into 3 tissue types based on their greyscale, i.e. <200 for soft tissues, between 200 and 360 for tissues with low mineralization (cartilage callus, woven bone) and >360 for tissues with high mineralization (lamellar bone), according to Gongroft et al. [18]. The analysis was performed at highest resolution with a voxel size of 6 μm.

Histology

After MicroCT analysis, the sections were embedded in MMA as described previously [19]. Thereafter, sections, which were subsequently ground down to approx. 200 μm, were prepared using an annular saw (Leica SP1600, Leica Microsystems, Glattbrugg, CH). The sections were polished and stained using McNeal's tetrachrome [20,21]. Microphotographs were taken on a Nikon Eclipse E800 microscopy system (Nikon Inc., Switzerland, Egg, CH).

Quantitative RT-PCR

To analyze the cellular composition of the repair tissues, quantitative RT-PCR was performed, using pre-synthesized Assays-on-Demand (AoD, Life Technologies/ABI, Rotkreuz, CH). After excision, the tissue of interest was stored in RNAlater (Qiagen, Basle, CH) until use. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Basle, CH) according to the recommendations of the manufacturer. After reverse transcription with MMuLV RT (Roche Diagnostics, Rotkreuz, CH), PCR was performed on an ABI PRISM 7500 System (ABI, Rotkreuz, CH). The reaction mixes contained Taqman Fast Universal PCR Master Mix (ABI, Rotkreuz, CH) and cDNA between 5 ng and 10 ng. The mixes were preincubated for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 5 sec at 95 °C and 15 sec at 60 °C each. The data was evaluated using the sequence detection software SDS V2.3. The following AoD were employed: COL1A1 (Mm00801666_g1), COL2A2 (Mm01309565_m1), COL10A1 (Mm00487041_m1), ACAN (Mm00545794_m1), ALP-1 (Mm00475834_m1), BGLAP (BGP, Mm03413826_mH), CSF-1R (cFMS Mm01266652_m1), F4/80/EMR-1 (Mm00802529_m1), MYH2 (Mm01332564_m1), GUSB (Mm00446953_m1). The expression of transcripts encoding the members of the WNT family of growth factors was also assessed by quantitative RT-PCR, the respective AoD used in this analysis are listed in Table 3. To compare the transcript levels, the values obtained from rigidly fixed *MouseFix*™ tissues after 3 weeks were set as "1", and the relative expression levels were calculated against *MouseFix*™ 3 weeks.

Low density arrays

Custom made low density arrays (LDA), which were loaded with pre-selected AoD, were used to analyze transcript levels of 9 housekeeping genes for standardization and 87 genes of interest (Suppl. Table 1). Before performing the LDAs, the quality of the RNA was controlled on an Agilent 2100 Bioanalyzer (Agilent, Basle, CH). The PCR

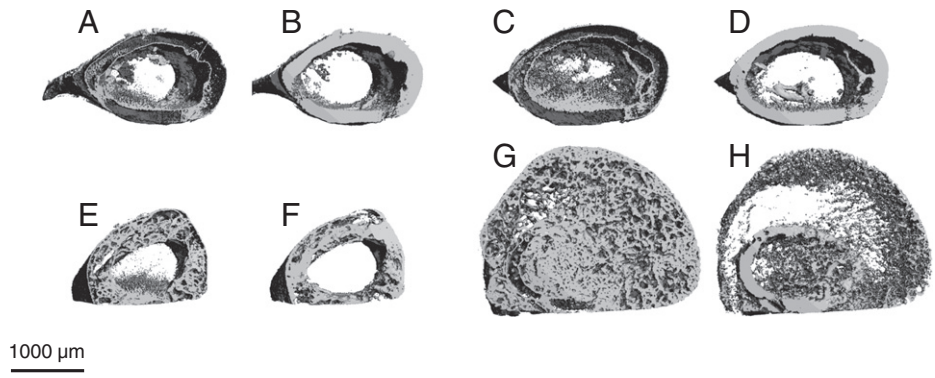

Fig. 1. *MicroCT analysis of repair site*. MicroCT analysis of the repair site, i.e. the area between the 2 central screws of the *MouseFix*™ (A, B, C, D) and *FlexiPlate*™ (E, F, G, H) implantation systems, was performed 1 week (A, B, E, F) and 3 weeks (C, D, G, H) after surgery. Mineralized tissues with low mineral density (A,C,E,G) and high mineral content (B, D, F, H) are separated. After 3 weeks, the femoral defect bridged with the *FlexiPlate*™ System is stabilized by formation of a fracture callus. No callus was found in femoral defects stabilized with the *MouseFix*™ System.

reaction (with 1 ng cDNA/well) was performed on an ABI PRISM 7900 System (ABI, Rotkreuz, CH) and the data were analyzed with the sequence detection software SDS V2.1 as described previously [22]. The PCR mixes were preincubated for 2 min at 50 °C and 10 min at 95 °C, and PCR was performed for 45 cycles of 15 sec at 95 °C and 1 min at 60 °C each.

Statistical analysis

For statistical analysis, one-sided ANOVA with Bonferroni's *post hoc* test was performed using GraphPad Prism Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Faxitron analysis

Three days after surgery, high-resolution X-ray imaging revealed defects looking identical, independently whether the *MouseFix*™ and *FlexiPlate*™ systems, respectively, were used (Suppl. Fig. 1A, B). After 3 weeks, however, the gap in the rigidly fixed bones was closed, and virtually no callus was present (Suppl. Fig. 1C), while upon fixation with the *FlexiPlate*™ System, the defect was stabilized by formation of a soft callus (Suppl. Fig. 1D).

MicroCT analysis of the defect site

One week after surgery, MicroCT analyses (Fig. 1) demonstrated that the volume of lamellar bone (T2) at the defect site was similar for both fixation systems (1.39 ± 0.23 for *MouseFix*™ and 1.22 ± 0.04 for *FlexiPlate*™), while low density mineralized tissues (T1) were slightly but significantly increased ($0.43\ mm^3 \pm 0.02$ and $0.51\ mm^3 \pm 0.03$, $p<0.05$, respectively) in the *FlexiPlate*™ stabilized defects (Fig. 1A, B and C, D; Table 1). Three weeks after surgery, the volume of the lamellar bone was still identical in bones stabilized with either fixation system ($1.50\ mm^3 \pm 0.66$ and $1.64\ mm3 \pm 0.29$, respectively; Fig. 1F, H). The volume of woven bone and of mineralized cartilage was increased by a factor 3 in defects stabilized with the *FlexiPlate*™ System ($1.34\ mm^3 \pm 0.35$ and $4.23\ mm^3 \pm 0.61$, $p<0.01$, respectively), corroborating the previous observations made by X-ray (Fig. 1E, G; Table 1). After 3 weeks, the BV/TV of the low density mineralized tissues was significantly increased in non-rigidly fixed osteotomies as compared to rigidly fixed defects (0.19 ± 0.03 *vs.* 0.30 ± 0.03), while neither the BV/TV of lamellar bone nor cortical density differed among the two fixation protocols (Table 1).

Histological analysis

One week after surgery, the osteotomy gaps were well visible with both fixation systems (Suppl. Fig. 2A,C) upon embedding in MMA and staining with McNeill tetrachrome. After 3 weeks, the defects stabilized with the *MouseFix*™ System (Suppl. Fig. 2E) were filled with new bone, with virtually no callus formation, while a considerable callus was formed after fixation with the *FlexiPlate*™ System (Suppl. Fig. 2G). The same course of bone repair was seen by MicroCT analysis, the osteotomy being visible after 1 week with both fixation systems (Suppl. Fig. 2B,D). The closure of the defect was confirmed 3 weeks after surgery with the *MouseFix*™ System (Suppl. Fig. 2F)

Table 1
MicroCT analysis. Volumes and BV/TV of mineralized tissues and Cortical Density at the defect site.

Thresholding											
T1: 200–360		"woven" bone									
T2: >360		"lamellar" bone									
		Volume T1 (mm^3)		Volume T2 (mm^3)		BV/TV T1		BV/TV T2		Cortical Density (mg HA/cm^3)	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1 week	*MouseFix*™	0.43	0.02	1.39	0.23	0.10	0.01	0.31	0.04	1446	12
	FlexiPlate™	0.51	0.03	1.22	0.04	0.12	0.00	0.30	0.02	1499	22
	$p<$	0.018		n. s.		n. s.		n. s.		n. s.	
3 weeks	*MouseFix*™	1.34	0.35	1.50	0.66	0.19	0.03	0.21	0.08	1373	7
	FlexiPlate™	4.23	0.61	1.64	0.29	0.30	0.03	0.12	0.03	1387	12
	$p<$	0.0021		n. s.		0.011		n. s.		n. s.	

Volumes of woven bone (T1) and lamellar bone (T2), BV/TV for woven and lamellar bone, and cortical density at the femoral defect site, as determined by MicroCT analysis. The values are presented as the mean ± SD from 3 animals. Differences with a p-value <0.05 are considered significant.

Table 2
Gene expression during fracture repair. Relative expression levels of transcripts. Values given are the average from two independent animals.

AoD	Relative levels of transcripts					
	MouseFix™ (no osteotomy)		*MouseFix™*		*FlexiPlate™*	
	1 week	4.5 weeks	1 week	4.5 weeks	1 week	4.5 weeks
col Iα1	3400	9700	22,000	6200	18,200	25,800
col IIα1	38	20	910	16	1730	45
col Xα1	6	0	315	1	225	3
Aggregan	5	3	164	3	415	7
ALPL	18	39	161	27	120	48
Bglap	410	1'040	750	430	165	2'210
c-fms	2	2	3	1	1	2
F4/80	39	48	49	35	26	74
Myh2	0	0	2	2	1	4

Relative expression levels at the repair site of transcripts encoding components of bone and cartilage extracellular matrix and of markers of the cells of osteoblastic, osteoclastic and monocytic cell lineages.

and callus formation was seen in bone defects fixed with the *FlexiPlate*™ System (Suppl. Fig. 2H).

Gene expression in repair tissues

To characterize the activities of the repair tissue and the invasion of the site by inflammatory cells, levels of transcripts encoding markers of osteoblast activity and differentiation (COL1A1, ALP, BGP), of chondrocyte activity and hypertrophy (COL2A1, ACAN, COL10A1) and of monocyte/macrophage lineage cells (*c-fms*, F4/80) were determined. To ascertain the absence of muscle tissues, the detection of transcripts encoding Myosin-2 (Myh2) was included. These studies were performed for bones stabilized with *MouseFix*™ (± osteotomy, since mounting the implant by itself may induce an anabolic response) and *FlexiPlate*™ Systems (Table 2). After 1 week, an increase in the levels of transcripts encoding COL1A1 was detected in the osteotomized bones (increased by a factor of 5–6 as compared to control, non-osteotomized bones). Transcripts encoding COL2A1 were increased by a factor of 20 (*MouseFix*™) and 40 (*FlexiPlate*™), transcripts encoding ACAN by factors of 30 and 80, and transcripts encoding COL10A1 by factors of 50 and 35, respectively. ALP as a marker for osteoblast activity was increased in both groups of osteotomized bones, but BGP, a marker for late osteoblast differentiation was reduced in osteotomized bones with *FlexiPlates*™ (relative expression levels normalized to GUSB: non-osteotomy/*MouseFix*™ control bones: 410; osteotomy/*MouseFix*™: 750; osteotomy/*FlexiPlate*™: 165). After 4.5 weeks, transcript levels were virtually normalized to non-osteotomy/*MouseFix*™ levels in the bones whose osteotomy was stabilized with the *MouseFix*™ System. Levels of transcripts encoding COL1A1 and BGP, however, were increased over control values (by factors of 2.5 and 2, respectively) in bones whose osteotomy was non-rigidly fixed with the *FlexiPlate*™ System. Neither after 1 week, nor after 4.5 weeks could an increase in the monocyte/macrophage lineage markers cFMS (M-CSF receptor) and F4/80 (monocyte/macrophage lineage marker) be detected. MYH2 transcript levels were not detectable in any of the samples.

Analysis of gene expression by low density arrays

The kinetics of gene expression during fracture healing was further analyzed using LDA. The genes were grouped according to their functions. Groups 1 and 2 encompass genes relevant for osteoblast (Fig. 2) and chondrocyte (Fig. 3) development and function, group 3 includes genes regulating bone resorption and vascularization (Fig. 4), groups 4 and 5 contain genes of the BMP family of growth factors and their antagonists (Fig. 5) and regulators of WNT signaling (Fig. 6).

Levels of transcripts encoding bone matrix proteins were expressed at highest levels in osteotomized bones stabilized with the *FlexiPlate*™ System 21 days after surgery. At the same time point, mRNAs encoding the transcription factors RUNX2 and Osterix/SP7 were significantly elevated in comparison to the contralateral, untreated bones, or to osteotomized bones stabilized with the *MouseFix*™ System 21 days after surgery (Fig. 2). Expression of cartilage specific transcripts was highest in the *FlexiPlate*™ stabilized defects, 1 and 3 weeks after surgery (Fig. 3), while some induction of the same group of genes was also observed at 7 days in stably fixed bones. Transcripts encoding the hematopoietic growth factors GM-CSF and M-CSF, and transcripts encoding tartrate resistant acid phosphatase (TRAP) and cFMS were found at elevated levels 3 weeks after surgery in osteotomized bones stabilized with the *FlexiPlate*™ system (Fig. 4). At the same time point, MMP9 and MMP13 mRNA levels were induced as well, while transcripts encoding VEGFA and VEGFC were not significantly elevated when compared to untreated control bones.

Levels of transcripts encoding BMPs 4, 6, and 7 were significantly increased in bones stabilized with the *FlexiPlate*™ System when compared to control bones or osteotomized bones stabilized with the *MouseFix*™ System (Fig. 5). BMP2 transcript levels were elevated as well, though these changes did not reach the level of significance. In parallel with the increased levels of BMP mRNAs, an increase in

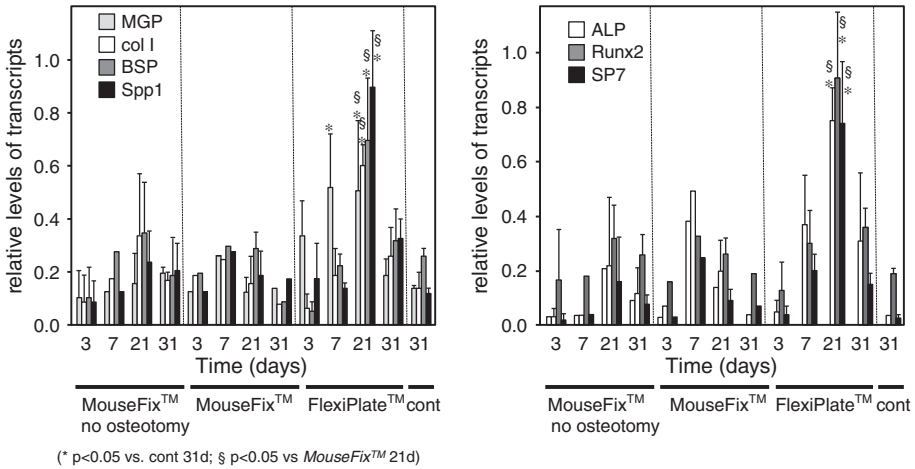

Fig. 2. Levels of transcripts encoding bone-related proteins. To assess the osteogenic response during the repair of the femoral defect, levels of transcripts encoding bone matrix proteins and osteoblastic transcription factors were quantitated. No significant upregulation of this group of transcripts was observed in rigidly fixed defects. In defects stabilized with *FlexiPlate*™, transcript levels were significantly increased 3 weeks after surgery. (* $p<0.05$ vs. cont 31d; § $p<0.05$ vs *MouseFix*™ 21d; $n=3$).

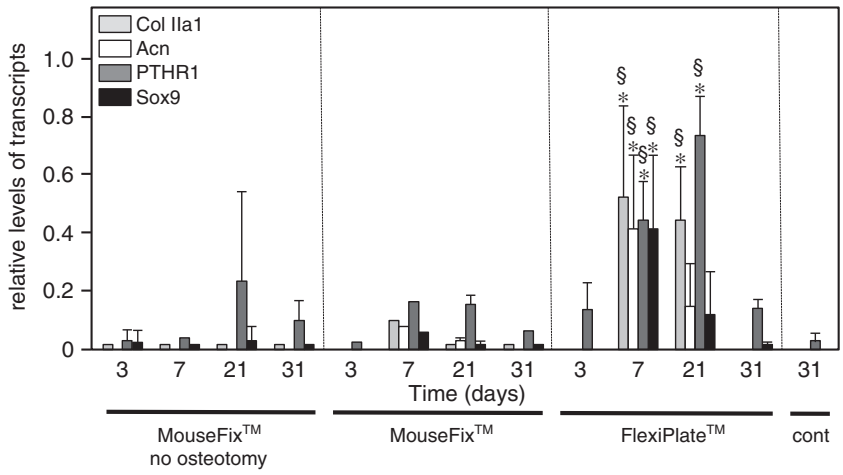

Fig. 3. Levels of transcripts encoding cartilage-related proteins. To assess the chondrogenic response during the repair of the femoral defect, levels of transcripts encoding COL02A1 and ACAN, the transcription factor SOX9 and PTHR1 were quantitated. No significant upregulation of transcripts was observed in rigidly fixed defects. In defects stabilized with *FlexiPlate*™, transcript levels were significantly increased 1 week and 3 weeks after surgery. (* $p<0.05$ vs. cont 31d; § $p<0.05$ vs *MouseFix*™ 21d; $n=3$).

transcripts encoding BMP antagonists, mainly Chordin and BMP3 was detected.

Levels of transcripts encoding the members of the WNT family of growth factors (WNT2b, WNT3a, WNT5a, and WNT7a) were not altered during fracture healing in the two models employed in this study (data not shown). Transcripts encoding the inhibitors of WNT signaling pathways, namely SFRP2, SFRP4, DKK1 and DKK2, however, were significantly increased in *FlexiPlate*™ stabilized bones after 7 days and after 21 days, respectively (Fig. 6).

At day 31 after surgery, most transcripts approached control levels again, even though osteoblast activity still tended to be increased in comparison to 31 days *MouseFix*™ and control bones.

Analysis of levels of transcripts encoding WNT proteins

Since only few members of the WNT family of growth factors were included on the LDA, and since several inhibitors of WNT signaling pathways were upregulated during the healing of non-rigidly fixed osteotomies, levels of transcripts encoding 19 members of this gene family were quantitated (Table 3). While the levels of most transcripts encoding WNT proteins were not changed during the healing process, transcripts encoding WNT5b, WNT9a, and WNT11 were increased in tissues from non-rigidly fixed osteotomies 1 week (WNT9a, WNT11) and 3 weeks (WNT5b, WNT9a, WNT11) after surgery. Transcript levels of DKK2 and SFRP2 were included in this study as positive controls for genes that were upregulated during the healing process of non-rigidly fixed osteotomies (Fig. 6).

Discussion

Within the present study the gene expression in the developing repair tissues of rigidly and non-rigidly fixed small femoral defect was investigated. Emphasis was placed on the kinetics of the expression of genes related to fracture healing. The study took advantage of a newly developed internal fixation system (*MouseFix*™/*FlexiPlate*™; AO Development Institute, Davos, CH), achieving rigid and non-rigid fixation [17]. In accordance with previous results [18], fixation of the osteotomy with the flexible *FlexiPlate*™ System resulted in an altered healing process and an extended time until healing was completed. Despite the small difference in the period required to reach

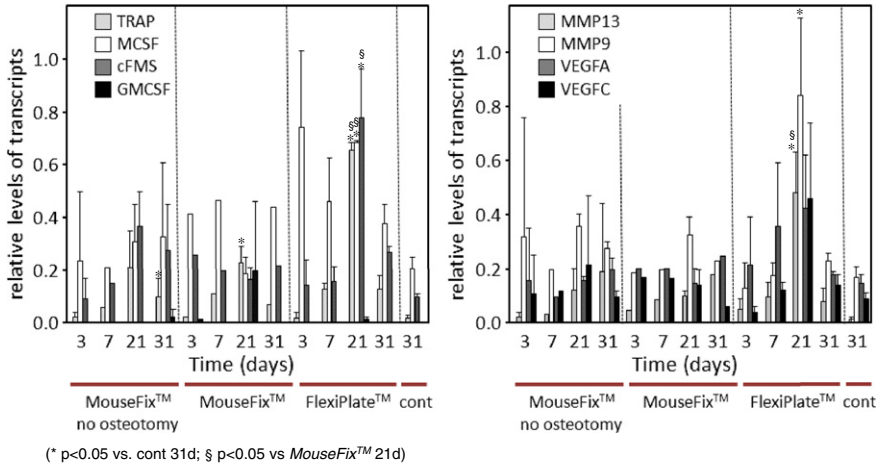

Fig. 4. Levels of transcripts encoding osteoclast/monocyte-related proteins. TRAP and cFMS expression were increased in non-rigidly fixed femoral fractures 3 weeks after surgery. At the same time point, transcripts encoding M-CSF were significantly increased, while mRNA encoding GM-CSF was virtually not detectable. Transcripts encoding the metalloproteinases MMP9 and MMP13 were increased 3 weeks after surgery as well. At no time was a significant upregulation of VEGF observed. (* $p<0.05$ vs. cont 31d; § $p<0.05$ vs *MouseFix*™ 21d; $n=3$).

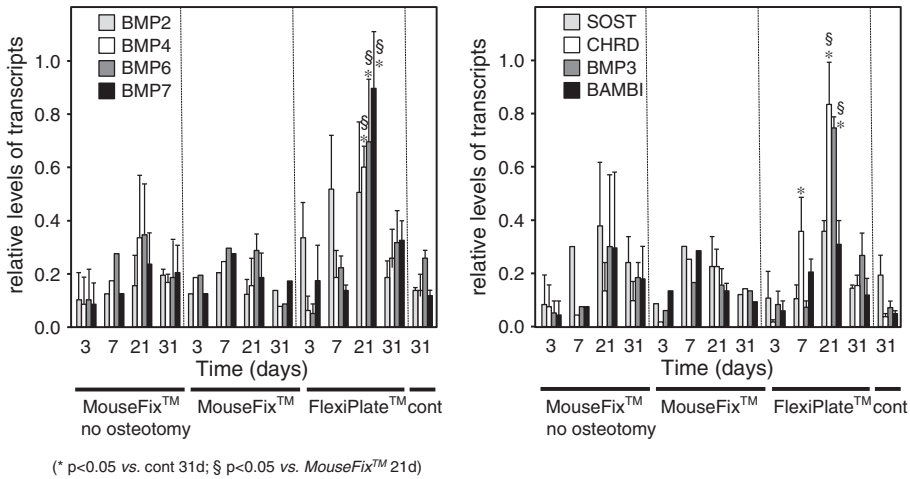

Fig. 5. Levels of transcripts encoding members of the BMP family of growth factors and antagonists. To assess, whether BMP signaling might be affected during fracture repair, transcripts encoding members of the BMP family of growth factors and their antagonists were quantitated. No significant changes in transcript levels were detected at any time point in fractures fixed with the *MouseFix*™ System. Using the *FlexiPlate*™ System for non-rigid fixation of the femoral defect lead to a significant upregulation of both BMP mRNAs as well as of the transcripts encoding BMP antagonists 3 weeks after the surgical treatment of the animals. (* $p<0.05$ vs. cont 31d; § $p<0.05$ vs *MouseFix*™ 21d; $n=3$).

radiologic healing of the fracture, the process of repair, as assessed by MicroCT and histological analyses, varied considerably. Non-rigid fixation of osteotomized femora resulted in the development of a stabilizing callus and endochondral bone formation. After rigid fixation, healing by membranous bone formation, without the development of a callus, was observed. The observed differences in the two models confirm that the stability of fixation exerts profound effects on the biological process of fracture healing in this small animal model, Thus, the *MouseFix*™ and *FlexiPlate*™ Systems are suitable to investigate the aberrant mechanisms leading to alterations in the repair process.

Investigation of the time-dependent expression of genes encoding bone and cartilage extracellular matrix proteins revealed an upregulation in the levels of transcripts encoding COL01A1 in osteotomized bones within 1 week with both fixation systems as compared to controls. Already after 1 week, however, markers for cartilage development (COL02A1and ACAN) were upregulated in bones whose osteotomies were non-rigidly fixed as compared to bones stabilized with *MouseFix*™ Systems. In non-rigidly fixed bones, repair was found to be a two-step process, the expression of bone specific proteins being preceded and paralleled by the expression of cartilage proteins. The expression of Sox9, the transcription factor directing mesenchymal progenitor cells towards chondrogenic differentiation [10], was upregulated in the repair tissues of bones from non-rigid fixations within 7 days after surgery. Similarly, transcripts encoding PTHR1, which has previously been shown to be critical in cartilage development [23], are expressed in osteotomized bones stabilized with *FlexiPlate*™ Systems 7 days after surgery, its expression being further increased after 3 weeks. Upon rigid fixation, however, transcripts encoding PTHR1 were detected at low levels only during the duration of the experiment. Therefore, within 7 days of surgery, the switch towards membranous or endochondral bone formation has been turned.

Furthermore, levels of transcripts encoding inhibitors of the BMP and WNT signaling pathways were found to be increased in osteotomized bones stabilized with the *FlexiPlate*™ System. Both signaling systems were previously suggested to be critically involved in the process of fracture repair [10,24–26], suggesting that it is not necessarily the lack of growth factors that is responsible for the delay in bone repair but rather it is the excess of inhibitory molecules that causes aberrant healing. The

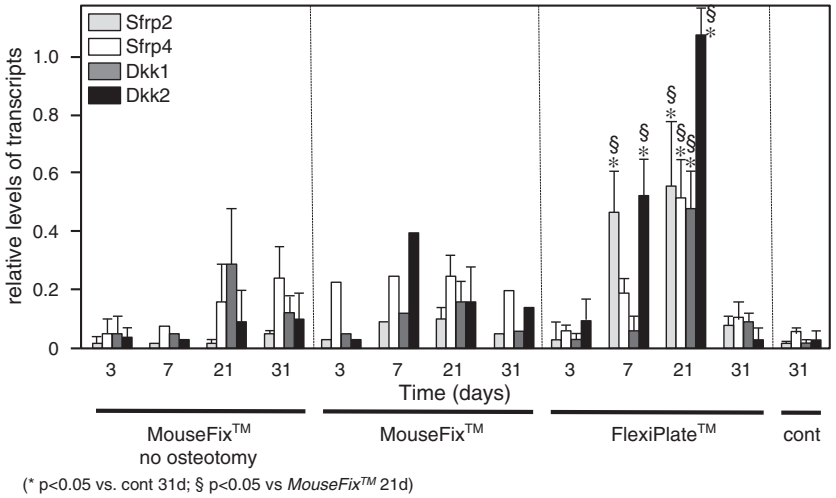

Fig. 6. Levels of transcripts encoding inhibitors of Wnt signaling. To assess whether WNT signaling might be affected during fracture repair, transcripts encoding SFRP5, DKK1 and DKK2 were quantitated. While no significant changes in transcript levels were detected in fractures fixed with the *MouseFix*™ System, the use of the *FlexiPlate*™ System for non-rigid fixation of the femoral defect leads to a significant upregulation of these mRNAs within 3 weeks after surgery. (* $p<0.05$ vs. cont 31d; § $p<0.05$ vs *MouseFix*™ 21d; $n=3$).

Table 3
Relative expression of transcripts encoding the members of the WNT family of growth factors.

WNT family member	AoD number	MouseFix (no osteotomy)			MouseFix			Flexiplate		
		1w	3w	4.5w	1w	3w	4.5w	1w	3w	4.5w
WNT1	Mm01300555g1	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT2	Mm00470018_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT2b	Mm00437330_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT3	Mm00437336_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT3a	Mm00437337_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT4	Mm01194003_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT5a	Mm00437347_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT5b	Mm01183986_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	↑↑	n.s.
WNT6	Mm00437353_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT7a	Mm00437355_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT7b	Mm01301717_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT8a	Mm00436822_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT8b	Mm00442107_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT9a	Mm00460518_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	↑↑	↑↑	n.s.
WNT9b	Mm00457102_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT10a	Mm00437325_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT10b	Mm00442104_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
WNT11	Mm00437328_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	↑↑	↑↑	n.s.
WNT16	Mm00446420_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	n.s.	n.s.	n.s.
DKK2	Mm00445025_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	↑↑	↑↑	n.s.
SFRP2	Mm00485986_ml	n.s.	n.s.	n.s.	n.s.	1	n.s.	↑↑	↑↑	n.s.

Relative expression of transcripts encoding the members of the WNT family of growth factors. From the 19 WNT transcripts quantified, those encoding WNT5b, WNT9a, and WNT11 were significantly increased in non-rigidly fixed osteotomies when compared to rigidly fixed defects after 3 weeks. DKK2 and SFRP2 were included as controls for genes, whose transcription was dependent on the defects' fixation (n.s.: no significant changes compared to *FlexiPlateTM* 21 days; significant increase in transcript levels compared to *FlexiPlateTM* 21 days ($p<0.05$); included on LDA).

critical roles of BMP antagonists in the response of bone in pathophysiology has been shown before, when it was demonstrated that osteolytic or osteoblastic bone metastases from prostate or mammary cancers do not induce the respective bone response by the secretion of growth factors for osteoblast and osteoclast lineage cells, but rather by the secretion of the BMP antagonist Noggin [27,28]. Lack of Noggin expression by the tumor cells induced an osteoblastic response, while expression of Noggin subdued the osteoblastic response, allowing resorption to take over.

Previously, an increase in the expression of BMPs and BMP antagonists during fracture repair and distraction osteogenesis [29,30] has been reported, suggesting that blocking inhibitory proteins might be a possible strategy to increase the endogenous BMPs' bioactivity. More recently, a proof of principle study was provided by our group, demonstrating that inhibition of BMP antagonists with a molecularly engineered BMP2 variant indeed increased the efficacy of external and endogenous BMP2 in rat femoral defect healing [31]. *In vitro*, the ability of L51P to suppress the inhibition of osteoblastic differentiation mediated by Noggin was demonstrated [32]. Thus, the induction of BMP antagonists, which is caused as a reaction to locally increased concentrations of BMPs [33], may contribute to the lack of osteogenic and an excess of chondrogenic differentiation.

Similarly to the BMP signaling cascade, the WNT system is a key regulatory system in skeletal development [34,35]. Ever since the finding that loss of function and gain of function mutations in LRP5 would either lead to a decrease [36] or an increase [37,38] in bone mass due to its interaction with DKK1, WNT signaling and its role in regulating bone mass have attracted interest, in particular also in the search for new therapeutic targets for the treatment of osteoporosis [39]. Experimentally, inhibition of the WNT signaling cascade, either by targeted overexpression of SFRP4 in osteoblasts [40] or by forced adenoviral expression of DKK1 at bone injury sites [41], lead to osteopenia and a delay in bone repair. Thus the observed upregulation of transcripts encoding SFRP2, SFRP 4, DKK1 and DKK2 in non-stably fixed defect sites suggests an inhibition of the chondro- andosteogenic WNT signaling pathways, which may subsequently cause a delay in fracture healing. The upregulation of the expression of inhibitors of WNT signaling may also blunt the observed upregulations of some members of the WNT family of growth factors, WNT5b, WNT9a, WNT11, all of which were previously suggested to play a role in chondrogenesis [42].

While WNT and BMP signaling pathways have been discussed separately, it must not be overlooked that during development and in the maintenance of skeletal homeostasis there is a close interaction among numerous signaling pathways such as WNT, BMP, Hedgehog and Notch [43,44]. Inhibition of one or the other will exert effects on the remaining pathways, which will lead to unexpected and wide-reaching effects on developmental processes.

Further, a group of genes involved in regulating monocyte/macrophage lineage cells, and their respective growth factors, was investigated. Three weeks after surgery, the levels of transcripts encoding TRAP, cFMS, MMP9 and MMP13 were increased in the repair tissues from non-rigidly stabilized defects. The induction of the local expression of TRAP and cFMS suggests recruitment and development of osteoclast lineage cells, necessary for the removal of the mineralized cartilage of the callus, to the repair site. Also in accordance with endochondral bone formation, expression of the metalloproteinases MMP9 and MMP13 was induced at this stage, these proteases having previously been shown to be necessary for vascularization and osteoclast invasion of bone anlagen during development [45].

In conclusion, we have shown that non-rigid fixation of femoral defects causes an alteration of the process of bone healing as compared to rigidly fixed defects. At the molecular level, the changes in the biological processes are characterized by increases in the levels of transcripts encoding inhibitors of the BMP and WNT families of growth factors, two of the major osteogenic signaling pathways. Based on the present data it is suggested that not the lack of growth factors, but the excess of antagonistic proteins, are the cause for the different paths of fracture healing in rigidly vs. non-rigidly fixed osteotomies.

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