Human scaphoid non-unions exhibit increased osteoclast activity compared to adjacent cancellous bone

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Received: April 23, 2015; Accepted: August 3, 2015

Abstract

Scaphoid bones have a high prevalence for non-union. Even with adequate treatment, bone regeneration may not occur in certain instances. Although this condition is well described, the molecular pathology of scaphoid non-unions is still poorly defined. In this study, gene expression of osteogenic and angiogenic growth and transcription factors as well as inflammatory mediators were analysed in human scaphoid non-unions and intraindividually compared to adjacent autologous cancellous bone from the distal radius. In addition, histology and immunohistochemical stainings were performed to verify qRT-PCR data. Gene expression analysis revealed a significant up-regulation of RANKL, ALP, CYCLIN D1, MMP-13, OPG, NFATc1, TGF-β and WNT5A in scaphoid non-unions. Interestingly, RANKL and NFATc1, both markers for osteoclastogenesis, were significantly induced in non-unions. Moreover, WNT5A was highly up-regulated in all non-union samples. TRAP staining confirmed the observation of induced osteoclastogenesis in non-unions. With respect to genes related to osteogenesis, alkaline phosphatase was significantly up-regulated in scaphoid non-unions. No differences were detectable for other osteogenic genes such as RUNX-2 or BMP-2. Importantly, we did not detect differences in angiogenesis between scaphoid non-unions and controls in both gene expression and immunohistochemistry. Summarized, our data indicate increased osteoclast activity in scaphoid non-unions possibly as a result of the alterations in RANKL, TGF-β and WNT5A expression levels. These data increase our understanding for the reduced bone regeneration capacity present in scaphoid non-unions and may translate into the identification of new therapeutic targets to avoid secondary damages and prevent occurrence of non-unions to scaphoid bones.

Keywords: non-union ● osteoclasts ● scaphoid ● RANKL ● WNT5A

Introduction

Bone fracture healing is typically completed 6–8 weeks after the initial injury without scar formation. Certain circumstances could result in delayed fracture healing or non-unions which lead to pain and arthritis. Scaphoid bones have by far the highest incidence of fractures among all carpal bones and show a 90–95% union rate. However, those fractures with dislocations greater than 1 mm are associated with a 55% incidence of non-union [1]. In general, non-unions may result from the instability of the fracture, disrupted vascularity, loss of bone and cyst formation. However, factors and molecular mechanisms that lead to failure of bone regeneration are not well defined [2]. Blood supply in scaphoids depends on distal branches of the radial artery, which could result in interrupted blood supply of the proximal scaphoid pole and avascular necrosis after fracture. Non-unions of the scaphoid are predominantly atrophic, which are historically defined by hypovascularization and little callus formation around a non-mineralized fibrous tissue-filled fracture gap [3]. Treatment of atrophic non-unions is difficult and often includes three steps: resection of scar tissue, grafting of autologous bone and internal fixation for mechanical stability.

Bone is a dynamic organ with tightly regulated continuous bone remodelling. Differentiation of bone resorbing osteoclasts (OCs) which share several regulatory molecules with immune cells is mainly regulated by tumour necrosis factor (TNF) superfamily member

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doi: 10.1111/jcmm.12677
Thus, the molecular pathology of non-unions in general is still poorly defined. Our extensive analysis focuses on the late events of scaphoid non-unions including osteogenesis and osteoclastogenesis, angiogenesis as well as immune response-related genes compared to cancellous bone from the radius controls in a large cohort, excluding interindividual differences. Our results indicate chronic OC activation in non-unions, potentially as a result of the altered regulation of WNT5A and TGF-β expression which may inhibit bone regeneration, whereas angiogenesis seems to be unaltered in non-unions.

Materials and methods

Human specimens

Tissue harvest and experiments were performed in accordance with the ethical committees, and informed consent was obtained from the patients. Patients with scaphoid non-unions defined as non-united fractures >3 months with a resorption zone wider than 1 mm (as determined by a mandatory CT-scan) with no apparent potential to heal without surgical intervention were selected to participate in the study. In total, 80 patients from two regional hand trauma centres were recruited and the tissue was processed for RNA and/or histology and intraindividually compared. Non-union tissue (excluding the cortex) and cancellous bone from the ipsilateral radius has been obtained at the time of operative repair. Patients with previous surgeries on the same scaphoid or conservative treatments were excluded from the study. Seventy-seven patients were male, three were female. The average age of the patients was 24.6 years (range between 18 and 71 years). The average time that elapsed between fracture and operation was 18.3 months (range 3–100 months).

Tissue processing

After removal, tissue was immediately washed in ice-cold PBS to avoid contaminations from blood cells and either frozen at −80°C until RNA preparation or directly processed for histology.

RNA preparation and cDNA synthesis

Homogenization of the tissue was achieved with Polytron® homogenizer (Kinematica, Eschbach, Germany) in 1 ml TRIzol reagent (Life Technologies, Darmstadt, Germany) on ice. Subsequently, homogenates were incubated at room temperature for 5 min. and 200 μl chloroform (Merck, Darmstadt, Germany) was added and mixed for 5 sec. Samples were centrifuged at 15,300 g for 15 min. at 4°C. The aqueous phase was proceeded for RNA isolation and 1 μl glycerol (Roche, Mannheim, Germany) was added as a carrier. About 250 μl of 100% isopropanol was added and incubated at −80°C overnight. After centrifugation at 12,000 r.p.m. for 30 min. at 4°C, samples were incubated and supernatants were removed. Pellets were washed with 1 ml 75% ethanol, centrifuged at 12,000 r.p.m. for 5 min. at 4°C and air-dried for 20 min. RNA was resuspended in 100 μl RNase-free water and incubated at
60°C for 10 min. Subsequently, RNA clean up was performed with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions including DNase digestion (RNase free DNase Kit; Qiagen) to avoid genomic DNA contaminations. To limit heterogeneity in the patient population, only young male patients (between 18 and 33 years old) were included. Moreover, only those patients that had high quality RNA (260/280 >1.8, 17 in total) in both tissue samples were included for qRT-PCR analysis. Synthesis of cDNA was performed by means of the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Life Technologies) using 200 ng total RNA per reaction.

Quantitative real-time PCR

Quantitative determination of relative gene expression was performed on Applied Biosystems 7900HT Fast Real-Time PCR System (384 well plates) using TaqMan™ gene expression assays (genes and assay IDs are listed in Table 1) and TaqMan Universal master mix (Applied Biosystems, Darmstadt, Germany). For each reaction, 2 ng cDNA were used. Data were analysed according to the manufacturer’s ΔΔCt method (Applied Biosystems). 18S was used as a reference gene. Each non-union sample was related to the corresponding cancellous bone sample control.

Table 1 List of genes and TaqMan™ gene expression assay IDs examined in the study

| Gene | Assay ID       | Assay ID       | Assay ID       |
|------|----------------|----------------|----------------|
| 18S  | Hs99999901_s1  | MMP9           | Hs00234579_m1  |
| ALP  | Hs01029144_m1  | NFATc1         | Hs00542678_m1  |
| ATF4 | Hs00909569_g1  | NFkB1          | Hs00765730_m1  |
| BGLAP/OCN | Hs01587814_g1 | Noggin         | Hs00271352_s1  |
| BMP2 | Hs00154192_m1  | Osterix/SP7    | Hs01866874_s1  |
| BMP7 | Hs00233476_m1  | PECAM1         | Hs00169777_m1  |
| CCND1| Hs00765553_m1  | RUNX2          | Hs00231692_m1  |
| CSF1 | Hs00174164_m1  | SPP1/OPN       | Hs00959010_m1  |
| DKK1 | Hs00183740_m1  | TGFB1          | Hs0098133_m1   |
| FGFR | Hs00826677_m1  | TNF-α          | Hs01113624_g1  |
| FGF2 | Hs00266645_m1  | TNFRSF11A/RANK | Hs00921372_m1  |
| FGF9 | Hs00181829_m1  | TNFRSF11B/RANKL| Hs00900358_m1  |
| IFNG | Hs00989291_m1  | TNFSF11/ RANKL | Hs00243522_m1  |
| IL1B | Hs01555410_m1  | Wnt3a          | Hs00263977_m1  |
| MMP13| Hs00233992_m1  | Wnt5a          | Hs0098537_m1   |

Histology and immunohistochemical staining

For histological analyses, tissue was shortly washed with cold PBS to get rid of blood cells, fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C and decalcified in 19% ethylenediaminetetraacetic acid (Applichem, Darmstadt, Germany) for 7 days. Thirty-four patients were analysed. Afterwards, samples were dehydrated and embedded in paraffin. Bone tissue was cut into serial sections (thickness 9 μm). Pentachrome staining was performed as previously described [27]. Tartrate-resistant acid phosphatase (TRAP) staining was performed with a leucocyte acid phosphatase kit (Sigma-Aldrich). Immunohistochemistry for PECA1-1 (#IS610; Dako, Hamburg, Germany) to evaluate blood vessels within the tissue was performed with heat antigen retrieval in citrate buffer (pH 6.0) as previously described [28]. Immunohistochemistry for alkaline phosphatase (ALP; #sc166261; Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:50) was performed after antigen retrieval with 0.1% proteinase K, followed by incubation with anti-mouse secondary antibody and detection with Vector ABC kit and Nova Red. Immunofluorescence for phosphorylated SMAD2/3 (#8828; Cell Signaling, Frankfurt a. M., Germany, dilution 1:200) was performed overnight at 4°C after antigen retrieval with proteinase K followed by incubation with anti-rabbit Alexa Fluor 594 secondary antibody (Life Technologies) for 2 hrs at RT and DAPI counterstaining. Immunohistochemistry was performed on samples from at least 13 patients.

Statistics

Results of qRT-PCR experiments are presented as mean ± S.E.M. P values were calculated with Wilcoxon signed rank test and statistical significances were set at a P < 0.05.

Results

Regulation of osteogenesis-related genes

Scaphoid non-unions are a common problem encountered in clinical practice [29]; however, the underlying molecular mechanisms are still poorly defined. To gain insight into the gene expression profiles of bone remodelling and immune response-related genes of scaphoid non-unions in comparison to adjacent healthy cancellous bone we performed qRT-PCR analyses. Osteogenesis and osteoclastogenesis regulating genes as well as pro- and anti-inflammatory markers were included. In scaphoid non-unions, RUNX-2 which is a key transcription factor regulating osteoblastic differentiation, showed similar expression levels compared to control cancellous bone (Fig. 1A). The zinc-finger containing transcription factor osterix known to act down-stream of RUNX-2 which is essential for bone development [30], was hardly detectable in both tissues (data not shown). Interestingly, OB differentiation marker ALP was significantly up-regulated across all non-unions (Fig. 1B). In contrast, late OB differentiation markers osteopontin (OPN) and osteocalcin (OCN) showed similar expression patterns in both tissues (Fig. 1C and D). Expression of BMP-2 in non-
unions was not differentially regulated as compared to cancellous bone (Fig. 1E). Interestingly, the BMP antagonist noggin was moderate, but down-regulated across all analysed non-unions except for two patients resulting in overall significant different gene expression (Fig. 1F). In contrast, BMP-7 as well as pro-osteogenic fibroblast growth factors FGF-9 and FGF-18 [31, 32] were neither detectable in non-unions nor in control cancellous bone (data not shown). FGF-2, essential for OB proliferation and function [33] was not differentially expressed (Fig. 1G). Cyclin D1 required for cell cycle progression [34] was found to be significantly up-regulated in non-unions (Fig. 1H). WNT3A expression was not detectable in both tissues (data not shown). Interestingly, WNT5A, which could interact with canonical Wnt3a pathway, was up-regulated in non-unions (mean: 6.7-fold) (Fig. 1I). Moreover, expression of the matrix metalloproteinases MMP-9 and MMP-13, genes related to both angiogenesis and bone remodelling, were investigated. Both, MMP-9 (Fig. 1J) as well as MMP-13 (Fig. 1K) expression were found to be significantly up-regulated in non-unions.

**Osteoclastogenesis- and immune response-related genes**

Osteoclastogenesis is primarily activated by RANKL which regulates OC differentiation processes by induction of transcription factor NFATc1 and M-CSF known to promote proliferation of monocyctic precursor cells. We were interested whether these key molecules of osteoclastogenesis are differentially regulated in non-unions in comparison to cancellous bone. Quantitative RT-PCR analysis revealed that RANKL was significantly up-regulated in scaphoid non-unions (mean: 20-fold; Fig. 2A). Importantly, RANKL expression was signifi-

![Fig. 1](image1.png)  
**Fig. 1** Regulation of osteogenesis-related genes in human scaphoid non-unions compared to control adjacent cancellous bone. Quantitative RT-PCR determination of osteogenesis-related genes revealed that the majority of genes were significantly up-regulated (ALP (B); cyclin D1 (H); WNT5A (I); MMP-9 (J); MMP-13 (K)) compared to controls. Expression levels of RUNX-2 (A), OPN (C), OCN (D), BMP-2 (E) and FGF-2 (G) were similar in both tissues. The BMP inhibitor noggin was significantly reduced in non-unions (F). Gene expression determinations were performed relative to 18S expression and data are presented as mean ± S.E.M.; Wilcoxon signed rank test (*P < 0.05, **P < 0.01, ***P < 0.001).

![Fig. 2](image2.png)  
**Fig. 2** Osteoclastogenesis- and immune response-related genes expressed in human scaphoid non-unions in comparison to adjacent cancellous bone. Quantitative RT-PCR determination of RANKL (A), NFATc1 (D) and TGF-β (G) revealed significantly increased expression in scaphoid non-unions, which are known to have impact on osteoclastogenesis and immune response. Gene expression of RANK (B), M-CSF (C) and ATF-4 (E) were only slightly increased in non-unions. Expression analysis of OPG (F) revealed enhanced expression of the RANKL antagonist. Gene expression determinations were performed relative to 18S expression and data are presented as mean ± S.E.M.; Wilcoxon signed rank test (*P < 0.05, **P < 0.01, ***P < 0.001).
cantly up-regulated in all samples regardless of the time elapsed between trauma and surgery. The RANKL receptor RANK was slightly but not significantly up-regulated in non-unions compared to controls (Fig. 2B). M-CSF was found to be moderately, but not significantly induced in non-unions (Fig. 2C) which is because of the high variance of the patient’s gene expression. NFATc1, a downstream effector, was up-regulated in non-unions (Fig. 2D). Recently, ATF4 was identified as an upstream activator of NFATc1. Moreover, ATF4 is critical for RANKL activation and a crucial factor for M-CSF induction of RANK expression [35]. However, in non-unions the expression level of an upstream activator of RANKL was unaltered (Fig. 2E). Interestingly, expression of the soluble decoy receptor RANKL OPG was likewise significantly up-regulated (Fig. 2F).

Transforming growth factor β was shown to maintain and enhance the OC-forming potential of OC precursors [14]. In scaphoid non-unions, TGF-β1 was significantly up-regulated compared to cancellous bone (Fig. 2G). NFKB1, which can be induced by TNF-α, was neither detected in non-union nor in cancellous bone (data not shown) which could be because of low TNF-α expression levels which were similar in both tissues (Fig. 2H). Moreover, other pro-inflammatory cytokines such as interleukin-1 (IL-1) and interferon-γ (IFN-γ) were not detected in both tissues.

All results revealed by means of quantitative RT-PCR analysis are summarized in Table 2.

Table 2 Summary of results. Gene expression up- and down-regulation in scaphoid non-unions compared to control is indicated

| ALP   | ↑*** | NFATc1 | ↑*** |
|-------|------|--------|------|
| ATF4  | n.s. | NFKB1  | n.d. |
| BGLAP/OCN | n.s. | Noggin | ↓*  |
| BMP2  | n.s. | Osterix/SP7 | n.d. |
| BMP7  | n.d. | PECAM1 | n.s. |
| CCND1 | ↑** | RUNX2  | n.s. |
| CSF1  | n.s. | SPP1/OPN | n.s. |
| DKK1  | n.s. | TGFb1  | ↑*  |
| FGF18 | n.d. | TNF-α  | n.s. |
| FGF2  | n.s. | TNFRSF11A/RANK | n.s. |
| FGF9  | n.d. | TNFRSF11B/OPG | ↑*** |
| IFNG  | n.d. | TNFSF11/RANKL | ↑*** |
| IL1B  | n.d. | Wnt3a  | n.d. |
| MMP13 | ↑*  | Wnt5a  | ↑*** |
| MMP9  | ↑*  |        |      |

*P < 0.05, **P < 0.01, ***P < 0.001. n.s.: not significant; n.d.: not detectable.

Altered architecture, bone remodelling and TGF-β signalling of scaphoid non-unions

Pentachrome staining revealed marked differences between scaphoid non-unions and healthy cancellous bone tissue. Non-unions exhibited a heterogeneous mix of different tissues, with a domination of connective tissue, whereas osteoid was the dominant tissue in cancellous bone (Fig. 3A). Gene expression data were supplemented with histological analysis of TRAP-positive OCs. Comparison of scaphoid non-unions with control tissue revealed high levels of TRAP staining in non-unions, indicating increased activity of OCs and confirming gene expression data (Fig. 3B). Of note, OCs were mainly localized in the areas of connective tissue. Moreover, immunohistochemical staining of ALP showed increased activity in scaphoid non-unions (Fig. 4A) mirroring results obtained in the qRT-PCR analysis (Fig. 1B) and emphasizing the remaining osteogenic potential of scaphoid non-unions. Immunohistochemical staining of pSMAD 2/3, a downstream effector of TGF-β1 revealed highly increased levels in scaphoid non-unions as compared to control tissue, which further highlights the potential role of TGF-β1 signalling in scaphoid non-unions (Fig. 4B).

Angiogenesis is unaltered in atrophic scaphoid non-unions

As angiogenesis is important for bone development and repair, we compared gene expression of PECAM-1 in non-unions and cancellous bone revealing equal expression levels (Fig. 5A). Concordantly, immunohistochemical staining of PECAM-1 did not reveal differences between non-unions and control tissue (Fig. 5B).

Discussion

Regular fracture healing has been extensively studied but causes for non-union formation still remain to be elucidated. Here, we performed histological and gene expression analysis for osteogenesis, osteoclastogenesis and immune-related genes in human atrophic scaphoid non-unions compared to adjacent cancellous bone. High expression levels of TGF-β, RANKL and NFATc1 as well as increased TRAP-positive OCs indicate that although trauma may have occurred more than a year before, osteoclastogenesis is constantly induced in non-unions. Furthermore, our results revealed that non-unions still have at least a partial regenerative capacity, which seems to be inhibited by increased OC activity.

The structure of non-unions and cancellous bone is markedly different revealed by pentachrome stainings indicating a dense connective tissue in non-unions which mainly consists of fibroblasts which is in strong agreement with previous studies [24]. As MSC differentiate along osteoblastic and chondrocytic as well as fibroblastic lineages, we speculate that immediately after fracture, MSC differentiation is mainly directed towards the fibroblasts lineage. Ini-
tial inflammation after fracture leads to the invasion of macro-
phages and platelets thereby releasing TGF-β. Transforming growth
factor β was described to enhance fibroblast migration and prolif-
eration in different contexts (reviewed in [36]) acquiring an activated
phenotype [37]. Furthermore, enhanced expression of TGF-β may
lead to sustained fibroblast differentiation and dense persisting
fibrous tissue in the fracture gap in an autocrine manner [38, 39].
In addition, enhanced TGF-β expression suggests increased OC
survival and differentiation [14]. On the contrary, TGF-β co-ordi-
nates bone formation by inducing migration of MSC [40] indicating
that levels of TGF-β have to be precisely regulated during bone
regeneration. Interestingly, a sheep femoral non-union model trea-
ted with bone allografting indicated that increased numbers of OCs
as well as fibroblasts and connective tissue were associated with
failure of bone regeneration [36, 41]. Our study lets us suggest,
that failure of bone regeneration in general is accompanied by
connective tissue formation and fibroblast invasion as well as
increased OC differentiation as a result of altered TGF-β and
increased phosphorylated SMAD expression.

A key role in the failure of bone regeneration could be increased
expression of RANKL, its receptor RANK and NFATc, accompanied
by OC invasion as indicated by TRAP staining, which could manifest
an imbalance of bone formation and bone resorption. Moreover, com-
parable to a previous study showing elevated OPG serum levels in
patients with long bone atrophic non-union fractures [26], OPG
expression was highly up-regulated in scaphoid non-unions poten-
tially indicating an intact negative feedback loop in response to
increased RANKL activity. Furthermore, MMP9 and MMP13 which are
important for vascularization, turnover of mineralized cartilage [42]
as well as degradation of extracellular matrix in inflammatory responses
showed elevated expression levels in scaphoid non-unions indicating
that bone remodelling could occur, but MMPs may also lead to imbal-
ance towards bone resorption.

Fig. 3 Architecture of scaphoid non-unions and osteoclast activity. Pentachrome
staining revealed a heterogeneous mix of
different tissues, with a domination of
connective tissue and fibroblasts in non-
unions, while osteoid was the dominant
tissue in cancellous bone (A). Representa-
tive TRAP staining of control cancellous
bone and scaphoid non-unions (B) revealed enhanced osteoclasts activity in
non-unions. Scale bars: 200 μm (A) and
50 μm (B).

Fig. 4 ALP and pSMAD2/3 activity. (A) Immunohistochemistry for ALP
revealed higher levels in scaphoid non-unions as opposed to cancellous
bone. Likewise, immunofluorescence for phosphorylated SMAD2/3
revealed increased activity in scaphoid non-unions. Scale bars (A and
B): 50 μm.

Fig. 5 Angiogenesis in scaphoid non-unions is similar to cancellous
bone. (A) Quantification of gene expression levels of PECAM-1 by qRT-
PCR revealed similar expression levels in non-unions and controls.
Blood vessels and endothelial cells were detected by immunohistochem-
ical staining of PECAM-1 in non-unions and controls (B) revealing simi-
lar levels of angiogenesis in both tissues; scale bar: 50 μm.

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Interestingly, our experiments revealed that WNT5a expression is up-regulated in scaphoid non-unions compared to adjacent cancellous bone. In rodents, it was demonstrated that during normal fracture healing Wnt5a is up-regulated at early stages and down-regulated to basal levels at later stages of bone healing as compared to non-injured contralateral tissue [43]. Our study revealed that even in long-term scaphoid non-unions WNT5A gene expression is highly up-regulated. As WNT5A has been shown to indirectly induce RANK expression in OCs thereby enhancing RANKL-induced osteoclastogenesis, it has been proposed that WNT5A is a new co-stimulatory cytokine for osteoclastogenesis [22] indicating that increased RANK gene expression in scaphoid non-unions could also result from increased WNT5a expression. On the other hand, WNT5a is up-regulated during osteoblastic differentiation of MSC thereby regulating expression of RUNX-2, osterix and ALP [44]. In that respect, up-regulation of ALP in scaphoid non-unions may be a consequence of increased WNT5A activation, suggesting a certain amount of differentiation capacity of OB progenitor cells. However, as RUNX-2 was not differentially regulated, we speculate that WNT5A rather induces osteoclastogenesis than OB differentiation. In another context, WNT5A was shown to stimulate fibroblasts [45] and could be induced by TGF-β [46, 47] which led us to speculate that WNT5A expression is at least partially induced by enhanced TGF-β1 expression which could lead to fibroblast proliferation and activation. Thus, to this date the exact role of up-regulated WNT5A in established human scaphoid non-unions is unclear, however, in the light of our results, we speculate that activation of RANKL and induction of WNT5A expression seems to be one major route of action.

In contrast to WNT5A, we neither detected WNT3A in scaphoid non-unions nor in cancellous bone demonstrating that WNT3A plays a minor role in established scaphoid non-unions which does not exclude a role at the early beginning of non-union development. In addition, DKK1, a Wnt antagonist known to inhibit fracture healing [48] is not differentially regulated in late non-unions, indicating that bone regeneration is not inhibited by DKK1. Interestingly, low levels of β-catenin as a downstream effector of Wnt3a lead to enhanced OC differentiation and cause osteoporosis [49]. Thus, the absence of WNT3A could further enhance osteoclastogenesis in scaphoid non-unions.

We further investigated inflammation-related which had low expression levels or were neither detectable in non-unions nor cancellous bone demonstrating that WNT3A plays a minor role in established scaphoid non-unions which does not exclude a role at the early beginning of non-union development. In addition, DKK1, a Wnt antagonist known to inhibit fracture healing [48] is not differentially regulated in late non-unions, indicating that bone regeneration is not inhibited by DKK1. Interestingly, low levels of β-catenin as a downstream effector of Wnt3a lead to enhanced OC differentiation and cause osteoporosis [49]. Thus, the absence of WNT3A could further enhance osteoclastogenesis in scaphoid non-unions.

In contrast to osteoclastogenesis-related genes, osteogenesis-related genes were moderately but not significantly up-regulated. Although other studies compared BMP-2 expression levels to regular fracture healing showing down-regulation of BMP-2 [53, 54], comparison of BMP-2 expression to healthy cancellous bone revealed no significant difference. Hence, detection of BMPs in non-unions depends on timing of the analysis, location, type of the defect [24] as well as on type of control tissue. Noggin directly binds BMPs which prevents interaction with their receptors resulting in inhibition of BMP signalling [55]. Noggin expression was significantly down-regulated in non-unions as PECAM-1 gene expression as well as blood vessel numbers were similar compared to control tissue which is in agreement with some previous experimental and non-comparative data [50–52]. For instance, in a rat model of atrophic non-union, blood vessel formation was found to be delayed but reaches the same level at later time-points [51].

In contrast to osteoclastogenesis-related genes, osteogenesis-related genes were moderately but not significantly up-regulated. Although other studies compared BMP-2 expression levels to regular fracture healing showing down-regulation of BMP-2 [53, 54], comparison of BMP-2 expression to healthy cancellous bone revealed no significant difference. Hence, detection of BMPs in non-unions depends on timing of the analysis, location, type of the defect [24] as well as on type of control tissue. Noggin directly binds BMPs which prevents interaction with their receptors resulting in inhibition of BMP signalling [55]. Noggin expression was significantly down-regulated in

![Image](2848.png)
scaphoid non-unions compared to the healthy bone which suggests that noggin does not inhibit BMP signalling. Of note, Qu and von Schroeder demonstrated that the addition of recombinant BMPs increases the osteogenic potential of human scaphoid non-union cells in comparison to pelvic bone cultures [56] indicating at least a partial osteogenic differentiation potential of non-unions. Our data indeed indicate remaining osteogenic potential in scaphoid non-unions, which could possibly be exploited, in case the altered balance is readjusted. As studies with human materials are restricted to end-point analyses, conclusion related to the dynamics of scaphoid non-union formation are limited. For obvious ethical reasons, the ideal control, contralateral scaphoid cancellous bone, cannot be utilized. However, comparison of non-union tissue with adjacent cancellous bone excluded interindividual differences. The results revealed by the presented study are summarized in Figure 6.

Conclusions

In this study, we revealed an imbalance between bone formation and resorption in scaphoid non-unions. Non-unions show abnormally high amounts of connective tissue which could result from altered TGF-β signalling. In an autocrine manner, TGF-β could further increase fibroblast proliferation and activation. In consequence, fibroblasts express high amounts of RANKL which stimulates osteoclastogenesis. Furthermore, non-unions showed increased WNT5A expression levels which may also result from altered TGF-β expression. In addition to TGF-β blockage and thereby preventing activation of WNT5A, alterations in RANKL and WNT5A expression might also offer therapy approaches. Furthermore, fibroblast proliferation and dense fibrous tissue may be modified. Our data reveal a detailed picture of the status quo of human scaphoid non-unions and may further accelerate efforts in the field to further understand, prevent and treat this potentially serious musculoskeletal disease.

Acknowledgements

This work was supported by a grant of the German Social Accident Insurance (DGUV) FR 201 to B.B. The funding organization does not take the responsibility for the content of this publication. The authors would like to thank the surgical teams for acquisition of the tissue samples in both centres.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

Author contribution

J.S. analysed quantitative RT-PCR data including statistics, prepared figures and wrote the manuscript; M.S. contributed to tissue analysis, to tissue collection and preparation; C.D. performed stainings; C.W. prepared figures and contributed to the manuscript; S.A. performed stainings and qRT-PCR; A.D, U.K and M.L. provided tissue and supervised the study; B.B. designed the research study, analysed stainings and wrote the manuscript.

References

1. Szabo RM, Manske D. Displaced fractures of the scaphoid. Clin Orthop Relat Res. 1988; 230: 30–8.
2. Panteli M, Pountos I, Jones E, et al. Biological and molecular profile of fracture non-union tissue: current insights. J Cell Mol Med. 2015; 19: 685–713.
3. Megas P. Classification of non-union. Injury. 2005; 36: S30–7.
4. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell. 1998: 93: 165–76.
5. Nakagawa N, Kinosaki M, Yamaguchi K, et al. RANK is the essential signalling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun. 1998; 253: 395–400.
6. Burgess TL, Qian Y, Kaufman S, et al. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. J Cell Biol. 1999; 145: S27–38.
7. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997; 89: 309–19.
8. Yarilina A, Xu K, Chen J, et al. TNF activates calcium-nuclear factor of activated T cells (NFAT)c1 signaling pathways in human macrophages. Proc Natl Acad Sci USA. 2011; 108: 1573–8.
9. Felix R, Cecchini MG, Fleisch H. Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. Endocrinology. 1990; 127: 2592–4.
10. Arai F, Miyamoto T, Ohneda O, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med. 1999; 190: 1741–54.
11. Li P, Schwarz EM, O’Keefe RJ, et al. RANK signaling is not required for TNFAlpha-mediated increase in CD11(hi) osteoclast precursors but is essential for mature osteoclast formation in TNFAlpha-mediated inflammatory arthritis. J Bone Miner Res. 2004; 19: 207–13.
12. Gerstenfeld LC, Cho TJ, Kon T, et al. Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. J Bone Miner Res. 2003; 18: 1584–92.
13. Joyce MB, Roberts AB, Sporn MB, et al. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol. 1990; 110: 2195–207.
14. Fuller K, Lean JM, Bayley KE, et al. A role for TGFbeta1 in osteoclast differentiation and survival. J Cell Sci. 2000; 113: 2445–53.
15. Kon T, Cho TJ, Aizawa T, et al. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res. 2001; 16: 1004–14.
16. Kim JB, Leucht P, Lam K, et al. Bone regeneration is regulated by Wnt signaling. J Bone Miner Res. 2007; 22: 1913–23.
17. Cui Y, Niziolek PJ, MacDonald BT, et al. Lrp5 functions in bone to regulate bone mass. Nat Med. 2011; 17: 684–91.
27. Behr B, Leucht P, Longaker MT, et al. Fgf-9 is required for angiogenesis and osteogenesis in long bone repair. *Proc Natl Acad Sci USA* 2010; 107: 11853–8.
28. Behr B, Sorkin M, Manu A, et al. Fgf-18 is required for osteogenesis but not angiogenesis during long bone repair. *Tissue Eng.* 2011; 17: 2061–9.
29. Montero A, Okada Y, Tomita M, et al. Disruption of the fibrobast growth factor-2 gene results in decreased bone mass and bone formation. *J Clin Invest.* 2009; 126: 1085–93.
30. Baldin V, Lukas J, Marcotte MJ, et al. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.* 1993; 7: 812–21.
31. Cao H, Yu S, Yao Z, et al. Activating transcription factor 4 regulates osteoclast differentiation in mice. *J Clin Invest.* 2010; 120: 2755–66.
32. Moustakas A, Pardali K, Gaal A, et al. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett.* 2002; 82: 85–91.
33. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer.* 2006; 6: 392–401.
34. Kawakami T, Ihn H, Xu W, et al. Increased expression of TGF-beta receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-beta signaling to scleroderma phenotype. *J Invest Dermatol.* 1998; 110: 47–51.
35. Kajihara I, Jinnin M, Makino T, et al. Overexpression of hepatocyte growth factor receptor in scleroderma dermal fibroblasts is caused by autocrine transforming growth factor beta signaling. *Biosci Trends.* 2012; 6: 136–42.
36. Tang Y, Wu X, Lei W, et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med.* 2009; 15: 757–65.
37. Laird RK, Pavlos NJ, Xu J, et al. Bone allograft non-union is related to excessive osteoclastic bone resorption: a sheep model study. *Histol Histopathol.* 2006; 21: 1277–85.
38. Lehmann W, Edgar CM, Wang K, et al. Tumor necrosis factor alpha (TNF-alpha) coordinatey regulates the expression of specific matrix metalloproteinases (MMPs) and angiogenic factors during fracture healing. *Bone.* 2005; 36: 300–10.
39. Zhong N, Gersch RP, Hadijargyrou M. Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. *Bone.* 2006; 39: 5–16.
40. Guo J, Jin J, Cooper LF. Dissection of sets of genes that control the character of wnt5a-deficient mouse calvarial cells. *Bone.* 2008; 43: 961–71.
41. Vuga LJ, Ben-Yehudah A, Kovakova-Naukovska E, et al. WNT5A is a regulator of fibroblast proliferation and resistance to apoptosis. *Am J Respir Cell Mol Biol.* 2009; 41: 583–9.
42. Kumawat K, Menzen MH, Bos IS, et al. Noncanonical WNT-5A signaling regulates TGF-beta-induced extracellular matrix production by airway smooth muscle cells. *FASEB J.* 2013; 27: 1625–33.
43. Kumawat K, Menzen MH, Slepetenhorst RM, et al. TGF-beta-activated kinase 1 (TAK1) signaling regulates TGF-beta-induced WNT5A expression in airway smooth muscle cells via Sp1 and beta-catenin. *PLoS ONE.* 2014; 9: e94801.
44. Chen Y, Whetstone HC, Lin AC, et al. Beta-catenin signaling plays a disparate role in different phases of fracture repair; implications for therapy to improve bone healing. *PLoS Med.* 2007; 4: e249.
45. Wei W, Zeve D, Suh JM, et al. Biphasic and dosage-dependent regulation of osteostatogenesisis by beta-catenin. *Mol Cell Biol.* 2011; 31: 4706–19.
46. Reed AA, Jeynor CJ, Brownlow HC, et al. Human atrophic fracture non-unions are not avascular. *J Orthop Res.* 2002; 20: 593–9.
47. Reed AA, Jeynor CJ, Isseku S, et al. Vascularity in a new model of atrophic nonunion. *J Bone Joint Surg Br.* 2003; 85: 604–10.
48. Garcia P, Pierschak A, Klein M, et al. Temporal and spatial vascularization patterns of unions and nonunions: role of vascular endothelial growth factor and bone morphogenetic proteins. *J Bone Joint Surg Am.* 2012; 94: 49–58.
49. Kwong FN, Hoyland JA, Freemont AJ, et al. Altered relative expression of BMPs and BMP inhibitors in cartilaginous areas of human fractures progressing towards non-union. *J Orthop Res.* 2009; 27: 752–6.
50. Niikura T, Hak DJ, Reddi AH. Global gene profiling reveals a downregulation of BMP gene expression in experimental atrophic nonunions compared to standard healing fractures. *J Orthop Res.* 2006; 24: 1463–71.
51. Zimmerman LB, De Jesus-Escobar JM, Harland RM. The Spermann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell.* 1996; 86: 599–606.
52. Qu G, von Schroeder HP. The osteogenic potential of pseudoarthrosis tissue and bone from human scaphoid non-unions. *J Hand Surg Eur.* 2008; 33: 449–56.