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BONE FRACTURE

Unbiased gene expression analysis of the delayed fracture healing observed in Zucker diabetic fatty rats

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Aims

Impaired fracture repair in patients with type 2 diabetes mellitus (T2DM) is not fully understood. In this study, we aimed to characterize the local changes in gene expression (GE) associated with diabetic fracture. We used an unbiased approach to compare GE in the fracture callus of Zucker diabetic fatty (ZDF) rats relative to wild-type (WT) littermates at three weeks following femoral osteotomy.

Methods

Zucker rats, WT and homozygous for leptin receptor mutation (ZDF), were fed a moderately high-fat diet to induce T2DM only in the ZDF animals. At ten weeks of age, open femoral fractures were simulated using a unilateral osteotomy stabilized with an external fixator. At three weeks post-surgery, the fractured femur from each animal was retrieved for analysis. Callus formation and the extent of healing were assessed by radiograph and histology. Bone tissue was processed for total RNA extraction and messenger RNA (mRNA) sequencing (mRNA-Seq).

Results

Radiographs and histology demonstrated impaired fracture healing in ZDF rats with incomplete bony bridge formation and an influx of intramedullary inflammatory tissue. In comparison, near-complete bridging between cortices was observed in Sham WT animals. Of 13,160 genes, mRNA-Seq analysis identified 13 that were differentially expressed in ZDF rat callus, using a false discovery rate (FDR) threshold of 10%. Seven genes were upregulated with high confidence (FDR = 0.05) in ZDF fracture callus, most with known roles in inflammation.

Conclusion

These findings suggest that elevated or prolonged inflammation contributes to delayed fracture healing in T2DM. The identified genes may be used as biomarkers to monitor and treat delayed fracture healing in diabetic patients.

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Keywords: Fracture healing, Fracture nonunion, Zucker rats, Type 2 diabetes mellitus

Article focus

- To investigate messenger RNA (mRNA) gene expression (GE) in the fracture callus of diabetic and control rats three weeks post-femoral osteotomy.
- Delayed fracture healing in diabetic patients may be related to increased proinflammatory GE.

Key messages

- Fracture healing was delayed in diabetic rats, indicated by histological and radiological analysis.
- Unbiased mRNA sequencing identified potentially causative elevated proinflammatory GE patterns in Zucker diabetic fatty (ZDF) compared to wild-type (WT) samples.

Strengths and limitations

- Unbiased GE analysis identified differentially expressed genes in the fracture callus of ZDF rats compared to WT, providing new insight into the pathogenesis of delayed fracture healing in type 2 diabetes mellitus.

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- This study could not determine if GE changes were the cause of the impaired fracture healing observed.

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by chronic hyperglycaemia, leading to decreased sensitivity to insulin. According to the World Health Organization (WHO), more than 422 million people currently have diabetes, with increasing prevalence and mortality.[1] Among its many complications, T2DM is known to pose a higher risk for fracture, particularly for hip fracture, and is associated with delayed fracture healing.[2] In diabetic patients, fracture healing can be prolonged by 87%,[3] with increased risk of other orthopaedic complications such as delayed union, nonunion, dislocation, or pseudoarthrosis.[4,5]

The detrimental effect of T2DM on the regenerative ability of bone is postulated to be acting at cellular, molecular, and biomechanical levels.[6] This includes increased osteoclastogenesis and prolonged and elevated inflammation, with difficulty downregulating inflammation once induced.[7-9] A variety of pharmacological treatments are available to control blood glucose levels, including metformin, sulfonylurea, and insulin.[10] However, there is no specific treatment to normalize fracture healing in diabetic patients.

Previous studies have examined gene expression (GE) associated with T2DM in patient-derived tissues, such as peripheral blood and skeletal muscle.[11,12] The Zucker diabetic fatty (ZDF) rat is a useful experimental model of human T2DM. ZDF rats carry recessive *fa*/*fa* mutation for the leptin membrane receptor, which results in a shortened leptin-receptor protein that does not interact with leptin effectively.[13] Animals homozygous for the *fa* allele become noticeably obese by three to five weeks of age, becoming hyperlipidaemic, hypercholesterolaemic, hyperinsulinaemic, and developing adipocyte hypertrophy and hyperplasia.[14] Liu et al[15] observed an upregulation of ANXA3 in patients with T2DM and fracture nonunion, leading to the conclusion that elevated ANXA3 expression potentially contributes to fracture nonunion in T2DM by modulating neutrophil activity. Although research involving altered expression of microRNA during fracture healing in diabetic rats has been performed previously,[16] to our knowledge specific messenger RNA (mRNA) expression during fracture healing in ZDF rats has not been reported.

This study aimed to improve the molecular understanding of the pathophysiology of delayed fracture healing in diabetes by examining the differential GE in the fracture callus of ZDF compared to wild-type (WT) littermate controls.

Methods

Animals. The authors of this study adhered to the ARRIVE guidelines for reporting in vivo experiments. We selected a small sample size because the mRNA GE in fracture callus of ZDF rats was evaluated in vivo for the first time. Therefore, the purpose was to gather basic evidence regarding the specific GE seen in the diabetic cohort. A total of 20 rats, consisting of 14 male ZDF rats (ZDF-Lepr$^{fa/fa}$) and six leptin receptor-intact Zucker rats (ZDF-Lepr$^{+/+}$), were randomly assigned to the diabetic and control groups, respectively. From four weeks of age, all were fed a high-fat (7.55%) diet (SF06-019, Purina 5008 Equivalent; Speciality Feeds, Australia) ad libitum. At ten weeks of age, rats were subjected to a mid-diaphyseal transverse osteotomy in the left femur, as described below. Three weeks after osteotomy, animals were culled by CO_2 exposure and cervical dislocation and the femora were removed for analysis. At the experiment end, the mean weight of ZDF rats was 546.8 g (standard deviation (SD) 27.5), compared to control rats, which weighed an average of 349.5 g (SD 4.0), confirming the differential acquisition of a diabetic phenotype in the ZDF group. This study was approved by the University of South Australia Animal Ethics Committee (Approval No. U15-16).

Surgical procedure. All procedures were performed using a previously reported technique.[17] Briefly, the left leg area where the surgery was performed was shaved, and the limb was cleaned using ethanol and betadine. A 3- to 4-cm curved incision was made through the skin parallel to the femur. An incision was then made through the fascia, and blunt dissection was used to separate the muscles to expose the femur. An external fixation device to the left femur was applied using a non-articulating fixator by hand-drilling four 1 mm holes in the cortical bone, so as not to cause overheating (and necrosis) of the surrounding tissue. Threaded stainless steel pins (1.2 mm diameter) were inserted into these holes. The original curved incision was made to stretch the skin and fit over the surgical site with the pins in situ. This also resulted in the incision being suitably distant from the pins to minimize the risk of infection. An external fixator device was then fitted to interlock the pins securely. Mid-diaphyseal transverse osteotomy in the left femur was performed using a diamond-tipped bone hand saw, and a 1 mm gap was created. Finally, the incised muscle and skin were sutured, and Betadine solution was applied to sterilize the wound. Vetergesic (0.1 cc; Alstoe, UK) for analgesia and cephalosporin (0.05 cc; Sandoz Ltd, UK) were given postoperatively for one day to prevent infection, and the animals were then returned to their cages. The external fixator remained attached, effectively keeping the fractured bone aligned and supported for the postoperative period. Due to the gross weight and size difference of the two groups of rats, the surgeon (PJS) could not be completely blinded to whether the animals operated on were from a diabetic or a control group.

Radiological analysis of fracture repair. Once the animals were culled, dual energy X-ray absorptiometry (DXA) radiographs of the osteotomized femur were acquired using a Faxitron cabinet (Hologic, USA). Radiological images were evaluated for evidence of fracture healing and fracture union, which was defined as bridging of the fracture by bone, callus, or trabeculae, bridging of the

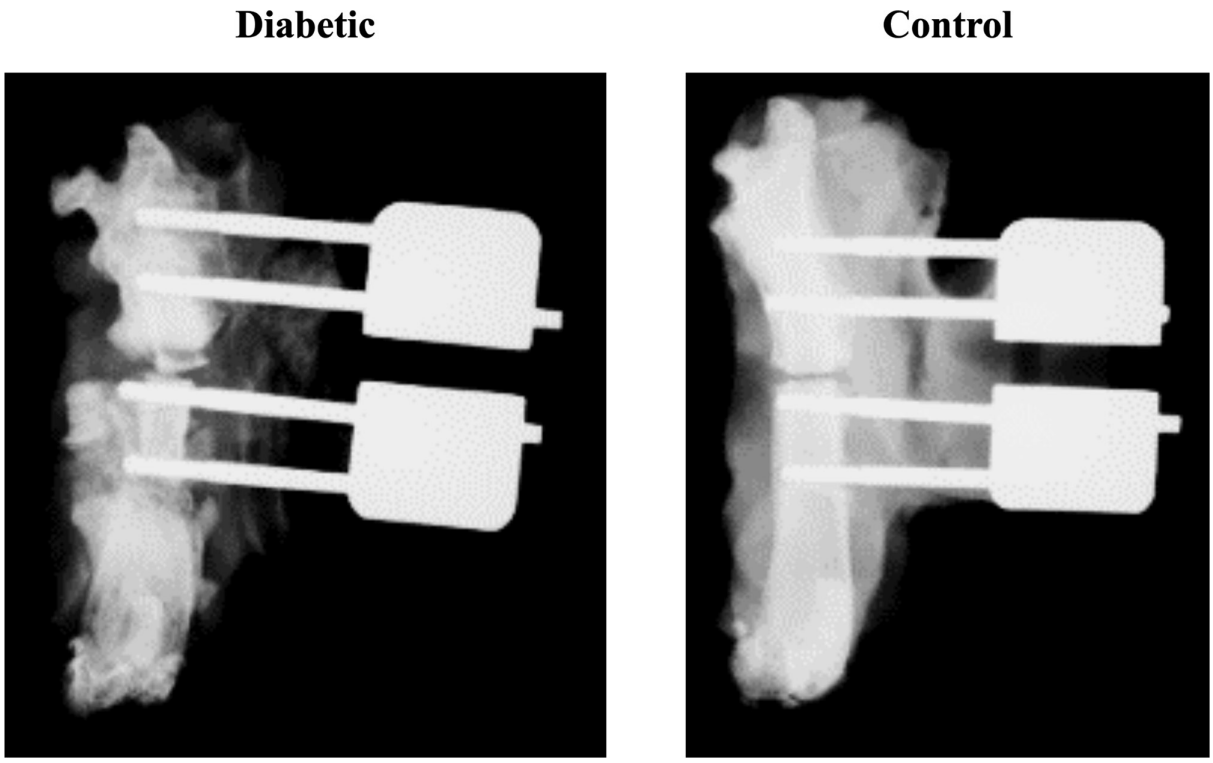

Fig. 1

A representative radiograph showing fracture healing of the type 2 diabetes mellitus group compared to non-diabetic controls three weeks post-femoral osteotomy, with external fixators in situ.

fracture at three of four cortices, and obliteration of the fracture line and/or cortical continuity.

Histology of fracture sites. For histology, non-decalcified sagittal sections of the femur were prepared from formalin-fixed, polymethyl methacrylate (PMMA) resin-embedded bone as previously described,[18] and stained with Toluidine blue.[19] Histological sections were imaged using a digital slide scanner (NanoZoomer 2.0-HT; Hamamatsu Photonics, Japan).

Gene expression analysis. For GE analysis, whole femora (n = 3/group) were submerged in ten volumes of RNALater (Invitrogen, Thermo Fisher Scientific, USA), and stored overnight at 4°C before freezing at -80°C. Frozen whole femora were then pulverized before extraction of total RNA using the TRIzol extraction method, as previously described.[20]

Following RNA purification, rat total mRNA sequencing (mRNA-Seq) was performed (Australian Genome Research Foundation, Australia). Sequencing data quality was assessed using ngsReports,[21] with reads being trimmed using AdapterRemoval v2.2.1[22] before transcript-level counts were obtained using kallisto v0.43.1.[23] Gene annotations for Rnor_6.0 were obtained from Ensembl Release 96 (European Bioinformatics Institute, UK). Transcript-level counts were summarized to the gene level, then analyzed using voom-precision weighs method, incorporating additional sample-level weights.[24] Differentially expressed (DE) genes were considered to be those with a false discovery rate (FDR)-adjusted p-value < 0.05.

FDR thresholds of 5% and 10.0% were used to identify genes as potentially DE. Gene set enrichment analysis (GSEA) was also performed[25] as implemented in the fgsea package,[26] which has no requirement for formal consideration of genes as DE. For this, genes were ranked by their respective t-statistic and genes from hallmark gene sets were tested for enrichment at either end of the ranked list.[27] A Bonferroni-adjusted p-value < 0.05 was used to consider a gene set as enriched within the ranked list of genes. All analytic code is available at https://github.com/UofABioinformaticsHub/20180328_Atkins_RatFracture.

Statistical analysis. Statistical analyses of differences between the ZDF and WT rats were generated using voom-precision weights and moderated t-statistics as implemented in the limma package.[28] An FDR-adjusted p-value of < 0.05 was considered to be statistically significant.

Results

Delayed healing in fractured ZDF rats. At three weeks post-femoral osteotomy, radiological analysis of the callus formation of the fractured femur demonstrated delayed healing in the diabetic cohort (Figure 1). Callus formation was delayed in all six of the diabetic cohort compared to the six non-diabetic controls. The quality of the callus formed within the diabetic group clearly shows inconsistent fracture healing via increased areas

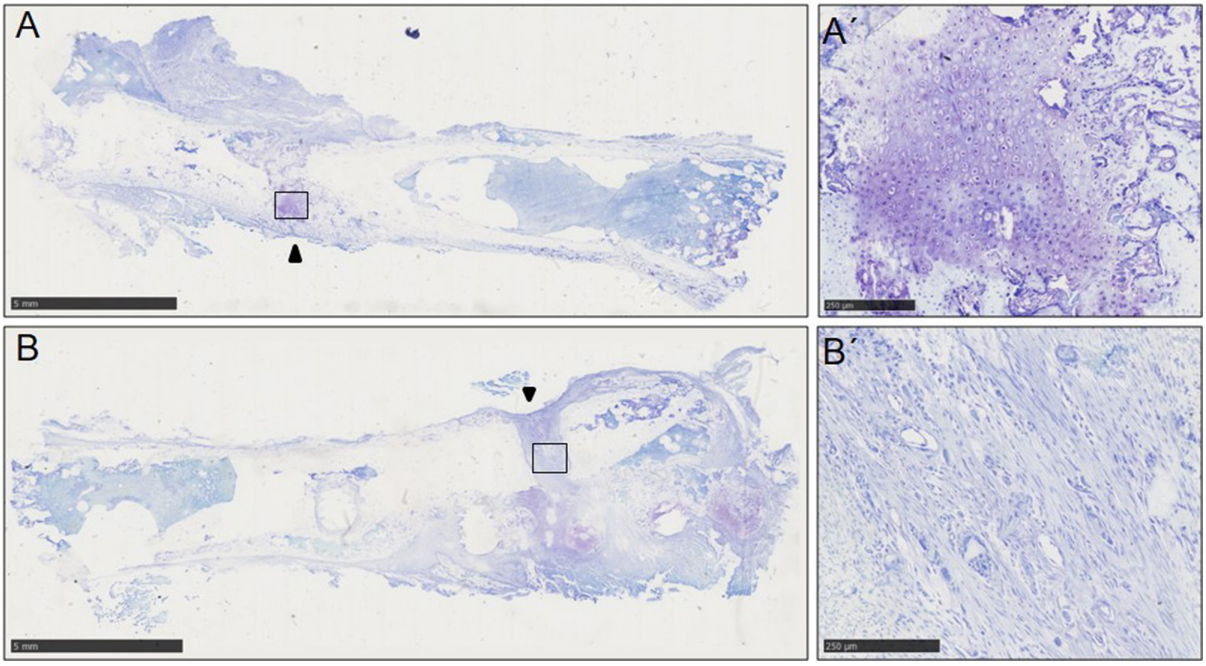

Fig. 2

Toluidine blue histology of whole femora of control (A) and Zucker diabetic fatty (B) rats three weeks post-fracture fixation. A´ and B´ show respective magnified regions indicated by black rectangles. Osteotomy sites are indicated by black triangles. The black arrow indicates the location of the fracture site. The black bars in A and B represent a length of 5 mm, while in A´ and B´ they represent 250 µm.

of translucency within the callus formed. Delayed union is clearly evident, as suggested by irregular joint fracture space compared to the control rats. External fixators of the fractured femora are in situ, as demonstrated by the radio-opacity (Figure 1).

Histological analysis of fracture repair. WT control fracture sites showed clear evidence of near complete healing, with endochondral ossification tissue traversing the intramedullary space (Figures 2A and 2A´). ZDF fractures showed incomplete or little repair, with large fibrotic ingrowths to the fracture site (Figures 2B and 2B´).

Gene expression analysis. Using differential mRNA-Seq analyses, 13,160 genes were considered as detected from the fracture site (Figure 3). Using a FDR of 0.05, only seven genes were found to be DE in ZDF callus, including *Patatin-like phospholipase domain containing protein 2* (*Pnpla2*), downregulated 7.39-fold (FDR = 0.006), and *LOC100909761*, now identified as *Myot*, the gene encoding the myocyte axon guidance product myotilin, which was upregulated 6.26-fold (FDR = 0.006).

Using a less stringent FDR threshold of 10.0% gave weak-to-moderate support for a further six genes as potentially DE (Table I). This includes proinflammatory genes such as the *Cxcl* group (*Cxcl1, Cxcl2, Cxcl3), Ccl20, interleukin (IL)-6,* and *TNFSF8,* which were significantly elevated. Only two genes were downregulated in this analysis, *Rcor2* and *Pnpla2* (Table I).

To gain further insight into the GE pathways differentially regulated in this model, GSEA was also performed (Table II). Of the 13 hallmark pathways identified as enriched, nine were enriched for upregulated genes, including interferon-gamma response, tumour necrosis factor alpha (TNF-α) signalling via nuclear factor kappa B (NF-κB), inflammatory response, and IL-6 JAK/STAT3 signalling, and all were associated with increased inflammation in the ZDF fracture callus. Three other pathways enriched for upregulated genes were epithelial-mesenchymal transition, myogenesis, and hypoxia, potentially indicating a disrupted cell differentiation or tissue repair response. Of the four pathways enriched for downregulated genes, G2M checkpoint, Myc targets V1, and E2f targets suggest impaired cell cycle progression. Overall, the GE analysis suggests a proinflammatory response associated with impaired or inappropriate tissue regeneration in the ZDF fractures.

Discussion

In this study, ZDF rats showed delayed or deficient fracture healing upon radiological and histological analysis of the callus. Radiological analyses (Figure 1) demonstrated disorganized mineralization and lack of union in ZDF fracture sites compared to WT littermates three weeks post-femoral osteotomy, indicated by unequal callus thickness and a polymorphic appearance. Histological analysis showed extensive fibrotic ingrowth within the callus of ZDF rats, compared to evidence of the expected endochondral fracture repair process in WT rats.

We sought to identify localized molecular mechanisms underlying aberrant fracture healing in this

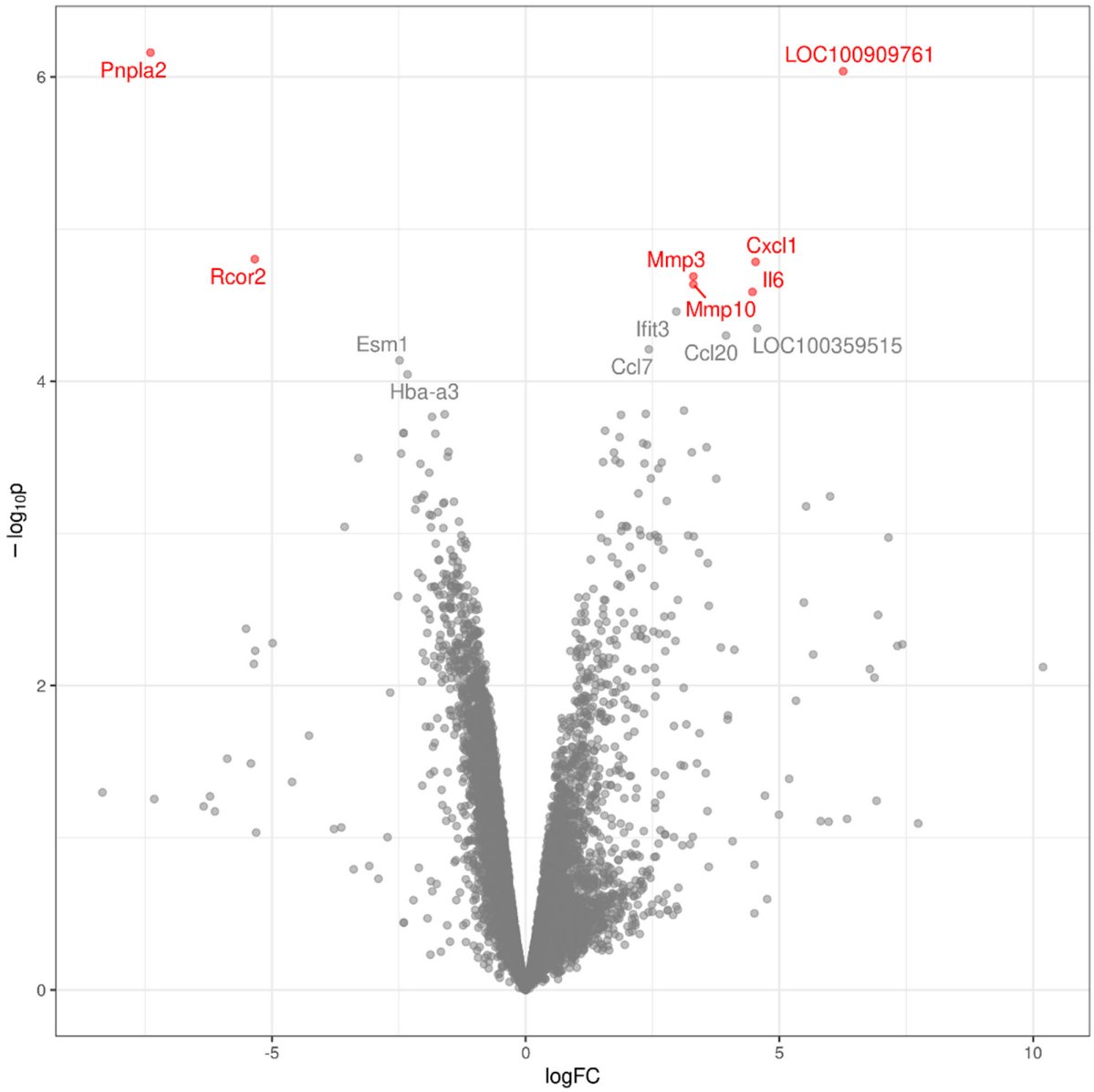

Fig. 3

Volcano plot with significant genes shown in red (FDR < 0.05). Genes to a FDR of 10.0% are additionally labelled in grey. FDR, false discovery rate; Mmp, matrix metallopeptidase; Pnpla2, Patatin-like phospholipase domain-containing protein 2.

diabetic model. mRNA-Seq analysis revealed seven DE genes (FDR < 0.05), with the two most highly-ranked being *Pnpla2* and *LOC100909761/Myot. Pnpla2* is a gene that encodes patatin-like phospholipase domain-containing protein 2, also known as adipose triglyceride lipase (ATGL), an enzyme responsible for intracellular hydrolysis of stored triglycerides.[29] *Pnpla2* was associated with non-alcoholic fatty liver disease in an obese population and developing renal complications within a diabetic population.[30,31] Kim et al[32] demonstrated that ATGL/*Pnpla2* expression is downregulated by insulin and TNF-α in adipocytes at both protein and mRNA levels. This correlates with the findings of our study, with the downregulation of *Pnpla2* mRNA likely reflecting the hyperinsulinaemic and hyperinflammatory state of diabetic rodents. *LOC100909761/Myot* was strongly upregulated in the ZDF callus. The significance of this is unclear but could reflect inappropriate mesenchymal differentiation away from osteogenic lineages (chondroblast-osteoblast) towards myoblast, as the gene product myotilin is best characterized as a protein involved in striated muscle contraction.[33] Further evidence for aberrant cell differentiation was obtained when GSEA of the entire gene set was performed (Table II). Of the top 13 hallmark gene pathway sets identified, epithelial-mesenchymal transition and myogenesis were positively associated with the tissue response in ZDF callus.

Table I. The top 13 differentially expressed genes in whole femur rat messenger RNA sequencing of Zucker diabetic fatty compared to wild-type three weeks post-fracture fixation.

Gene name	Gene symbol	Expression in ZDF	logFC	p-value	FDR
Patatin-like phospholipase domain-containing protein 2	*Pnpla2*	down	7.39	6.92E-07	0.006
LOC100909761/myotilin	*Myot*	up	6.26	9.18E-07	0.006
REST corepressor 2	*Rcor2*	down	5.34	1.58E-05	0.049
C-X-C motif chemokine ligand 1	*Cxcl1*	up	4.53	1.64E-05	0.049
Matrix metallopeptidase 3	*Mmp3*	up	3.30	2.05E-05	0.049
Matrix metallopeptidase 10	*Mmp10*	up	3.31	2.31E-05	0.049
Interleukin-6	*IL-6*	up	4.47	2.59E-05	0.049
Interferon-induced protein with tetratricopeptide repeats 3	*Lfit3*	up	2.97	3.48E-05	0.057
LOC100359515/nitric oxide synthase 2, pseudogene 1	*Nos2-ps1*	up	4.56	4.49E-05	0.066
C-C motif chemokine ligand 20	*Ccl20*	up	3.95	5.01E-05	0.066
C-C motif chemokine ligand 7	*Ccl7*	up	2.43	6.17E-05	0.074
Endothelial cell specific molecule 1	*Esm1*	down	2.48	7.29E-05	0.080
Hemoglobin alpha, adult chain 3	*Hba-a3*	down	2.33	9.02E-05	0.091

FDR, false discovery rate; logFC, log fold-change; ZDF, Zucker diabetic fatty.

When applying a less stringent FDR of 0.1, six additional DE genes were identified (Table I). A striking feature from this analysis was evidence of an inappropriate inflammatory response in the ZDF callus. Inflammation is an expected feature of fracture and of the healing response; under normal physiological conditions, inflammatory signals result in a local increase in macrophages, which release proinflammatory mediators such as IL-6 and CXCL chemokines that promote fracture healing.[34,35] Proinflammatory cytokines are known to show peak expression within the first 24 hours after fracture, depressed levels during cartilage formation, and their levels increase again during bone remodelling.[36] Indeed, activating this initial inflammatory pathway is pivotal in fracture healing. The deficiency of inflammation caused by therapeutic agents such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids is known to delay fracture healing time and increase the risk of nonunion.[37–39] IL-6 is pivotal in the local immune response after fracture by regulating the recruitment of immune cells to the fracture haematoma, and by inducing regenerative processes augmenting bone repair.[40] IL-6 also regulates the differentiation of both osteoblasts and osteoclasts, and promotes angiogenesis by stimulating the release of vascular endothelial growth factor (VEGF).[41] The importance of this cytokine in fracture healing was exemplified by studies in IL-6 knockout mice, which demonstrated delayed fracture healing with decreased osteoclastogenesis and impaired callus formation.[42,43] Therefore, it is indisputable that inflammation is crucial in the bone healing process and that suppression of inflammatory cytokine expression forms an unfavourable microenvironment in the fracture site to prolong healing.

In contrast, a prolonged proinflammatory phase, as evident in the fracture healing cascade in ZDF rats at three weeks post-fracture in this study, is associated with impaired bone repair. Acute inflammation due to severe injuries such as polytrauma and open fracture can lead to pronounced TNF-α and IL-6 expression, and has a detrimental effect on bone healing.[44–46] GSEA in our study indicated that within the diabetic rat cohort, upregulation of the inflammatory gene sets interferon-gamma response, TNF-α signalling via NF-κB, inflammatory response, and IL-6 JAK/STAT3 occurred or persisted even after three weeks. Furthermore, the analysis showed significantly upregulated individual GE of *Il6* and *Mmp3* with a log fold-change value of 4.47 and 3.30, respectively. As aforementioned, while IL-6 has an important role in fracture healing, over-production can inhibit canonical Wnt/β-catenin activity, an essential pathway for osteoblast differentiation.[47] Wu et al[48] also suggested that IL-6 enhances osteoclastogenesis by excessive promotion of JAK2 and RANKL activity. Moreover, IL-6 has been shown to stimulate osteoclastogenesis and bone resorption directly,[49] with IL-6-overexpressing mice found to have osteopenia and defective ossification.[50]

An increased inflammatory state within the ZDF fracture callus is further supported by the increased GE of *Angptl4* and *Ifit3*, which are involved in inflammation in patients with T2DM and interferon-gamma signalling.[51,52] Alvarez-López et al[53] described the association between *Rcor2* downregulation and increased inflammation, consistent with our findings. GE of the CXC chemokine group, including *Cxcl1* (FDR = 0.049), *Cxcl2* (FDR = 0.188), and *Cxcl3* (FDR = 0.164), was also upregulated in the diabetic rat cohort (Table I). These chemokines are collectively known as potent neutrophil chemoattractants and are involved in cancer metastasis, angiogenesis, and wound healing.[54–56] CXCL1 forms complex interactions with glycosaminoglycans (GAG) on endothelial cells and the extracellular matrix (ECM), resulting in chemotactic gradients that modulate neutrophil recruitment to the site of inflammation.[57] Although neutrophils have a crucial role in the systemic immune response, and despite being the most abundant immune cell type in the early

Table II. Top 13 enriched Hallmark gene sets using a Bonferroni-adjusted p-value < 0.01. The normalized enrichment score indicates which end of the ranked list appeared to be enriched for each gene set, with negative values indicating the downregulated genes. The number of genes in the gene set is also indicated, as is the number of genes at the point of maximum enrichment. The most highly ranked genes before the point of maximal enrichment are given in the final column.

Hallmark pathway	p-value	Adjusted p-value	NES	Size, n	Size at max, n	Genes
Epithelial- mesenchymal transition	8.83E-28	1.72E-23	3.27	173	89	*Cxcl1; Mmp3; Il6; Cxcl3; Cxcl2; Cxcl6; Acta2; Fbln2; Tgfbi; Htra1; Timp1; Mgp; Ptx3; Tagln; Mfap5; Col5a3; Inhba; Tfpi2*
TNF-α signalling via NF-κB	1.73E-27	3.35E-23	3.25	173	83	*Cxcl1; Il6; Ccl20; Cxcl3; Il1b; Cxcl2; Cxcl6; Tlr2; Ptgs2; Tnfaip6; G0s2; F3; Sod2; Olr1; Il1a; Pde4b; Ptx3; Gfpt2*
Inflammatory response	7.75E-23	1.51E-18	3.10	156	59	*Il6; Ccl20; Ccl7; Il1b; Cxcl6; Tlr2; Has2; Tnfaip6; F3; Olr1; Slc7a2; Il1r1; Il1a; Pde4b; Timp1; Ifitm3; Inhba; Hif1a*
E2f targets	6.24E-20	1.21E-15	2.40	193	129	*Birc5; Ran; Srsf2; Kif18b; Cdc25b; Rrm2; Rad51ap1; Cdca3; Pold2; Ncapd2; Stmn1; Ppm1d; Dclre1b; Ccne1; E2f8; Prps1; H2afx; Gins3*
Heme metabolism	3.51E-19	6.82E-15	2.40	175	98	*Bpgm; Minpp1; Mkrn1; Klf1; Foxo3; Abcb6; Cdr2; Fbxo7; Fech; Alas2; Ctse; Epb41; Gclm; Epb42; Ranbp10; Prdx2; Cpox; Hagh*
Myogenesis	2.93E-16	5.69E-12	2.67	165	61	*Mylpf; Myl1; Myoz1; Tnni2; Myh1; Myom2; Eno3; Ckm; Pgam2; Tnnt3; Tagln; Tnnc2; Cox6a2; Pvalb; Mb; Col3a1; Actn3; Hspb8*
Coagulation	3.11E-13	6.04E-09	2.78	93	33	*Mmp3; Mmp10; Ctsl; Cfi; C1s; F3; Plat; Olr1; Htra1; Timp1; LOC100911545; Tfpi2; Maff; C1qa; Mmp2; Ctsk; Serpine1; C2*
IL-6 JAK/STAT3 signalling	1.09E-12	2.12E-08	2.85	75	20	*Cxcl1; Il6; Ccl7; Cxcl3; Il1b; Cxcl2; Tlr2; Il1r2; Cxcl13; Il1r1; LOC100911545; Osmr; Cd14; Tnfrsf12a; Il2ra; Il4r; Csf3r; Pf4*
G2M checkpoint	3.08E-12	5.98E-08	2.11	189	107	*Hira; Birc5; Srsf2; Ccna2; Tent4a; Cdc25b; Stmn1; Tgfb1; H2afx; Aurka; E2f3; Racgap1; E2f4; Hnrnpd; Mcm5; Ccnf; Cul4a; Cdkn2c*
Complement	7.26E-11	1.41E-06	2.30	158	37	*Cxcl1; Il6; Cxcl3; Ctsl; Cxcl2; C1s; F3; Plat; Olr1; Ctss; Timp1; Ctsc; Tfpi2; Maff; C1qa; Col4a2; Pla2g7; Zfpm2*
Hypoxia	5.24E-09	1.02E-04	2.14	171	49	*Il6; Angptl4; F3; Errfi1; Eno3; Slc2a1; Tgfbi; Pgam2; Plin2; Ddit4; Ier3; Atf3; Maff; Pygm; Prdx5; Cdkn1a; Fos; Tgm2*
Myc targets V1	1.08E-08	2.10E-04	1.90	194	101	*Ran; Srsf2; Pabpc4; Ccna2; Tufm; Pold2; Pabpc1; Ppm1g; Phb2; Cstf2; Snrpa1; Hnrnpd; Mcm5; Mcm7; Trim28; Impdh2; Prdx3; Rpl14*
Interferon-gamma response	3.29E-08	6.40E-04	2.03	173	39	*Il6; Ifit3; Ccl7; Ptgs2; C1s; Tnfaip6; Sod2; Pde4b; Ifitm3; Upp1; Mx1; Hif1a; Cdkn1a; Cd274; Il4r; Ifit2; C2; Icam1*

IL, interleukin; NES, normalized enrichment score; NF-κB, nuclear factor kappa B; Size, number of genes in the gene set; Size at max, number of genes at the point of maximum enrichment; TNF, tumour necrosis factor alpha.

fracture haematoma, excessive or prolonged influx of neutrophils into the fracture haematoma can have a negative impact on fracture healing after systemic inflammation.[58] Furthermore, rats with a 60% decrease in neutrophils from the administration of neutrophil-neutralizing antiserum showed enhanced osteoblastic differentiation, supporting the conclusion that the neutrophil-mediated inflammatory response appears

to suppress osteoblastic differentiation.[59] In the ZDF fractured femora, there was clear evidence of a pannus-like fibrotic ingrowth at the fracture site, containing polymorphs likely to be neutrophils. Together, the data from our study suggest that delayed bone healing in ZDF fractures is likely to be caused by elevated inflammatory mediator expression.

This study has some limitations. We only compared GE profiles between fractured ZDF and WT bone, and did not adjust for pre-existing differences in GE between these genotypes. Furthermore, we only examined GE at a single timepoint, albeit at the highly informative timepoint of three weeks postoperative, providing a snapshot rather than a progression of GE profiles throughout the healing phase. In this sense, we consider our study to be a pilot, giving proof of the concept of the model. The samples examined by mRNA-Seq contained various cell types, which varied between genotypes thus limiting the statistical power of the study; in future studies, it may be possible to use techniques such as laser capture microdissection to analyze cell type-specific GE changes. We also could not determine the cause or effect of GE changes from this analysis. Furthermore, we only examined mRNA changes and did not consider alterations in non-coding RNA types or resulting protein expression levels. However, to our knowledge, this is the first study of GE in the ZDF model during the fracture healing phase. By using unbiased GSEA, we were able to show significantly upregulated inflammatory gene pathway expression within the fracture callus. As the GE profiles for the ZDF rats are highly consistent with those observed in human patients,[60] we believe that this study will aid in understanding the delayed fracture healing phenomenon seen in diabetic patients. Further study is required to confirm whether these same genes are similarly associated in humans, and whether the identified genes are causative of delayed healing, or a product of it.

The findings have implications for the management of fracture healing in diabetic patients. The aberrant healing process and molecular mechanisms identified could potentially serve as targets for future therapeutic interventions to enhance fracture repair in this patient population. Further research should investigate whether modulation of the identified pathways (Pnpla2 and LOC100909761/Myot) can improve outcomes. Additionally, the potential links highlighted in this study between diabetes, aberrant localized mesenchymal differentiation, and impaired fracture healing may stimulate further exploration into the broader effects of diabetes on tissue regeneration and repair.

In conclusion, ZDF rodent callus showed persistent upregulation of pro-inflammatory and pro-chemotactic GE, such as *IL-6*, *Cxcl1*, *Ccl7*, and *Ccl20*. Such increased inflammation can delay fracture healing by complex mechanisms involving excessive neutrophil migration and increased osteoclastogenesis, resulting in disorganized mineralization of the callus.

Supplementary material

An ARRIVE checklist is included to show that the ARRIVE guidelines were adhered to in this study.

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