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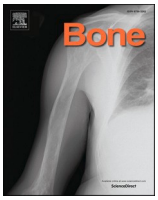

Full Length Article

# Human nonunion tissues display differential gene expression in comparison to physiological fracture callus

Leonidas Salichos [a,b,1], Rishika Thayavally [a,b,1], Peter Kloen [c,d], Michael Hadjiargyrou [b,e,*]

[a] Department of Biological & Chemical Sciences, New York Institute of Technology, New York, NY 10023, USA
[b] Center for Biomedical Data Science, New York Institute of Technology, New York, NY 10023, USA
[c] Department of Orthopedic Surgery and Sports Medicine, Amsterdam UMC location, Meibergdreef 9, the Netherlands
[d] Amsterdam Movement Sciences, (Tissue Function and Regeneration), Amsterdam, the Netherlands
[e] Department of Biological & Chemical Sciences, New York Institute of Technology, Old Westbury, NY, 11568, USA

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ABSTRACT

The healing of bone fractures can become aberrant and lead to nonunions which in turn have a negative impact on patient health. Understanding why a bone fails to normally heal will enable us to make a positive impact in a patient's life. While we have a wealth of molecular data on rodent models of fracture repair, it is not the same with humans. As such, there is still a lack of information regarding the molecular differences between normal physiological repair and nonunions. This study was designed to address this gap in our molecular knowledge of the human repair process by comparing differentially expressed genes (DEGs) between physiological fracture callus and two different nonunion types, hypertrophic (HNU) and oligotrophic (ONU). RNA sequencing data revealed over ~18,000 genes in each sample. Using the physiological callus as the control and the nonunion samples as the experimental groups, bioinformatic analyses identified 67 and 81 statistically significant DEGs for HNU and ONU, respectively. Out of the 67 DEGs for the HNU, 34 and 33 were up and down-regulated, respectively. Similarly, out of the 81 DEGs for the ONU, 48 and 33 were up and down-regulated, respectively. Additionally, we also identified common genes between the two nonunion samples; 8 (10.8 %) upregulated and 12 (22.2 %) downregulated. We further identified many biological processes, with several statistically significant ones. Some of these were related to muscle and were common between the two nonunion samples. This study represents the first comprehensive attempt to understand the global molecular events occurring in human nonunion biology. With further research, we can perhaps decipher new molecular pathways involved in aberrant healing of human bone fractures that can be therapeutically targeted.

## 1. Introduction

Bone nonunions are indicative of bone healing failure and constitute a medical problem for millions of people as they cause prolonged pain and disability [1]. In fact, each year in the United States, millions of people fracture a bone and in approximately 7–9 %, the bone fails to heal and becomes a nonunion [2]. The most common nonunion types are oligotrophic or hypertrophic [3] and they often affect the tibia, scaphoid and humerus, as a result of factors such as high-energy injury, open fracture, segmental or comminuted fracture in combination with specific patient behavior such as alcoholism, smoking, medication or illicit drug use, diabetes mellitus, age [4], and even genetics [5]. Others are suboptimal surgical technique, lacking stability of fixation, or soft tissue stripping leading to devascularization. Each type of nonunion is define by a specific radiographic appearance which enables the physician to treat, usually with surgery. Recent developments have also included addition of mesenchymal stem cells, growth factors, hormones, demineralized bone, grafts, etc., but these have not yet proven to be very successful [6]. Given this, we must continue to search for additional factors [7], but to do so, we must first have a better understanding of the biology of nonunions.

In order to fully understand the biology of nonunions, we must also explore physiological fracture repair so that the results can be compared directly to those of nonunions. By no means is this a trivial task, given that physiological fracture repair involves multiple major processes such as inflammation, angiogenesis, osteogenesis, chondrogenesis,

* Corresponding author at: Department of Biological & Chemical Sciences, New York Institute of Technology, Old Westbury, NY 11568, USA.
*E-mail address:* mhadji@nyit.edu (M. Hadjiargyrou).
[1] These authors have contributed equally.

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endochondral ossification and remodeling [8]. As one can easily imagine, each of these processes involves hundreds, if not thousands of genes that are expressed by multiple cell types [9] as well as regulatory RNAs [4,10,11]. While a number of global gene expression studies have been conducted with animal models, there are only a handful of studies dealing with human callus samples. In fact, there is only one recent study that reported on the global gene expression of isolated and cultured osteoprogenitor cells from fracture calluses [12]. In contrast, there are a number of studies reporting on the global expression of miRNAs [13,14], including one from our laboratory [4]. Thus, there is an unmet need to fully examine global mRNA expression from human physiological fracture repair as compared to those of nonunion samples.

Given the scant human fracture callus and nonunion global mRNA expression data, we sought to explore this by comparing normal human fracture callus and nonunion tissues (both hypertrophic and oligotrophic). We hypothesized that we would identify common and unique differentially expressed genes (DEGs) in these human tissues. We tested this hypothesis by analyzing RNA sequence data from samples isolated from human physiological fracture callus and tissues from hypertrophic and oligotrophic nonunions as described in La Manna et al., (2023). As this study focused more on the comparison of osteoprogenitor cells isolated from intact bone with that of fracture callus and nonunion samples, we utilized the same RNA sequence data but performed a different analysis. We decided to use the RNA data from sequencing of tissue samples from human fracture callus and nonunions. Specifically, the callus sample was used as the control and we compared it to both, heterotrophic and oligotrophic nonunion samples separately. We decided to use the fracture callus as a control because it represented normal physiological healing and thus comparing it to the nonunion samples would identify DEGs related to these pathophysiological processes. Herein, we describe that there are differences between the mRNAs that are present in these samples, but there are also many that are the same which is also the case with biological processes. Taken together, these data provide the first complete elucidation of the transcriptome derived from normal human physiological fracture repair callus as well as two types of nonunions (hypertrophic and oligotrophic). We hope that this opens the door to continue to explore individual genes and the impact they may have on normal physiological repair or its failure.

## 2. Materials and methods

### 2.1. Human samples and RNA extraction and sequencing

The human samples, patient characteristics and RNA extraction and sequencing (GEO dataset GSE226568) have been previously reported by La Manna et al. [12] as well as by our laboratory [4]. From these data, we have used a total of 23 RNA seq samples for our analyses; physiological fracture callus (Callus, $n = 6$), hypertrophic nonunion (HNU, $n = 8$), and oligotrophic nonunion (ONU, $n = 9$). The total number of samples used was smaller than those reported by La Manna et al. [12] since in that study they had used additional samples to isolate cells, rather than mRNA. All patients were treated by a fellowship trained orthopedic trauma surgeon (PK), between June 2016 and July 2020. Consent for removal of the tissue and its storage in the tissue bank in a coded fashion for research purposes was obtained from each patient per Institutional Review Board (IRB) guidelines (W20_075 #20.103, Academic Medical Center, Amsterdam). The used patient sample details are shown in Table 1.

**Table 1**
Patient/facture characteristics.

| Tissue type | Age/ sex | Location | Time since fracture | Co-morbidities |
|---|---|---|---|---|
| Fracture Callus (Callus) | 15/M | Ulna | 0.5 mo | None |
| | 16/M | Tibia | 0.65 mo | None |
| | 58/M | Wrist | 2 mo | None |
| | 54/M | Radius | 2 mo | None |
| | 27/M | Ulna | 3 mo | None |
| | 53/M | Wrist | 0.6 mo | Gout |
| Hypertrophic nonunion (HNU) | 57/M | Tibia | 95 mo | None |
| | 53/M | Tibia | 21 mo | None |
| | 53/M | Femur | 7 mo | Hypertension/ hypothyroidism |
| | 74/F | Humerus | 180 mo | Hypertension |
| | 58/F | Femur | 24 mo | Knee arthrosis |
| | 62/M | Tibia | 25 mo | None |
| | 28/M | Ulna | 13 mo | None |
| | 54/F | Femur | 27 mo | Rheumatoid arthritis; obesity |
| Oligotrophic nonunion (ONU) | 95/F | Femur | 5 mo | None |
| | 69/F | Humerus | 18 mo | None |
| | 19/M | Humerus | 6 mo | None |
| | 81/F | Femur | 7 mo | None |
| | 26/M | Femur | 35 mo | None |
| | 47/F | Tibia | 13 mo | None |
| | 57/F | Humerus | 13 mo | Asthma |
| | 32/M | Femur | 28 mo | None |
| | 78/F | Humerus | 13 mo | None |

Abbreviations: mo = months; M = male; F = female.

### 2.2. Bioinformatic analyses

Mapping was conducted using the Spliced Transcripts Alignment to a Reference (STAR) software version 2.7.10a [15]. Comprehensive gene annotation data and the human genome sequence of the GRCh38 human reference genome assembly (release 40) accessed from the GENCODE database [16] were used to generate the genome index for mapping. Mapping was performed using paired-end read sequences and STAR default parameters. The aligned reads were sorted by genomic coordinates using the SAMtools package (version 1.13, [17]) and gene expression was quantified using the HTSeq package (version 2.0.1, [18]) to count reads against gene regions.

An independent differential gene expression analysis was conducted, where we utilized physiological fracture callus as the control. A count matrix was created to present, for each sample, the number of raw read counts assigned to each gene. The DESeq2 R package (version 1.40.1, [19]) was used to perform the DEG analysis. The DESeq2 data set object was created using the count matrix and a table containing sample information as inputs, with the control (fracture callus) assigned as the reference level. The Cook's cutoff parameter was set as false. DEGs possessed a $p$-value $\leq 0.05$ (adjusted by the Benjamini-Hochberg method). Those DEGs were further used for gene, reactome and pathway enrichment analyses using DAVID Bioinformatics Resources [20–22].

## 3. Results

### 3.1. mRNA expression in fracture callus and nonunion tissues

RNA-sequence and differential expression analysis of HNU and ONU samples compared to callus control yielded thousands of DEGs. Initially, we obtained 18,124 and 18,863 DEGs for HNU and ONU, respectively (Fig. 1A, B, Supplementary Tables 1 and 2). Volcano plots also indicate the expression of the DEGs for each condition (Fig. 1C, D). Further statistical analyses (with an adjusted $p$-value threshold of 0.05) reduced the numbers of significant DEGs to 67 (0.37 %) for HNU and 81 (0.43 %) for ONU. Out of the 67 DEGs for the HNU, 34 and 33 were up and down-regulated, respectively. Similarly, out of the 81 DEGs for the ONU, 48 and 33 were up and down-regulated, respectively (Fig. 2A-D, Supplementary Table 3). Moreover, the ONU sample clearly shows a greater range of expression with both up and down-regulated DEGs in comparison to the HNU (Fig. 2A, B). Venn analysis also identified common genes between the two nonunion samples with 8 (10.8 %) upregulated and 12 (22.2 %) downregulated (Fig. 2C, D). The identity of these

common genes, their exact expression levels and *p* values are shown in Table 2. The expression level of the upregulated genes ranges from ~0.5 to 2.2-fold while for downregulated genes, the range is ~ −0.9 to −24.1-fold (Table 2). The Venn analysis showing the identity of all up and down-regulated and common DEGs for both HNU and ONU are shown in Supplementary Figs. 1 and 2, respectively.

### 3.2. Biological significance and identification of molecular pathways

To explore the biological significance of the DEGs in the nonunion samples, we conducted biological process and pathway enrichment analyses using DAVID Bioinformatics Resources. Gene Ontology (GO) of significant DEGs from HNU and ONU revealed 39 and 67 biological processes, respectively, (Supplementary Tables 4 and 5). Fig. 3 also shows the results of these analyses and it reveals the common significant biological processes between the two nonunion tissue types. For example, many skeletal muscle related processes were identified and include, muscle contraction, sarcomere organization, circadian rhythm, circadian regulation of gene expression, circadian regulation of translation, positive regulation of transcription from RNA polymerase II promoter, negative regulation of inflammatory response (Fig. 3). Statistical analyses revealed that only the muscle-related processes were significant (Fig. 4A and Supplementary Tables 6 and 7). Further, there are some biological processes, albeit insignificant, that are unique to each sample type; i.e. anterior/posterior pattern specification, negative regulation of transcription from RNA polymerase II promoter, response to ischemia, etc., for HNU (Fig. 3) and cellular response to fibroblast growth factor stimulus, intracellular receptor signaling pathway, cellular response to tumor necrosis factor, negative regulation of NF-kappaB transcription factor activity, ossification involved in bone maturation, etc., for ONU (Fig. 3). Further, this Venn analysis clearly shows that both HNU and ONU share 13 (14 %) common biological processes as well as individual ones, 26 (28 %) for HNU and 54 (58.1 %) for ONU (Fig. 3).

We also used another enrichment approach to identify biological processes in these two nonunion samples. REACTOME analysis revealed common molecular pathways between the two experimental samples. Similar to the aforementioned analysis, REACTOME also identified significant muscle-related processes (Fig. 4B). In addition, there were other processes, albeit insignificant, that were identified that were specific to each nonunion sample. For example, there were several neurotransmitter and related receptors processes for the HNU sample, while the ONU sample showed transcriptional processes such as nuclear receptor transcription pathway, PPARA activates gene expression, signaling by interleukins, and PI3K/AKT signaling (Fig. 5). This Venn analysis also shows the 3 (13.6 %) common pathways but also the 6 (27.3 %) and 13 (59.1 %) that are unique for HNU and ONU, respectively (Fig. 5). We also present data (KEGG analysis) that shows greater details of a significant and common biological process involved in nonunions, skeletal muscle related (Fig. 6). In the schematic, the gene indicated by a blue or red star represents a DEG identified in HNU and ONU, respectively. The genes related to skeletal muscle were all downregulated in the nonunion samples (Fig. 6).

## 4. Discussion

This study was designed to identify DEGs between physiological fracture repair and two nonunion types, hypertrophic and oligotrophic. Overall, the significant molecular differences between physiological fracture callus and nonunion samples were not that different and this

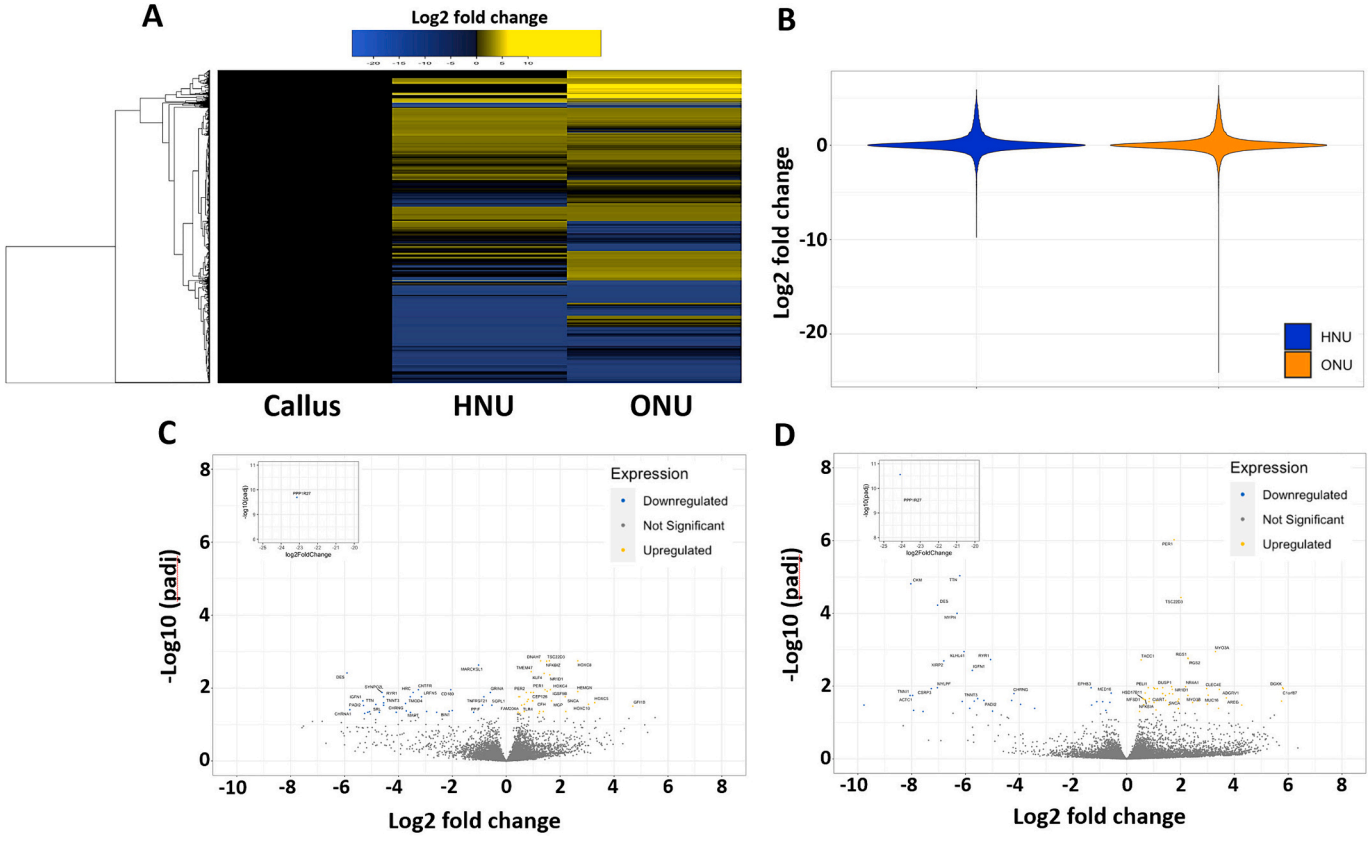


**Fig. 1.** Differential human mRNA expression between control (fracture callus) and nonunion tissues (HNU and ONU). A. Heatmap of all human DEGs identified by RNA sequencing (the average expression signal of the control callus sample was set as baseline). B. Violin graph of expression signals of the human DEGs for each experimental tissue type in comparison to control. C and D. Volcano plots of all DEGS expressed in HNU and ONU, respectively. Plots were generated using R Version 2023.06.0 + 421 (2023.06.0 + 421) and R package gplots version 3.1.3.

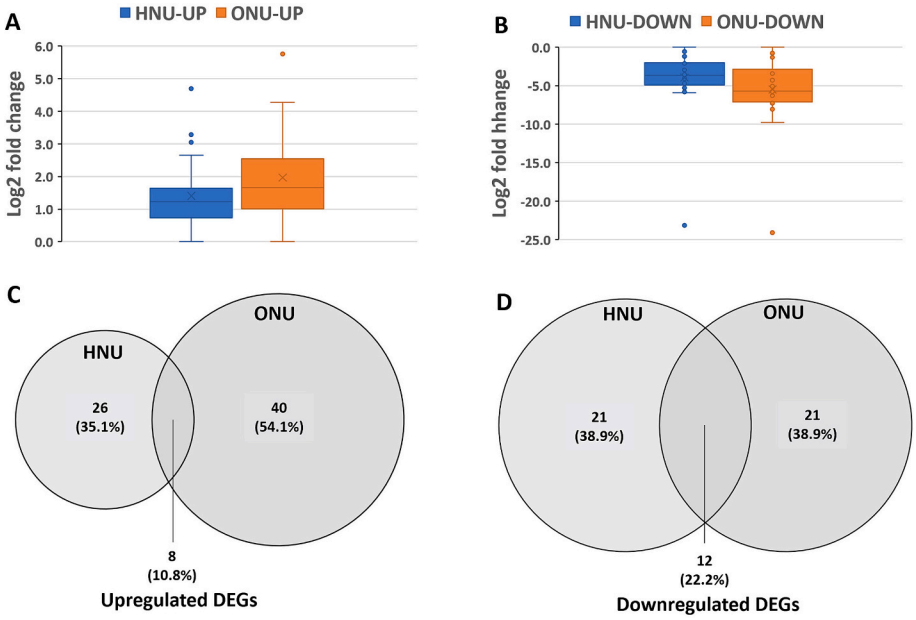


**Fig. 2.** Differential human mRNA expression between control (callus) and nonunion tissues (HNU and ONU). A and B. Boxplot graphs of expression signals of the human miRNAs with either up or down-regulated expression between each experimental tissue type in comparison to control. C and D. Venn diagram indicating common up (C) and down-regulated (D) mRNAs between the two experimental samples (HNU and ONU). Numbers in both C and D indicate the common mRNAs between the two samples. We also show the number of mRNAs that were exclusive to each experimental sample. Plots were generated using R Version 2023.06.0 + 421 (2023.06.0 + 421) and R package ggvenn version 0.1.10.

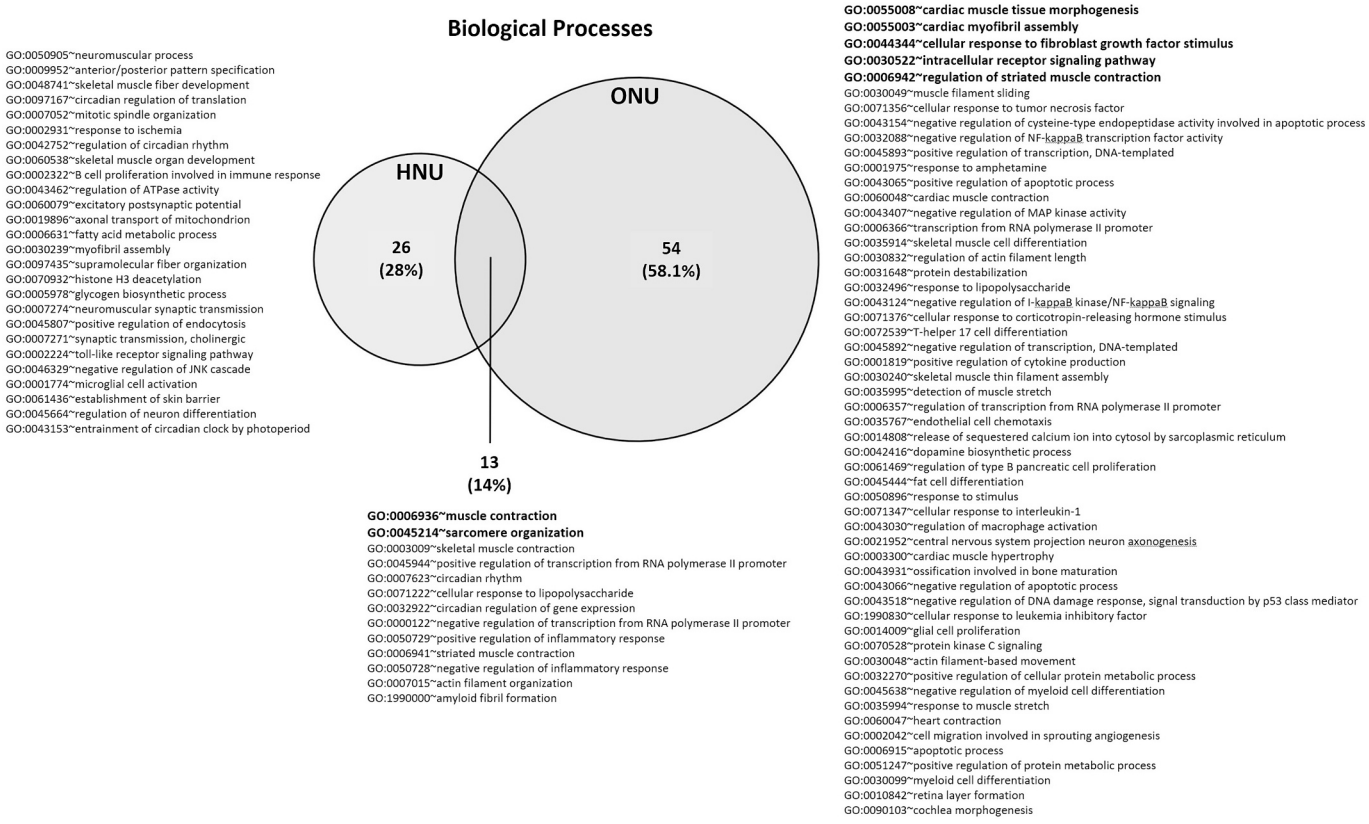


**Fig. 3.** Nonunion-related biological processes. Venn diagram indicating the related biological processes for the two experimental samples (HNU and ONU) as determined by GO. Numbers indicate the biological processes identified between the two samples. We also show both, the exact number and identity of each biological process that were common as well as exclusive to each experimental sample. Bold/larger font indicates statistically significant pathways. Plots were generated using R Version 2023.06.0 + 421 (2023.06.0 + 421) and R package ggvenn version 0.1.10.

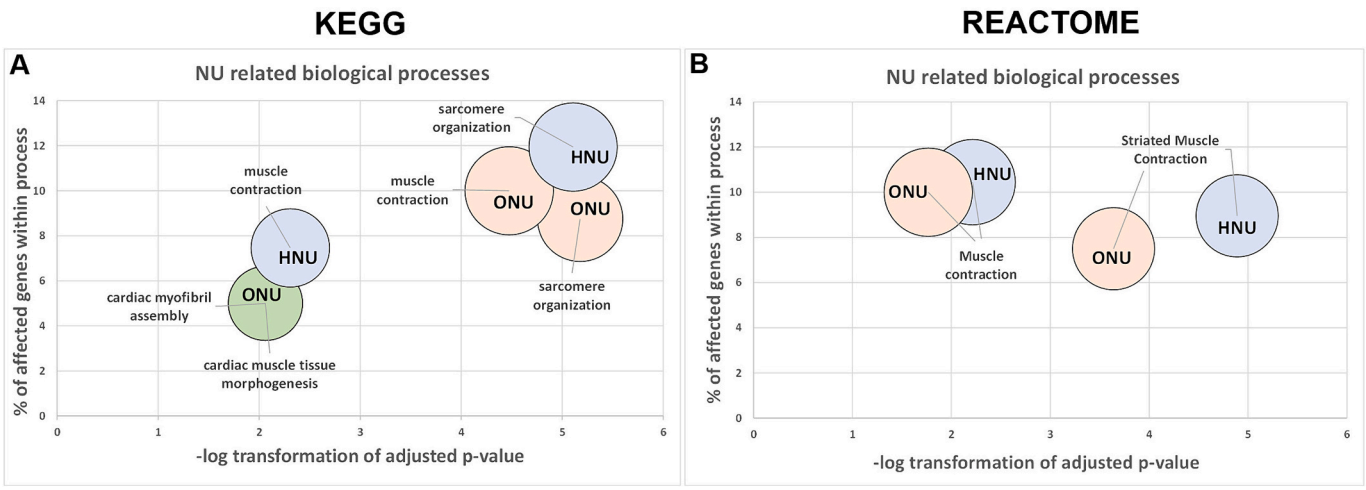


**Fig. 4.** Nonunion-related biological processes. Plots showing biological processes affected by DEGs expressed (both up and down-regulated) in HNU and ONU using two different approaches, KEGG (A) and REACTOME (B) analysis. Salmon/blue color indicates similar processes between the two samples, whereas green is unique to ONU. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Significant and common DEGs between callus vs. NU samples.

| Common Upregulated DEGs | HNU log2 fold change | p value | ONU log2 fold change | p value |
|---|---|---|---|---|
| DUSP1 | 1.216 | 0.00021813 | 1.667 | 9.93E-06 |
| KLF4 | 1.404 | 2.39E-06 | 1.585 | 3.09E-05 |
| NFKBIZ | 1.498 | 6.11E-07 | 1.337 | 1.13E-05 |
| NR1D1 | 1.615 | 2.90E-06 | 1.709 | 3.43E-05 |
| PER1 | 1.438 | 9.23E-06 | 1.759 | 1.08E-10 |
| SNCA | 2.190 | 3.12E-05 | 1.935 | 6.15E-05 |
| TACC1 | 0.496 | 3.36E-05 | 0.536 | 1.40E-06 |
| TSC22D3 | 1.598 | 3.76E-07 | 2.015 | 1.04E-08 |

| Common Downregulated DEGs | HNU log2 fold change | p value | ONU log2 fold change | p value |
|---|---|---|---|---|
| CHRNG | −3.742 | 6.73E-05 | −4.194 | 3.06E-05 |
| DDN | −5.258 | 0.0002176351 | −5.672 | 8.29E-05 |
| DES | −5.911 | 2.06E-06 | −7.034 | 2.03E-08 |
| IGFN1 | −5.323 | 4.95E-05 | −5.742 | 3.13E-06 |
| KLHL41 | −5.079 | 0.0001683932 | −6.050 | 5.76E-07 |
| MARCKSL1 | −1.030 | 9.37E-07 | −0.896 | 9.20E-05 |
| MYPN | −5.142 | 0.0001831169 | −6.311 | 3.97E-08 |
| PADI2 | −5.298 | 9.26E-05 | −5.312 | 7.31E-05 |
| PPP1R27 | −23.134 | 1.34E-14 | −24.101 | 1.56E-15 |
| RYR1 | −4.554 | 3.13E-05 | −5.064 | 1.27E-06 |
| TNNT3 | −4.553 | 6.28E-05 | −5.545 | 5.76E-05 |
| TTN | −4.843 | 7.67E-05 | −6.208 | 1.58E-09 |

was not a surprise since we know that the same tissues (fibrocartilage, cartilage, bone, etc.) are present in all fracture calluses [4,8]. This significant molecular difference between the control (physiological fracture callus) and experimental samples, HNU and ONU, amounted to essentially 0.37 % and 0.43 %, respectively. Thus, we can assume that these samples are essentially the same, but yet the callus tissue in the nonunion samples is not effective in healing the fractured bones of these patients, some suffering with years-long nonunions (Table 1). Despite this high similarity between the different samples, we did identify some specific DEGs and they are discussed below in detail.

In one of the earliest studies, Zimmermann et al. [61], examined differential gene expression of osteogenic and chondrogenic genes in patients with regular and failed fracture healing (did not specify type of nonunion). The authors identified eight genes (CDO1, COMP, FMOD, FN1, CLU, TCS22D1, ACTA2, PDE4DIP) that were significantly elevated two-fold or more in the group with failed fracture healing relative to the normal controls. Similarly, we also found that CDO1, COMP, FMOD, FN1, and CLU were also upregulated in both nonunion samples but the differences in comparison to the control physiological callus were not statistically significant. In contrast, we found that ACTA2 and PDE4DIP were downregulated. TCS22D1 was not present in our samples.

More recently, another study reported on single cell sequencing of intramedullary canal tissue obtained from human patients with femoral nonunions and compared it to native bone controls [23]. Results identified twenty-three distinct cell clusters, with higher monocytes and CD14+ dendritic cells (DCs), and lower proportions of T cells, myelocytes, and promyelocytes in the nonunion samples. Similarly, some of the biological processes and their corresponding genes in our analyses, were indeed immune-related. Examples include, GO:0002322 ~ B cell proliferation involved in immune response, GO:0050729 ~ positive regulation of inflammatory response, and GO:0050728 ~ negative regulation of inflammatory response for HNU and GO:0071356 ~ cellular response to tumor necrosis factor, GO:0050728 ~ negative regulation of inflammatory response, GO:0001819 ~ positive regulation of cytokine production, GO:0071347 ~ cellular response to interleukin-1, GO:0050729 ~ positive regulation of inflammatory response, GO:0045638 ~ negative regulation of myeloid cell differentiation, and GO:0030099 ~ myeloid cell differentiation, for ONU. Although our analyses used physiological fracture callus as a control while the Avin study used native bone, the fact that we identified immune-related genes and processes indicate that inflammation is still active in these nonunion tissues as well, even months and years after the fracture has occurred.

In examining our results collectively, despite the small difference in

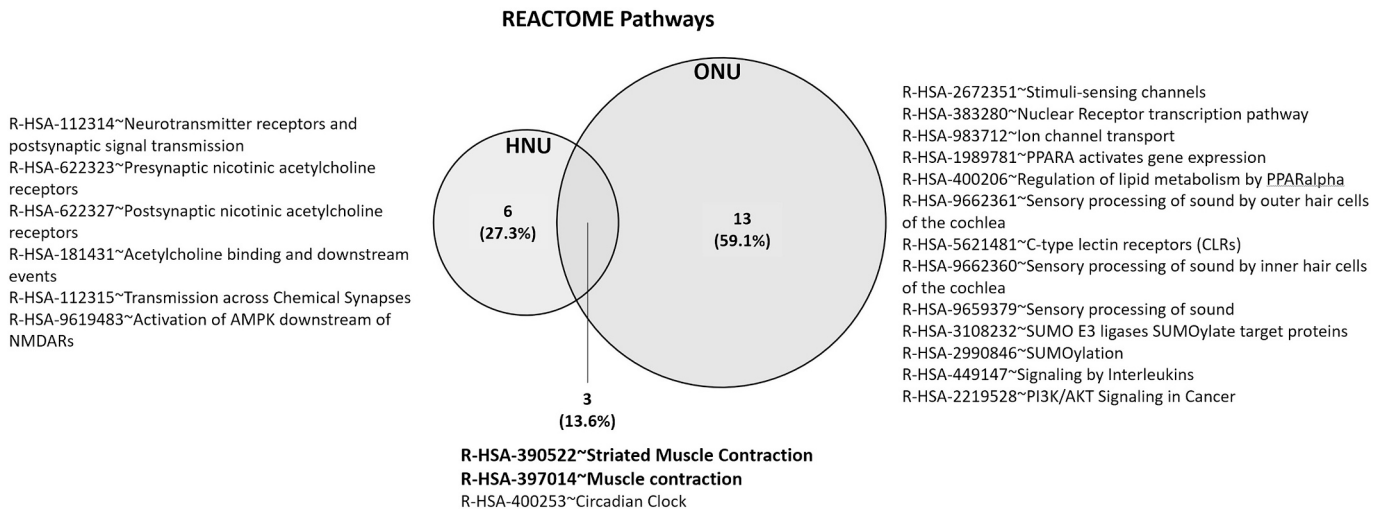


**Fig. 5.** Nonunion-related biological processes. Venn diagram indicating the related biological processes for the two experimental samples (HNU and ONU) as determined by REACTOME. Numbers indicate the biological processes identified between the two samples. We also show both the exact number and identity of each biological process that were common as well as exclusive to each experimental sample. Bold/larger font indicates statistically significant pathways. Plots were generated using R Version 2023.06.0 + 421 (2023.06.0 + 421) and R package ggvenn version 0.1.10.

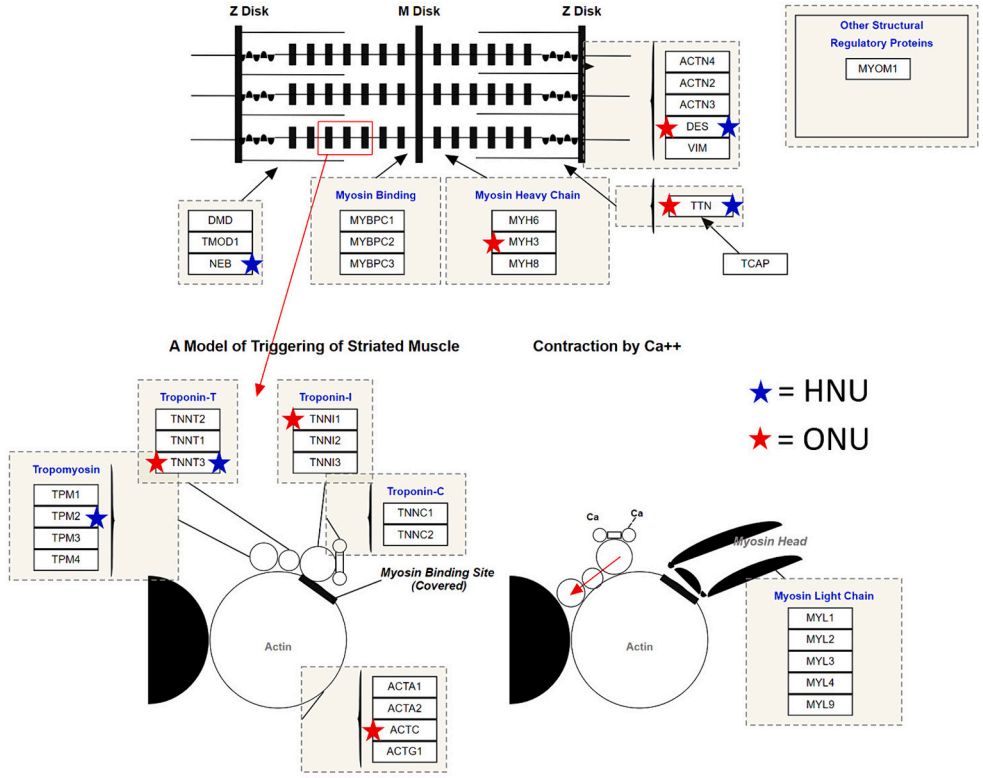


**Fig. 6.** Identification of nonunion DEGs related to a biological process and pathway. Based on the identified DEGs, a Muscle-related pathway is presented. All genes were upregulated. Gene names denoted by a red and blue star indicate genes that were identified in HNU and ONU, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DEGs, we did identify a number of common genes in the nonunion samples that were either up or down-regulated in comparison to physiological fracture callus. The upregulated genes were DUSP1, KLF4, NFKBIZ, NR1D1, PER1, SNCA, TACC1, and TSC22D3. DUSP1 is a dual specificity phosphatase that dephosphorylates MAP kinase MAPK1/ERK2 [24] and was showed to play a role in the regulation of anti-inflammatory genes [25]. In the skeletal system, DUSP1 was shown to be strongly downregulated in patients with rheumatoid arthritis and osteoarthritis [26]. KLF4, Krüppel-like factor 4 is a conserved zinc finger-containing transcription factor that was recently demonstrated to be involved in osteoblast differentiation by serving as a cofactor to RUNX2 to regulate downstream genes. Moreover, KLF4 heterozygous mice displayed decreased osteogenic differentiation resulting in fewer osteoblasts [27].

The NFKBIZ gene encodes the NFKB Inhibitor Zeta (IκB-ζ) protein (principal mediator downstream of NF-κB) which plays a role in

cytokine expression and regulates mononuclear phagocyte system function as well as in NK cells, T cells and B cells [28]. More recently, a couple of studies demonstrated that IκB-ζ is a critical pro-inflammatory mediator in chondrocytes. Specifically, Arra et al. [29] showed that during osteoarthritis, inflammation shifts cell metabolism towards aerobic glycolysis via NF-κB and that lactate dehydrogenase A (LDHA) induces production of reactive oxygen species (ROS) in chondrocytes and that by inhibiting LDHA activity, it results in potent anti-inflammatory and anti-catabolic events via increasing degradation of IκB-ζ by the proteasome. More recently, the same group also demonstrated in a model of osteoarthritis, that bone particle and IL-1β-induced inflammation and subsequent oxidative stress and senescence activate IκB-ζ, which in turn regulates expression of RANKL, inflammatory, catabolic, and senescence-associated secretory phenotype (SASP) genes [30].

NR1D1 (Nuclear receptor subfamily 1 group D member 1, also known as REV-ERBα) and PER1 (Period 1), code for transcriptional repressors which control biological rhythms [31,32]. Recently, it was established that bone metabolism is closely related to mammalian circadian rhythms [33]. In bone, Schilperoort et al. [34] demonstrated the diurnal expression patterns of clock (including REV-ERBα and PER1) and bone-related genes and that if the circadian rhythm is disturbed by shifts in light-dark cycles, it perturbs osteoclast and osteoblast numbers and function. This in turn leads to decreased bone turnover and changes bone mineralization and overall structure that may negatively affect bone strength. REV-ERBα has recently been shown to negatively regulate both osteoblast and osteoclast differentiation through the p38 MAPK signaling pathway [35]. Further, organ culture of fracture femurs from PER2:luciferase knock in mice revealed bioluminescence rhythms of 24-h intervals, and when PTH was administered to these fractured femurs, the peak time of activity in the callus and growth plates was altered, indicating the presence of a PTH-responsive circadian clock [36]. Although this experiment focused on PER2, PER1 was also shown to be responsive to PTH treatment in osteoblasts [37]. Lastly, circadian regulation has been established in various models of tissue regeneration, including, skin, intestinal, and hematopoietic systems [38].

SNCA, Alpha-synuclein, is a member of the synuclein family, neuronal proteins that are associated with the pathology of several neurodegenerative diseases [39]. Although nothing links SNCA to fracture repair, several studies have implicated this gene in ovariectomy-induced bone loss. Calabrese et al. [40], using ovariectomized mice coupled to co-expression network analysis, demonstrated that SNCA is a crucial mediator of specific network module expression and the bone's response to estrogen deficiency. The same research group also demonstrated that SNCA deletion in mesenchymal progenitors (Prrx1+) partially protected mice against weight gain post ovariectomy, but did not prevent bone loss [41]. Lastly, previous research using a mouse strain carrying mutated SNCA and multimerin-1 genes, showed that these mice display significantly lower trabecular bone mass, as well as an increase in osteoclast number and decreased osteoblast mineralization, suggesting that, one or both genes, are involved in bone metabolism [42].

TACC1, Transforming Acidic Coiled-Coil Containing Protein 1, function is related to centrosome and microtubule-associated events and dysregulation of TACC1 has been shown to be associated with multiple malignancies [43]. More recent research revealed that in many cancers, chromosomal translocations result in joining in-frame members of the fibroblast growth factor receptor-TACC gene families (FGFR-TACC gene fusions) and that these gene fusions can generate growth-promoting oncogenes by activating signaling pathways [44]. TSC22D3, also known as glucocorticoid-induced leucine zipper (GILZ), was previously reported to play a role osteogenic differentiation [47,48], as well as in increasing bone mass as a result of enhanced bone formation via interaction with C/EBPs as well as by disrupting C/EBP-mediated Pparγ2 gene transcription [45]. Similarly, when GILZ was overexpressed in bone marrow mesenchymal stem and progenitor cells, it offered protection from TNF-α-induced inflammatory bone loss and subsequently improved bone integrity in mice [46].

As for the twelve downregulated genes, eight, CHRNG, DES, IGFN1, KLHL41, MYPN, RYR1, TNNT3, and TTN, are related to muscle. Why these muscle related genes are expressed in the nonunion calluses and why they are downregulated in comparison to physiological fracture callus remains to be determined. As for the other four downregulated genes, DDN, encodes for dendrin, a kidney related podocyte protein that has been shown to translocate to the nucleus in injured podocytes [49] and attenuates their loss [50]. MARCKSL1, Myristoylated Alanine-Rich C-kinase Substrate like 1, is a ubiquitous membrane-associated protein that plays a role in many processes, including, regulation of the actin cytoskeleton and thereby affecting, motility, adhesion, chemotaxis, phagocytosis and exocytosis [51]. More importantly, this protein plays a role in development and regeneration [52]. And as it relates to bone regeneration, it was shown that this protein can be released extracellularly and induces the initial cell cycle response during axolotl appendage and tail regeneration, especially in muscle-derived cells. As such, it induces cell proliferation and ultimately affects blastema length [53].

PADI2 encodes for peptidyl arginine deiminase 2 which catalyze the post-translational deamination (citrullination) of proteins, converting arginine into citrulline [54]. A number of studies have been published on the expression of PADI2 in bone, specifically in bone marrow CD34+ cells and rheumatoid arthritis [55], and in osteoblasts where it was also showed that PADI2 is a regulator of ROS-accelerated senescence [56]. Lastly, in another study, the same research group demonstrated that PADI2 deficiency in osteoblasts results in decreased bone mass, cleidocranial dysplasia and delayed calvarial ossification and clavicular hypoplasia, all due to impaired osteoblast differentiation [57]. The PPP1R27 gene is by far the most downregulated (~24–25 fold) significant gene in both nonunion samples. It encodes for protein phosphatase 1 regulatory subunit 27 and only three transcriptomics studies appear in the literature, one dealing with expression in horse muscle [58], hair follicle development in sheep [59], and lung squamous cell carcinoma [60]. Lastly, for some of these DEGs (e.g. TACC1, TSC22D3, DDN, MARCKSL1, PPP1R27) nothing is known about their temporal and spatial expression nor function in the skeleton, thus, their investigation during normal bone development and especially, fracture repair is warranted.

Despite the interesting data we generated, our study also exhibits several limitations. For example, our samples were not all of the same sex and age, nor was the duration of the callus (time since fracture), leading to tissue/patient variability within each group and also between groups. Further, our samples only consisted of hypertrophic and oligotrophic, but not atrophic (which are rarer) nonunions. Similarly, the tissue samples were collected from various bones (e.g. femur, tibia, ulna, wrist, humerus) as opposed to a single type which make the calluses more heterogeneous. Unfortunately, these are difficulties we encounter when working with human samples as they are harvested as patients become available for surgery. Experimental verification should also be performed for the significantly DEGs identified; specifically, their spatial localization within the fracture callus so that they can be linked to a cell type and thereby, a specific process (i.e. inflammation, angiogenesis, osteogenesis, chondrogenesis, ossification, remodeling, etc.). Experiments in our lab are underway now. Extending these results to more controlled experiments using rodents will verify the veracity of this human data and enable us to further probe the significant role of some of these DEGs during fracture repair. Lastly, as some of these genes have never been examined in the skeleton, their investigation could potentially lead to new bone and cartilage-related molecular discoveries intrinsically linked to human fracture repair.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117091.

## CRediT authorship contribution statement

**Leonidas Salichos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Formal analysis, Data curation. **Rishika Thayavally:** Software, Methodology, Formal analysis, Data curation. **Peter Kloen:** Writing – review & editing, Writing – original draft, Resources. **Michael Hadjiargyrou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

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*Declaration of generative AI in scientific writing*

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