## Title

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# Age-related decrease in periostin expression may be associated with attenuated fracture healing in old mice

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## Abstract

Older adults suffer more bone fractures with higher rates of healing complications and increased risk of morbidity and mortality. An improved understanding of the cellular and molecular mechanism of fracture healing and how such processes are perturbed with increasing age may allow for better treatment options to manage fractures in older adults. Macrophages are attractive therapeutics due to their role in several phases of fracture healing. After injury, bone marrow-derived macrophages are recruited to the injury and propagate the inflammatory response, contribute to resolution of inflammation, and promote bone regeneration. A tissue resident population of macrophages named osteal macrophages are present in the periosteum and are directly associated with osteoblasts and these cells contribute to bone formation. Here, we utilized bulk RNA sequencing to analyze the transcriptional activity of osteal macrophages from old and young mice present in primary calvarial cultures. Macrophages demonstrated a diverse transcriptional profile, expressing genes involved in immune function as well as wound healing and regeneration. Periostin was significantly downregulated in macrophages from old mice compared to young. Periostin is an extracellular matrix protein with important functions that promote osteoblast activity during bone regeneration. An age-related decrease of periostin expression was verified in the fracture callus of old mice compared to young. Young periostin knockout mice demonstrated attenuated fracture healing outcomes that reflected what is observed in old mice. This work supports an important role of periostin in fracture healing, and

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Author Contribution:

Daniel Clark, Theodore Miclau, Mary Nakamura, and Ralph Marcucio contributed to study conception. Daniel Clark, Mary Nakamura, and Ralph Marcucio designed the experiments. Daniel Clark, Jeffrey Doelling, and Diane Hu executed the experiments. Daniel Clark, Jeffrey Doelling, Diane Hu, Mary Nakamura, and Ralph Marcucio conducted data analysis and interpretation. Theodore Miclau, Mary Nakamura, and Ralph Marcucio provided financial support and materials via grant funding. Daniel Clark prepared initial draft of manuscript. All authors have read and approved the final submission.

therapeutically targeting the age-related decrease in periostin may improve healing outcomes in older populations.

**Keywords**

periostin; macrophage; fracture healing; RNA sequencing

## 1. Introduction

Fracture healing is initiated by a robust inflammatory response that is followed by a tightly regulated and complex process of tissue regeneration and bone remodeling. The contribution of immune cells throughout these processes has been shown to be vital for successful healing outcomes.[1] The role of macrophages in the propagation and resolution of inflammation during fracture healing has been well characterized.[2,3] In addition, macrophages have been shown to contribute to regeneration of bone, in part, through direct interactions between osteal macrophages and osteoblasts.[4] Osteal macrophages are the resident macrophage population within bone, residing largely in the periosteum.[4] Primary calvarial cultures of osteoblasts contain a population of osteal macrophages, comprising approximately 15% of the total cells in culture.[4] Depletion of macrophages results in decreased mineralization in vitro and attenuated fracture healing in vivo, which supports the role of osteal macrophages contributing to bone regeneration.[4,5] However, the mechanisms by which osteal macrophages promote osteoblast activity during regeneration are not fully understood. The diverse roles of macrophages make this cell type an attractive target for therapeutic intervention, but more research is necessary to better understand how macrophages contribute to successful fracture healing.

Poor fracture healing outcomes are associated with increased age.[6,7] The reason for the age-related healing perturbation is likely multifactorial and inflammatory dysregulation, altered progenitor cell populations, and decreased blood supply have been thought to contribute to the poor outcomes.[6,7] We have previously shown that age-related changes to macrophages result in local inflammatory dysregulation that perturbs successful fracture healing.[8] However, we have not examined to what extent age-related changes to macrophages affect their contribution to bone regeneration during fracture healing.

To improve our understanding of macrophage activity during bone regeneration, we utilized high throughput RNA sequencing (RNAseq) to identify the transcriptional activity of osteal macrophages during mineralization. Here, osteal macrophages are defined as the macrophages isolated from primary calvarial cell cultures, as previously shown.[4] Comparison of the transcriptional activity of osteal macrophages isolated from old mice versus young mice identified significant age-related changes including a marked decreased periostin expression. Periostin is a key extracellular matrix protein that provides structural support in bone and acts as a signaling molecule that promotes osteoblast recruitment, migration, and function.[9,10] We further validated the age-related decrease of periostin expression in the fracture callus of old mice and demonstrated that old wild-type mice and young periostin null mice ($Postn^{-/-}$) had similar delays in fracture healing. Such findings

demonstrate a potentially important contribution of macrophages to fracture healing and suggest that periostin may be a possible therapeutic target to promote fracture healing in aging populations.

## 2. Methods

### 2.1 Animals

All procedures were approved by the UCSF Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines for humane animal care. All results have been reported in accordance with the ARRIVE guidelines. Old and young wild type mice (C57B6/J) were obtained from the National Institute on Aging's Aged Rodent Colony. Periostin null mice ($Postn^{-/-}$) were obtained from Jackson Laboratory (Stock No: 009067). All mice were maintained and bred in barriers within our colony and utilized for experiments at 24 months (old adult mice) or 3 months (young adult mice) of age.

### 2.2 Primary cell isolation and culture

Cells were isolated from the calvaria of old and young mice. Cells were not pooled and were cultured as separate samples for each mouse. Mice were first euthanized and the calvaria was exposed. Cuts were made along the parietal bones and the calvaria was isolated free from overlying connective tissue and placed in sterile PBS. Bone samples were transferred to a 1mg/ml Collagenase A solution (Roche, Basel, Switzerland) in α-MEM media (Gibco, Thermo Scientific, Waltham, MA) and digested at 37° C in a shaking water bath for 30 minutes. The supernatant from the first round of digestion was discarded. Digestion was repeated 4 times. After each digestion period the supernatant was collected, strained over a 70 μm cell strainer, pelleted by centrifugation, and then resuspended in complete α-MEM media (10% FBS, 100U/ml penicillin, 100 ug/ml streptomycin). Cells were cultured to approximately 70% confluency and then passaged. After the first passage, cells were seeded in duplicate in 6 well plates and were cultured in osteogenic media (complete α-MEM with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate). Media was replaced every 2 days for 10 days. At 10 days of culture in osteogenic media, one well from each sample was used for Von Kossa staining to confirm the presence of mineralization. In the other well for each sample, cells were detached from the surface with the addition of 0.05 % Trypsin-EDTA solution (Thermo Scientific, Waltham, MA), washed, and isolated for Bulk RNA sequencing as described below.

### 2.3 Bulk RNA Sequencing

Cells were removed from culture and resuspended in incubation buffer (0.5% BSA in PBS). Flow cytometry was utilized to isolate the macrophages. Cells were first blocked using 10% rat serum for 10 minutes and then stained with directly conjugated fluorescent antibodies: CD45 (clone 30-F11), CD3 (145–2C11), B220 (RA3682), NK1.1 (PK136), Ly6G (clone 1A8), F4/80 (clone BM8), and CD11b (clone M1/70) (Biolegend, San Diego, CA). Staining with Fixable Red Dead (Thermo Fisher, Waltham, MA) was used for the detection of dead cells. Isotype controls and fluorescence minus one controls were used to gate for background staining. For macrophage selection, CD3+, B220+, NK1.1+, and Ly6G+ cells

were excluded and CD45+, CD11b+, F4/80+ cells were collected. Cells were sorted on a FACSAria (BD Biosciences, San Jose, CA). FlowJo Software 9.6 (Treestar, Ashland, OR) was used for analysis. Supplementary Figure 1 demonstrates representative flow diagrams used for macrophage isolation.

Macrophages that were collected were prepared for RNA sequencing as described previously[8]. RNA was extracted using Invitrogen RNA aqueous Micro Kit (AM1931). The library was prepared using Illumina Truseq Stranded mRNA Library Prep Kit and Single-end 50 bp RNAseq was performed on Illumina HiSeq 4000. An average read depth of 53.3 million reads per sample was generated. Reads were aligned using STAR_2.4.2a to the mouse genome (Ensemble Mouse GRCm38.78). Reads that mapped uniquely to known mRNAs were used to assess differential expression. Differential gene expression was assessed using DEseq2. Gene ontology and pathway analysis was performed using DAVID (http://david.abcc.ncifcrf.gov). The normalized read counts generated from the RNA sequencing study data are openly available at https://figshare.com/s/f979a8b89f9a6621ba69.

### 2.4 Fractures

Mice were anesthetized with a 1:1 mixture of ketamine (60 mg/kg) and dexmedetomidine (0.3 mg/kg) delivered intraperitoneally and subjected to closed, non-stable fractures of the right tibia created by three-point bending, as previously described.[2,8] Analgesics (0.05–0.01 mg/kg buprenorphine) were administered subcutaneously post-surgery and mice were permitted to ambulate freely with no stabilization of the fractured tibia. Mice were allowed to heal for 5 or 10 days, euthanized, and tissue was collected for analysis as described below. Health and welfare of mice were monitored throughout healing period and no adverse events were reported.

### 2.5 qRT-PCR

Young, old, and Postn$^{-/-}$ mice (n=5/group) were euthanized at 5 days post fracture. The fracture callus was dissected and placed in Trizol and homogenized. RNA extraction was performed with isopropanol. cDNA was reverse transcribed using Superscript III (Invitrogen, Thermo Scientific, Waltham, MA). Quantitative real time polymerase chain reaction (qRT-PCR) was performed on technical triplicates. Relative gene expression of Periostin, Twist, Bglap, Runx2, Col1a1, Col2a1, and Col10a1 were quantified. The ΔCT was calculated between each gene of interest and GAPDH, which was used as the house keeping gene. Data is presented as fold change ($2^{-\Delta\Delta CT}$). See Supplementary Table 1 for full list of primers used.

### 2.6 Histology and Immunohistochemistry

Young, old, and Postn$^{-/-}$ mice (n=6/group) were euthanized at 10 days post fracture. The fractured tibiae were collected and fixed for 24 hours in 4% paraformaldehyde and then decalcified in 19% EDTA for 14 days. Samples were subsequently prepared and embedded in paraffin. Serial sagittal sections (10μm) were made using a microtome (Lecia, Bannockburn, IL) and mounted on glass slides. Sections were stained using Hall Brunt Quadruple Stain (HBQ) to visualize bone and cartilage.

Identification of macrophages and osteoclasts followed protocols previously described by our group[12]. Briefly, for the identification of F4/80+ macrophages, the F4/80 primary antibody (rat anti-mouse, BD Biosciences) was incubated at a 1:200 dilution in 5% goat serum overnight at 4°C on representative sections. The secondary anti-body, HRP conjugated goat anti-mouse IgG, was applied at 1:500 dilutions and incubated for 1 hour. VectaStain ABC Kit (Vector) was applied and sections were stained with 3,3'-diaminobenzidine (DAB) substrate for visualization. Identification of osteoclasts was performed on representative histological sections via tartrate-resistant acid phosphatase (TRAP) staining using the acid phosphatase leukocyte kit (Sigma-Aldrich) according to manufacturer's recommended protocol.

Immunohistochemistry for periostin expression was completed on representative histological sections. Antigen retrieval was first performed using 10 mM sodium citrate buffer (20 min, 100°C). Endogenous peroxidase blocking in 3% H2O2 (30 min, room temperature) was next completed, followed by nonspecific epitope blocking with 5% goat serum (1 h, room temperature). The primary antibody (periostin/OSF-2, RD Systems) was incubated at 1:100 dilution in 5% goat serum overnight at 4 C. The secondary antibody (Rabbit anti-goat, Alexa Fluor 594) was applied at 1:100 dilution and incubated for 1 hour.

### 2.7 Stereology

To quantify the callus volume and the volume of bone and cartilage within the callus, an Olympus CAST system (Center Valley, PA) and software by Visiopharm (Hørsholm, Denmark) was used to quantify tissue volumes according to stereological methods developed by Howard and Reed.[11] Low magnification of the entire fracture site was utilized to first outline the region of interest. Using 20x magnification, the bone and cartilage tissue were identified and labeled. The Cavalieri formula was used to quantify the absolute volume of the total callus, bone, and cartilage tissue as previously described.[8]

### 2.8 Statistics

Between group comparisons were made by first using a 2-way ANOVA multiple comparisons test followed by a 2-tailed Student's t test. Statistical significance was considered at $P<0.05$. For the data generated from bulk RNA sequencing, differential gene expression was considered significant at FDR<0.05. For term enrichment in gene ontology and KEGG pathway analysis the level of significance was set using a modified Fisher Exact P-value of $p<0.05$. GraphPad Prism v.7 software was used for analysis.

## 3. Results

### 3.1 Macrophages in culture with osteoblasts during mineralization demonstrate a heterogenous transcriptional profile

Primary cell cultures were obtained from the calvaria of old and young mice and placed in osteoblastic media. After 10 days in culture, extracellular matrix deposition and mineralization was observed via Von Kossa staining in both old and young mice. At day 10 of culture, the osteal macrophages were isolated and analyzed via bulk RNA sequencing. Analysis was completed on 3 old and 4 young samples. One sample was removed from

analysis after quality control measures demonstrated contamination of macrophages with other cell types.

Analysis of the RNA sequencing demonstrated that there was significant transcriptional heterogeneity of osteal macrophages in culture with osteoblasts during periods of mineralization. The top 10,000 expressed genes were identified and used for further analysis (Supplementary Table 2). Gene ontology (GO) was utilized to analyze the biological processes and pathways upregulated in the transcriptome of osteal macrophages. GO analysis demonstrated an enrichment of genes involved in adaptive and innate immune response and other processes characteristic of the immune functions of macrophages (Figure 1A). Additionally, regenerative processes such as cell migration, osteoblast differentiation, and angiogenesis were enriched in the transcriptome (Figure 1A). Individual genes expressed by osteal macrophages that are involved in immune function and regeneration are shown in Figure 1B,C. The immune function genes included chemokines (Ccl2, Ccr2), cytokines (Il1β), and receptors for pro-inflammatory pathways (Tlr2, Tlr4) and anti-inflammatory pathways (IL10ra, Trem2). Genes expressed by osteal macrophages that are involved in regenerative process include those involved in bone and cartilage formation (BMP2, BMP7, Runx2), extracellular matrix formation (Col1a1, Col2a1, Periostin), and angiogenesis (Vegf, Pdgf). Taken together, the genes expressed by osteal macrophages in co-culture with osteoblasts during mineralization have a heterogenous profile that includes the promotion of immune functions and the promotion of functions that support bone regeneration.

### 3.2 Significant transcriptional age-related changes are present in osteal macrophages

We next analyzed how the gene expression in osteal macrophages derived from young mice compared to old. Differential gene expression analysis demonstrated that 126 genes were significantly differentially expressed in osteal macrophages from old mice compared to young (FDR<0.05) (Figure 2A) (Supplementary Table 2). 123 of the differentially expressed genes (DEGs) were significantly upregulated in old mice compared to young (Figure 2B). GO analysis demonstrated that the DEGs upregulated in osteal macrophages from old mice were enriched for immune functions including inflammatory response and T cell receptor signaling, suggesting an age-related upregulation of pro-inflammatory functions of macrophages (Figure 2C). There were 3 DEGs that were significantly downregulated in old mice compared to young (Cd209a, Mest, Periostin) (Figure 2B). Periostin is an extracellular matrix protein that has been shown to promote osteoblast recruitment, attachment, and differentiation.[9] The implications of an age-related decrease in periostin expression during fracture healing was unknown.

### 3.3 Periostin expression within the fracture callus is decreased in old mice compared to young

We next validated the age-related decrease of periostin within the fracture callus. Nonstable closed fractures were created in old and young mice (n=5/group). The fracture callus was isolated at 5 days post fracture and analyzed via immunohistochemistry (IHC) and qRT-PCR. Periostin expression was present in the fracture callus of old and young mice at the periosteal and endosteal surfaces of long bone and the newly forming trabecular

structures (Figure 3A). The expression of periostin was significantly less in the callus of old mice compared to young ($p<0.05$) (Figure 3B). The expression of the transcription factor regulating periostin activity, Twist, was not affected as a function of age (Figure 3B). An age-related decrease in other genes that encode for extracellular matrix proteins and involved in osteochondral regeneration (Col1a1, Col2a1, Col10a1) was also observed in the fracture callus of old mice (Figure 3C). Expression of Cola1a1, Col2a1, and Col10a1 in the callus of young periostin null mice ($Postn^{-/-}$) was comparable to young wild type mice (Figure 3D). Similar to the young wild type mice, $Postn^{-/-}$ mice had significantly increased expression of the same collagen genes compared to old mice (Figure 3E).

### 3.4 Fracture healing is attenuated in old and young $Postn^{-/-}$ mice

To understand the effect of decreased periostin expression on fracture healing, we compared the extent of fracture healing in young, old, and young $Postn^{-/-}$ mice. Histological analysis shows that endochondral ossification occurred in the $Postn^{-/-}$ mice (Figure 4A,B,C). Higher magnification demonstrates evidence of new bone formation and a transition from cartilage to bone in $Postn^{-/-}$ mice (Figure 4C). Representative histological slides of the fracture callus in young, old, and $Postn^{-/-}$ mice illustrate the observable differences in healing at 10 days post fracture (Figure 4D,E,F). Stereological analysis of serial histological sections of the healing callus at 10 days post fracture showed that $Postn^{-/-}$ mice had significantly smaller callus volume with significantly decreased bone and cartilage volume compare to the age-matched wild type young mice ($p<0.05$) (Figure 4G,H,I). The extent of fracture healing in old mice was similar to $Postn^{-/-}$ mice with a significant decrease in bone and cartilage volume compared to young mice (Figure 4H,I). Macrophage infiltration into the fracture callus and osteoclast activity appeared unaffected by age or by knockout of periostin, as demonstrated by F4/80 and TRAP staining of histological sections from the fracture callus (Supplementary Figure 2).

## 4. Discussion

In this study, we utilized next generation sequencing to characterize the heterogeneous gene signature of osteal macrophages that support osteoblastic activity in vitro. These findings further support the important role of macrophages in contributing to bone fracture healing. The study also confirms the important role of periostin in fracture healing[9], and, for the first time, identifies an age-related decrease in periostin expression as a potential contributing factor to age-related attenuation of fracture healing.

Primary calvarial cell cultures have previously been shown to contain a vital osteal macrophage population.[4,13] The support of mineralization by these macrophages is important, as studies have shown that removal of these macrophages from the primary calvarial culture reduces the osteogenic potential of the remaining osteogenic cells.[4] Similarly, other studies have shown that depletion of macrophages resulted in the reduction of osteoblast differentiation and an associated perturbed fracture healing outcome.[5,14,15] However, the mechanism through which macrophages support osteoblastic activity is not fully understood.

Towards a better understanding of how osteal macrophages support osteogenic cells, we utilized RNAseq to characterize the transcriptional activity of macrophages during periods of mineralization in vitro. We found that the transcriptional profile of the cells supports immune and regenerative functions. GO analysis demonstrated that genes were enriched for immune functions that are known to be characteristic of macrophage activity, including both the propagation and resolution of inflammation. Such immune functions are critical during the early phases of fracture healing.[16] Additionally, genes were enriched for regenerative functions that can support wound healing and bone regeneration. These cells were shown to express Bmp2 and Bmp7, which are known to promote osteoblast differentiation and activity.[17,18] Additionally, osteal macrophages expressed genes that promote angiogenesis (Pdgf, Vegf), which is critical for angiogenesis and adequate fracture repair.[19,20] Other studies have similarly shown that macrophages express these genes.[21–24] Interestingly, we found that osteal macrophages expressed genes encoding extracellular matrix proteins (Col1a1, Col2a1, Col3a1). It is unknown to what extent macrophage-derived collagen contributes to fracture repair as there is little research on the subject. However, collagen expression and production by macrophages has been shown in other tissues.[22,25,26]

The in vitro cell culture system used here allows us to examine more closely the interaction of osteal macrophages and osteogenic cells during mineralization. However, it is limited, as all in vitro systems are, by omitting the highly complex in vivo environment. During fracture healing, macrophages arise from a variety of sources and acquire different phenotypes as multiple subpopulations have been described with diverse functions and developmental origins. In addition to diverse origins, they appear to have different activities.[27] How the transcriptional profile of macrophages analyzed here is representative of the diverse subpopulations and phenotypes of macrophages present throughout fracture repair in vivo is unknown.

This study also examined the effect of age on macrophages. Age-related changes in macrophages have been implicated in the pathogenesis of multiple diseases.[28] Here, we demonstrated significant age-related differences in osteal macrophage transcriptional profiles during mineralization in vitro. In the RNAseq dataset, GO analysis of the DEGS upregulated in macrophages from old mice showed that they were enriched for pro-inflammatory functions. This finding is consistent with our previous work showing that age-related changes to macrophages result in inflammatory dysregulation that was detrimental to fracture healing outcomes in experimental mouse models.[8] Only three genes were significantly downregulated as a function of age (Cd209a, Mest, Periostin). Cd209a is the murine homologue for dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), which functions in the initiation of adaptive immunity.[29] Mest is a protein coding gene for a member of the alpha/beta hydrolase superfamily with nonspecific enzymatic function.[30] Periostin is an extracellular matrix protein which has an important role in the early regenerative phase of fracture healing[9]. Periostin is a structural protein of the bone extracellular matrix and interacts with osteoblast to promote migration and differentiation.[10] In mouse models, periostin expression increases significantly within bone cells in response to injury.[31]

Here, we demonstrated attenuated fracture repair in old mice compared to young. A large body of research using animal models and data from human studies strongly support attenuated fracture repair associated with increased age, but the reason for the attenuated repair is not fully understood and is likely multifactorial.[2,6,32–34] We further showed that the attenuated fracture repair was associated with decreased periostin expression within the callus tissue. An age-related decrease in periostin expression has previously been demonstrated in adipose tissue[35] and skin[36]. This study is the first to demonstrate an age-related decrease of periostin within bone and its potential contribution to attenuated fracture repair in aged animals. Macrophages have previously been shown to be a source of periostin.[22] We were unable to directly link the reduced periostin expression in the callus to the reduced expression by the macrophages that was demonstrated in the RNAseq analysis. There are multiple cellular sources of periostin within bone, including periosteal cells that may have been affected by age in a similar manner.[31] Future studies may better demonstrate the specific role of macrophage-derived periostin by isolating macrophages from the facture callus via flow cytometry and quantifying periostin transcription or protein expression and by using conditional inactivation to remove periostin expression directly from macrophages.

Interestingly, knockout of periostin in mice demonstrated similar fracture healing outcomes as old mice. As we showed that periostin was decreased in old mice, this finding supports an age-related decrease in periostin contributes to the attenuation of fracture healing. Others have also evaluated fracture healing in Postn$^{-/-}$ mice and have shown similar delayed fracture healing compared to controls.[31] Figure 4 shows that endochondral ossification was still occurring in mice lacking periostin. However, there was significantly less cartilage and bone within the healing fracture callus of Postn$^{-/-}$ mice compared to their aged-matched controls, suggesting that the defect in healing was in the accretion of callus tissues. The analysis was performed at a single time point, and analysis of additional time points in future work may provide interesting insight into the attenuated healing response throughout the dynamic stages of fracture healing.

Periostin has been shown to regulate collagen cross linking within tissue.[10] However, the reduction in the expression of the collagen genes in the fracture callus of old mice was independent of decreased periostin expression, as Postn$^{-/-}$ mice demonstrated a similar level of collagen expression as the young wild type controls. The age-related reduction in the extracellular matrix genes measured within the callus (Periostin, Col1a1, Col2a1, Col10a1) points to a possible age-related perturbation of the formation of extracellular matrix of bone after injury that may be either an important contributor, or just a measure of the age-related attenuation of fracture repair.

Any attenuation or delay in fracture healing in older adult populations can have severe consequences, as rates of morbidity and mortality are increased with longer hospital stays and periods of immobility.[7] Therefore, there is a clinical need to prevent the age-related attenuation of fracture healing in older adults in order to increase their health span and lifespan. Towards this, a better basic molecular and cellular understanding of the age-related changes to fracture healing is needed. Understanding the multifactorial nature of the changes that contribute to reduced healing in aged animals may lead to a more complete understanding of how age affects fracture healing outcomes in older adults.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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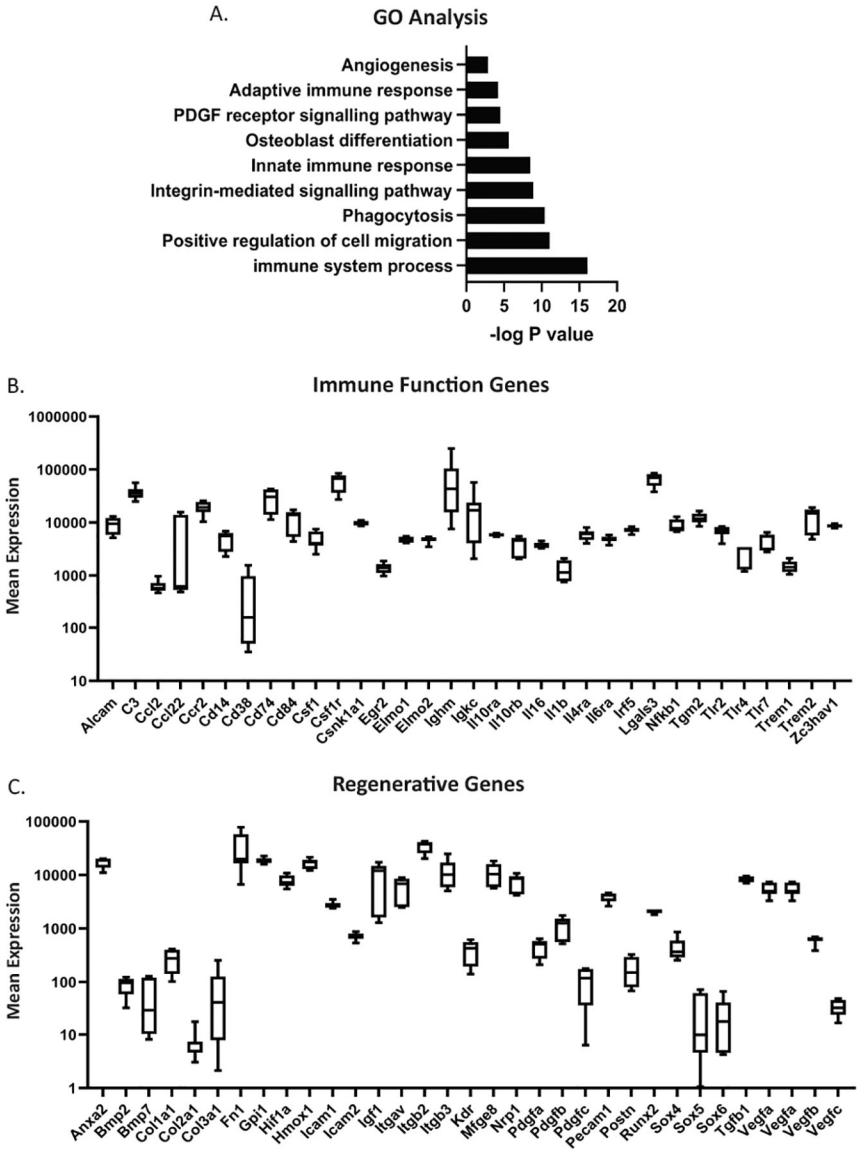


**Figure 1.**

Macrophages in culture with osteoblasts during mineralization demonstrate a heterogenous transcriptional profile. Macrophages isolated from primary calvarial cell cultures after 10 days in osteogenic medium were analyzed via bulk RNAseq. **(A)** Gene ontology demonstrated biological processes and pathways upregulated in the transcriptome of macrophages. **(B)** Mean expression of genes involved in immune function and **(C)** regeneration are shown. Mean expression was calculated across all old and young samples (n=3 old, 4 young).

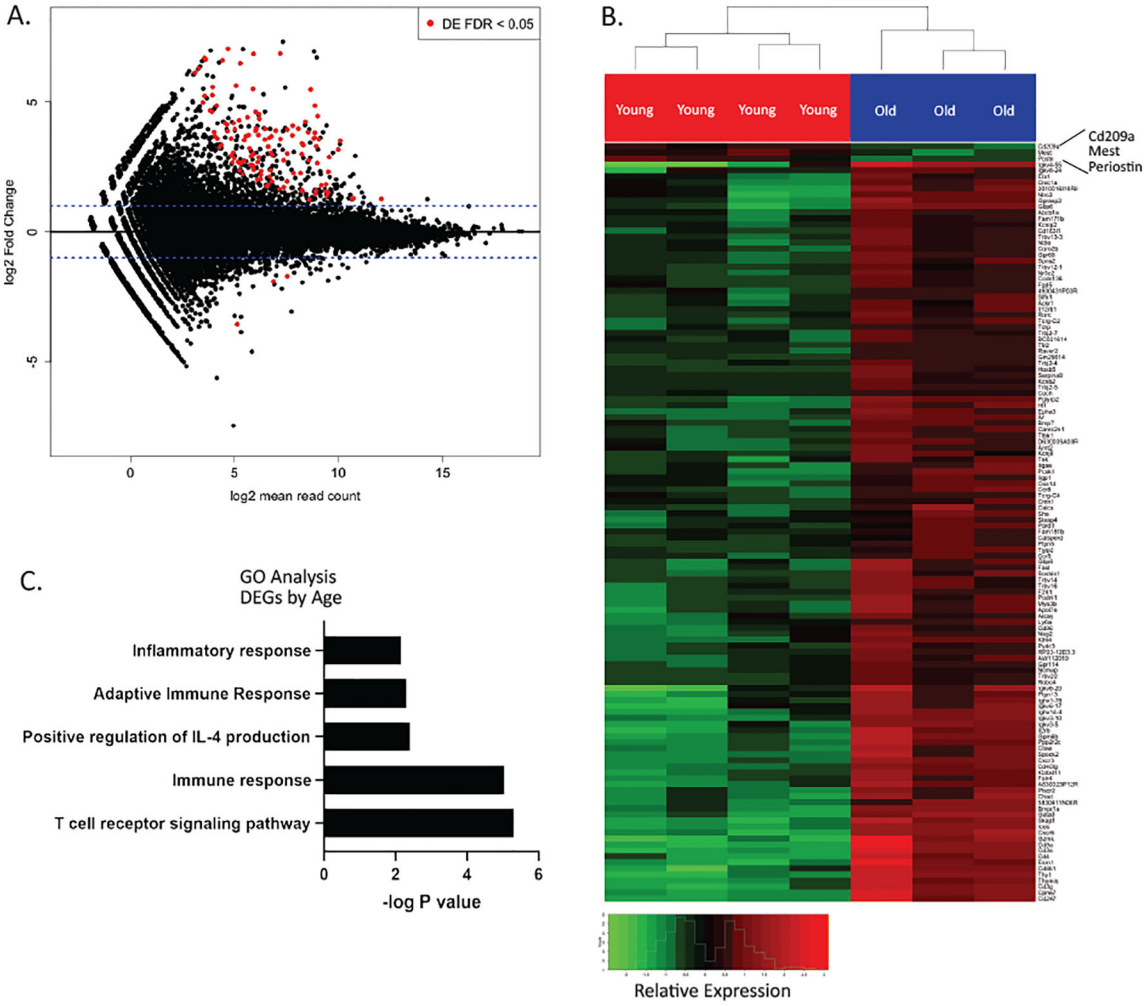


**Figure 2.**

Significant transcriptional age-related changes are present in macrophages. **(A)** Differentially expressed genes (DEGs) were analyzed in macrophages from old mice compared to young via bulk RNAseq. MA plot demonstrates 126 genes were significantly differentially expressed (red dots) (FDR<0.05). **(B)** Heatmap demonstrates hierarchical clustering of old and young mice based a relative expression of each DEGs across all samples. Only 3 genes (Cd209a, Mest, Postn) were significantly downregulated in macrophages from old mice compared to young. **(C)** Gene ontology analysis of the DEGs upregulated in macrophages from old mice were enriched for immune functions.

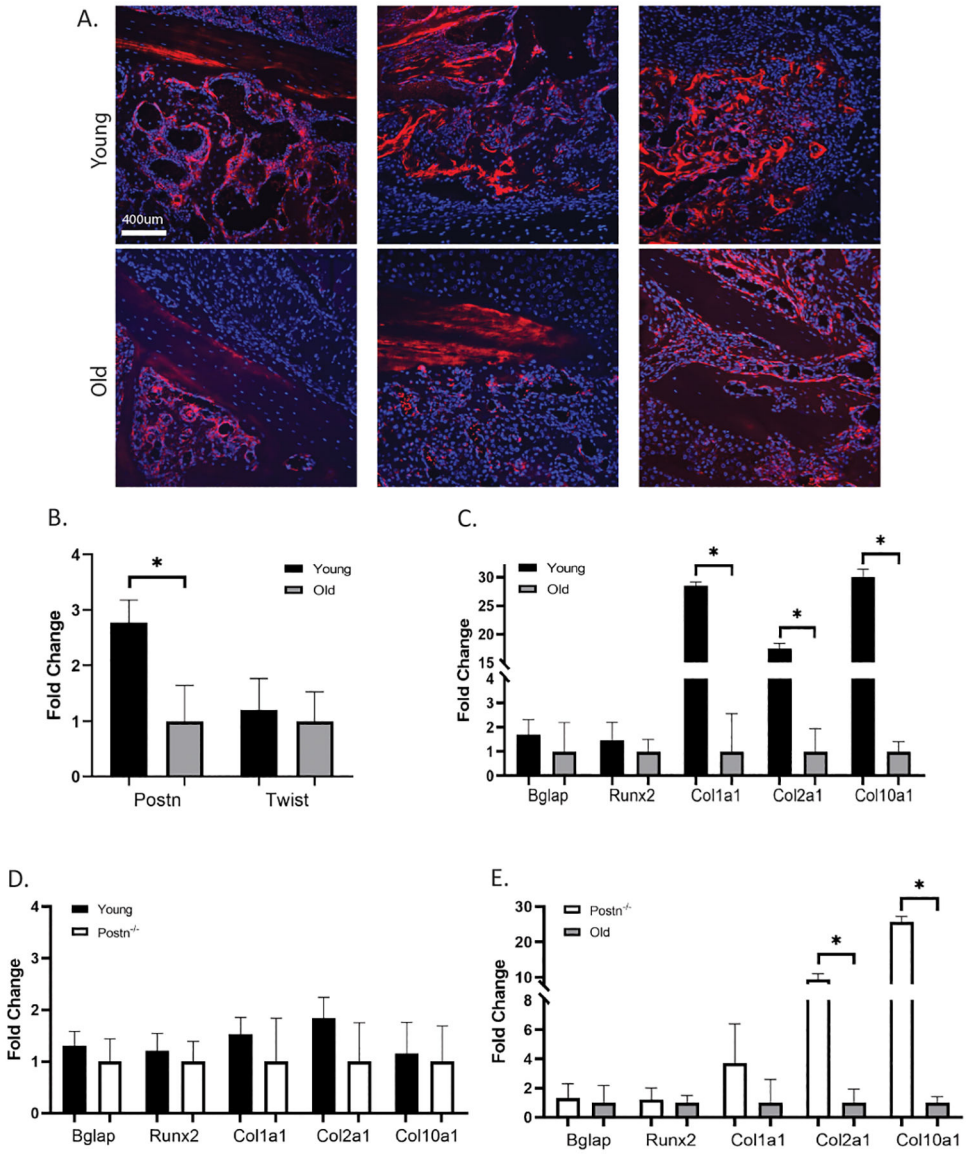


**Figure 3.**

Periostin expression within the fracture callus is decreased in old mice compared to young. Immunohistochemistry (IHC) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze the expression of periostin and other key osteogenic genes within the healing callus at day 5 post fracture. **(A)** Representative images of the fracture callus of three old and young mice stained for periostin (red). Perisotin localizes to the periosteal and endosteal surfaces of long bones adjacent to the fracture callus and to the surfaces of the trabecular bone structures within the callus. **(B)** Periostin expression was significantly downregulated in the callus of old mice compared to young (n=5/group). **(C)** Genes that encoded other extracellular matrix molecules (Col1a1, Col2a1, Col10a1) were also downregulated in the fracture callus of old mice compared to young. **(D)** In young Postn−/− mice there was no difference in expression of Col1a1, Col2a1, Col10a1

compared to young wild type mice. **(E)** Similar to young wildtype mice, young Postn−/− mice had increased expression of Col2a1, Col10a1 compared to wildtype old mice. *p<0.05.

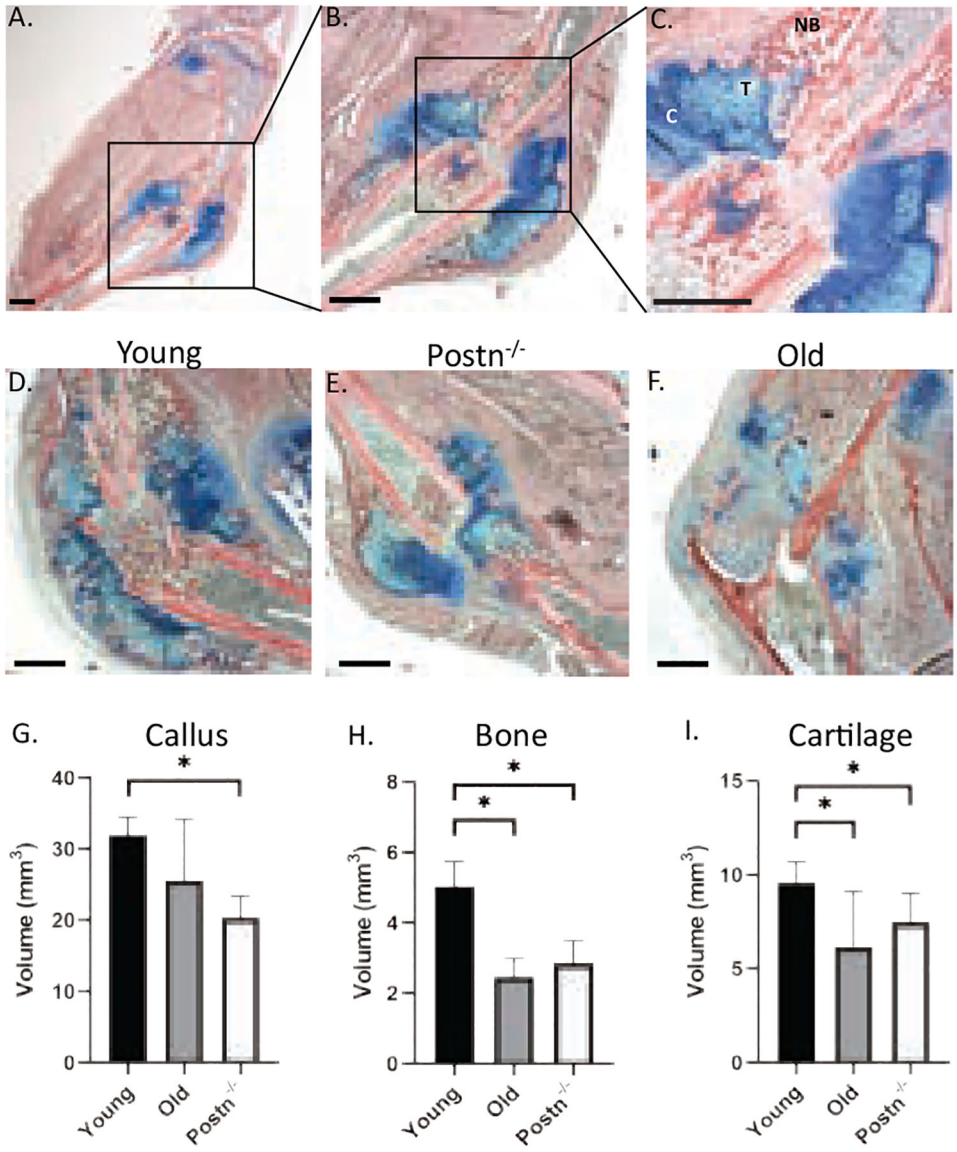


**Figure 4.**

Fracture healing is attenuated in old and young Postn−/− mice. Histological analysis was completed on fracture calluses at 10 days post fracture in old, young, and young Postn−/− mice (n=6/group). Sections were stained using Hall Brunt Quadruple Stain (HBQ) to visualize bone (red) and cartilage (blue). **(A-C)** Postn$^{-/-}$ mice demonstrated osteochondral ossification. Panel C demonstrates the presence of cartilage (C), new bone (NB), and a transition zone (T) within the Postn$^{-/-}$ mice. **(D-E)** Representative sections of the callus in young, Postn$^{-/-}$, and old mice show differences in tissue composition. Stereological analysis was performed to quantify the volume of the **(G)** callus and **(H)** the volume of the bone and **(I)** cartilage within the callus. The callus of Postn$^{-/-}$ mice demonstrated significantly smaller volume compared to wildtype young mice. Both Postn$^{-/-}$ mice and old

mice demonstrated significantly less bone and cartilage volume compared to wildtype young mice. Scale bar=1mm, *p<0.05.