Mechanobiology in Medicine 3 (2025) 100125

Contents lists available at ScienceDirect

Mechanobiology in Medicine

journal homepage: www.sciencedirect.com/journal/mechanobiology-in-medicine

Full Length Article

Swimming induces bone loss via regulating mechanical sensing pathways in bone marrow

Shaotian Fu [a,1], Yahong Lu [b,1], Wenkun Sun [c], Wugui Chen [d], Chengshou Lin [d,*], An Qin [a,**]

[a] Shanghai Key Laboratory of Orthopedic Implants, Department of Orthopedics, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, 200011, China
[b] The Fifth Affiliated Hospital of Wenzhou Medical University, Lishui Municipal Central Hospital, 289 Kuocang Road, Zhejiang, 323000, China
[c] Shanghai University of Medicine & Health Sciences, Shanghai, 201318, China
[d] Department of Orthopaedics, Mindong Hospital Affiliated Fujian Medical University, Fujian, China

ARTICLE INFO

Keywords:
Swimming
Mechanical stress
Mechanical sensing
Single-cell RNA sequencing
Osteoprosis

ABSTRACT

Bone is an organ capable of perceiving external mechanical stress in real time and responding dynamically via mechanosensing proteins such as Piezo1 and YAP/TAZ. Upon sensing the mechano-signals, cells within the bone matrix collaborate to coordinate bone formation and resorption, while bone marrow cells are also stimulated and mobilized. High-load exercise stimulates osteoblast differentiation and bone formation. However, the mechanism through which the low-load exercises affect bone homeostasis is still unclear. In this work, we established a long-term swimming training model to unload the mechanical stress in mice. Throughout the training model, we observed a significant loss in trabecular bone mass, as evidenced by microCT scanning and histological staining. Single-cell sequencing of the tibial bone marrow tissue revealed a significant increase in the percentage of bone marrow neutrophils, along with alterations in Integrins and the ERK1/2 signaling pathway. Notably, the changes in both Integrins and the ERK1/2 signaling pathway in macrophages were more pronounced than in other cell types, which suggests a mechanical adaptive response in these cells. Moreover, the involvement of Integrins is also critical for the crosstalk between monocyte precusors and macrophages during swimming. Together, this study provides a resource of the alterations of bone marrow cell gene expression profile after swimming and highlights the importance of Integrins and the ERK1/2 signaling pathway in the bone marrow microenvironment after swimming.

1. Introduction

Bone is a dynamic organ that responds to external mechanical stress.[1] Mechanical stress coordinates the activities of osteoblasts and osteoclasts, which engaging in bone formation and bone resorption to meet the needs of human growth and development.[2,3] Mechanical stress is transmitted to cells through the extracellular matrix, cytoskeleton, and Integrins to cellular mechanical stress receptors such as Piezo1, YAP/-TAZ, and TRPs,[4] ultimately leading to the activation of downstream key pathways like Wnt/β-catenin and ERK1/2.[5–9] This process precisely accomplishes the mechanical feedback of bone.[10] In addition to stimulate osteoblasts, osteoclasts and other bone matrix cells, mechanical stress also mobilizes cells within the bone marrow tissue. Previous studies have shown that exercise can enhance the secretion of reticulocalbin-2 by bone marrow macrophages, promoting fat metabolism and mobilizing lymphocytes.[11] However, research on dynamic cellular changes, interactions, and pathway alterations within the bone marrow during exercise is lacking.[12,13]

Ground reaction force (GRF) is crucial for maintaining bone tissue homeostasis. Unloading the force inhibits bone formation, subsequently impairing the development of the musculoskeletal system.[14] Studies show that swimming, with low weight-bearing, doesn't promote osteoblast bone formation as much as high-load exercises like running and jumping.[15,16] Compared to other athletes, swimmers exhibit a significant decrease in bone density in the femoral neck and lower limbs.[17,18] However, in animal experiments, long-term swimming has been shown to alleviate muscle pain, ameliorate atherosclerosis, and increase levels of pipecolic acid, which inhibits the release of inflammatory cytokines by

This article is part of a special issue entitled: Bone Mechanobiology published in Mechanobiology in Medicine.

* Corresponding author.
** Corresponding author.
E-mail addresses: chengs_lin@sina.com (C. Lin), dr_qinan@shsmu.edu.cn (A. Qin).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.mbm.2025.100125
Received 31 January 2025; Received in revised form 27 February 2025; Accepted 4 March 2025
Available online 12 March 2025

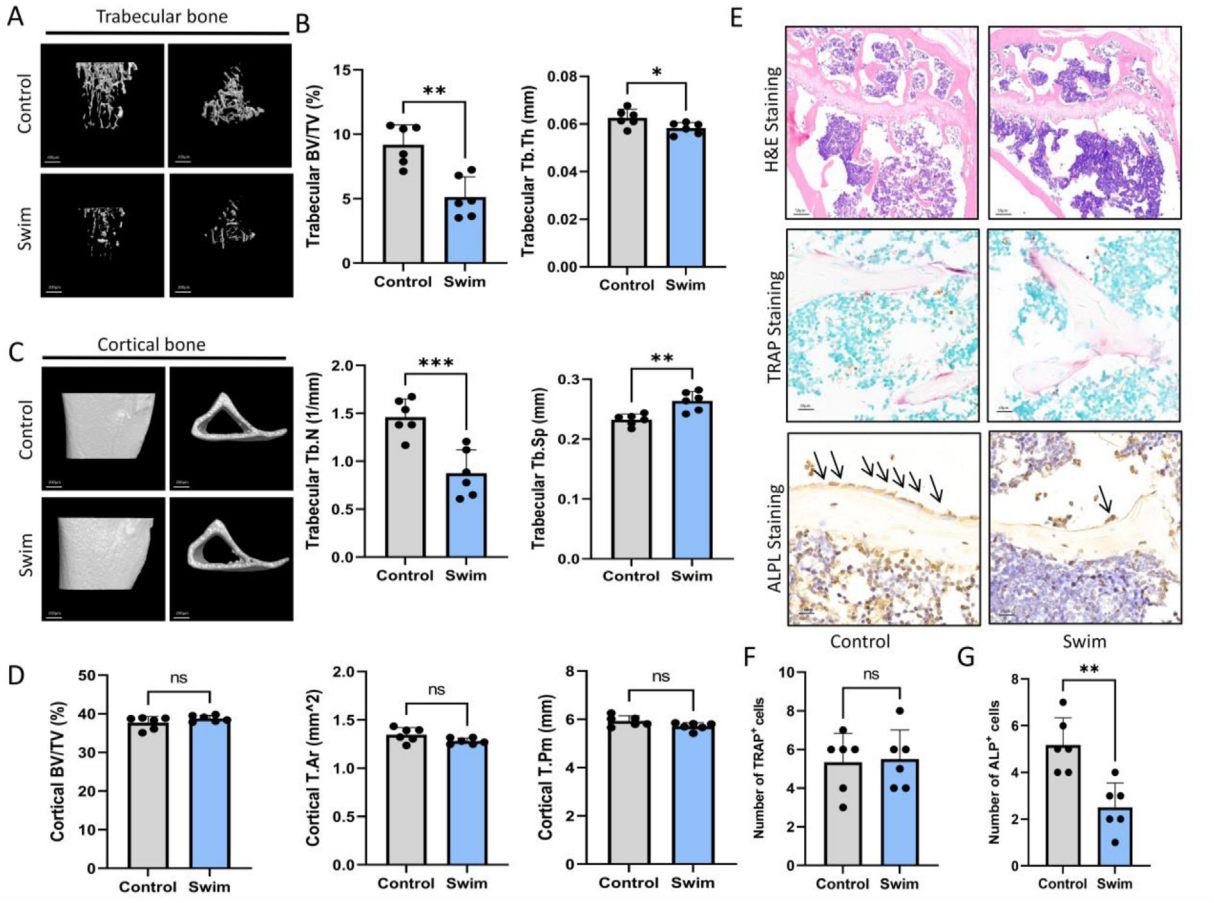

Fig. 1. Swimming leads to bone loss in mice. (A) 3D Reconstruction Images of the cancellous Bone of the Tibia in Swimming Group Mice and Control Group Mice. (B) BV/TV, Tb.Th, Tb.N, and Tb.Sp of the cancellous bone in both groups (n = 6). (C) 3D Reconstruction Images of Tibial Cortical Bone in Swimming Group Mice and Control Group Mice. (D) BV/TV, T.Ar, T.Pm of the Tibial Cortical bone in both groups (n = 6). (E) H&E staining, TRAP staining and ALPL staining of tibias from both groups of mice. (F) The number of $TRAP^+$ cells in each tibial cancellous bone area of the two groups of mice (n = 6). (G) The number of ALP^+ cells in each tibial cancellous bone area of the two groups of mice (n = 6). The bar graph represents the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

bone marrow-derived macrophages.[19–23] The health benefits of swimming are of interest and the understanding of swimming-induced signaling pathways in bone cells and their interactions are needed.[24,25]

Swimming training in mice serves as a classic animal model for studying human swimming exercise and mechanical stress unloading.[26] Through long-term swimming training of mice, we observed a significant loss of trabecular bone mass, as proved by microCT and staining of tissue sections. Therefore, we further investigated the cellular and molecular changes of bone marrow cells through single-cell sequencing analysis. Surprisingly, the results indicate that Integrins and the ERK1/2 signaling pathway play pivotal roles in regulating the changes within the bone marrow microenvironment after swimming. Furthermore, there are significant differences in the responses of different cell types to mechanical stress unloading. In summary, we found the changes in various cell types within the bone marrow after swimming and provided an explanation for the bone metabolism changes caused by swimming, focusing on multiple cells and signaling pathways related to mechanical stress.

2. Materials and methods

2.1. Animals and exercise protocol

The animal experiments were approved by the Animal Care and Experiment Committee of the Ninth People's Hospital [No. SH9H-2023-A489-SB]. Twelve 2-month-old male C57bl/6j mice, sourced from Shanghai Ninth People's Hospital, were housed in SPF conditions with free access to food and water, under a 12-h light/dark cycle (07:00–19:00), at 22 °C and 30–70 % humidity. They were divided into swimming (n = 6) and non-swimming (n = 6) groups. The swimming group mice starting with 10 min of swimming on the first day and gradually increasing to 60 min per day within a week. Afterward, they swam for 60 min every day, five times a week, for a total of 12 weeks. All swimming training sessions were conducted in the afternoon. The non-swimming group mice did not undergo swimming training.

2.2. MircoCT analysis

The tibiae of the mice were dissected and immersed in 4 % paraformaldehyde. The 3D reconstruction of the tibiae was completed as previously described.[27] Cortical bone morphometric parameters including bone volume fraction (BV/TV), trabecular area (T.Ar), and trabecular perimeter (T.Pm) were analyzed. For cancellous bone, the parameters analyzed included BV/TV, trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp).

2.3. Histological staining

After decalcification in 10 % EDTA for two weeks, the mouse tibiae were embedded in paraffin. Following sectioning, the slices underwent alkaline phosphatase (ALPL), tartrate-resistant acid phosphatase (TRAP), and hematoxylin and eosin (H&E) staining. The stained slices were then imaged under an optical microscope (ZEISS, Germany).

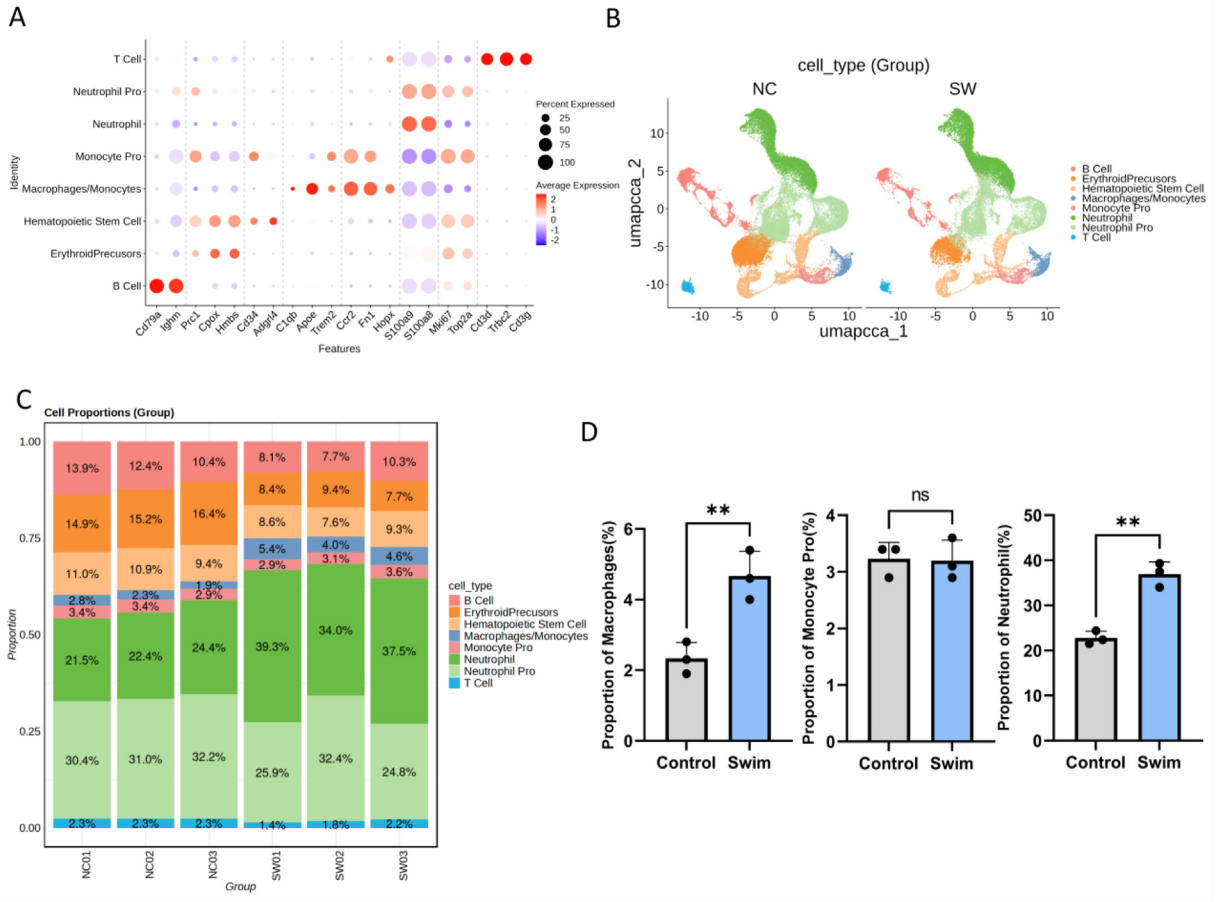

Fig. 2. The proportion of immune cells in bone marrow changed. (A) Cellular markers used for cell population segregation in bone marrow. (B) t-Distributed stochastic neighbor embedding (UMAP) visualization of major bone marrow cell populations of two groups of mice. (C) Changes in the proportions of different cell types in the tibial bone marrow of two groups of mice (n = 3). (D) Changes in macrophages, monocyte progenitor cells, and neutrophils between the two groups (n = 3).

2.4. Single-cell RNA sequencing

After the previous training, we collected one tibia from each of six mice in each group and flush out the bone marrow tissue using PBS. The samples from the same group are then mixed pairwise, ultimately yielding three mixed samples per group. After dispersing the bone marrow tissue, dead cells were removed using a Dead Cell Removal Kit (130-090-101; Miltenyi Biotec) according to the manufacturer's instructions. Single-cell RNA sequencing (scRNA-seq) analysis of the tibial bone marrow cells was then performed using the 10x Genomics Chromium platform. The data were normalized using the LogNormalize method and 2000 highly variable genes (HVGs) were identified through variance-stabilizing transformation (VST) for subsequent Gene Set Enrichment Analysis, Cell–Cell Communication Analysis, and Pathway Activity Scoring Analysis. All raw data are accessible under the GEO accession number GSE289853.

2.5. Statistical analysis

The data were collected from three independent experiments and presented as mean ± standard deviation (SD). GraphPad Prism version 9.0 software (GraphPad Software, San Diego, CA, United States) was used to performe Student 's t-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Swimming leads to bone loss in mice

The micro-CT reconstruction and analysis of the tibiae indicated that the swimming group mice exhibited decreases in trabecular bone volume fraction, trabecular number, trabecular thickness, as well as increases in trabecular separation compared to the non-swimming group mice (Fig. 1A and B). For example, the bone volume fraction decreased to half of its original level after swimming, suggesting a reduction in trabecular bone in the swimming group. However, there were no significant changes in cortical bone-related indices such as bone volume fraction, mean total cross-sectional area and mean tissue perimeter of the cortical bone (Fig. 1C and D). Subsequently, we observed the distribution of cells within the bone matrix using hematoxylin and eosin (H&E) staining of sections and found a significant decrease in trabecular bone under the tibial epiphysis (Fig. 1E). To distinguish the changes in the distribution of osteoblasts and osteoclasts, we further confirmed through TRAP staining and ALPL staining. There was no increase in osteoclasts, but a significant decrease in the distribution of osteoblasts (Fig. 1E–G). These results suggest that low-mechanical-load exercise has a greater impact on osteoblasts than osteoclasts, which is the main reason for tibial bone loss in mice.

3.2. Swimming leads to changes in the proportion of various cell types within the bone marrow

Based on cell surface markers, we classified the cell types within the bone marrow into T cells, B cells, neutrophil precursors, neutrophils, macrophage precursors, macrophages, mesenchymal stem cells and erythroid precursors (Fig. 2A and B). Among the changes in the proportions of cell types, there was no significant change in the proportion of monocyte progenitor cells between the two groups, but a significant increase in the proportions of neutrophils and macrophages. The number of neutrophils increased nearly two-fold (Fig. 2C and D). This could be due

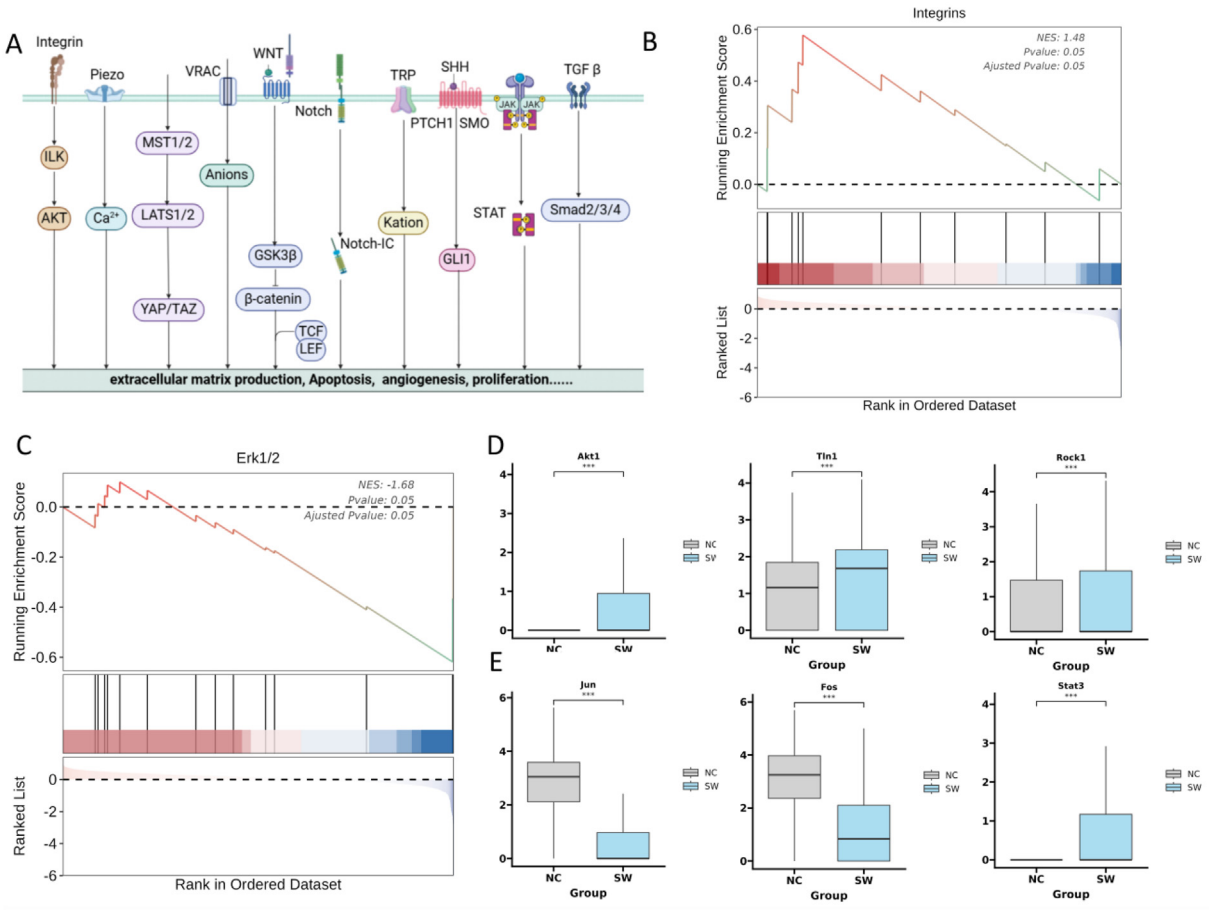

Fig. 3. The changes of mechanical perception pathway in neutrophils. (A) Diagram of mechanical stress receptors and signaling pathways. (B) GSEA of genes related to mechanical stress receptors Integrins in neutrophils. (C) GSEA of genes related to the mechanical stress signaling pathway Erk1/2 in neutrophils. (D) The comparison of genes related to the mechanical stress receptor Integrins between two groups of mice (n = 3). (E) Comparison of genes related to the mechanical stress signaling pathway Erk1/2 between two groups of mice (n = 3). The bar graph represents the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

to differences in the proliferation and differentiation capabilities of different cells in the bone marrow under low mechanical stress stimulation.

3.3. Alteration of multiple mechanical stress receptors and signaling pathways within neutrophils

The reception and transduction of mechanical stress signals require the involvement of various receptors and downstream transcription factors.[28] To investigate how the mechanical unloading of swimming influences changes in neutrophils within bone marrow tissue, we classified the currently known receptors into five categories: Piezo, Integrins, YAP/TAZ, TRP, and VDRC signaling cascades. The mechanical stress-related pathways were further categorized into eight pathways and their associated transcription factors, namely RhoA/ROCK, TGFβ/Smad, JAK/STAT, Wnt/β-catenin, ERK1/2, Hedgehog, PI3K/AKT, and Notch, for analysis within neutrophils (Fig. 3A). Through Gene Set Enrichment Analysis (GSEA), we found that among the receptors responsive to mechanical stress, the changes in Integrin receptor-related genes were the most significant (Fig. 3B and Supplement Fig. 1). For instance, the downstream-related genes Akt, Tln1 and Rock1 showed a marked increase in the swimming group (Fig. 3D). Among the mechanical response pathways and transcription factors, the ERK1/2 pathway showed significant changes in neutrophils and ERK1/2-related transcription factors such as Fos, Jun and Stat3 changed notably (Fig. 3E and F and Supplement Fig. 2). The Integrin and ERK1/2 pathway has been demonstrated to be involved in the chemotaxis and adhesion processes of neutrophils and macrophages.[29] Our study indicates a strong correlation between the increase in neutrophil proportion after swimming and the effector integrin receptors as well as the ERK1/2 pathway.

3.4. Alteration of mechanical force related genes in bone marrow tissue after mechanical stress unloading

Previous studies have shown that integrin receptors can activate downstream ERK1/2 and RhoA/ROCK signaling pathways, there also exists potential crosstalk with Piezo[30–32] (Fig. 4A). After integrating the above-mentioned five mechanical receptors, eight mechanical signaling pathways, and related transcription factors, we found Piezo, Integrins, RhoA/ROCK, ERK1/2 showed significant changes (Fig. 4B and C). The changes in the above mechanical stress-related receptors and signaling pathways were most evident in macrophages and monocyte progenitor cells, while the changes in erythroid precursor cells were the least significant (Supplement Fig. 3). In macrophages and monocyte progenitor cells, the changes in Integrin effectors were the most pronounced. The transcription factors in the RhoA/ROCK pathway increased significantly after swimming, but the ERK1/2 pathway decreased significantly (Fig. 4D and E). This suggests that macrophages and monocyte progenitor cells are comprehensively regulated by multiple mechanical stress pathways, which also indirectly confirms the interaction between immunity and exercise.[33]

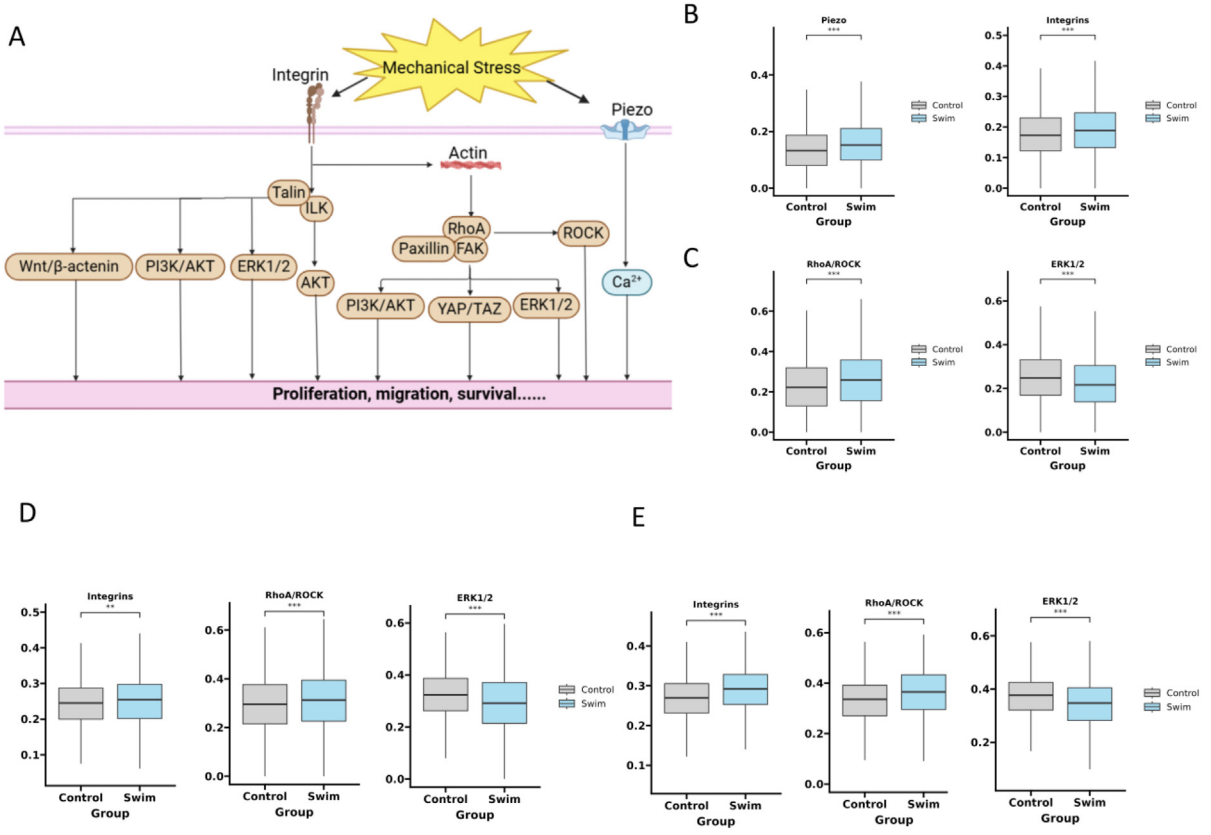

Fig. 4. The changes of mechanical perception pathway in bone marrow. (A) Diagram of downstream-related pathways of the mechanical stress receptor Integrin. (B) Differences in the expression levels of Piezo and Integrins in bone marrow cells between two groups of mice. (C) Differences in the expression levels of RhoA/ROCK and ERK1/2 in bone marrow cells between two groups of mice. (D) Differences in the expression levels of Integrins, RhoA/ROCK, and ERK1/2 in bone marrow macrophages between two groups of mice. (E) Differences in the expression levels of Integrins, RhoA/ROCK, and ERK1/2 in bone marrow monocyte progenitor cells between two groups of mice. The bar graph represents the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control group.

3.5. mechanical force alters the interactions among various cell types in the bone marrow

The reduction of trabeculae bone involves not only the influence of mechanical stress but also crosstalk among various cell types in the bone marrow. To further elucidate the mechanism, we quantified the potential intercellular interactions between all cell types within the bone marrow. By calculating the number of pairings between each mechanosensor and signaling pathway, we identified potential intercellular interaction relationships that may exist among them. The interaction network revealed a decrease in interactions among Erythroid Precursors, B cells, T cells, Neutrophil progenitors, Hematopoietic Stem cells, but a significant enhancement in interactions between macrophages, monocyte progenitor cells and neutrophils. Taking macrophages as an example, after swimming training, the interactions between them and Erythroid Precursors, as well as T cells, significantly decreased, whereas the interactions with monocyte progenitor cells and neutrophils markedly increased (Fig. 5A and B). In the analysis of the interaction between macrophages and monocyte progenitor cells, genes related to Integrin receptors, such as Cd44, Itgb1, Itgb2, Itga4, Itgb7, Itgal, and Itgam, exhibit significant changes, and these genes are associated with Fn1, C3, and Icam2 signaling pathways (Fig. 5C). Fn1 is involved in the interactions between macrophages, macrophage precursors, B cells, T cells and hematopoietic stem cells (Supplement Fig. 4). This aligns with the notion that there is an increase in macrophages and neutrophils in muscles and peripheral blood after exercise, especially as these two cell types have been shown to jointly regulate muscle oxidative metabolism post-exercise.[13] Combining our previous analysis, Integrins and the ERK1/2 signaling pathway not only undergo significant changes in expression levels after mechanical stress unloading but also interfere with cellular interactions within the bone marrow tissue.

4. Discussion

Bone is a tissue composed of mineralized bone, bone matrix cells, and bone marrow cells.[34] The homeostasis of the bone and bone marrow niche is dynamically regulated by hormones and mechanical force.[35] In addition to the chemical and biomolecular signals, mechano-signals are also vital for the cell and tissue development.[36] Besides osteocytes in bone matrix, which participate in mechanical stress sensing and coordinating the functions of osteoblasts and osteoclasts, various blood cells such as neutrophils in the bone marrow cavity also play a role in regulating the process of bone remodeling.[37,38] Aging and disuse are the most common causes of osteoporosis.[39] Current research on age-related osteoporosis has found that with the decline of sex hormones, senescent cells accumulate in the bone marrow cavity, releasing a large number of cytokines. Additionally, the decreased expression of Wnt in osteoblasts leads to an imbalance between bone formation and bone resorption, resulting in decreased bone remodeling.[40–44] Prolonged disuse leads to a lack of external mechanical stimulation, which results in decreased osteogenic capacity of osteoblasts and increased fat content in the bone marrow cavity, causing an inflammatory state.[45] This enhances bone resorption

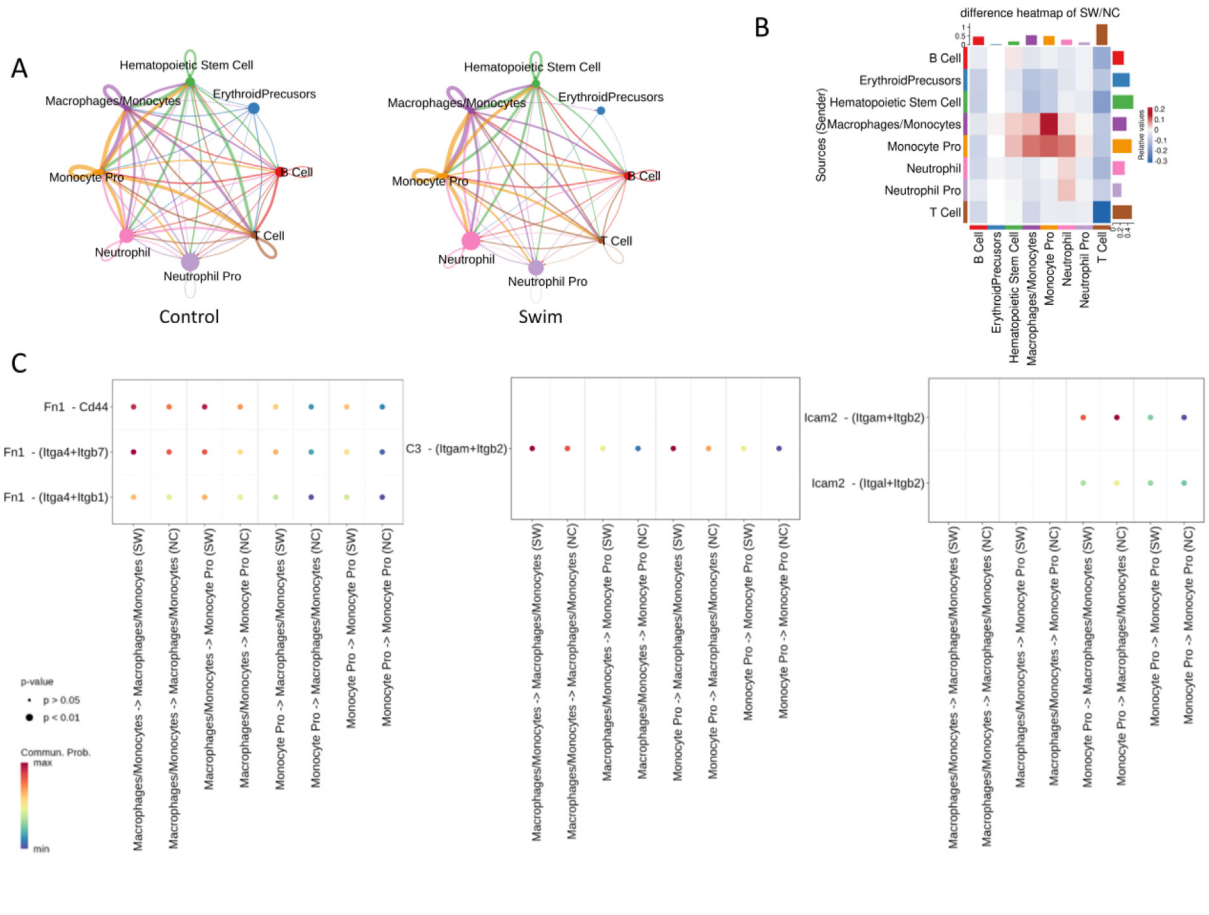

Fig. 5. Swimming changes the interaction between cells in the bone marrow. (A) Capacity for intercellular communication between mouse cell types of bone marrow. The line thickness is proportional to the number of ligand–receptor pairs. (B) Heatmap of changes in the interaction degrees among different types of cells in the bone marrow after swimming training. (C) Changes in ligands and receptors relate to Fn1, C3 and Icam2 between the two groups.

and inhibits the differentiation of mesenchymal stem cells and hematopoietic stem cells.[46,47] High-intensity exercise has therapeutic effects on osteoporosis caused by both of the above reasons, as it can enhance the osteogenic potential of mesenchymal stem cells and weaken their adipogenic potential. However, swimming, as a low-load and low-mechanical impact exercise, leads to unloading of multiple mechanical pathways, inhibiting the function of osteoblasts and being detrimental to bone formation. Our research has also proven this phenomenon.[35,48]

Swimming exercise, besides altering the function of bone matrix cells, also changes the mechanical sensing of bone marrow cells and the proportion of various cell types.[49] Through single-cell analysis of bone marrow tissue from mice with or without swim training, we found a significant increase in the proportion of macrophages and neutrophils in the bone marrow, as well as significant changes in the ERK1/2 pathway of the mechanical sensor Integrins. On one hand, this suggests that swimming enhances the mobilization of granulocytes, which can strengthen the body's immunity. On the other hand, there is a bias towards the ERK1/2 pathway in the mechanosensing pathway of granulocytes. When mechanical unloading occurs, it may reduce the pathway's response, leading to cell proliferation for timely immune feedback. After analyzing classic mechanoreceptors and transcription factors related to mechanical signaling pathways, we found that although the proportion of macrophages and macrophage precursor cells did not change significantly after mechanical stress unloading, the changes in mechanosensors and signaling pathways within these cells were the most pronounced compared to other cells. Similarly, low intensity mechanical signals have been proved to promote proliferation in a cell-specific manner.[50] Combining this with our previous findings, it suggests that the process of mechanical stress regulating the bone marrow environment is a complex one involving multiple cell types. In addition to causing osteoporosis and mobilizing cells within the medullary cavity, the unloading of mechanical stress during swimming also alters the interactions among various cell types, particularly promoting the interaction between macrophage precursor cells, macrophages, and neutrophils. Notably, these mechano-activated cells could also communicate with more cells by releasing extracellular vesicles.[51] In this process, Cd44, Itgb1, Itgb2, Itga4, Itgb7, Itgal, and Itgam play crucial roles. These proteins have also been proven to be involved in a process where muscles, through the Integrin signaling pathway, promote autophagy in liver cells after exercise.[52] From the perspectives of changes in cell proportions, alterations in gene expression levels, and cellular interactions, we have demonstrated that Integrins and the ERK1/2 signaling pathway play crucial roles in the adaptive process of bone marrow tissue after swimming. We have linked the mechanical response in the bone marrow microenvironment to specific cellular actions, explaining the correlation between osteoporosis caused by swimming and mechanical responses from the perspective of bone marrow cells.

However, our research still has limitations. Although we studied various immune cells in bone marrow tissue through mechanosensing pathways, we lacked exploration into their inflammation or activation status of other pathways. Neither swimming training in mice nor animal models of mechanical stress unloading such as femoral nerve resection, tail suspension, and hindlimb immobilization can exclude the influence of sympathetic nerve activation on bone loss.[53] Existing research shows that T cells in the process of aging release RANKL to induce osteoclast differentiation. In our study, the proportion of T cells did not change

significantly. But given that swimming can alter local and systemic immune environments, we will further explore the relationship between exercise and immunity.

5. Conclusion

Swimming, as an aerobic exercise that requires the participation of muscles throughout the body, does not promote bone formation but is more likely to lead to osteoporosis.[54,55] The osteoporosis associated with swimming differs from age-related or disuse osteoporosis in that it arises from the lack of mechanical stress stimulation.[56,57] Swimming leads to a reduction in trabecular bone, which is constantly undergoing dynamic changes within the bone marrow blood environment. However, due to the limited cellular resolution of transcriptome analysis, the exact molecular mechanism of cellular phenotypic transitions and regulatory patterns remains unclear. Therefore, we employed single-cell analysis methods to investigate the correlations between various cell types, mechanical stress effectors, and signaling pathways in the bone marrow tissue of mice, comparing those that swam to those that did not. The mechanical signaling analysis specifically focusing on neutrophils has shown the most significant changes in proportion. The changes are mainly in mechanical response receptors and pathways, represented by the effectors Integrins and the ERK1/2 signaling pathway, across multiple cell types from the perspective of cells regulated by mechanical stress. In summary, our research provides molecular insights into the heterogeneity of cellular responses to mechanical stress stimuli in different bone marrow tissues and the molecular mechanisms underlying cell-mechanical stress interactions within bone marrow tissues. It also lays a foundation for future explanations of the interactions between bone marrow and bone tissues via mechanical signals.

CRediT authorship contribution statement

Shaotian Fu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yahong Lu:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Wenkun Sun:** Writing – review & editing, Investigation, Data curation. **Wugui Chen:** Writing – review & editing, Visualization, Methodology, Investigation. **Chengshou Lin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition. **An Qin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Ethics declarations

The animal experiments and the related experimental protocols, used in the present study, were approved by the Animal Care and Experiment Committee of the Ninth People's Hospital [No. SH9H-2023-A489-SB].

Declaration of competing interest

The authors report no potential conflicts of interest.

Acknowledgement

This study was funded by Shenzhen Medical Research Fund (B2302005) and the Fujian Provincial Natural Science Foundation (Grant No. 2022J011513). We appreciate Yang Xiao for the drawing of the scheme using BioRender.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbm.2025.100125.

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