# Integration of Proteomics and Intact N-Glycoproteomics Reveals the Mechanisms Underlying BMSC Osteogenesis in the Context of Fracture Hematoma

*Original article*


ABSTRACT

*Background:* The initial hematoma formed at the fracture site-often colloquially referred to as the "first crucial bleed"-plays a critical role in initiating and orchestrating the bone healing process. Among its components, plasma proteins and circulating bioactive factors significantly influence osteogenic activity. Many of these proteins undergo N-glycosylation, a post-translational modification that modulates their expression, stability, and function-making N-glycosylation a promising target for investigation due to its prevalence and detectability.

*Methods:* Hematoma samples were collected from both young and elderly patients with patellar fractures, and subjected to integrated proteomic and intact N-glycoproteomic profiling. Western blotting (WB), RT-qPCR, β-galactosidase staining, alizarin red staining, alkaline phosphatase (ALP) staining, and immunofluorescence (IF) staining were employed to examine the roles of Tenascin-C (TNC) and Joining Chain of Multimeric IgA and IgM (JCHAIN) in BMSC senescence and subsequent osteogenesis.

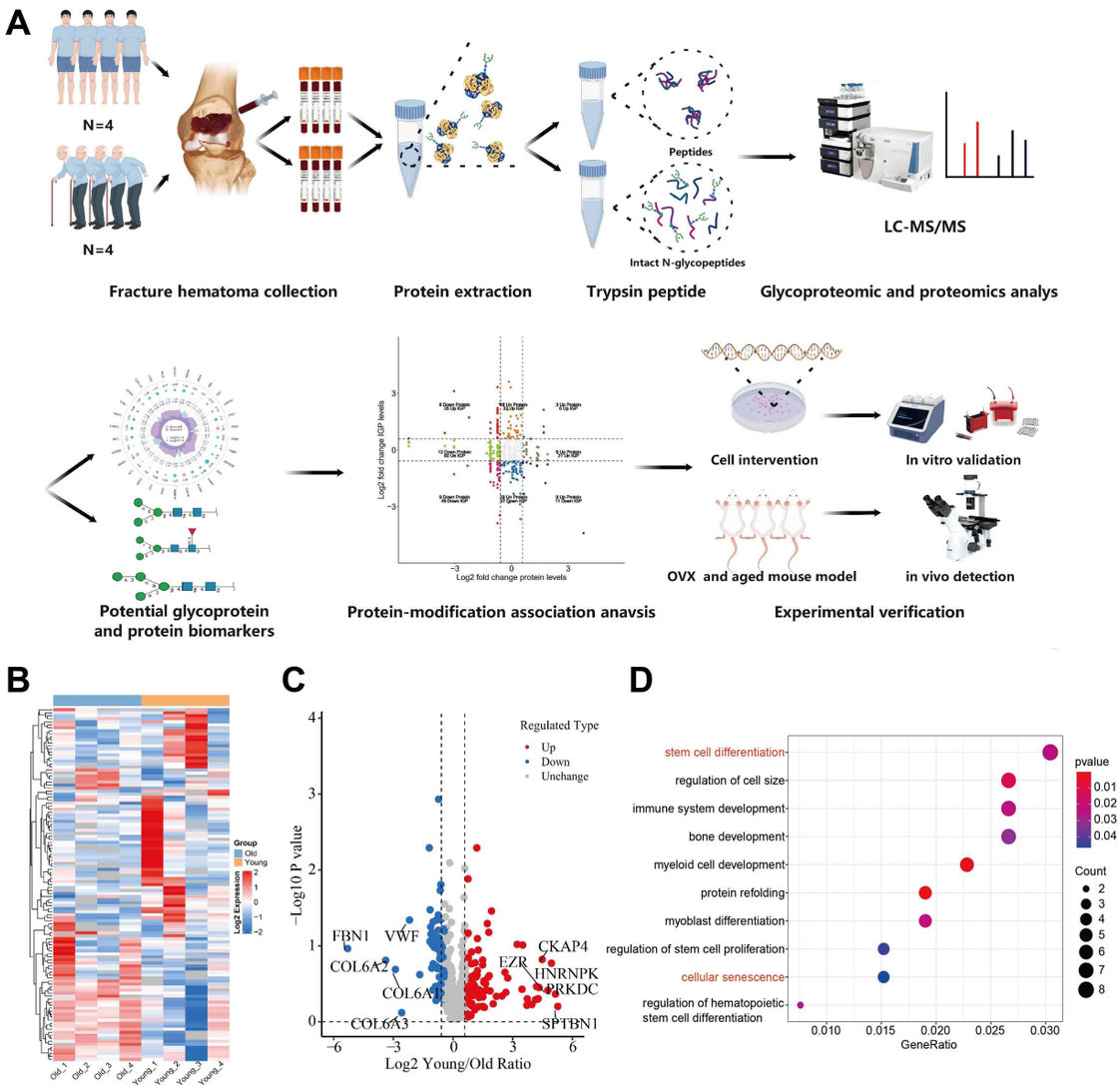
*Results:* A total of 550 differentially expressed proteins (DEPs) were identified, and pathway enrichment analysis indicated their strong association with bone marrow mesenchymal stem cell (BMSC) senescence and osteogenic differentiation. Additionally, we detected 1,289 intact N-glycopeptides (IGPs) corresponding to 407 N-glycosylation sites across 272 glycoproteins. Among these, 191 IGPs exhibited significant abundance differences between the young and elderly groups. By integrating proteomic and glycoproteomic datasets, 32 DEPs were found to also contain significantly altered glycopeptides. N-glycosylation modifications of TNC and JCHAIN contribute to the upregulation of their protein levels in hematomas from younger patients. These molecular alterations enhance the osteogenic differentiation capacity of BMSCs by modulating cellular senescence.

*Conclusion:* Our study reveals age-dependent differences in the expression and glycosylation patterns within fracture hematomas, provides new insight into the molecular mechanisms underpinning age-related differences in bone regeneration, and highlights N-glycosylation as a potential regulatory target in fracture healing.

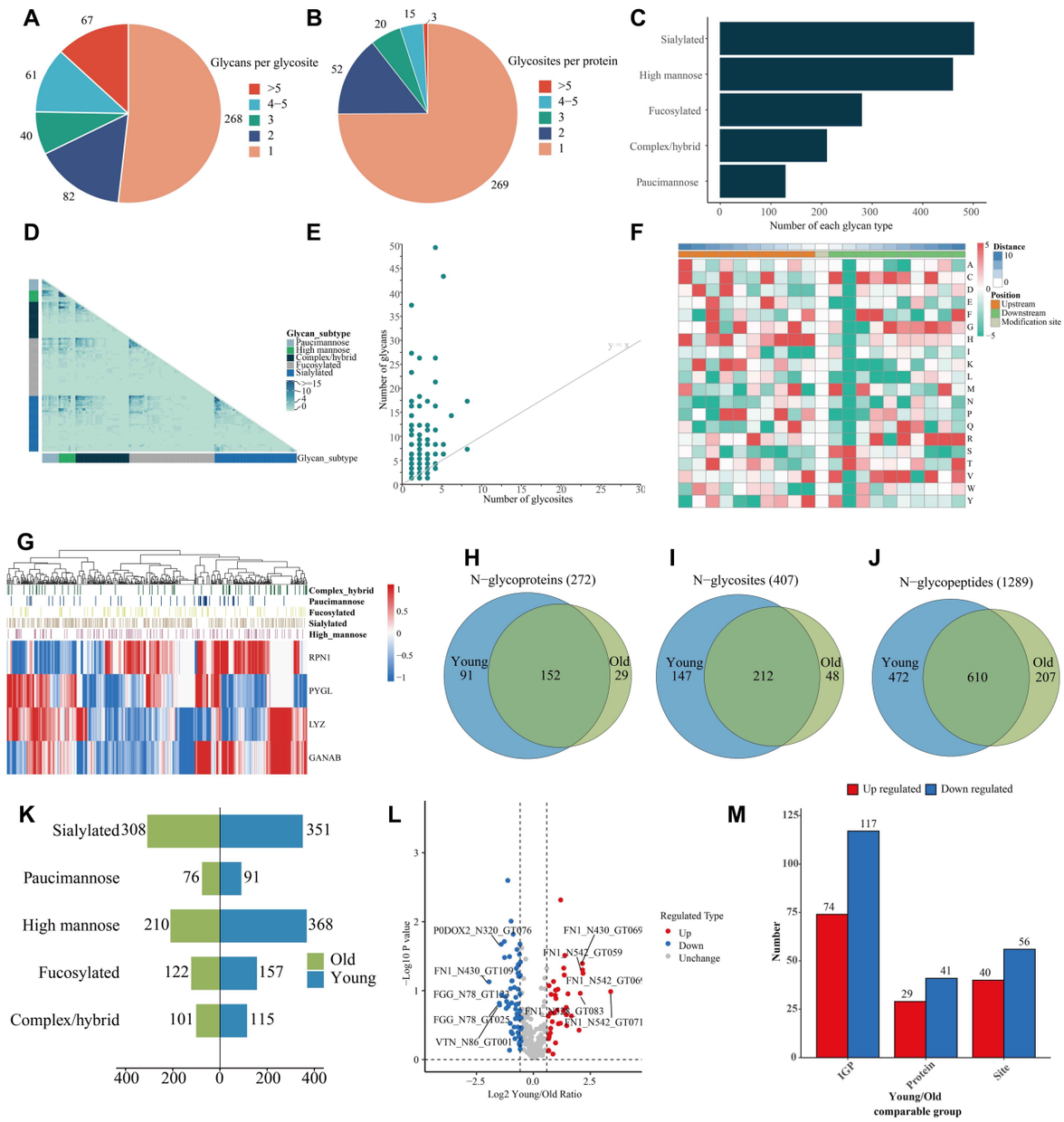
*The Translational Potential of this Article:* The age-related differences in both protein expression and N-glycosylation patterns within fracture hematomas hold strong potential as prognostic biomarkers for evaluating bone healing efficiency. Modulating these specific post-translational modifications offers a promising therapeutic strategy to counteract cellular senescence and enhance fracture repair. Furthermore, developing targeted glycoprotein-based therapeutics could provide innovative treatments to improve bone regeneration outcomes in elderly patients.



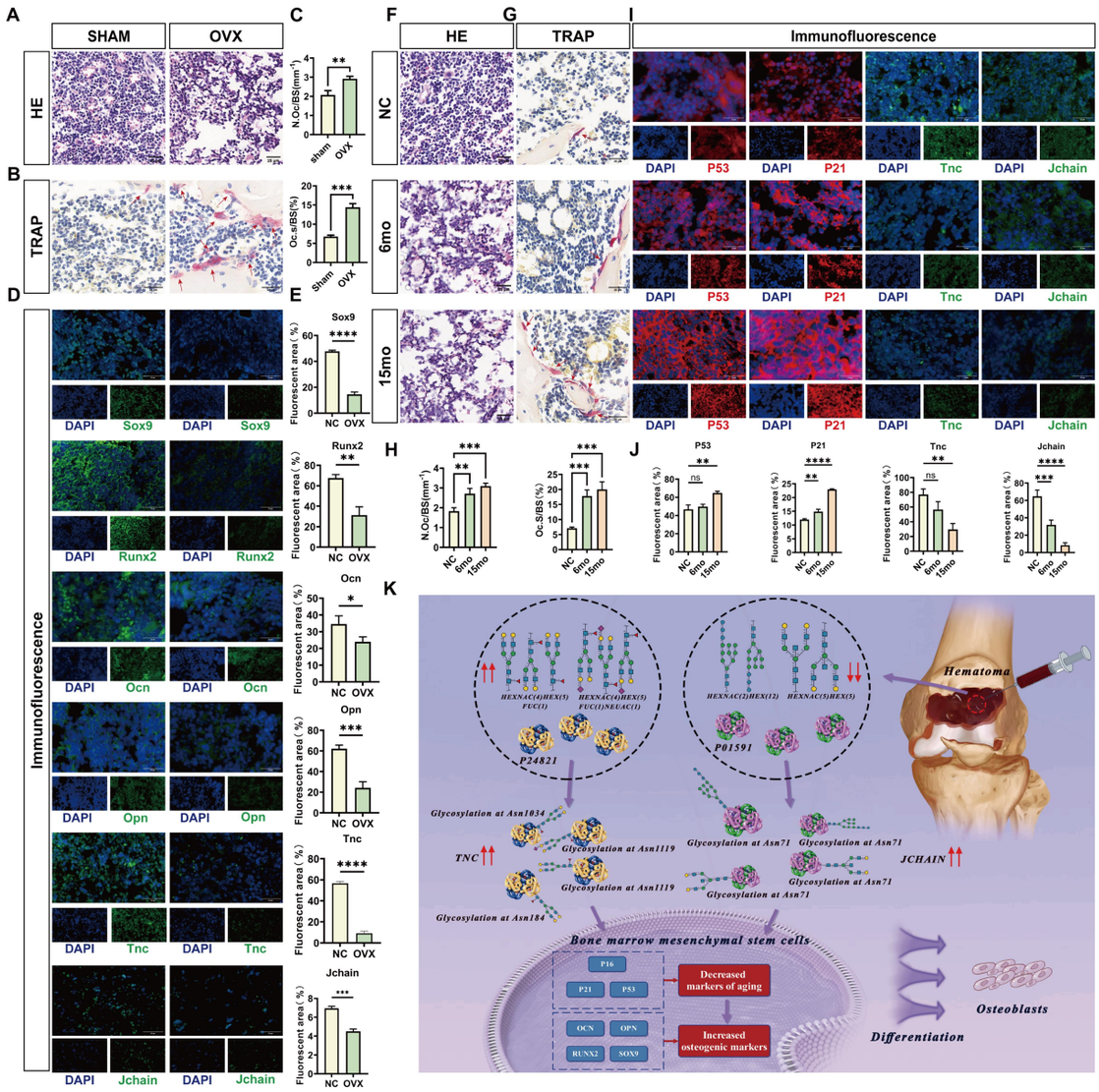
*Keywords：* Osteogenesis; Hematoma; Proteomic; Intact N-glycopeptides; Mesenchymal stem cells.




**Quantitative proteomics analysis of fracture hematoma in young and elderly patients.** (A) The workflow of quantitative proteomics and N-glycoproteomics analyses of human fracture hematoma. (B) Heatmap showing DEPs between young and elderly patients. Each column represents a sample, and each row represents a protein. Red indicates high expression levels, blue indicates low expression levels, and grey denotes proteins that could not be quantitatively measured in the corresponding samples. (C) Volcano plot showing 265 upregulated and 285 downregulated proteins in young patients (median ratio (young/old) >1.5 or <0.67). (D) GO analysis of differentially expressed proteins in fracture hematoma of young patients in terms of biological process (BP).




**Characteristics of intact N-glycopeptides in fracture hematomas in young and elderly patients.** (A-B) Pie charts depict the percentage of N-glycoproteins with the identified number of N-glycosites per protein (A) and the percentage of N-glycosites with the identified number of N-glycans per glycosite (B). (C) Percentage of the five types of glycans in all N-glycosites. (D) Heatmap showing the co-occurrence frequency between glycoforms. Each row/column in the map represents a glycoform. (E) Scatter plot depicting the number of modification sites and glycoforms on each protein between young and old patients. Each point in the map represents a protein. (F) Heat map showing the frequency change score of amino acids near the modification site (DS). (G) Heatmap showing the glycosylation-associated transferase and hydrolase expression based on the glycopeptides data. (H-J) Venn diagram showing identified N-glycoproteins (H), N-glycosites (I), and N-glycopeptides (J) in young and old patients. (K) The different glycosylation modifications on the N-glycosites of hematomas in young and old patients. (L) Volcano plot showing 74 upregulated and 117 downregulated N-glycopeptides in young patients (median ratio (young/old) > 1.5 or < 0.67). (M) Bar graph showing the difference between young and old patients complete glycopeptide distribution.




**TNC and JCHAIN play an important role in BMSC anti-aging and promoting osteogenesis in vivo.** (A) HE staining of the distal femoral epiphysis in 2 different groups (Sham and OVX) (n=5) Scale bars, 25μm. (B-C) TRAP staining of the distal femoral epiphysis indicates osteoclasts (red arrows) in 2 different groups (Sham and OVX). Parameters including N.Oc/BS and Oc.S/BS were quantified (n=5). Scale bars, 25 μm. (D-E) Representative IF images of Sox9, Runx2, Ocn, Opn, Tnc and Jchain of sham and OVX group and quantitative analysis. Data are presented as mean ± SD, n = 5. green: Sox9, Runx2, Ocn, Opn, Tnc and Jchain; blue: DAPI. Scale bars, 25μm. (F) Representative images of HE staining of the distal femoral epiphysis in 3 different groups (NC, 6-month-old and 15-month-old) (n=5) Scale bars, 25μm. (G-H) TRAP staining of the distal femoral epiphysis indicates osteoclasts (red arrows) in 3 different groups (NC, 6-month-old and 15-month-old), and parameters including N.Oc/BS and Oc.S/BS were quantified (n=5). Scale bars, 25 μm. (I-J) Representative IF images of P21, P53, Tnc and Jchain in control and aged model group (6-month-old and 15-month-old) and quantitative analysis. Data are presented as mean ± SD, n =5. Red: P21, P53; green: Tnc and Jchain; blue: DAPI. Scale bars, 25 μm. (K) Schematic diagram illustrating the osteogenic mechanisms of TNC and JCHAIN in promoting fracture healing within the hematoma microenvironment. *P < 0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not statistically significant. OVX, ovariectomy.