KCa3.1: A key regulatory molecule of ferroptosis in chondrocytes, it is crucial for cartilage degradation and bone destruction in rheumatoid arthritis

Renpeng Zhou, Shufang Li, Ke Wang, Wei Hu

Department of Clinical Pharmacology, The Second Affiliated Hospital of Anhui Medical University

zhourenpeng@ahmu.edu.cn

Disclosure: None

Introduction: Rheumatoid arthritis (RA) is a debilitating inflammatory joint disorder characterized by progressive cartilage destruction, yet the molecular mechanisms underlying chondrocyte dysfunction remain poorly elucidated. Our previous investigations revealed that ferroptosis, an iron-dependent form of regulated cell death-plays a pivotal role in RA-associated cartilage degradation (Zhou et al., ***Redox Biology***, 2022). Moreover, ion channels are an important component of membrane signal transduction and are related to cartilage degradation, inflammation and pain (Zhou et al., ***Nature Reviews Rheumatologyy***, 2024). Building on these findings, we identified the calcium-activated potassium channel KCa3.1 as a key mediator of chondrocyte ferroptosis in RA pathogenesis. Through integrated multi-omics analysis of human RA cartilage tissues, we discovered that KCa3.1 expression is markedly upregulated in ferroptotic chondrocytes and strongly correlates with disease severity. The present study was designed to clarify the functional significance of KCa3.1-mediated ferroptosis of chondrocytes and cartilage degradation in the pathogenesis of RA.

Materials & Methods: To identify ferroptosis-associated regulators, we performed RNA-sequencing. Further confirmed the expression of KCa3.1 and ferroptosis-related proteins in primary RA cartilage. CRISPR/Cas9 technology was employed to knockout KCa3.1 in human chondrocytes.The significance of KCa3.1 in regulating chondrocyte ferroptosis was assessed through: Mitochondrial ultrastructure, Lipid peroxidation (C11-BODIPY staining), Mitochondrial morphology (TEM), Mito-ROS/Fe^{2+} accumulation (MitoSox/FerroOrange), protein expression (Western blot). In vivo studies were conducted through H&E, safranin O and green staining, immunofluorescence staining, and micro-CT.

Results: KCa3.1 was identified as a novel regulatory molecule of ferroptosis in chondrocytes and is upregulated in the articular cartilage of RA. To investigate the key regulatory mechanisms underlying chondrocyte ferroptosis, we performed comprehensive RNA sequencing analysis comparing control, erastin-treated, and erastin + 2-APB rescue groups (**Fig. 1a, b**). Based on the known calcium-inhibitory function of 2-APB, we focused our analysis on calcium-related differentially expressed genes (DEGs), which identified the calcium-activated potassium channel KCa3.1 as a prominently regulated candidate (**Fig. 1c**). Remarkably, western blot analysis showed that KCa3.1 was upregulation while ferroptosis marker GPX4 was downregulated in RA cartilage (**Fig. 1d, e**). Molecular analysis demonstrated that CIA-derived articular chondrocytes displayed significantly elevated KCa3.1 expression concurrent with dysregulated ferroptosis markers: increased ACSL4 accompanied by decreased Gpx4, Acan, and Col2a1 expression. These findings establish KCa3.1 as a critical regulator of chondrocyte ferroptosis in RA progression.

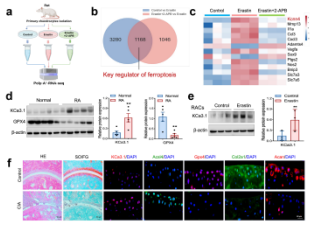

Fig. 1. KCa3.1 is associated with ferroptosis of chondrocytes and is involved in the RA progression. (a) Flowchart of transcriptome sequencing. (b, c) Venn and heat map illustrating the expression of KCa3.1, ferroptosis and RA-related molecules in RACs treated with 2-APB. (d) The expression level of KCa3.1 and GPX4 in normal and RA cartilages were determined by western blot. (e) Western blot analysis for KCa3.1 in RACs treated with erastin. (f) Representative images of H&E and SO/FG staining and immunostaining of KCa3.1, Acsl4, Gpx4, Col2a1, Acan in CIA mice cartilage chondrocytes.

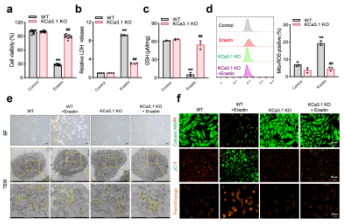

Fig. 2. KCa3.1 inhibited and deficiency negatively regulates chondrocyte ferroptosis in vitro. (a, b) Cell viability and LDH release in KCa3.1 KO C28/I2 cells. (c) Meraurement of GSH levels and ATP content in KCa3.1 KO C28/I2 cells. (d) Flow cytometry analysis of Mito-ROS levels in KCa3.1 KO C28/I2 cells. (e) Visualization and morphological analysis (TEM) of KCa3.1 KO C28/I2 cells. The yellow dotted shape indicate mitochondria. (f) Live/dead cell staining (Calcein AM/PI), ΔΨm (JC-1) and Fe^{2+} levels were assessed in KCa3.1 KO C28/I2 cells.

Gene knockout of KCa3.1 protect against chondrocyte ferroptosis in vitro. To investigate the functional role of KCa3.1 in chondrocyte ferroptosis, we generated KCa3.1-knockout C28/I2 cells using CRISPR/Cas9 technology. Genetic ablation of KCa3.1 significantly improved cell viability and reduced cytotoxicity upon ferroptosis induction (**Fig. 2a, b**). Furthermore, KCa3.1 knockout reversed key ferroptotic biochemical alterations, including normalization of GSH levels and reduction of mitochondrial ROS (Mito-ROS) (**Fig. 2c, d**). TEM analysis revealed that KCa3.1 deficiency protected against ferroptosis-associated mitochondrial damage, as evidenced by preserved membrane integrity, cristae structure, mitochondrial membrane potential (ΔΨm) and Fe^{2+} levels (**Fig. 2e, f**). Collectively, these results indicate that KCa3.1 serves as a key mediator of RA chondrocyte ferroptosis.

KCa3.1 knockdout attenuate chondrocyte ferroptosis, relieve cartilage injury and bone destruction in vivo. To assess whether KCa3.1 deficiency protects chondrocytes from ferroptosis and mitigating cartilage damage in vivo, we established the collagen antibody-induced arthritis (CAIA) model in both $KCNN4^{f/f}$ and $KCNN4^{f/f,Col2a1}$ mice (**Fig. 3a**). Compared with $KCNN4^{f/f}$ mice, $KCNN4^{f/f,\ Col2a1}$ mice exhibited reduced paw swelling and arthritis scores following CAIA (**Fig. 3b**). H&E and SO/FG staining results showed that KCa3.1 deficiency reversed the reduction of chondrocyte counts and proteoglycan loss induced by CAIA model (**Fig. 3c, d**). Immunofluorescence staining revealed that KCa3.1 deletion in CAIA mice significantly upregulated Col2a1 and Gpx4 expression while downregulating Mmp13 and Acsl4 levels in articular chondrocytes (**Fig. 3e**). Micro-CT analysis showed that deletion of KCa3.1 effectively mitigated cartilage damage and bone destruction in CAIA mice (**Fig. 3f**). Collectively, these results reinforce KCa3.1 as a critical mediator in regulation of chondrocytes ferroptosis and cartilage protection against RA pathogenesis.

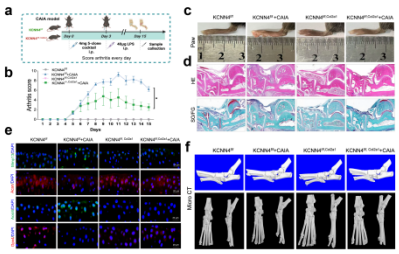

Fig. 3. KCa3.1 knockout attenuated CAIA mice progression and protected cartilage destruction and bone erosion. (a) Flowchart for experiment design of CAIA model in $KCNN4^{f/f}$ and $KCNN4^{f/f,Col2a1}$ mice. (b) Arthritis swelling severity scores in CAIA mice. (c, d) Representative of ankle joints, H&E staining and safranin O/fast green staining on cartilages. (e) Immunostaining of catabolism (Mmp13), anabolism (Acan) and ferroptosis (Acsl4, Gpx4) markers in CAIA mice articular cartilage. (f) Micro-CT analysis for the ankles and representative micro-CT images of the trabecular in bone.

Conclusions: In this study, we identified KCa3.1 as a critical mediator of chondrocyte ferroptosis in RA pathogenesis, demonstrating that KCa3.1 is upregulated in RA cartilage and essential for ferroptosis-associated cartilage degradation.

Significance: The identification of KCa3.1 as a key regulator of chondrocyte ferroptosis provides novel insights into RA-associated cartilage destruction. These findings position KCa3.1 as a promising therapeutic target for mitigating ferroptosis-driven joint degeneration in RA and other inflammatory arthritic conditions.

References: 1. Zhou et al., Redox Biology, 2022; 2. Zhou et al., Nature Reviews Rheumatology, 2024