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The role of the NOTCH1 signaling pathway in the maintenance of mesenchymal stem cell stemness and chondrocyte differentiation and its potential in the treatment of osteoarthritis

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Abstract

Objective This study aims to explore the potential role of mesenchymal stem cells (MSCs) in the treatment of osteoarthritis (OA), particularly the function of the NOTCH1 signaling pathway in maintaining the stemness of MSCs and in chondrocyte differentiation.

Methods Utilizing diverse analytical techniques on an osteoarthritis dataset, we unveil distinct gene expression patterns and regulatory relationships, shedding light on potential mechanisms underlying the disease. Techniques used include the culture of MSCs, induction of differentiation into chondrocytes, establishment of stable cell lines, Western Blot, and immunofluorescence. Through the construction of lentiviruses overexpressing and knocking out NOTCH1, the effects of NOTCH1 on the stemness of MSCs and chondrocyte differentiation were investigated. Additionally, the effects of NOTCH1 on chondrocyte homeostasis and apoptosis were evaluated by adding the EZH2 inhibitor GSK126 and the endoplasmic reticulum stress inducer tunicamycin.

Results Experimental results demonstrated that NOTCH1 expression can influence the maintenance of MSC stemness and chondrocyte differentiation by regulating EZH2. Knockout of NOTCH1 decreased the expression of chondrocyte markers, while overexpression increased their expression. Under conditions of endoplasmic reticulum stress, NOTCH1 expression helped reduce the expression of stress-related proteins, maintain chondrocyte homeostasis, and inhibit apoptosis.

Conclusion The NOTCH1 signaling pathway plays a crucial role in maintaining the stemness of MSCs, differentiating into chondrocytes, and in the treatment of osteoarthritis. NOTCH1 influences the differentiation fate of MSCs and the homeostasis of chondrocytes by regulating EZH2 and other related genes, offering new targets and strategies for the treatment of diseases like osteoarthritis.

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Keywords Mesenchymal stem cells, Osteoarthritis, NOTCH1 signaling pathway, EZH2, Chondrocyte differentiation, Endoplasmic reticulum stress

Introduction

Osteoarthritis (OA) is a debilitating chronic condition that not only causes intense pain but also profoundly impacts individuals' lives, significantly diminishing their quality of life. This disease is especially prevalent among the elderly, making it an escalating concern as the global population continues to age. Research into OA has illuminated that Mesenchymal Stem Cells (MSCs), pluripotent progenitor cells naturally found in the body, may offer promising avenues for treatment. These cells have the remarkable ability to differentiate into various musculoskeletal tissues, a trait that holds significant promise for regenerative strategies in OA therapy. MSCs are renowned for their exceptional immunomodulatory properties, allowing them to regulate inflammatory responses primarily through paracrine signaling mechanisms. This feature highlights the immense potential of MSCs as a cutting-edge cell therapy option for OA patients. Current research is focused on identifying the most optimal sources from which MSCs can be derived, given their inherent capacity to regenerate multiple tissue types [1].

The role of mesenchymal stem cells (MSCs) in musculoskeletal diseases has been a hot research topic in recent years. MSCs have multi-directional differentiation potential and can differentiate into osteoblasts, chondrocytes, adipocytes, etc., which is of great significance for the regeneration and repair of the musculoskeletal system. Studies have shown that MSCs can promote fracture healing. They can migrate to the fracture site, participate in bone formation and angiogenesis, thereby accelerating the healing process. MSCs can secrete anti-inflammatory factors, regulate immune responses, and have the potential to regenerate cartilage. Clinical studies have shown that MSC injections can improve joint function and reduce pain. MSCs can form new bone tissue through differentiation and have the potential to be used in the treatment of osteoporosis. Some animal studies have shown that MSC-based treatments can increase bone density and strength. In addition, MSCs can secrete a variety of biologically active molecules that can inhibit inflammatory responses and regulate the immune system through paracrine effects, which has a positive effect on the treatment of many musculoskeletal diseases [2–5]. Central to the regulation and differentiation of stem cells, including MSCs, the NOTCH1 signaling pathway emerges as a key regulator. This pathway not only governs the stem cell niche but also profoundly influences the maintenance and differentiation of these cells into various lineages [6]. In the specific context of MSC differentiation into chondrocytes, essential for cartilage formation, NOTCH1 plays a particularly crucial role. It facilitates the activation of pathways, such as those mediated by transforming growth factor beta 1 (TGF-β1), which subsequently activate other molecular cascades like p38 and ERK-1. These pathways enhance the expression of SOX9, a gene indispensable for the initiation of cartilage-specific gene transcription and the early stages of cartilage development [7, 8].

When NOTCH1 levels are elevated, SOX9 expression is correspondingly increased, playing a crucial role in chondrocyte differentiation and overall cartilage health. Conversely, elevated NOTCH1 may inhibit EZH2, which is known to suppress SOX2 expression. The human homolog 2 of the Drosophila Zeste gene enhancer (enhance of zeste homolog 2, EZH2) belongs to the PcG family (polycomb group) of proteins and is a key epigenetic regulatory factor. EZH2 is a histone methyltransferase that can methylate the lysine at position H3K27 (H3K27me3), thereby silencing the expression of specific genes. This gene silencing mechanism is essential for maintaining the stability of the cell cycle and controlling cell proliferation and differentiation. The pathway involving CAMK2A leads to the epigenetic derepression of SOX2 by reducing the presence of H3K27me3 and EZH2 at the SOX2 regulatory regions, fostering an environment conducive to proper gene expression [9]. Meanwhile, EZH2 suppresses Sox9 by enhancing the H3K27me3 mark and binding to the Sox9 promoter region, highlighting the dynamic interplay of these molecular players. Furthermore, EZH2 enhances the expression of METTL3, which in turn stabilizes EZH2 mRNA through m6A modification, establishing a positive feedback loop crucial for cellular functions [10, 11]. During endoplasmic reticulum stress, misfolded proteins may trigger a cellular response known as the Unfolded Protein Response (UPR), aiming to restore protein homeostasis and prevent potential cell damage. In such scenarios, key molecules such as ATF4 are activated, further influencing cellular processes [12]. For instance, during chondrocyte differentiation, ATF4 along with C/EBPβ can elevate RUNX2 expression, essential for chondrocyte formation and function [13]. Lastly, the interaction between MSCs' NOTCH1 and HES1 influences the promoters of SOX9 and EZH2, adjusting their expression levels appropriately, alluding to the complex networks of genetic regulation pivotal for effective treatment and understanding of OA [14–16].

Experimental methodology

Bioinformatics analysis

Utilizing R version 4.2.1, employing R packages: GEOquery [2.64.2], limma [3.52.2], ggplot2 [3.3.6], and ComplexHeatmap [2.13.1]. Fetching dataset GSE98918 from the GEO database via the GEOquery package. Conducting differential analysis on expression profile data, subsequently normalizing the data again utilizing the normalizeBetweenArrays function from the limma package. Removing duplicate probes corresponding to multiple molecules; in cases where probes correspond to the same molecule, retaining only the probe with the maximal signal intensity. Operating under R version 4.2.1, utilizing R packages: clusterProfiler [4.4.4], GOplot [1.0.2], ggplot2 [3.3.6], and ID conversion package: org.Hs.eg.db. Species: Homo sapiens. After converting the input molecule list IDs, perform enrichment analysis using the clusterProfiler package. Calculating the z-score values corresponding to each enrichment entry via the GOplot package, utilizing the provided numerical values of molecules. Filtering out gene clusters and pathways exhibiting biological feature disparities among differentially expressed genes. Visualizing the enrichment analysis results using the ggplot2 package, with a significance cut-off set to corrected p-value < 0.05 for GO and KEGG enrichment analysis. Employing R version 4.2.1, with R package ggplot2 [3.3.6], exploring the correlation between variables ATF4 (Activating Transcription Factor 4), C/EBPβ (CCAAT enhancer binding protein β), NOTCH1 (Neurogenic locus notch homolog protein 1), RUNX2 (Runt-related transcription factor 2), EZH2 (enhancer of zest homolog), and SOX9 (Transcription factor SOX-9). Visualizing the results of variable correlation analysis using the ggplot2 package, creating a scatter plot of gene co-expression.

Culture of mesenchymal stem cells

Mesenchymal stem cells (MSCs), primarily derived from bone marrow, are cultured in a medium enriched with specific components such as fetal bovine serum (FBS), growth additives, penicillin, and streptomycin. At an incubation condition of 37℃, alongside a 5% CO2 atmosphere and saturated humidity, MSCs demonstrate fibroblast-like morphology. They are expanded through passaging culture, typically up to 3–5 generations, with the initial three generations being in an optimal cellular state, at a passaging ratio of 1:2. For facilitating passaging or experimental manipulation, cells are digested using 0.25% trypsin. Moreover, to maintain the requisite nutrients for cell growth and a sterile environment, the culture medium is replaced every 2–3 days.

Induction of mesenchymal stem cells differentiation into chondrocytes

BMSCs are transferred into a medium containing chondrogenic inducers. Kartogenin (KGN): A small heterocyclic molecule, it accelerates the differentiation of human bone marrow multipotent stem cells into chondrocytes. In experiments, varying concentrations of KGN (100, nM) are employed to treat human bone marrow multipotent stem cells for two weeks, intended for the subsequent part of the assay. Before successful induction of differentiation, the EZH2 inhibitor, GSK126, is added, acting for 48 h, aiming for the third part of the assay. Concurrently, chloramphenicol is included in the fourth part of the assay.

Establishment of stable cell lines

The CDS region of the NOTCH1 mRNA sequence from the NCBI is synthesized onto the enzyme digestion site of the pCDH vector, using the human NOTCH1 gene as an illustration. A three-plasmid packaging system is utilized, comprising the transfer plasmid pCDH-EV/pCDH-NOTCH1, pCDH-sh-NOTCH1, the packaging plasmid pSPAX2, and the envelope plasmid pMD2.G. Initially, 293T cells are seeded in a 6 cm culture dish, and the medium is replaced when cell density reaches 70-80%. Then, 2.5 μg of pCDH-EV/pCDH-NOTCH1 plasmid, 1.5 μg of pSPAX2, and 1.5 μg of pMD2.G are incorporated into opti-MEM, forming solution A of 100 μL; 30 μL of DNA transfection reagent PolyJet is added to opti-MEM, forming solution B of 100 μL. After a brief rest, solution B is gently mixed into solution A, which is then slowly dripped into the 293T cell culture supernatant. After 12 h, the medium is replaced, and after 48 h, the supernatant is collected and centrifuged at 2000 rpm for 10 min, discarding the cell precipitate, then filtered through a 0.45 μm membrane, thus obtaining a viral liquid containing viral particles. Cells in the logarithmic growth phase are digested, resuspended, and spread in a 6-well plate, adding DMEM medium containing varying concentrations (0~10 μg/mL, setting 6 gradients) of puromycin when cell density reaches 80-90%. After 24 h, cells are observed to determine the lowest concentration (2 μg/mL) at which all cells are deceased, which is then used as the selection concentration for stable clones. The logarithmic growth phase of SU-DHL-2 cells is seeded in a 6-well plate, and when cell density achieves 70%, the medium is replaced. Each well is infected with a 3:1 ratio of viral liquid to complete culture medium. After infection for 48 h, the medium is replaced with fresh medium containing 2 μg/mL puromycin, continuing the culture. The constructs include LV-OE-NC, LV-NOTCH1-OE, LV-KD-NC, LV-NOTCH1-KD.

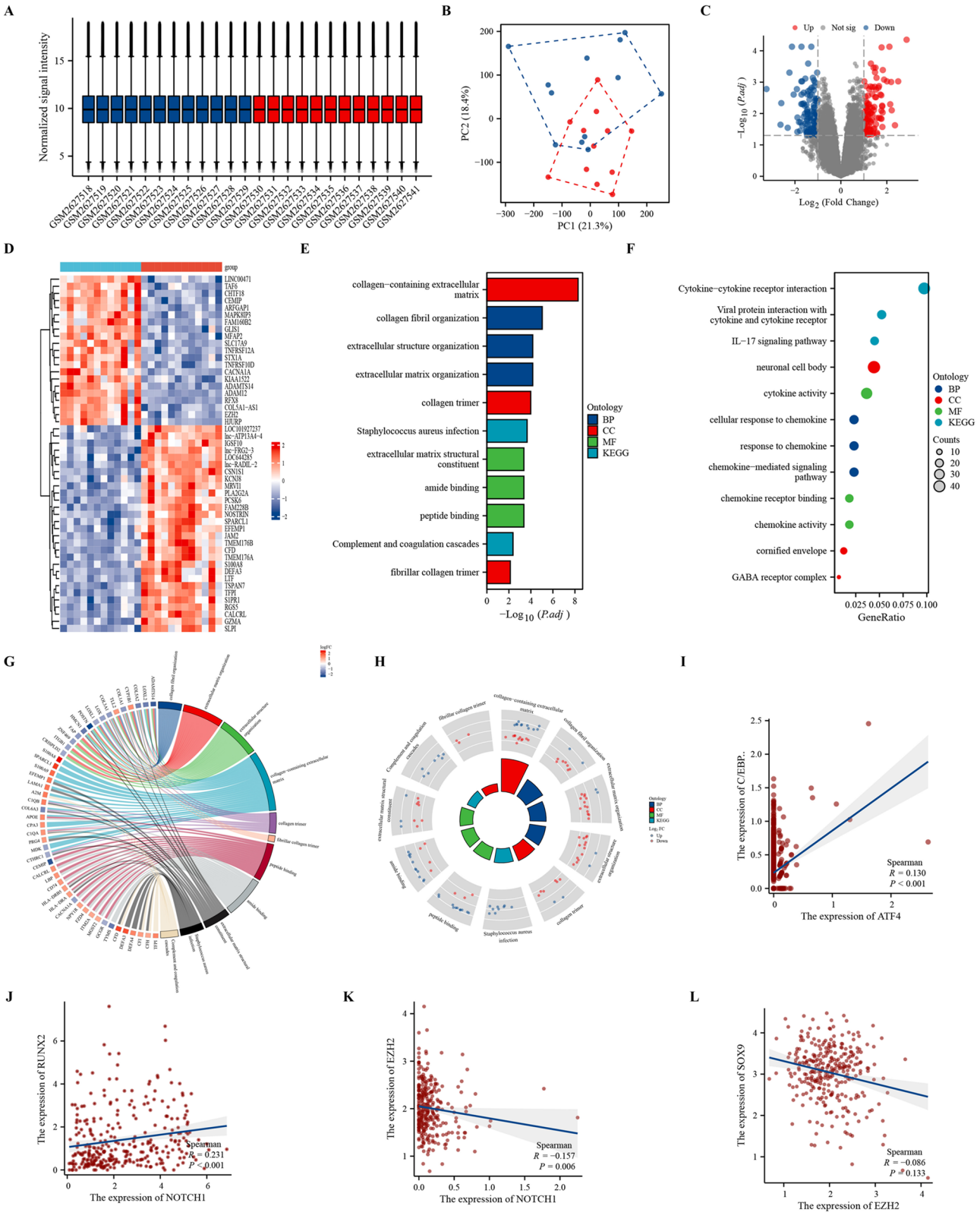

Fig. 1 (See legend on next page.)

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Fig. 1 Bioinformatics analysis. **A**. Box plots illustrating the distribution of 24 samples from the OA-related dataset GSE98918, comprising 12 osteoarthritis patients and 12 non-osteoarthritis subjects. **B**. PCA plot displaying clustering patterns between osteoarthritis patients and non-osteoarthritis subjects based on the dataset GSE98918. **C**. Volcano plot representing the outcomes of the limma package's analysis, showing differential gene expression between osteoarthritis patients and non-osteoarthritis subjects. **D**. Heatmap exhibiting the expression profiles of the top 50 differentially expressed genes in osteoarthritis patients compared to non-osteoarthritis subjects. **E**. Bar plots illustrating the top 12 enriched pathways of differentially expressed genes in biological processes, cellular components, and molecular functions. **F**. Bubble plots visualizing the top 12 enriched pathways of differentially expressed genes in biological processes, cellular components, and molecular functions. **G**. Chord plots depicting the top 12 enriched pathways of differentially expressed genes in biological processes, cellular components, and molecular functions. **H**. Circular plots showcasing the top 12 enriched pathways of differentially expressed genes in biological processes, cellular components, and molecular functions. **I**. Scatter plot illustrating the co-expression relationship between ATF4 and C/EBPβ, suggesting ATF4's promotion of C/EBPβ expression. **J**. Scatter plot depicting the co-expression relationship between NOTCH1 and RUNX2, implying NOTCH1's facilitation of stemness expression. **K**. Scatter plot demonstrating the co-expression relationship between NOTCH1 and EZH2, elucidating NOTCH1's inhibition of EZH2 expression. **L**. Scatter plot showing the co-expression relationship between EZH2 and SOX9, indicating EZH2's suppression of SOX9 expression

Western blot

Initially, protein extraction is carried out using RIPA lysis solution and protease inhibitor treatment on the samples, followed by protein quantification through the BCA protein assay kit. Subsequently, SDS-PAGE is utilized for the electrophoretic separation of proteins, after which the proteins are transferred from the gel to a PVDF membrane. Post-transfer, the membrane is blocked using a 5% skim milk or 5% BSA solution to prevent non-specific binding. Thereafter, the membrane is incubated overnight at 4 °C with primary antibodies against Notch1, EZH2, SOX2, SOX9, KLF2, KLF4, OCT4, COL2A1, AGC-1, COMP, METTLE3, p-EIF2α, ATF4, C/EBPβ, CHOP, CASPASE3, and CASPASE9, followed by a 2-hour room temperature incubation with secondary antibodies. Finally, an ECL chemiluminescence reagent is used for the substrate luminescence reaction, and imaging is performed using an exposure device to capture and analyze the optical density of protein bands.

Immunofluorescence

Initially, chondrocytes, followed by the blockade of innate antibodies, are supplemented with a primary antibody and incubated overnight at 4 °C. Subsequently, after washing, a fluorescence-tagged secondary antibody is added and allowed to react at room temperature for one hour. Lastly, a mounting medium containing DAPI is used for mounting, and observations and photographs are taken under a fluorescence microscope.

Statistical analysis

Data analysis is conducted using GraphPad Prism version 9.0 and presented as mean ± standard deviation. The Student's t-test is employed to compare differences between two groups, and one-way ANOVA is used to compare differences among multiple groups. A $^{*}P<0.05$ is considered statistically significant. All experiments are independently repeated three times.

Results

Bioinformatics analysis

Through screening the OA-related dataset GSE98918 from the GEO database, 24 OA-related samples were obtained, comprising 12 osteoarthritis patients and 12 non-osteoarthritis patients. Employing box plots (Fig. 1A) for the correction of the 24 samples, the PCA plot (Fig. 1B) was utilized to observe the clustering between the two groups, indicating a relatively dispersed pattern. Utilizing the limma package for differential analysis between the two groups, the results of the differential analysis were visualized using a volcano plot (Fig. 1C), where each point represents a gene: blue points signify significantly downregulated genes, red points denote significantly upregulated genes, and black points represent genes with unchanged expression levels. Simultaneously, significant expression molecules were visualized in the form of a heatmap (Fig. 1D), displaying the top 50 differentially expressed genes in microarray data analysis results: red indicates significantly upregulated genes, and blue represents significantly downregulated genes. Corresponding differential expressed genes were subjected to GO analysis for integration of GO terms, followed by the creation of a biological process network. GO function annotation results were categorized into three classes: biological process (BP), cellular component (CC), and molecular function (MF). Through GOKEGG enrichment analysis, a total of 220 IDs were input, of which 179 Entrez IDs were successfully converted, with a conversion rate of 81.4%. After visualization of the results, bar plots (Fig. 1E), bubble plots (Fig. 1F), chord plots (Fig. 1G), and circular plots (Fig. 1H) were obtained. The top 12 enriched pathways of BP, CC, and MF differential expressed genes were in biological processes such as chemical synaptic transmission, cell adhesion, and potassium ion transport. A scatter plot of gene co-expression was depicted, with gene expression levels represented by color, and a blue line indicating the fitting line. Specifically, ATF4 and C/EBPβ co-expression relationship scatter plot (Fig. 1I) depicted ATF4 promoting C/EBPβ expression. The co-expression relationship scatter plot of NOTCH1 and RUNX2 (Fig. 1J) indicated NOTCH1

promoting stemness expression. Additionally, the co-expression relationship scatter plot of NOTCH1 and EZH2 (Fig. 1K) demonstrated NOTCH1 inhibiting EZH2 expression, while the scatter plot of EZH2 and SOX9 co-expression (Fig. 1L) illustrated EZH2 inhibiting SOX9 expression.

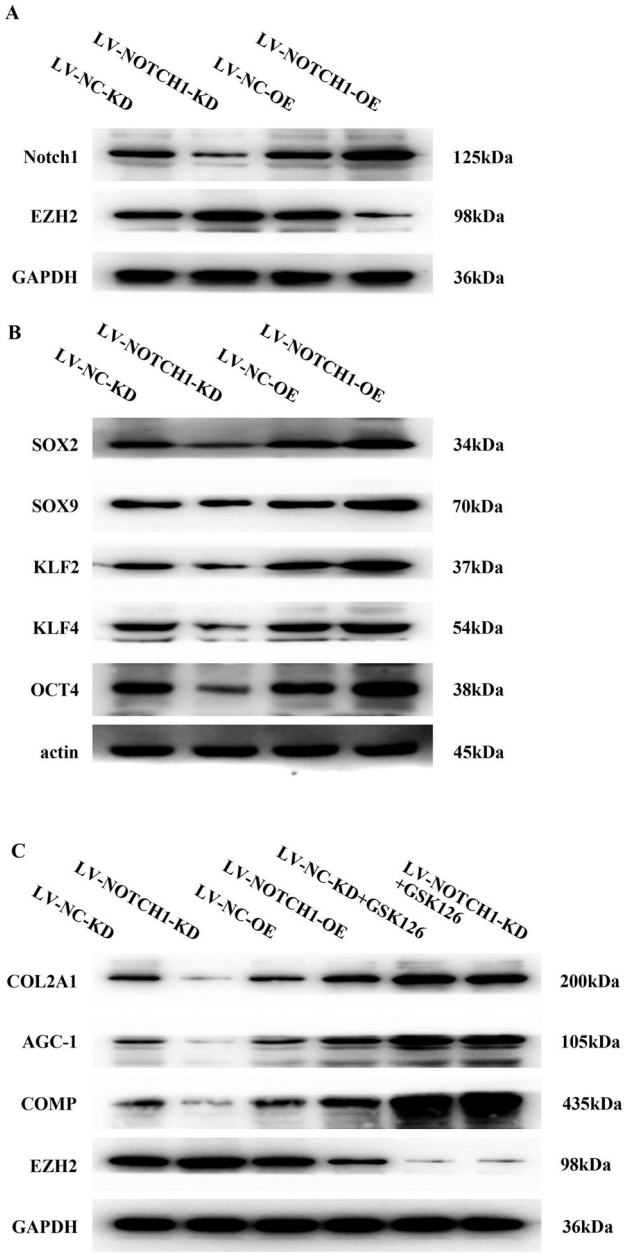

Fig. 2 The impact of NOTCH1 on the maintenance of stemness in MSCs and differentiation into chondrocytes. **A**. The protein bands of NOTCH1 and EZH2 in LV-NC-KD, LV-NOTCH1-KD, LV-NC-OE and LV-NOTCH1-OE groups were detected by Western blot. **B**. Western blot for SOX2, SOX9, KLF2, KLF4, and OCT4 in LV-NC-KD, LV-NOTCH1-KD, LV-NC-OE, and LV-NOTCH1-OE groups. **C**. Western blot for COL2A1, AGC-1, and COMP in LV-NC-KD, LV-NOTCH1-KD, LV-NC-OE, and LV-NOTCH1-OE groups and after addition of EZH2 inhibitor GSK126

NOTCH1 maintains the stemness of mesenchymal stem cells through EZH2 and promotes the differentiation of chondrocytes

To explore the influence of NOTCH1 on mesenchymal stem cells, experiments were conducted using lentiviruses for both knockdown and overexpression of Notch1. Human bone marrow mesenchymal stem cells were infected with these lentiviruses, and total protein from these cells was extracted. Western blot analysis revealed that, compared to the LV-NC-KD group, the expression levels of Notch1 significantly decreased in the LV-NOTCH1-KD group, while EZH2 levels significantly increased. Conversely, compared to the LV-NC-OE group, Notch1 expression levels markedly increased in the LV-NOTCH1-OE group, whereas EZH2 expression decreased significantly (Fig. 2A).

The effects of NOTCH1 knockdown or overexpression on key stem cell markers were then assessed. Western blot analysis also showed a notable decrease in the expression of SOX2, SOX9, KLF2, KLF4, and OCT4 in the LV-NOTCH1-KD group compared to the LV-NC-KD group. In contrast, these protein levels significantly increased in comparison to the LV-NC-OE group (Fig. 2B).

Finally, the effect of EZH2 inhibitors on chondrocyte differentiation under the regulation of NOTCH1 was assessed. Following the induction of differentiation into chondrocytes via Kartogenin in human bone marrow mesenchymal stem cells, the addition of the EZH2 inhibitor GSK126 after 48 h increased the protein expression levels of COL2A1, AGC-1, and COMP in both the LV-NC-KD and LV-NOTCH1-KD groups post-differentiation, though the increase was more pronounced in the LV-NOTCH1-KD group. In this set, EZH2 expression was nominally increased and not significant (Fig. 2C).

NOTCH1 enhances the chondrogenic potential through EZH2

Immunofluorescence staining was used to confirm how NOTCH1 enhances the chondrogenic potential of mesenchymal stem cells by regulating EZH2. Immunofluorescence staining experiments demonstrated no significant changes in NOTCH1 fluorescence intensity across all groups; however, the EZH2 fluorescence intensity notably decreased while COL2A1 and AGC-1 intensities significantly increased in the LV-NC-KD+GSK126 and LV-NOTCH1-KD+GSK126 groups compared to their respective controls (Fig. 3).

NOTCH1 strengthens chondrocyte homeostasis by stabilizing endoplasmic reticulum stress

First, differential gene expression in osteoarthritis was analysed using bioinformatics and the related molecular mechanisms were explored. The osteoarthritis dataset

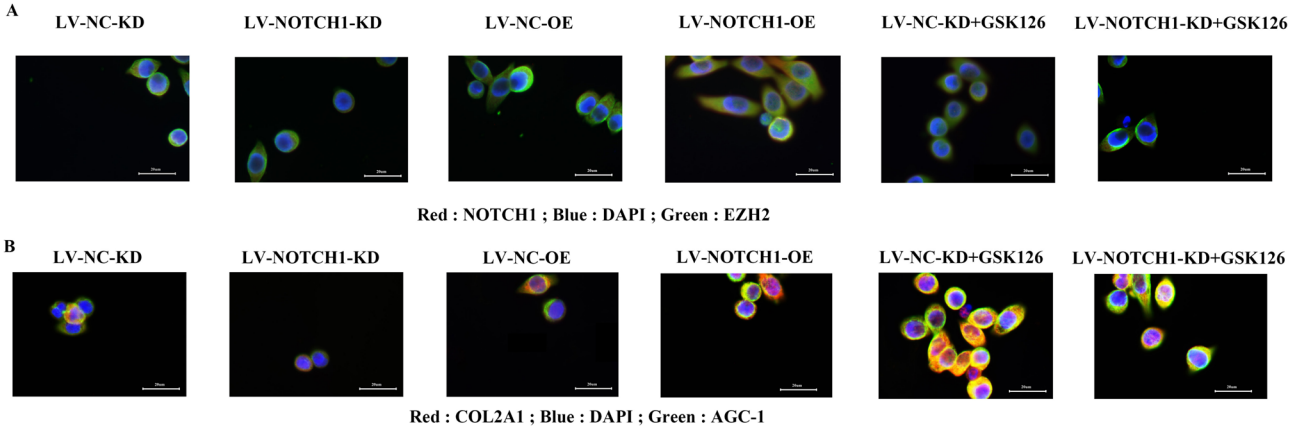

Fig. 3 Immunofluorescence analysis of NOTCH1's promotion of chondrocyte formation capability through EZH2. **A**. Results of immunofluorescence staining experiments to detect NOTCH1 and EZH2. **B**. Results of immunofluorescence staining experiments to detect COL2A1 and AGC-1

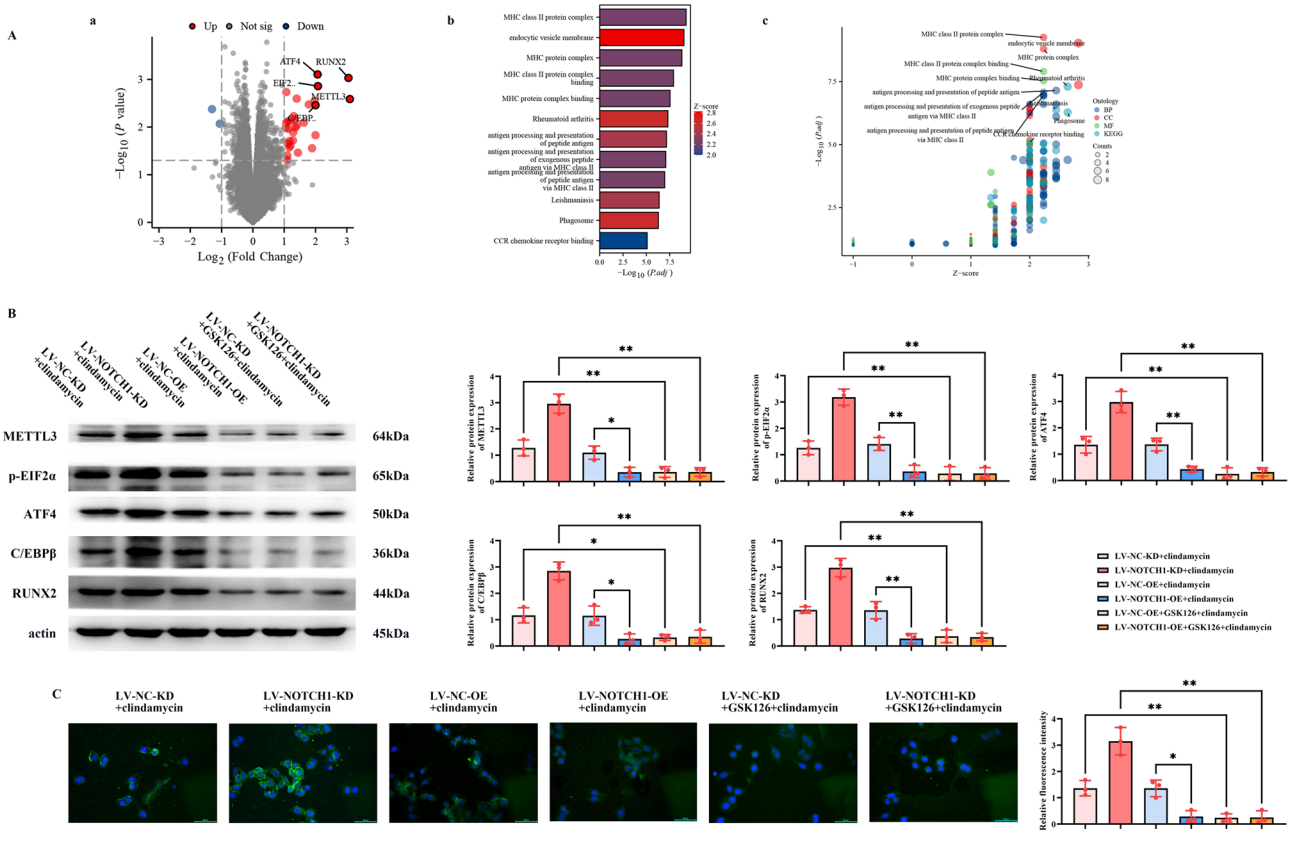

Fig. 4 NOTCH1's enhancement of chondrocyte homeostasis by stabilizing endoplasmic reticulum stress **A**. a. The volcano plot showed a significant increase in the expression of ATF4, C/EBPβ, RUNX2, METTL3, and EIF2α after NOTCH1 silencing. b&c. KEGG analysis showed that pathways related to inflammation response are closely associated with NOTCH1 silencing. **B**. Western blot experiments show the impact of changes in NOTCH1 expression on the expression of proteins such as METTLE3, p-EIF2α, ATF4, and C/EBPβ. **C**. Immunofluorescence experiments display changes in COL2A1 fluorescence intensity. *P<0.05, **P<0.01

GSE98918 was downloaded from the GEO database by the GEOquery package, and 12 of the samples were grouped into the normal expression group and the silent expression group, and the two groups were analyzed by differential analysis and enrichment analysis. Twenty-nine differentially expressed genes were identified by differential analysis, of which 27 genes were up-regulated and 2 genes were down-regulated.Of particular note, ATF4, C/EBPβ, RUNX2, METTL3 and EIF2α were identified as significantly up-regulated genes, and these differentially expressed genes were visualized using a volcano plot (Fig. 4Aa), which clearly showed the changes

in gene expression. The results of enrichment analysis showed that these differential genes were significantly enriched in biological processes such as MHC class II protein complex, endocytic vesicle membrane, and MHC protein complex, which were visualized using bar graph representation (Fig. 4Ab) and bubble graph (Fig. 4Ac), and these results not only reveal the underlying molecular mechanisms of osteoarthritis, but also provide new targets for future therapeutic strategies.

To scrutinize the role of endoplasmic reticulum stress in maintaining the homeostasis of chondrocytes, each group was simultaneously induced with tunicamycin to incite endoplasmic reticulum stress response. Western blot experiments conducted by extracting the total protein from the aforementioned chondrocytes revealed a significant decrease in the protein expression levels of METTLE3, p-EIF2α, ATF4, and C/EBPβ in the LV-NC-KD + GSK126 + tunicamycin group compared to the LV-NC-KD + tunicamycin group. Likewise, a conspicuous reduction in the protein expression levels of METTLE3, p-EIF2α, ATF4, and C/EBPβ was observed in the LV-NOTCH1-KD + GSK126 + tunicamycin group in contrast to the LV-NOTCH1-KD + tunicamycin group. Conversely, the protein expression levels of METTLE3, p-EIF2α, ATF4, and C/EBPβ were notably elevated in the LV-NOTCH1-KD + tunicamycin group when compared to the LV-NC-KD + tunicamycin group. Moreover, the LV-NOTCH1-OE + tunicamycin group exhibited a marked decrease in the protein expression levels of METTLE3, p-EIF2α, ATF4, and C/EBPβ compared to the LV-NC-OE + tunicamycin group (Fig. 4B).

Immunofluorescence staining experiments were subsequently used to assess changes in COL2A1 expression under different NOTCH1 regulation and endoplasmic reticulum stress conditions. Immunofluorescence experiments unveiled that the fluorescence intensity of COL2A1 was significantly reduced in the LV-NC-KD + GSK126 + tunicamycin group as opposed to the LV-NC-KD + tunicamycin group. Similarly, a notable decrease in the fluorescence intensity of COL2A1 was evident in the LV-NOTCH1-KD + GSK126 + tunicamycin group compared to the LV-NOTCH1-KD + tunicamycin group. Conversely, the fluorescence intensity of COL2A1 was markedly increased in the LV-NOTCH1-KD + tunicamycin group when compared to the LV-NC-KD + tunicamycin group. Furthermore, the LV-NOTCH1-OE + tunicamycin group exhibited a significant decrease in the fluorescence intensity of COL2A1 compared to the LV-NC-OE + tunicamycin group (Fig. 4C).

NOTCH1 suppresses apoptosis induced by hypertrophy in chondrocytes through the stabilization of endoplasmic reticulum stress

Apoptosis and endoplasmic reticulum stress in chondrocytes under the treatment conditions of NOTCH1 regulation and EZH2 inhibitor GSK126 were assessed by Western blot and flow cytometry analysis. Flow cytometry results showed that the apoptosis rate and the expression of CHOP, CASPASE3 and CASPASE9 in the LV-NC-KD + GSK126 + tunicamycin group were significantly lower than those in the LV-NC-KD + tunicamycin group. Similarly, the apoptosis rate and the expression of CHOP, CASPASE3 and CASPASE9 in the LV-NOTCH1-KD + GSK126 + tunicamycin group were significantly lower, while the opposite was true in the LV-NOTCH1-KD + tunicamycin group. In contrast, the apoptosis rate and the expression of CHOP, CASPASE3 and CASPASE9 in the LV-NOTCH1-KD + tunicamycin group were significantly higher than those in the LV-NC-KD + tunicamycin group. In addition, the apoptosis rate and the expression of CHOP, CASPASE3 and CASPASE9 in the LV-NOTCH1-OE + tunicamycin group were significantly lower than those in the LV-NC-OE + tunicamycin group. This indicates that NOTCH1 has a protective effect on hypertrophy-induced apoptosis. This protective effect is associated with a decrease in endoplasmic reticulum stress and the levels of apoptosis markers, such as CHOP, CASPASE3 and CASPASE9 (Fig. 5), indicating that NOTCH1 stabilises endoplasmic reticulum stress, thereby reducing the apoptotic activity of chondrocytes. In summary, NOTCH1 affects the expression of SOX9 and EZH2 by binding to their promoters in conjunction with HES1, and further affects the hypertrophy of chondrocytes and the treatment of osteoarthritis through the METTL3 and ATF4/C/EBPβ pathways (Fig. 6).

Discussion

Osteoarthritis (OA) is recognized as a degenerative joint disorder that progressively compromises the structural integrity of cartilage tissue. Currently, conventional treatments relying on pharmaceutical interventions or surgical procedures have shown limited efficacy. In contrast, cellular therapies have emerged as a highly promising avenue for cartilage regeneration. The pioneering therapy developed to address cartilage defects was autologous chondrocyte implantation [17], with stem cell therapy evolving as an innovative treatment paradigm for individuals suffering from osteoarthritis. Research into stem cell therapy for this debilitating condition predominantly focuses on elucidating the cells' pathogenic mechanisms and their therapeutic effects [18].

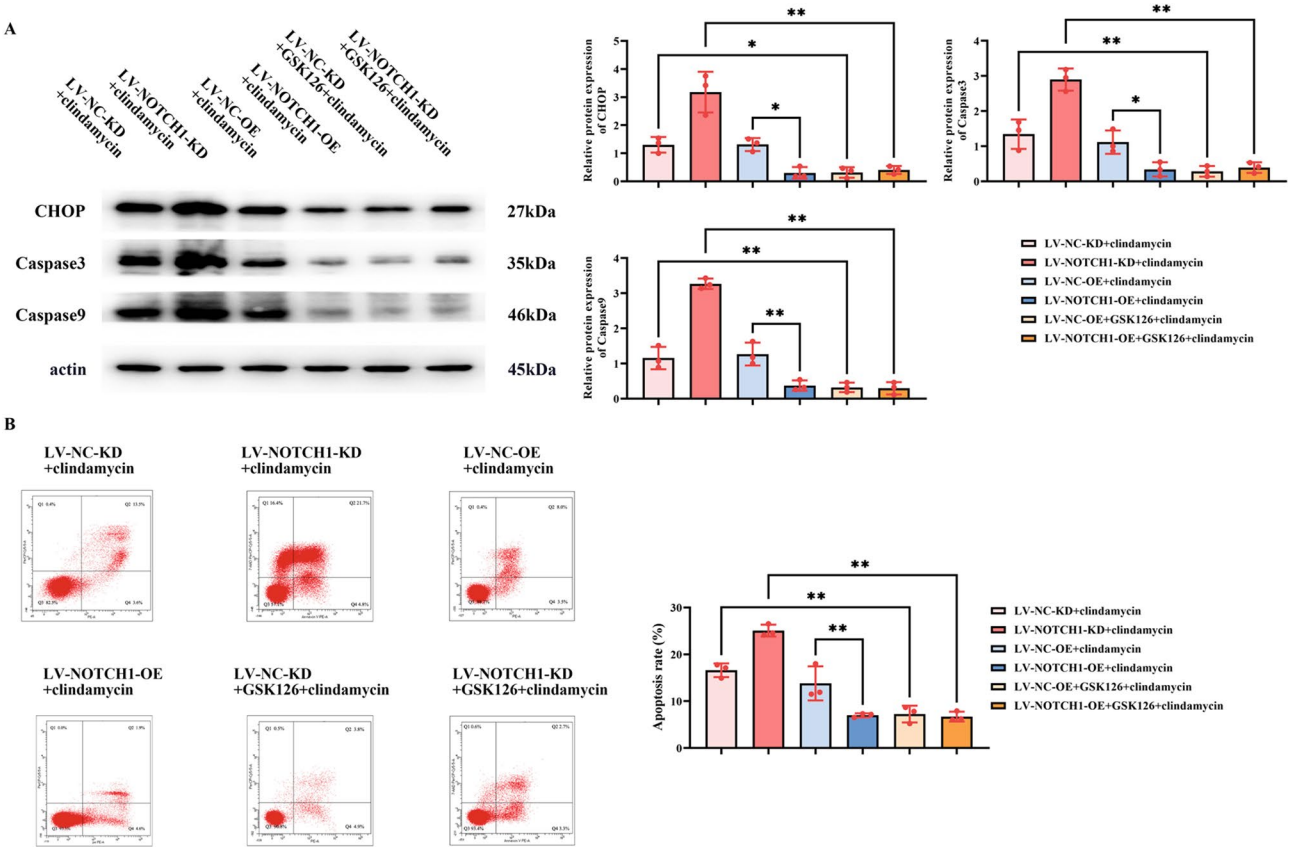

Fig. 5 The inhibitory effect of NOTCH1 on apoptosis induced by chondrocyte hypertrophy. **A**. Protein banding plots and relative protein expression statistics of NOTCH1 expression on the expression of endoplasmic reticulum stress marker CHOP and apoptosis markers CASPASE3 and CASPASE9 detected by Western blot. **B**. Flow cytometry measures changes in the ratio of early and late apoptotic cells. **P*<0.05, ***P*<0.01

In the advanced stages of osteoarthritis, the repair tissues of human joints exhibit a distinctive population of progenitor cells known as Cartilage Progenitor Cells (CPCs), which demonstrate stem cell characteristics and a marked capacity for chondrogenesis [19]. Compared to OA chondrocytes, both categories of OA-MSCs show significantly elevated expression levels of hypertrophic OA cartilage markers, such as COL10A1 and RUNX2. The initiation of chondrogenesis in OA-MSCs further enhances COL10A1 expression and MMP-13 secretion, underscoring their role in the OA phenotype. It has been determined that merely suppressing RUNX2 is insufficient to reduce COL10A1 in OA-MSCs, necessitating the concurrent knockdown of NOTCH1, suggesting a paradigm shift in gene regulation within OA stem cells relative to chondrocytes [20]. The NOTCH1 pathway has been observed to enhance BMP9-driven osteogenesis in cardiac tissue engineering by promoting the coupled processes of osteogenesis and angiogenesis [21]. Detailed bioinformatics analysis has revealed the intricate associations of the NOTCH1 signaling pathway with the maintenance of stemness in Mesenchymal Stem Cells (MSCs) and their differentiation into chondrocytes. A reduction in NOTCH1 (designated as the LV-NOTCH1-KD group) led to an increase in EZH2 expression, while an elevation in NOTCH1 (denoted as the LV-NOTCH1-OE group) resulted in decreased EZH2 expression patterns. These results put forward the idea that NOTCH1 may exert influence on the stemness of MSCs by managing the regulation of EZH2. Amid Kartogenin-induced differentiation of MSCs into chondrocytes, the silencing of NOTCH1 dwindled the protein manifestation of chondrocyte markers (such as COL2A1, AGC-1, COMP), while the amplification of NOTCH1 bolstered the expression of the cited markers. There seems to be a positive correlation between the expression of NOTCH1 and the expression of chondrocyte markers, whereas a contrasting negative correlation is observed with the expression of EZH2. This further substantiates NOTCH1's capacity to regulate the differentiation of chondrocytes via EZH2. During periods of endoplasmic reticulum stress induction, NOTCH1 expression can depress the expression of stress-concomitant proteins (such as METTLE3,

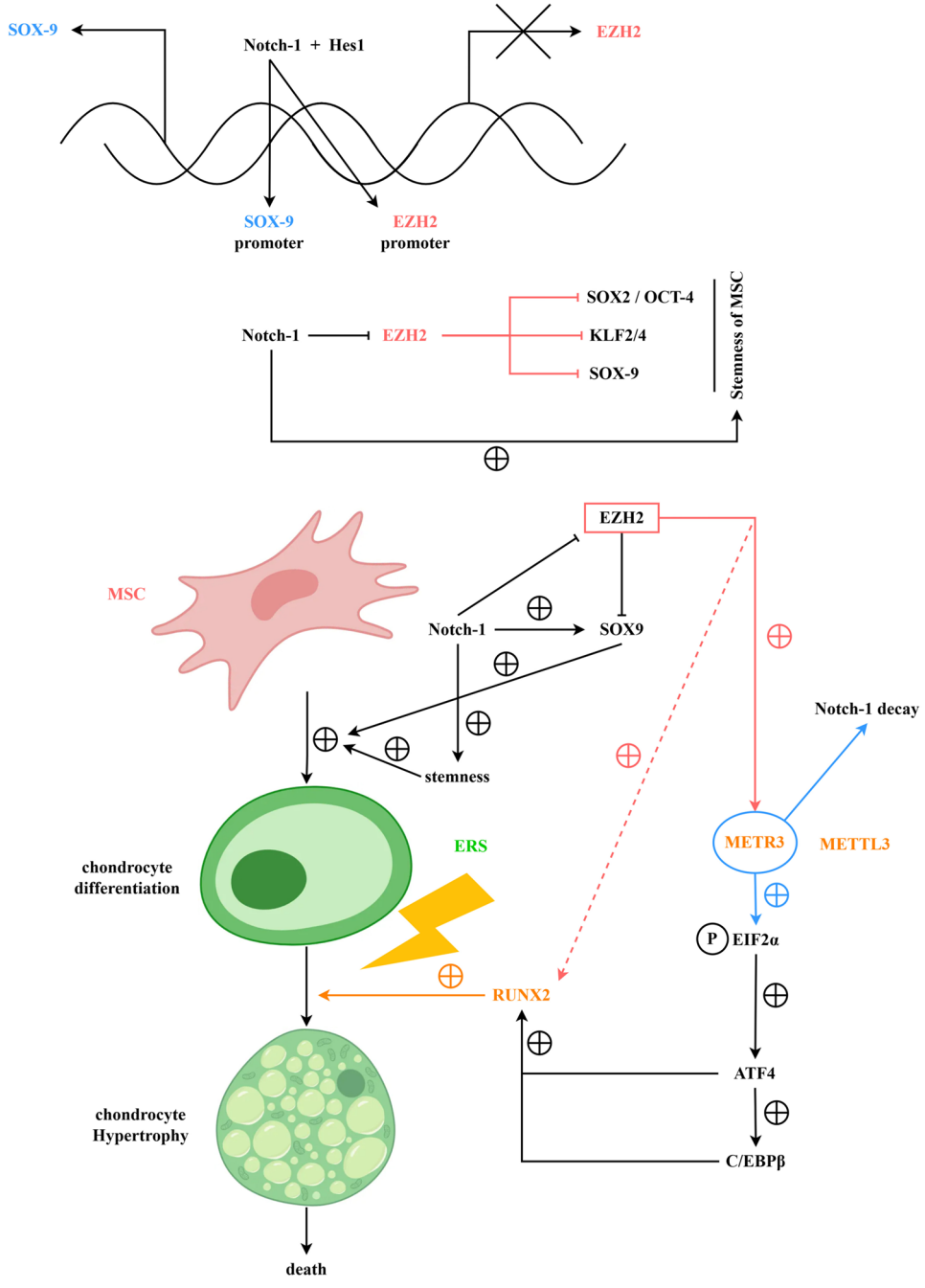

Fig. 6 A schematic diagram of NOTCH1 in the therapeutic conversion of mesenchymal stem cells into chondrocytes for treating osteoarthritis. The diagram illustrates how NOTCH1 influences its expression by acting on the promoters of SOX9 and EZH2 through binding to HES1 and further affects the hypertrophic process of chondrocytes through pathways involving METTL3 and ATF4/C/EBPβ

p-EIF2α, ATF4, C/EBPβ), thus contributing to the maintenance of chondrocyte's homeostasis.

During endoplasmic reticulum stress induction, NOTCH1 expression suppresses stress-related proteins (such as METTL3, p-EIF2α, ATF4, C/EBPβ), contributing to the maintenance of chondrocyte homeostasis. While an environment with 9% oxygen promotes osteogenic differentiation, a reduced oxygen level of 1% markedly inhibits such differentiation in PBMSCs, a result linked to increased NOTCH1 expression [22, 23]. The repression of histone deacetylases (HDACs) and polycomb group genes (PcGs) like EZH2, alongside the increased expression of Jumonji domain-containing 3 (JMJD3), elucidates the aging processes in adult stem cells. HDAC activity preserves the self-renewal capacity of MSCs by balancing the

expression of PcGs and JMJD3 [24]. The epigenetic regulation by EZH2 in the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (BM-MSCs) has been demonstrated. Studies reveal that inhibiting EZH2 enhances the osteogenic differentiation of MSCs [25]. Additionally, research into the impact of EZH2 on the osteogenic differentiation of MSCs found that EZH2 overexpression leads to the downregulation of key osteogenesis-related genes [26]. Through meticulous bioinformatics analysis, the complexities of the NOTCH1 signaling pathway have been established as having notable associations with maintaining stemness in Mesenchymal Stem Cells (MSCs) and their differentiation into chondrocytes. A reduction in NOTCH1 (classified as the LV-NOTCH1-KD group) led to an increase in EZH2 expression, while an increase in NOTCH1 (indicated as the LV-NOTCH1-OE group) resulted in a decrease in EZH2 expression patterns. These findings suggest that NOTCH1 may influence MSC stemness by regulating EZH2. During Kartogenin-induced differentiation of MSCs into chondrocytes, the silencing of NOTCH1 reduced the protein expression of chondrocyte markers (such as COL2A1, AGC-1, COMP), while the enhancement of NOTCH1 increased the expression of these markers. There appears to be a positive correlation between the expression of NOTCH1 and chondrocyte markers, whereas an inverse correlation is observed with the expression of EZH2. This further corroborates NOTCH1's role in regulating chondrocyte differentiation through EZH2. During endoplasmic reticulum stress induction, NOTCH1 expression can suppress the expression of stress-related proteins (such as METTLE3, p-EIF2α, ATF4, C/EBPβ), thereby contributing to the maintenance of chondrocyte homeostasis.

NOTCH1 acts as an active agent in reducing the expression of endoplasmic reticulum stress-induced apoptotic markers (including CHOP, CASPASE3, and CASPASE9), effectively obstructing the apoptosis pathway of chondrocytes. NOTCH1 acts on the promoters of SOX9 and EZH2 by binding to HES1, thereby increasing the expression of SOX9 while decreasing that of EZH2. The reduction in EZH2 triggers an increase in the expression of critical genes for maintaining MSC stemness, including KLF2 and KLF4. During chondrocyte differentiation, NOTCH1 suppresses EZH2, subsequently promoting the expression of METTL3, which is beneficial in the hypertrophic process of chondrocytes.

In summary, the NOTCH1 signaling pathway assumes paramount importance in preserving MSC stemness, facilitating chondrocyte differentiation, and informing osteoarthritis interventions. The regulation of EZH2 and other relevant genes by NOTCH1 has a substantial impact on the differentiation fate of MSCs and the equilibrium of chondrocytes. This presents unexplored targets and strategic approaches for treating conditions such as osteoarthritis.

Supplementary Information

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Supplementary Material 1

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Author contributions
Y.H.Q and W.F.M assumed responsibility for the conceptualization and design of the study. W.F.M engaged in manuscript composition, bioinformatics analysis, western blot analysis, cellular experiments, immunofluorescence, and flow cytometry assays.

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Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval
None.

Competing interests
The authors declare no competing interests.

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