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Gene Expression Patterns and Phenotypic Associations in Chondrogenesis: Insights into Skeletal Dysplasia Nosology

Kondrogenezde Gen İfade Kalıpları ve Fenotipik İlişkiler: İskelet Displazisi Nozolojisine Dair Yaklaşımlar

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Abstract: The differentiation of mesenchymal stem cells (MSCs) into chondrocytes, known as chondrogenesis, is a complex process that plays a fundamental role in cartilage formation and skeletal development. This study elucidates the transcriptional dynamics and phenotypic correlations at various stages of chondrogenesis (early, mid, and late) using RNA-seq data. We focused on the differential expression of transcription factors (TFs) and RNA-binding proteins (RBPs). We identified critical genes during their highest expression periods and generated heatmaps to visualize these temporal patterns. Additionally, we conducted a comprehensive analysis of skeletal dysplasia nosology genes, determining their highest expression periods and phenotypic implications using the DisGeNET database. Our findings reveal that early-stage (D1) gene expression is linked to craniofacial development and limb formation anomalies, primarily involving genes responsible for extracellular matrix (ECM) organization and signal transduction. Mid-stage (D7) genes are associated with cartilage matrix composition and skeletal growth, highlighting roles in chondrocyte proliferation and matrix deposition. Late-stage (D21) genes are implicated in bone mineral density, cartilage integrity, and joint formation, ensuring the maturation and functionality of cartilage tissue. This study provides a detailed analysis of gene expression regulators and their phenotypic correlations during chondrogenesis, offering insights into the molecular mechanisms driving cartilage development and skeletal dysplasias. Understanding these temporal gene expression patterns enhances our knowledge of chondrogenesis and aids in developing targeted therapies for cartilage-related diseases. These findings underscore the significance of time-point analyses in capturing the dynamic regulation of gene expression throughout the differentiation process.
Keywords: Chondrogenesis, Gene Expression Profiling, Skeletal Dysplasia, Transcription Factors (TFs), RNA-Binding Proteins (RBPs).

Özet: Kondrogenez olarak bilinen mezenkimal kök hücrelerin (MKH) kondrositlere farklılaşması, kıkırdak oluşumu ve iskelet gelişiminde temel rol oynayan karmaşık bir süreçtir. Bu çalışma, RNA-seq verilerini kullanarak kondrojenezin çeşitli aşamalarındaki (erken, orta ve geç) transkripsiyonel dinamikleri ve fenotipik korelasyonları aydınlatmaktadır. Çalışmada, Transkripsiyon faktörlerinin (TF'ler) ve RNA-bağlayıcı proteinlerin (RBP'ler) diferansiyel ekspresyonuna odaklandık. En yüksek ifade dönemlerinde kritik genleri belirledik ve bu zamansal kalıpları görselleştirmek için ısı haritaları oluşturduk. Ayrıca, DisGeNET veri tabanını kullanarak iskelet displazisi nozoloji genlerinin kapsamlı bir analizini yaptık, en yüksek ekspresyon dönemlerini ve fenotipik etkilerini belirledik. Bulgularımız, erken evre (D1) gen ifadesinin kraniyofasiyal gelişim ve uzuv oluşumu anomalileri ile bağlantılı olduğunu, öncelikle ECM organizasyonu ve sinyal iletiminden sorumlu genleri içerdiğini ortaya koymaktadır. Orta evre (D7) genleri, kondrosit proliferasyonu ve matris birikimindeki rolleri vurgulayarak kıkırdak matris bileşimi ve iskelet büyümesi ile ilişkilidir. Geç evre (D21) genleri kemik mineral yoğunluğu, kıkırdak bütünlüğü ve eklem oluşumunda rol oynayarak kıkırdak dokusunun olgunlaşmasını ve işlevselliğini sağlar. Bu çalışma, kondrogenez sırasında gen ekspresyon düzenleyicilerinin ve fenotipik korelasyonlarının ayrıntılı bir analizini sunarak, kıkırdak gelişimi ve iskelet displazilerini yönlendiren moleküler mekanizmalar hakkında fikir vermektedir. Bu zamansal gen ifadesi modellerinin anlaşılması, kondrojenez hakkındaki bilgilerimizi artırmakta ve kıkırdakla ilgili hastalıklar için hedefe yönelik tedavilerin geliştirilmesine yardımcı olmaktadır. Bu bulgular, farklılaşma süreci boyunca gen ifadesinin dinamik düzenlemesini yakalamada zaman noktası analizlerinin öneminin altını çizmektedir.
Anahtar Kelimeler: Kondrogenez, Gen İfade Analizi, İskelet Displazisi, Transkripsiyon Faktörleri (TF), RNA-Bağlayıcı Proteinler (RBP).

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1. Introduction

Chondrogenesis is the process through which cartilage is formed from condensed mesenchymal stem cells (MSCs) differentiating into chondrocytes (1). This process provides the template for bone formation through endochondral ossification, which is crucial for skeletal development (2). Chondrogenesis stages start with MSC condensation, followed by chondrocyte differentiation, proliferation, matrix production, hypertrophy, and conclude with mineralization. Each stage is tightly regulated by a complex network of transcription factors and signaling pathways (3). The condensation of MSCs at specific sites in the developing embryo is the initial stage of chondrogenesis, mediated by cell-cell and cell-matrix interactions through cell adhesion molecules such as N-cadherin and integrins (4). This condensation progresses to chondrocyte differentiation influenced by various signaling molecules and transcription factors. During this stage, the commitment of MSCs to the chondrogenic lineage is marked by the expression of chondrocyte-specific markers such as SOX9, COL2A1, and ACAN. Among these, SOX9 is the cardinal regulator of chondrogenesis, essential for activating other chondrocyte-specific genes and maintaining the chondrogenic phenotype (5). For the proper differentiation and function of chondrocytes, the presence of SOX9 is crucial, along with other transcription factors such as RUNX2, SOX5, SOX6, MEF2C, and HIF-1α (6-8).

After the initial differentiation, chondrocytes rapidly proliferate and produce the cartilage- specific extracellular matrix (ECM) components, primarily composed of collagen type II, aggrecan, and other proteoglycans. This ECM provides cartilage's structural framework and biomechanical properties (2). In the maturation stage, chondrocytes exit the cell cycle and undergo hypertrophy, characterized by increased cell size and the expression of hypertrophic markers such as *COL10A1* and *MMP13* (2).

Skeletal dysplasias are a heterogeneous group of disorders characterized by abnormalities in the size and shape of the limbs, trunk, and skull, primarily caused by genetic mutations affecting cartilage and bone development (9). Mutations in genes involved in the chondrogenesis process can result in various disorders, such as achondroplasia, osteoarthritis, and other forms of skeletal dysplasia (10). Understanding the molecular mechanisms underlying chondrogenesis is essential to elucidate the pathogenesis of skeletal dysplasias and develop targeted therapies (11). Investigating and analyzing the transcriptional dynamics and phenotypic correlations of the chondrogenesis stages can provide insights into the development of the skeletal system and identify potential therapeutic targets for treating cartilage-related diseases.

Transcriptomic profiling during chondrogenesis can reveal critical regulatory mechanisms. Numerous studies have been conducted to unravel the complexities of chondrogenesis and its regulatory mechanisms (12-17). Our study was inspired by these conceptual works, aiming to contribute a comprehensive analysis that serves as a helpful guide for both in vitro and ex vivo modeling of chondrogenesis. By concentrating on the differential expression of transcription factors and RNA-binding proteins and integrating gene expression profiles with phenotypic data, we provide insights that enhance the understanding of cartilage development and skeletal dysplasias. This approach complements existing research and addresses the need for a systematic and detailed framework for further investigations into cartilage-related diseases.

RNA-seq data were collected from mesenchymal stem cells differentiating into chondrocytes at various times. Gene Ontology (GO) and functional enrichment analyses were conducted to pinpoint critical biological processes and pathways. By integrating gene expression profiles with

phenotypic data, this study provides a comprehensive view of the molecular mechanisms driving chondrogenesis and their implications in skeletal dysplasia. The findings highlight the potential clinical implications of understanding the molecular basis of skeletal dysplasias, which can guide the development of targeted therapies and ultimately enhance patient outcomes.

2. Materials and Methods

Data Acquisition

The gene expression data used in this study were obtained from the Gene Expression Omnibus (GEO) database. A suitable dataset relevant to chondrogenesis was selected based on the experimental conditions and sample availability. We selected RNA-seq data from the dataset (GEO accession number: GSE109503) published by Huynh et al. (2019) (17).

Identification of Differentially Expressed Genes (DEGs)

The preprocessed data were analyzed using the integrated Differential Expression and Pathway analysis (iDEP v1.13) tool, an online platform for RNA-seq analysis (18). Log transformation and scaling were applied to prepare the data for downstream analysis. Differential gene expression analysis was performed to identify significantly upregulated or downregulated genes at different stages of chondrogenesis. Statistical thresholds for significance were set (e.g., adjusted p-value < 0.05, |log2 fold change| > 1) to ensure the robustness of the identified DEGs.

Gene Ontology and Pathway Analysis

The identified DEGs were subjected to GO enrichment analysis using iDEP to determine the biological processes (BP), cellular components (CC), and molecular functions (MF) significantly enriched at each stage of chondrogenesis. The GO terms were visualized using dot plots and bar graphs to highlight the critical biological themes at each differentiation stage. The Enrichr platform mapped the DEGs to the DisGeNET terms to identify potential phenotypic outcomes associated with the expression changes (19, 20). This analysis provided insights into the clinical relevance of the gene expression patterns observed during chondrogenesis.

3. Results

In the present study, we utilized Principal Component Analysis (PCA) to investigate the transcriptomic evolution during the differentiation of MSCs over 21 days. The differentiation process was divided into three distinct stages: early (D1), mid (D7), and late (D21), corresponding to days post-induction of differentiation. PCA revealed a substantial variation in gene expression profiles, captured in the first two principal components, accounting for 62.33% of the total variance (PC1: 43.47%, PC2: 18.86%) (Figure 1). At the early stage (D1), the samples clustered closely together, indicating a homogenous transcriptomic profile that deviates from the undifferentiated state (D0). A sharp shift in the PCA plot was observed as the differentiation progressed to the mid-stage (D7), reflecting the onset of transcriptional changes associated with the mid-phase differentiation. By the late stage (D21), the samples were distinctly separated along both principal component axes, signifying extensive transcriptional remodeling that marks the advanced stages of cell differentiation. The trajectory of differentiation from D1 to D21 suggests a sequential transcriptomic reprogramming. Samples from D0 presented minimal dispersion, signifying a relatively uniform expression profile characteristic of undifferentiated MSCs. Conversely, the dispersion of points at D21 indicates high variability, suggesting heterogeneity in cell populations or a broad spectrum of differentiation states. The process of MSC differentiation is collectively accompanied by dynamic changes in gene expression, which are reflected in the PCA representation.

To further delineate the dynamic changes in gene expression associated with MSC differentiation, we performed a differential gene expression analysis comparing sequential stages: undifferentiated to early (D0 to D1), early to mid (D1 to D7), and mid to late (D7 to D21)., and. Our analysis identified a substantial number of DEGs between each stage transition, indicating active regulation of gene expression during differentiation. When comparing the early (D1) to mid (D7) differentiation stages, we found 2,038 DEGs, with 1,120 genes upregulated and 918 downregulated. This change denotes a robust transcriptional response as the cells progress from the early differentiation. In the transition from the mid (D7) to late (D21) stage, there was a decrease in the number of DEGs to 598, with 402 genes upregulated and 196 downregulated, indicating a consolidation phase of gene expression changes as differentiation progresses. The most pronounced changes were observed when comparing the undifferentiated (D0) to early (D1) stage, with 4116 DEGs. In this phase, there was an almost equal distribution of upregulation and downregulation, with 2062 genes upregulated and 2054 genes downregulated (Figure 1B). This reflects the extensive transcriptional reprogramming that cells undergo upon induction of differentiation.

To comprehensively assess the transcriptomic changes across different stages of MSC differentiation, we employed Venn diagrams to illustrate the overlapping genes that upregulated and downregulated between the transitions from undifferentiated to early (D0- D1), early to mid (D1-D7) and mid to late (D21-D7) stages. The analysis of upregulated genes revealed a core set of 13 consistently upregulated genes across all three transitions, highlighting potential critical regulators of MSC differentiation. The transition from D0 to D1 demonstrated the most extensive change, with 1067 unique genes upregulated, whereas the transition from D1 to D7 had 116 unique upregulations, and from D7 to D21, 898 unique genes were upregulated. Similarly, the analysis of downregulated genes identified a common set of 3 genes consistently downregulated through all the stages. The transition from D0 to D1 displayed 1164 unique downregulated genes, indicating a strong downregulation of genes associated with the undifferentiated state. The transitions from D1 to D7 and D7 to D21 exhibited 209 and 814 unique downregulated genes, respectively (Figure 1C). This distribution underscores the dynamic nature of gene expression regulation during MSC differentiation, with a significant transcriptional shift occurring at the initiation of differentiation and more refined changes as differentiation proceeds.

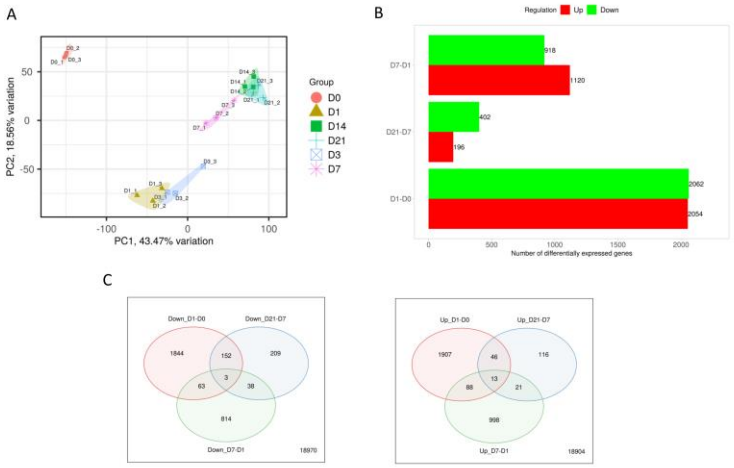

Figure 1. Comprehensive Transcriptomic Analysis and Differential Gene Expression Profiling.

PCA score plot showing the distribution of MSC samples at different stages of differentiation: D0 (red circles), D1 (yellow triangles), D3 (blue squares with a cross), D7 (purple crosses), D14 (green squares), and D21 (blue plus signs) (A). Each point represents a sample labeled with the differentiation day and replicate number (e.g., D0_1). The direction and distance of sample distribution from the origin (D0) to later time points reflect the magnitude of transcriptomic changes occurring at each stage of differentiation, with the trajectory from D1 (early phase) through D7 (mid-phase) to D21 (late phase) indicating progressive transcriptional reprogramming associated with MSC differentiation. The bar chart represents the number of DEGs between consecutive stages of differentiation: D7 vs. D1, D21 vs. D7, and D1 vs. D0 (B). Upregulation is indicated by red bars, while downregulation is indicated by green bars. The number of each bar indicates the count of DEGs in that category. The Venn diagrams represent the upregulated (left) and downregulated (right) gene numbers during MSC differentiation at transitions between undifferentiated to early (D0-D1), early to mid (D1-D7), and mid to late (D21-D7) stages (C). Each circle corresponds to a set of DEGs unique to or shared between the stages, with the total number indicated within. The numbers within the overlapping regions of the circles indicate the count of DEGs shared between the stages, reflecting genes commonly regulated during different phases of differentiation.

The initial differentiation phase from MSCs to D1 reveals critical changes in biological processes, cellular components, and molecular functions (Figure 2). Genes related to the ECM show significant upregulation, indicating that early differentiation involves substantial remodeling of the ECM. Specific components such as collagen are upregulated, suggesting initiating structural changes necessary for differentiation. Upregulation of genes involved in cell adhesion implies increased cell-cell and cell-matrix interactions. Downregulation of genes involved in transcription and DNA binding suggests a decrease in general transcriptional activity, possibly indicating a shift towards more specific gene expression patterns. Genes involved in metabolic processes are downregulated, which may reflect a metabolic shift as cells begin to differentiate. Early differentiation is characterized by significant ECM remodeling and a shift in metabolic activities. There is an initial increase in cell adhesion and structural component genes, with a concurrent decrease in general transcriptional and metabolic activity.

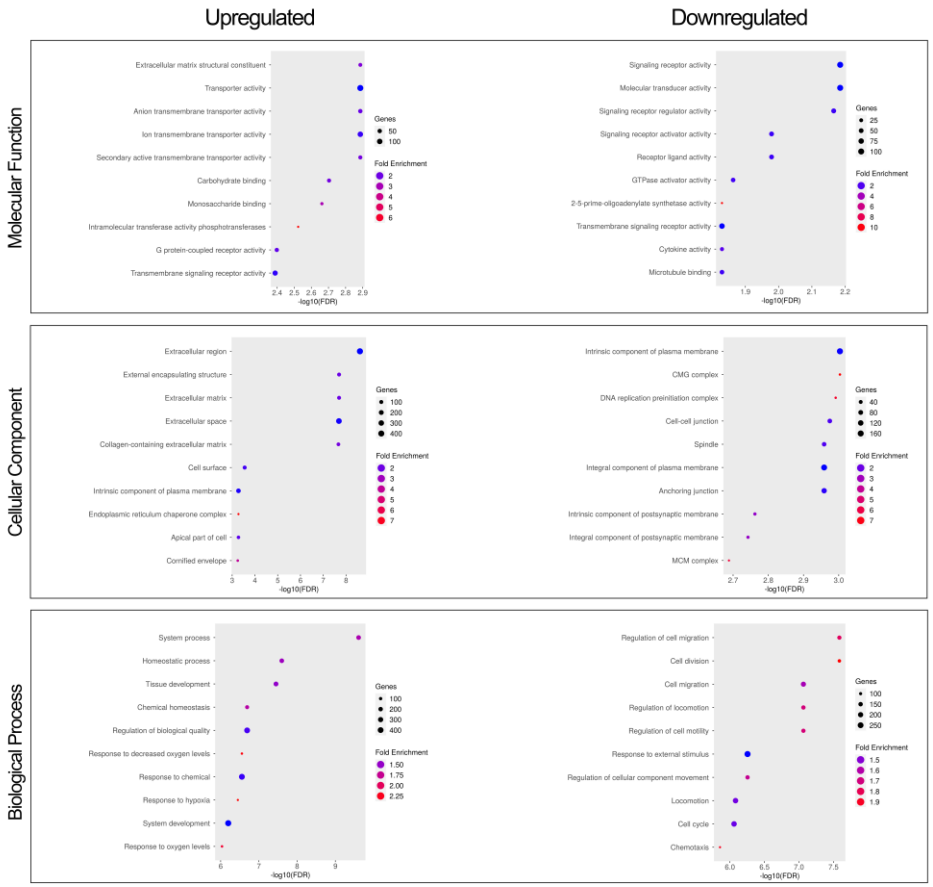

Figure 2. *Gene Ontology (GO) Enrichment Analysis for Genes Upregulated and Downregulated at D1 of MSC Differentiation.*

This set of dot plots displays the significant GO terms associated with genes upregulated (left panel) and downregulated (right panel) during the early stage of differentiation. Each dot represents a GO term, categorized by biological processes, cellular components, and molecular functions. The size of each dot indicates the number of genes involved, and the color depth signifies the fold enrichment. The x-axis shows the -log10(p-value), indicating the statistical significance of the enrichment. This analysis elucidates the shift towards extracellular matrix remodeling and decreased transcriptional activities as MSCs begin to differentiate.

Transition from D1 to D7 during MSC differentiation, our GO enrichment analysis identifies prominently regulated BP, CC, and MF. Continued upregulation of ECM-related genes, highlighting ongoing matrix remodeling (Figure 3). Increased expression of genes involved in collagen fibril organization suggests maturation and strengthening of the ECM. Upregulation of genes involved in receptor signaling indicates enhanced cell communication and signaling as differentiation progresses. Continued downregulation of metabolic process genes, reinforcing the metabolic shift towards differentiation-specific activities. Downregulation of genes involved in phosphorylation and protein kinase activity, suggesting reduced general signaling activity and a focus on specific pathways. Mid-stage differentiation emphasizes ECM development, particularly collagen organization, with enhanced cell signaling. Metabolic processes and general signaling activities continue to decrease, indicating a focus on differentiation-specific functions

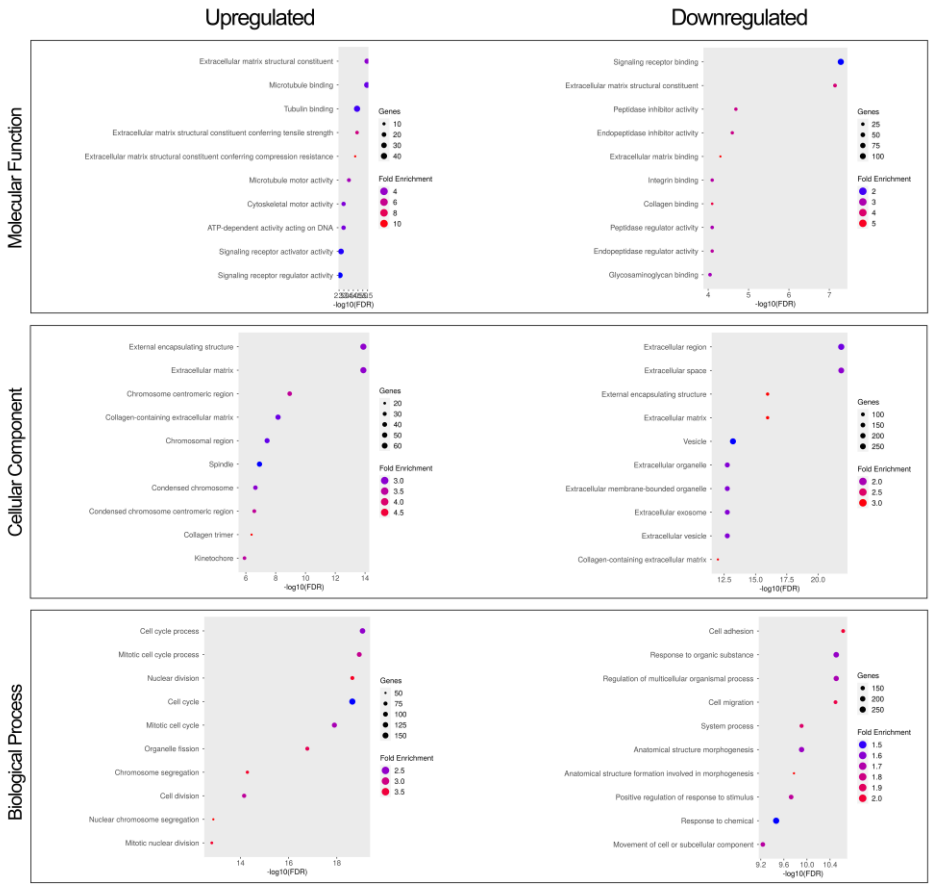

Figure 3. Gene Ontology (GO) Enrichment Analysis for Genes Upregulated and Downregulated from D1 to D7 in MSC Differentiation.

This figure illustrates the significant GO terms associated with genes that are upregulated (left panel) and downregulated (right panel) during the transition from D1 to D7. Each dot represents a GO term, with dot size indicating the number of genes involved and color intensity representing the fold enrichment. The x-axis shows the -log10(p-value), emphasizing the statistical significance of the enrichment. The upregulated genes primarily involved in extracellular matrix formation and collagen structure highlight a developmental focus on tissue architecture. In contrast, the downregulated genes are associated with decreased metabolic activities, indicating a fascinating redirection of cellular resources toward differentiation-specific functions. This novel insight adds to our understanding of MSC differentiation.

MSCs progress from D7 to D21, our analysis highlights significant regulation of critical BP, CC, and MF during the later stages of differentiation (Figure 4). Upregulation of ECM structural genes indicate ongoing ECM maturation and stability. Continued upregulation of collagen-related genes, emphasizing the importance of collagen in later stages of differentiation. Enhanced signaling receptor activity, reflecting increased cell signaling and communication. Protein kinase activity genes are downregulated, indicating a reduced reliance on kinase signaling as cells stabilize in their differentiated state. Downregulation of genes related to DNA binding and chromatin organization suggests a stable and less dynamic chromatin state as differentiation progresses. Late-stage differentiation maintains ECM stability and structural integrity with ongoing collagen involvement. Kinase signaling and DNA binding activities further decrease, suggesting a stabilization of the

differentiated state and reduced dynamic chromatin changes. These observations highlight the dynamic and sequential nature of gene regulation during MSC differentiation, with a clear progression from ECM remodeling and metabolic shifts in the early stages to structural stabilization and reduced general signaling in the later stages.

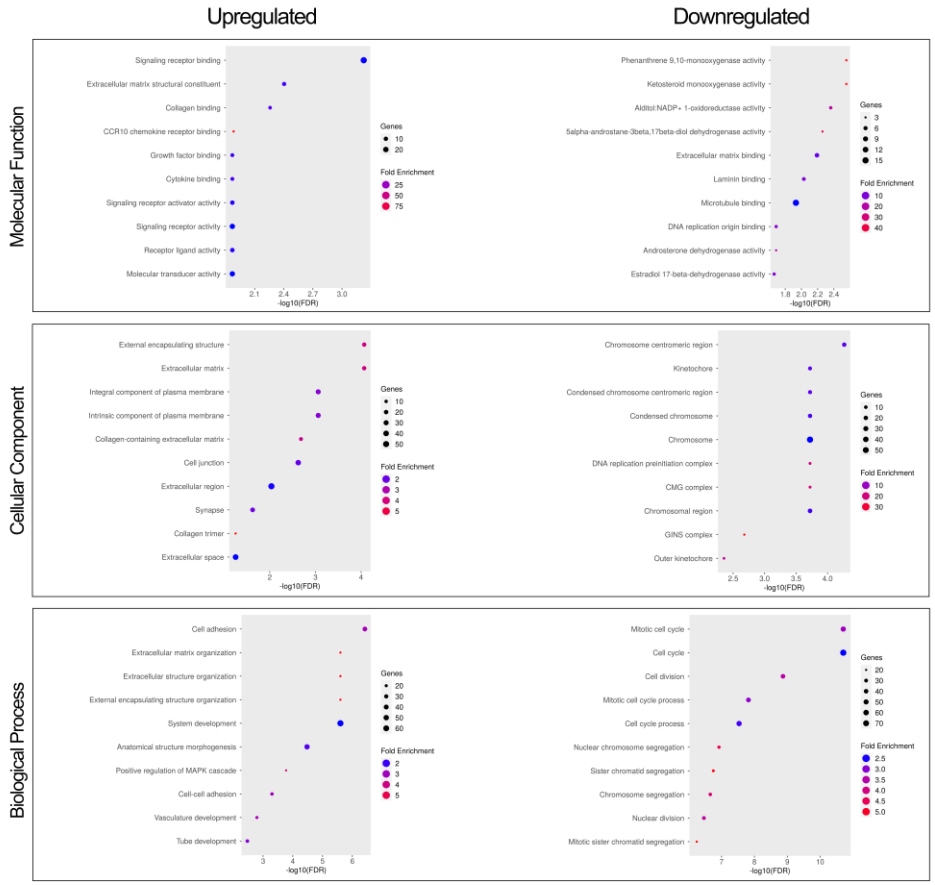

Figure 4. Gene Ontology (GO) Enrichment Analysis for Genes Upregulated and Downregulated from D7 to D21 in MSC Differentiation

This figure illustrates the significant GO terms associated with genes that are upregulated (left panel) and downregulated (right panel) during the transition from D7 to D21. Each dot represents a GO term, where the size of the dot corresponds to the number of genes involved, and the color intensity indicates the fold enrichment. The x-axis represents the -log10(p-value), showing the statistical significance of each term. Upregulated genes are primarily involved in extracellular matrix organization and collagen structure, indicating further maturation and development of the tissue architecture. Downregulated genes are associated with decreased metabolic and signaling activities, reflecting a shift towards maintaining the differentiated state and structural organization.

Next step, we focused on the role of transcription factors (TFs) and RNA-binding proteins (RBPs) as crucial regulators of gene expression during the chondrogenesis of MSCs. By analyzing their expression profiles, we aim to uncover the regulatory networks and molecular mechanisms that guide chondrogenesis. To understand the dynamics of gene expression regulation, we identified DEGs at various time points of chondrogenesis (D0, D1, D7, D21). Specifically, we focused on TFs and RBPs that showed significant changes in expression levels across these points (Figure 5A and B). The DEGs were categorized based on their highest expression levels at specific time points, and heatmaps were generated to visualize these expression patterns. The heatmaps of TFs and RBPs revealed distinct temporal expression patterns, indicating their roles in different phases of chondrogenesis.

Several key observations emerged from the analysis: At the early stage of differentiation (D1), several TFs such as AKNA, ARID5A, and ASCL2 were upregulated, suggesting their involvement in initiating the chondrogenic program. RBPs such as CHD1L and DDX41 also showed increased expression, indicating their potential roles in post-transcriptional regulation during the early differentiation phase. The mid-stage of differentiation (D7) was characterized by the upregulation of TFs such as E2F7 and FOXM1, which are known to regulate cell cycle progression and proliferation. RBPs like NBEAL2 and HELLS were upregulated, highlighting their role in maintaining cellular homeostasis and genomic stability during chondrogenesis. In the late stage of differentiation (D21), TFs such as NPAS3 and HESX1 showed the highest expression levels, indicating their roles in terminal differentiation and maintenance of the chondrocyte phenotype. RBPs such as SAMD4A and SRSF12 were prominently expressed, suggesting their involvement in stabilizing the differentiated state and ensuring proper mRNA processing and export. The heatmap analysis provided a comprehensive view of the dynamic changes in TF and RBP expression during chondrogenesis. These regulators likely play critical roles in orchestrating the complex gene expression programs required to differentiate MSCs successfully into chondrocytes.

Furthermore, a comprehensive analysis of the skeletal dysplasia nosology genes revealed their highest expression levels during chondrogenesis. The genes were categorized based on their highest expression levels, indicating their critical time points during differentiation (Figure 5C). Genes like SH3PXD2B, SMAD4, BMP1, NSMCE2, TBX3, PGM3, IFT43, NMNAT1, and others
showed highest expression levels at D1. This stage is characterized by enhanced signaling pathways and transcriptional activity necessary for initiating chondrogenesis. Genes including EFTUD2, TXNL4A, TBX6, CENPE, LRP4, TCF12, APC2, SIX2,B3GALT6, and others showed highest expression levels at D7. This stage involves matrix production and chondrocyte proliferation, highlighting genes that regulate ECM synthesis and cell cycle progression. Genes such as PEX7, COL9A3, MATN3, HSPG2, SLC39A13, WDR60, COL9A2,ARSE, SLC35B2, and others were predominantly expressed at D21. These genes are associated with terminal differentiation, matrix maturation, and the establishment of cartilage structural integrity.

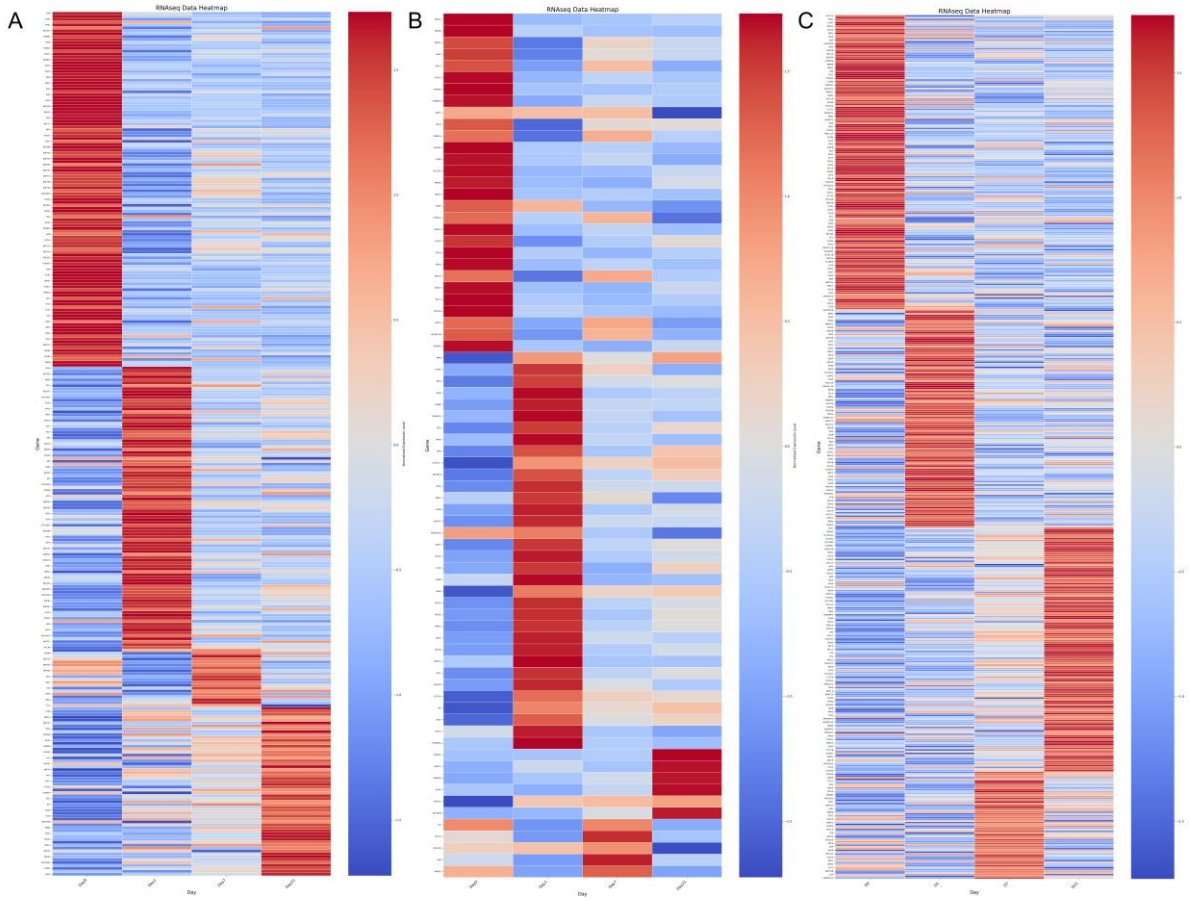

Figure 5. Heatmap of Gene Expression Levels for TFs (A), RBPs (B), and Skeletal Dysplasia (C) Genes During Chondrogenesis.

Heatmap illustrating the normalized gene expression levels of TFs (A), RBPs (B), and skeletal dysplasia nosology genes (C) at four distinct time points (D0, D1, D7, D21) during the chondrogenesis of MSCs. Each row represents a different gene, and each column represents a different time point. The color scale indicates the gene expression intensity, with red representing high expression and blue representing low expression.

The expression patterns of nosology genes and their correlation with phenotypic outcomes were analyzed using DisGeNET and Enrichr (Figure 6). This approach helped identify the phenotypic manifestations associated with the dysregulated genes at different stages of chondrogenesis. Genes at D1 showed strong associations with intellectual disability, short stature, brachydactyly, and osteogenesis imperfecta. D7 was characterized by associations with craniofacial abnormalities such as depressed nasal bridge, retrusion of the lower jaw, and hypoplastic mandible condyle, alongside skeletal anomalies like the curvature of the spine. In the late stage (D21), genes were prominently associated with skeletal conditions, including osteogenesis imperfecta, osteochondrodysplasias, and acquired scoliosis, indicating their critical role in the final stages of skeletal development and maintenance. These associations suggest a stage-specific influence of these genes on skeletal and craniofacial phenotypes.

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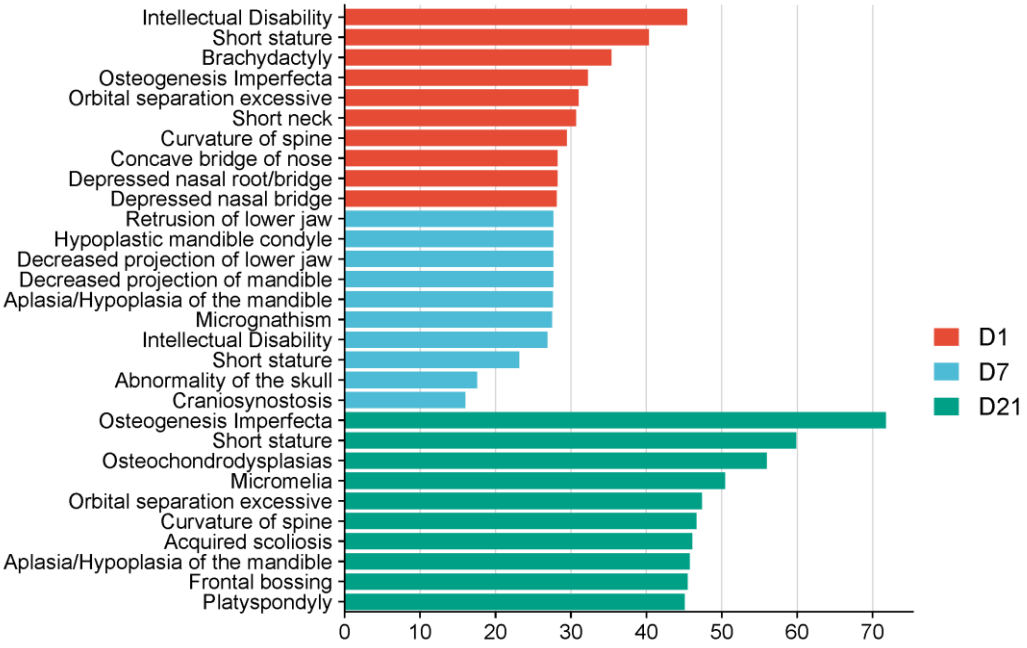

Figure 6. Bar Graph of Phenotypic Associations of Skeletal Dysplasia Genes at Different Stages of Chondrogenesis.

Bar graph showing the significant phenotypic associations of skeletal dysplasia nosology genes at different stages of chondrogenesis (D1, D7, D21). The x-axis represents the - log10 (p-value) of the phenotypic associations. Red bars indicate D1, blue bars indicate D7 and green bars indicate D21. Higher values on the x-axis represent stronger phenotypic associations. This figure highlights the dynamic roles of these genes across different stages of chondrogenesis, with distinct phenotypic implications at each stage.

analyzes the role of gene expression regulators such as TFs and RBPs, which are essential for modulating gene expression patterns that drive cellular differentiation and development. Using RNA-seq data at various stages of chondrogenesis (D0, D1, D7, D21), we identified DEGs, mainly focusing on the roles of TFs and RBPs and analyzed changes in their expression levels. This approach contributes to our understanding of the molecular mechanisms of cellular differentiation processes, highlighting the potential roles of transcription factors and

4. Discussion

This study comprehensively investigated the transcriptional dynamics and phenotypic correlations of MSCs during chondrogenesis. Huynh et al. (2019) reveal the gene expression profiles of MSCs during chondrogenesis through high-depth transcriptomic analysis
(20). Our study uses the same dataset to focus on Skeletal Dysplasia Nosology genes and pathway analysis. It comprehensively

RNA-binding proteins in developing therapeutic strategies for chondrogenic diseases.

We observed that genes highly expressed at the early stage of chondrogenesis (D1) are specifically related to ECM organization and signal transduction pathways. These findings suggest that genes observed at D1 are associated with craniofacial development and limb formation. At the early stage, the synthesis of ECM components and increased cell-cell interactions play critical roles in directing cells toward the chondrocyte

lineage. Furthermore, activation of signal transduction pathways enables cells to receive environmental signals necessary to initiate differentiation (21, 22).

Mid-stage (D7) genes are associated with cartilage matrix composition and skeletal growth, suggesting that these genes play essential roles in maintaining chondrocyte proliferation and matrix deposition. In particular, synthesizing matrix components such as collagen and proteoglycans are necessary for cartilage tissue to maintain its structural integrity (23, 24). In the mid-stage, cells' high proliferative capacity is essential for forming new and growing existing cells. Gene expression profiles observed at this stage show that genes related to cell cycle regulators and ECM production are highly expressed.

This study observed significant changes at the D21 stage of chondrogenesis, especially in the expression levels of splicing elements. Up- regulated splicing factors such as SAMD4A, SAMSN1, SRSF12, and STIM1 and down- regulated splicing elements such as CORO1A, DDX39A, DTL, HELLS, MYEF2, PCBP3, SRSF1, SRSF3, STRBP, and WDR62 stand out as critical components of cellular functions and gene regulation. Changes in these splicing elements can result in the appearance of different isoforms of genes, which play an essential role in regulating cellular phenotypes and functions (26). Studying late-stage genes at the isoform level may be critical for a better understanding these processes and a detailed analysis of genetic regulation in chondrogenesis. Late- stage (D21) genes are associated with bone mineral density, cartilage integrity, and joint formation phenotypes. These genes are critical in ensuring the maturation and functionality of cartilage tissue (26). In the late stage, hypertrophy of cells and initiation of mineralization processes mark the onset of endochondral ossification (27). This process is characterized by hypertrophy of chondrocytes and mineralization of the matrix. Among the genes expressed at this stage, hypertrophic chondrocyte markers and mineralization- related genes stand out.

The phenotypic associations observed in this study highlight the dynamic and stage-specific roles of skeletal dysplasia nosology genes during chondrogenesis. Early-stage genes (D1) are primarily linked to intellectual and skeletal development disorders, underscoring the foundational role of these genes in early cartilage formation and growth. The mid-stage (D7) associations with craniofacial anomalies, such as depressed nasal bridge and retrusion of the lower jaw, emphasize the critical period for detailed craniofacial morphogenesis. This stage is also marked by active cell proliferation and DNA replication, as evidenced by the involvement of genes in mitotic processes (28). Such activities are crucial for the rapid expansion and structural organization necessary for facial and mandibular development.

Additionally, skeletal anomalies like curvature of the spine during this period highlight the significant remodeling and growth occurring at this stage. In the late-stage (D21), genes showed strong associations with severe skeletal conditions like osteogenesis imperfecta and osteochondrodysplasias, highlighting their role in the final maturation and stabilization of skeletal tissues. The significant expression of genes associated with these conditions indicates ongoing differentiation and mineralization processes, ensuring the proper formation and maintenance of skeletal structures. These findings underscore the importance of temporal gene expression analysis in understanding the development of skeletal dysplasias. For future research, focusing on specific genes at different stages of chondrogenesis can provide targeted insights into therapeutic interventions. The mid-stage (D7) presents a unique window where interventions could influence craniofacial development and correct early skeletal anomalies. This stage's association with active DNA replication and mitosis suggests that treatments to modulate cell proliferation could be highly effective. In the context of in vitro and ex vivo

modeling, these results provide a framework for developing stage-specific models that can accurately replicate the dynamic intricate mechanisms of skeletal development and dysplasia.

By observing the expression of nosology genes during chondrogenesis, we aimed to gain insights into the phenotypic consequences of gene expression changes at different stages. We observed that genes highly expressed in the early stage are associated with craniofacial development and limb anomalies. In contrast, genes expressed in the middle stage are associated with cartilage matrix composition and skeletal growth. Genes highly expressed in the late stage are associated with bone mineral density, cartilage integrity, and joint formation phenotypes. These findings are essential in understanding the molecular basis of various skeletal dysplasias and developing targeted therapies for these diseases.

The findings of this study highlight the dynamic and stage-specific expression of gene expression regulators in chondrogenesis. New insights into the role of transcription factors and RNA binding proteins contribute to a better understanding the molecular mechanisms of chondrogenesis and skeletal dysplasias. Furthermore, the data obtained in this study may help identify new targets for diagnosing and treating cartilage-related diseases.

This study examined the gene expression dynamics and phenotypic correlations of human MSCs in chondrogenesis. However, focusing solely on chondrogenesis is a limitation. Simultaneously examining osteogenesis and chondrogenesis and their combined analysis in skeletal dysplasias would provide more comprehensive and valuable information. Osteogenesis and chondrogenesis are critically interrelatedprocesses in skeletal development (29). Analyzing the genes and signaling pathways involved in both processes may help us better understand the molecular basis of skeletal dysplasias and processes of chondrogenesis. Such models can be invaluable for testing potential therapeutic agents and understanding the develop more effective treatment strategies.

Future research should adopt integrative approaches that examine osteogenesis and chondrogenesis together. Such approaches should include detailed analyses of gene expression profiles, cellular and phenotypic changes, and functional analysis of gene regulators like transcription factors and RNA- binding proteins. This comprehensive study of gene regulatory networks and signaling pathways will better understand the molecular mechanisms underlying cell differentiation and contribute to developing new approaches to treat diseases such as skeletal dysplasias.

Furthermore, analyzing both processes together will allow a more detailed and accurate classification of different types of skeletal dysplasia. This information could contribute to developing new approaches to manipulating chondrogenesis and osteogenesis, enhancing our understanding of skeletal development and improving clinical applications. Understanding the dynamic regulation of gene expression throughout the differentiation process is vital, and time-point analyses are essential in capturing these changes.

This study has taken an essential step in understanding the molecular basis of cartilage development and skeletal dysplasias by comprehensively analyzing gene expression dynamics during chondrogenesis and their phenotypic implications. The findings provide a valuable reference point for future studies on chondrogenesis and skeletal dysplasias.

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Ethics

Ethics Committee Approval: This study did not require ethical approval as it exclusively utilized publicly available data from the Gene Expression Omnibus (GEO) database. No new data were collected, and no human or animal subjects were involved in this research. Therefore, no ethical approval was necessary.

Informed Consent: The authors declared that it was not considered necessary to get consent from the patients because the study was a retrospective data analysis.

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