The Effect of HEXIM1 Knockdown on Gene Expression and Its Biological Function in Undifferentiated Keratinocytes

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Abstract-HEXIM1 is a multifunctional protein that plays significant roles in various biological processes, including transcriptional regulation, cell cycle, and stress responses. It primarily functions by binding to the 7SK small nuclear ribonucleoprotein (7SK snRNP), thereby inhibiting the activity of the P-TEFb complex to regulate gene transcription. In the context of skin self-renewal and repair, the accurate differentiation of epidermal progenitor cells (EPCs) is essential. This study aims to elucidate the specific function and molecular mechanisms of HEXIM1 protein in this process through transcriptome data analysis. The results indicate that HEXIM1 knockdown leads to alterations in the gene expression profiles of undifferentiated keratinocytes. Furthermore, genes with upregulated expression are predominantly enriched in pathways related to "epidermal development" and "keratinization," suggesting that HEXIM1 can suppress the expression of differentiation-related genes, thus maintaining the progenitor cell state.

Keywords- HEXIM1; Transcriptome Analysis; Cell Differentiation

1. Introduction

The HEXIM1 (Hexamethylene Bisacetamide Inducible 1) gene, located on human chromosome 1 (1p36.33), encodes a multifunctional protein that plays a critical role in various cellular processes. HEXIM1 was initially identified as a protein induced by hexamethylene bisacetamide (HMBA), a compound known for its ability to induce differentiation in certain cell types, particularly in promyelocytic leukemia cells (PML). Since its discovery, HEXIM1 has been extensively studied for its diverse functions in transcriptional regulation, cell cycle control, and cellular stress responses. One of the primary functions of HEXIM1 is its involvement in the regulation of gene transcription. Previous studies have reported that HEXIM1 forms a complex with the 7SK small nuclear ribonucleoprotein (7SK snRNP), which is a non-coding RNA that serves as a scaffold for the assembly of regulatory proteins[1]. This complex sequesters the positive transcription elongation factor b (P-TEFb), a key component in the transition from transcriptional initiation to elongation. By inhibiting P-TEFb, HEXIM1 effectively blocks the phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II, thereby preventing the elongation of transcripts[2, 3]. This regulatory mechanism is crucial for controlling the expression of genes involved in cell growth, differentiation, and survival.

The epidermis, the outermost layer of the skin, serves as a critical barrier protecting the body from environmental insults, such as pathogens, UV radiation, and chemical agents[4]. The maintenance and homeostasis of the epidermis rely heavily on the continuous process of keratinocyte differentiation, which ensures the constant renewal of the skin surface. Skin epidermis, composed of 90% keratinocytes, is a type of highly accessible self-renewing somatic tissue[5, 6]. The differentiation process of keratinocytes is strictly regulated, including from proliferating basal cells to terminally differentiated stratum corneum cells.

Here, by analyzing RNA-seq data, we found that HEXIM1 knockdown significantly downregulates the expression of a key set of genes and differentiation-driving transcription factors in keratinocytes. Additionally, the upregulated genes after HEXIM1 knockdown were mainly concentrated in the "cell division" and "DNA replication" pathways. Moreover, the upregulated genes were also predominantly enriched in pathways related to "epidermal development" and "keratinization." These data

suggest that HEXIM1 plays an important regulatory role in keratinocyte differentiation and controls the differentiation process by regulating the expression of key genes. Understanding the detailed mechanisms by which HEXIM1 exerts its effects will provide valuable insights into the molecular basis of these processes and may open new avenues for skin-related diseases.

2. Methods

This study utilized sequencing data from Sample GSM5543712, GSM5543713, GSM5543714, GSM5543715 within the NCBI GEO dataset Series GSE182959 in undifferentiated keratinocytes[7]. To induce differentiation, keratinocytes were seeded in confluence and cultured with the addition of 1.2mM of calcium to growth medium. 10 uM of siRNA for each target were nucleofected. Each experimental condition was replicated twice.

To ensure the reliability of our data, we performed quality control assessments on the raw sequencing reads using FastQC (v0.12.1) and Cutadapt (v2.6) to eliminate low-quality sequences and adapter contaminants. The filtered reads were then mapped to the human reference genome (GENCODE Release 21, GRCh38) using the STAR aligner (v2.7.11b)[8]. Unique alignments were further processed and sorted with Samtools (v1.6). Principal component analysis (PCA) was employed to evaluate the similarity and variability among the samples. Differentially expressed genes (DEGs) between the control (NC) and HEXIM1 knockdown (HEXIM1-KD) groups were identified using the DESeq2 R package (v1.44.0), with a significance threshold of an adjusted p-value (padj) < 0.05 and a log2 fold change ≥ 0.58.

We utilized the clusterProfiler R package (v4.12.2) to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses[9]. These analyses were designed to uncover significant biological processes and key signaling pathways associated with the differentially expressed genes.

3. Results

3.1 DEGs in Undifferentiated Keratinocytes after HEXIM1 Knockdown

To determine the full spectrum of gene expression influenced by HEXIM1 knockdown, we performed RNA-seq data analyses. First, we performed principal component analysis (PCA) on the two replicates of both the control (NC) and HEXIM1 knockdown (KD) groups to assess the consistency and quality of the RNA-seq data. The PCA plot (Figure 1) clearly demonstrates the distinct clustering of the NC and KD groups, indicating a high degree of reproducibility between the replicates within each group. The first principal component (PC1) explains 48.8% of the variance and effectively separates the NC and KD samples, highlighting the significant impact of HEXIM1 knockdown on the global gene expression profile of the keratinocytes. The second principal component (PC2) accounts for 29.5% of the variance, further supporting the robustness of the data and providing additional insight into the variability within the samples.

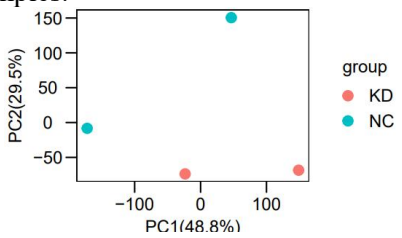

Figure 1. Analysis of Pricipal Componnent Anlysis (PCA)

Following the PCA, we conducted differential expression analysis to identify genes that were significantly upregulated or downregulated in the HEXIM1 knockdown (KD) group compared to the control (NC) group. Using the DESeq2 R package (version 1.44.0), we identified a total of 2000 differentially expressed genes (DEGs) with an adjusted p-value (padj) < 0.05 and a log2 fold change (FC) of 0.58 or higher (Figure 2). Specifically, 1200 genes were upregulated, and 800 genes were downregulated in the KD group.Among the upregulated genes, several key differentiation-activating transcription factors were identified, including GRHL3, OVOL1, PRDM1, and ZNF750. These transcription factors are known to play critical roles in the regulation of keratinocyte differentiation and epidermal development. The upregulation of these genes suggests that HEXIM1 knockdown may promote the differentiation of keratinocytes by activating these transcription

factors.

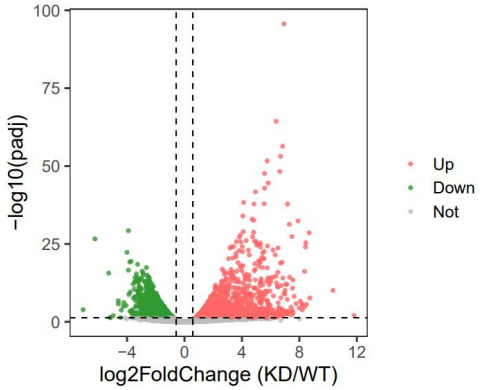

Figure 2. Volcano Plot Show the Upregulated and Downregulated Genes in the NC and HEXIM1 Knockdown Groups from RNA-seq Data.

3.2 Gene Ontology (Go) Enrichment Analysis

To further understand the biological processes, cellular components, and molecular functions associated with the differentially expressed genes (DEGs) identified in the HEXIM1 knockdown (KD) group, we performed Gene Ontology (GO) enrichment analysis using the clusterProfiler R package (version 4.12.2). The GO enrichment analysis revealed that the upregulated genes were primarily enriched in biological processes related to “keratinocyte differentiation”, “skin development”, and “keratinization”. These genes were also enriched in cellular components such as the cornified envelope and apical junction complex, and were significantly associated with molecular functions including structural constituents of the “skin epidermis” and “phospholipid binding” (**Figure 3**).

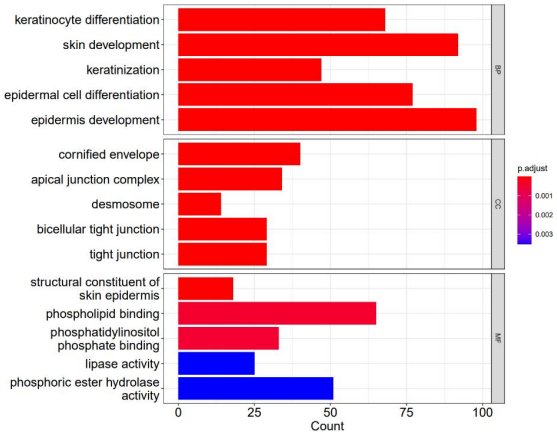

Figure 3. Top GO terms of upregulated genes in HEXIM1 knockdown RNA-seq.

In contrast, the downregulated genes were mainly enriched in biological processes related to “DNA replication” and “chromosome segregation”, and were associated with molecular functions related to DNA binding and chromatin organization (**Figure 4**). These results provide a comprehensive understanding of the biological impact of HEXIM1 knockdown, highlighting its role in promoting keratinocyte differentiation and altering cell cycle-related processes.

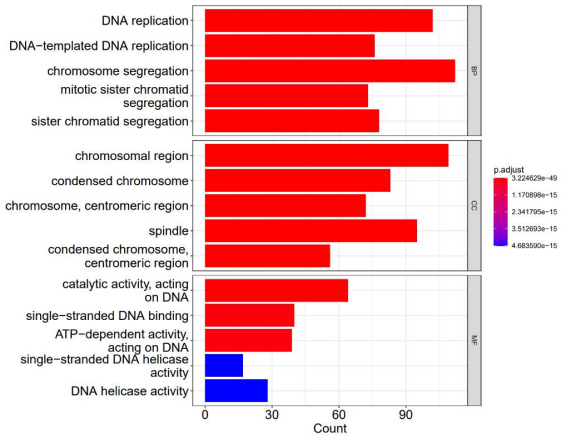

Figure 4. Top GO Terms of Downregulated Genes in HEXIM1 Knockdown RNA-seq.

3.3 KEGG Pathway Enrichment Analysis

Subsequently, we conducted KEGG pathway enrichment analysis to identify the key signaling pathways affected by the differentially expressed genes. The results showed that the upregulated genes were primarily enriched in pathways such as "Tight junction," "Autophagy − animal," and "p53 signaling pathway" (**Figure 5**).

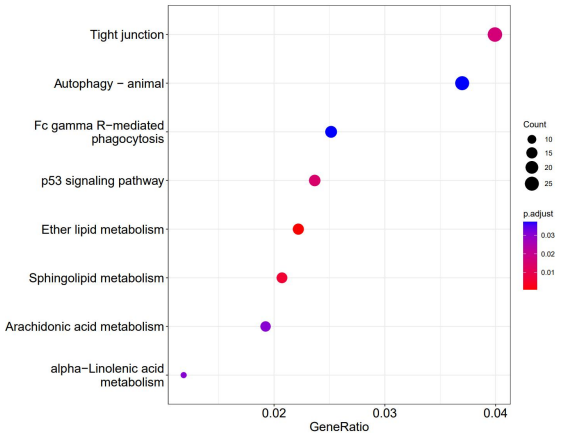

Figure 5. Top KEGG Terms of Upregulated Genes in HEXIM1 Knockdown RNA-seq.

On the other hand, the downregulated genes were mainly enriched in pathways related to "Cell cycle," "Carbon metabolism," and "DNA replication" (Figure 6). These findings provide

a comprehensive understanding of the biological impact of HEXIM1 knockdown, highlighting its role in promoting keratinocyte differentiation and altering cell cycle-related processes, as well as its involvement in tight junction formation, autophagy, and p53 signaling.

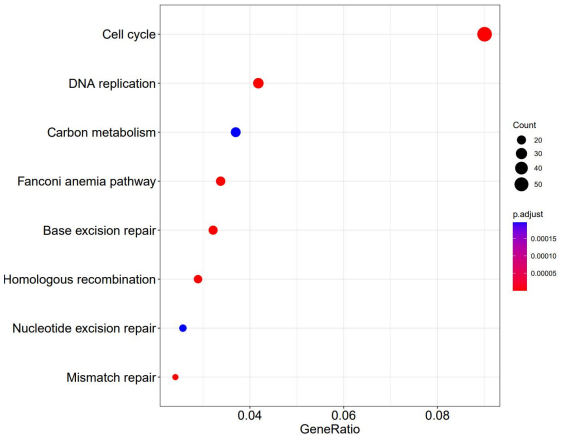

Figure 6. Top KEGG Terms of Downregulated Genes in HEXIM1 Knockdown RNA-seq.

4. Discussion

Our study identified significant changes in gene expression following HEXIM1 knockdown in keratinocytes. The upregulated genes, including key differentiation-activating transcription factors such as GRHL3, OVOL1, PRDM1, and ZNF750, suggest that HEXIM1 acts as a repressor of these genes. HEXIM1 is known to regulate gene expression by interacting with various transcriptional co-repressors and modulating chromatin structure. Therefore, its knockdown likely releases the repression on these differentiation-promoting genes, leading to their upregulation. The downregulated genes, primarily involved in DNA replication and cell cycle progression, indicate that HEXIM1 plays a role in maintaining cell proliferation. HEXIM1 can bind to and inhibit the activity of positive transcription elongation factor b (P-TEFb), which is crucial for the transcription of genes involved in cell cycle regulation. Thus, knocking down HEXIM1 may increase P-TEFb activity, leading to the downregulation of genes required for DNA replication and cell division.

The upregulated genes were enriched in pathways related to keratinocyte differentiation, skin development, and keratinization. These pathways are essential for the formation and maintenance of the epidermal barrier. The upregulation of genes involved in tight junction formation and the cornified envelope suggests that HEXIM1 knockdown promotes the terminal differentiation of keratinocytes, enhancing the barrier function of the skin.

Conversely, the downregulated genes were enriched in pathways related to cell cycle progression, DNA replication, and carbon metabolism. These findings indicate that HEXIM1 knockdown inhibits cell proliferation and shifts the cellular focus from growth to differentiation. The downregulation of cell cycle-related genes supports the idea that HEXIM1 is a key regulator of the balance between proliferation and differentiation in keratinocytes.

Our results demonstrate that HEXIM1 plays a critical role in regulating keratinocyte differentiation and cell cycle progression. The upregulation of differentiation-activating transcription factors and the downregulation of cell cycle-related genes following HEXIM1 knockdown highlight its dual function in maintaining epidermal homeostasis. These findings provide new insights into the molecular mechanisms underlying keratinocyte differentiation and suggest potential therapeutic targets for skin disorders. For example, for skin diseases caused by abnormal differentiation of keratinocytes, such as psoriasis and ichthyosis, the expression or activity of HEXIM1 can be regulated to restore the normal differentiation of keratinocytes, so it can improve skin symptoms. In addition, HEXIM1 may be a potential target for treating skin cancers. Studies have demonstrated that HEXIM1 is overexpressed in some skin tumor cells and that modulating HEXIM1 expression or activity can decrease the proliferation and differentiation of skin carcinoma cells, resulting in an anti-tumor impact.

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