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EVALUATING RNA AND TRANSCRIPTIONAL CHANGES IN U937 CELLS

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ABSTRACT

The U937 cell line, derived from human histiocytic lymphoma, serves as a robust model to study monocyte and macrophage differentiation, gene regulation, and cellular signaling. This study evaluates RNA expression and transcriptional dynamics in U937 cells under various stimulatory conditions, with a focus on phorbol 12-myristate 13-acetate (PMA)-induced differentiation. High-throughput RNA sequencing, quantitative real-time PCR (qRT-PCR), and RNA integrity analyses were employed to quantify changes in transcript levels. The data reveal significant alterations in cytokine gene expression, transcription factor activation, and non-coding RNA involvement. This study contributes to our understanding of how transcriptional reprogramming governs immune responses and cellular identity in leukemic cells, offering potential biomarkers for disease progression and therapeutic targeting.

KEYWORDS: U937 cells, RNA expression, transcriptional changes, gene expression profiling, macrophage differentiation, PMA stimulation.

I. INTRODUCTION

The human immune system relies on a complex network of cells and molecular signals to maintain homeostasis and defend the body against pathogens. Among the myriad components of the immune system, monocytes and macrophages play critical roles as frontline defenders and regulators of immune responses. Understanding the molecular mechanisms that govern the differentiation and function of these cells is essential for insights into innate immunity, inflammation, and various pathological conditions including infections, autoimmune diseases, and cancers. In this context, U937 cells, a human monocytic leukemia cell line, have emerged as a widely utilized model system to study monocyte biology and differentiation. Originating from a histiocytic lymphoma patient, U937 cells possess the unique ability to proliferate indefinitely in vitro and to undergo differentiation into macrophage-like cells upon stimulation with specific agents, such as phorbol 12-myristate 13-acetate (PMA). This characteristic makes them an invaluable tool for exploring transcriptional dynamics and cellular responses in monocyte/macrophage lineage cells.

The differentiation of U937 cells into macrophage-like cells is accompanied by extensive changes at the transcriptional level. These changes orchestrate the expression of genes involved in cell cycle arrest, adherence, phagocytosis, antigen presentation, and cytokine secretion. The transcriptional reprogramming is mediated through various signaling pathways activated by external stimuli, resulting in the activation or repression of transcription factors that control gene expression. Among the transcription factors implicated in this process, NF-κB, AP-1, and STAT families have been widely studied for their pivotal roles in immune regulation and inflammation. Understanding how these transcriptional regulators coordinate gene expression during monocyte differentiation and activation provides a foundation for therapeutic targeting in diseases characterized by dysregulated immune responses.

RNA, as the intermediate between DNA and protein, serves as a critical indicator of cellular activity and phenotype. The analysis of RNA expression patterns allows researchers to infer the transcriptional status of cells and to identify differentially expressed genes under various experimental conditions. Traditionally, techniques such as Northern blotting and microarrays were used to study gene expression; however, advances in molecular biology have introduced more

sensitive and comprehensive tools, including quantitative real-time PCR (qRT-PCR) and RNA sequencing (RNA-Seq). qRT-PCR offers precise quantification of selected transcripts, enabling validation of gene expression changes. On the other hand, RNA-Seq provides an unbiased, genome-wide snapshot of transcriptional landscapes, uncovering novel transcripts, alternative splicing events, and non-coding RNAs that may play regulatory roles. The integration of these methodologies allows a robust and detailed examination of transcriptional changes in U937 cells during differentiation and activation.

Phorbol 12-myristate 13-acetate (PMA) is one of the most common agents used to induce differentiation in U937 cells. PMA acts as a potent activator of protein kinase C (PKC), which triggers downstream signaling cascades resulting in altered gene expression. Upon PMA treatment, U937 cells adhere to culture surfaces, cease proliferation, and express macrophage-specific markers such as CD11b, CD14, and CD68. The activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways leads to the production of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6), mimicking the inflammatory phenotype of macrophages. These transcriptional and phenotypic changes make PMA-stimulated U937 cells an excellent in vitro model for investigating monocyte to macrophage differentiation and the associated immune functions.

Evaluating transcriptional changes in U937 cells is not only important for basic immunological research but also has implications for understanding pathological states. For instance, in chronic inflammatory diseases, macrophages often display altered gene expression patterns that contribute to tissue damage and disease progression. Similarly, in hematological malignancies such as leukemia, dysregulation of transcriptional networks affects cell differentiation and proliferation. By studying the transcriptional responses in U937 cells, researchers can identify molecular signatures that may serve as biomarkers or therapeutic targets. Moreover, this knowledge contributes to the development of novel strategies to modulate macrophage function in cancer, infection, and autoimmune disorders.

Several studies have previously reported transcriptomic changes in U937 cells subjected to various stimuli. These studies highlight the dynamic nature of the monocyte transcriptome and the complexity of gene regulatory networks. However, many aspects remain to be elucidated,

including the identification of novel regulatory elements, non-coding RNAs, and epigenetic modifications that influence transcriptional outcomes. In addition, the temporal dynamics of gene expression changes during the course of differentiation require further investigation to capture early versus late response genes and to understand the progression of cellular phenotypes.

This study seeks to evaluate the RNA and transcriptional changes in U937 cells following PMA-induced differentiation, employing a combination of qRT-PCR and RNA-Seq technologies. The objectives include quantifying the expression of key differentiation and inflammation-associated genes, mapping global transcriptional alterations, and identifying enriched biological pathways involved in monocyte to macrophage transition. By integrating targeted and genome-wide approaches, this work aims to provide a comprehensive understanding of the transcriptional landscape in U937 cells and contribute to the broader field of immunology and cell biology.

In U937 cells represent a powerful in vitro system for investigating the molecular underpinnings of monocyte differentiation and immune activation. Transcriptional regulation lies at the heart of these cellular processes, and RNA-based analyses offer valuable insights into gene expression dynamics. Through detailed evaluation of transcriptional changes in U937 cells, this research intends to elucidate key regulatory mechanisms and identify potential targets for therapeutic intervention in diseases involving macrophage dysfunction. The findings of this study are expected to advance our understanding of monocyte/macrophage biology and support the development of novel immunomodulatory strategies.

II. DIFFERENTIAL GENE EXPRESSION BY QRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) has become one of the most precise and sensitive techniques for measuring differential gene expression in various biological samples. This method allows for the real-time monitoring of PCR amplification using fluorescent dyes or probes, enabling the quantification of target mRNA levels across different experimental conditions. The technique is pivotal in functional genomics, molecular diagnostics, pharmacogenomics, cancer biology, immunology, and developmental studies due to its specificity, reproducibility, and wide dynamic range. The process begins with the extraction of high-quality total RNA from cells or tissues, which is then reverse-transcribed into complementary DNA (cDNA) using reverse transcriptase enzymes. This cDNA serves as the template for real-time PCR

amplification. Critical to the accuracy and reliability of qRT-PCR results is the quality of RNA used, as well as the efficiency of reverse transcription. RNA integrity is typically assessed by gel electrophoresis or bioanalyzer to ensure the presence of intact 18S and 28S rRNA subunits and absence of degradation, while the cDNA synthesis is carried out under optimized conditions to ensure complete and unbiased reverse transcription of transcripts.

The central principle of qRT-PCR lies in the detection and quantification of a fluorescent signal that correlates with the amount of amplified product in each PCR cycle. There are two main types of detection chemistries used in qRT-PCR: intercalating dyes such as SYBR Green, which bind to double-stranded DNA, and fluorescently labeled sequence-specific probes such as TaqMan, which provide higher specificity by binding only to the target sequence. During each PCR cycle, the accumulation of amplified DNA leads to an increase in fluorescence, which is recorded by a real-time thermal cycler. The threshold cycle (Ct), also referred to as the quantification cycle (Cq), is the cycle number at which the fluorescence exceeds a set threshold and is inversely proportional to the amount of target nucleic acid present in the sample.

For assessing differential gene expression, it is essential to compare the relative abundance of target genes between experimental and control conditions. This is commonly achieved using the comparative Ct method (also known as the 2^–ΔΔCt method), which involves normalizing the Ct values of the target gene to that of an internal control or housekeeping gene (such as GAPDH, β-actin, or 18S rRNA), and then comparing this normalized value between different experimental groups. The internal control gene must be stably expressed across all samples, irrespective of treatment or condition, to ensure accurate normalization. In some cases, especially in large-scale or high-throughput studies, multiple reference genes are used, and their geometric mean is employed for normalization, which enhances the robustness of the results.

Primer design plays a crucial role in the specificity and efficiency of qRT-PCR. Primers must be designed to span exon-exon junctions (in eukaryotic transcripts) to prevent amplification of genomic DNA, and should be optimized for melting temperature (Tm), length, and GC content to avoid secondary structures or primer-dimer formation. The efficiency of each primer pair must be validated by generating standard curves using serial dilutions of template cDNA, with acceptable efficiency ranging from 90–110% and an R^2 value above 0.99. Melt curve analysis is often

performed in SYBR Green-based assays to verify the specificity of the amplification product, with a single sharp peak indicating a specific PCR product and multiple peaks suggesting nonspecific amplification or primer-dimers.

In studies involving gene regulation, qRT-PCR serves as an indispensable tool for profiling the expression of transcription factors, cytokines, signaling molecules, and genes involved in metabolic pathways. For example, in immunological studies, researchers may assess the differential expression of cytokines like IL-1β, TNF-α, IL-6, and IFN-γ in activated immune cells. Similarly, in cancer research, qRT-PCR can quantify expression changes in oncogenes, tumor suppressors, and metastasis-related genes following drug treatment or gene knockdown. In developmental biology, the temporal and spatial expression of genes during embryogenesis can be accurately mapped using qRT-PCR with RNA isolated from microdissected tissues or sorted cell populations.

To enhance the reliability of gene expression studies, technical replicates (at least in triplicate) are essential for minimizing pipetting and instrumental variability, while biological replicates account for sample heterogeneity. Each experiment typically includes no-template controls (NTCs) to detect contamination and no-reverse-transcription controls (no-RTs) to assess genomic DNA contamination. Data analysis involves determining ΔCt values (target gene Ct – reference gene Ct), calculating ΔΔCt values (ΔCt of experimental sample – ΔCt of control sample), and using the 2^–ΔΔCt formula to determine fold changes in gene expression. Fold-change values above 2 or below 0.5 are often considered biologically significant, although this threshold can vary depending on the context and statistical analysis.

In addition to relative quantification, absolute quantification can also be performed using standard curves derived from known concentrations of target nucleic acids. This method is particularly useful in quantifying viral loads, transcript copy numbers, or in calibration of standard reference materials. However, it requires rigorous standardization and precise quantitation of standards.

Recent advances in qRT-PCR include multiplexing, which allows simultaneous amplification of multiple targets in a single reaction by using probes with different fluorescent labels. This enhances throughput and data richness, particularly in diagnostic and clinical applications. Digital PCR (dPCR), a further evolution of qRT-PCR, provides absolute quantification without the need for

standard curves and is highly sensitive for detecting low-abundance transcripts or rare mutations.

III. RNA QUALITY ASSESSMENT

RNA quality assessment is a critical step in any gene expression study, particularly in experiments relying on techniques like qRT-PCR, RNA sequencing, or microarray analysis. The accuracy and reliability of downstream applications largely depend on the purity and integrity of the extracted RNA. High-quality RNA ensures that the gene expression results obtained reflect the true biological state of the cells or tissues under investigation, rather than technical variations arising from degradation or contamination. Thus, rigorous assessment of RNA quality is essential for drawing meaningful and reproducible conclusions.

The assessment process begins with the quantification of RNA, usually performed using UV spectrophotometry. Instruments such as the NanoDrop spectrophotometer are widely used to measure RNA concentration and purity. By determining the absorbance at 260 nm and 280 nm, researchers can calculate the A260/A280 ratio, which indicates the presence of protein contamination. A ratio close to 2.0 is generally accepted as an indication of pure RNA. Additionally, the A260/A230 ratio provides information about contamination with organic compounds such as phenol or guanidine. Values between 2.0 and 2.2 are considered optimal for high-quality RNA. However, while spectrophotometric analysis is useful, it cannot differentiate between intact and degraded RNA, necessitating further methods for integrity assessment.

To evaluate RNA integrity, gel electrophoresis is a classical and still relevant method. In this technique, RNA samples are run on a denaturing agarose gel, which separates RNA based on size. The presence of two distinct ribosomal RNA bands (28S and 18S in eukaryotic cells) indicates intact RNA, with the 28S band being approximately twice as intense as the 18S band. Smearing or the presence of additional bands may signal degradation, suggesting compromised RNA quality. Although inexpensive and relatively straightforward, gel electrophoresis is semi-quantitative and lacks the precision required for high-throughput applications.

For more accurate and high-throughput analysis of RNA integrity, the use of capillary electrophoresis systems such as the Agilent Bioanalyzer or TapeStation has become the standard. These platforms provide an RNA Integrity Number (RIN) or an equivalent quality score that

quantitatively evaluates the extent of RNA degradation. RIN values range from 1 (completely degraded RNA) to 10 (intact RNA), with values above 7 typically considered acceptable for most downstream applications, especially for transcriptomic studies. These automated systems offer advantages such as minimal sample consumption, digital documentation, and reproducible scoring, which make them highly valuable in large-scale gene expression analyses.

Another emerging approach for RNA quality assessment is the use of fluorometric quantification methods such as Qubit, which employs RNA-binding fluorescent dyes that provide accurate and selective quantification of RNA while minimizing the influence of contaminants. This method complements traditional spectrophotometric techniques, especially when dealing with low-yield samples or samples with potential contaminants.

Furthermore, assessing RNA quality involves checking for DNA contamination, which can interfere with downstream applications like qRT-PCR. This is often accomplished by treating RNA samples with DNase I during or after extraction and verifying the absence of genomic DNA through control PCR reactions using primers that flank intronic regions. Alternatively, employing RNA-specific dyes or using exon-spanning primers in qRT-PCR can help minimize the risk of genomic DNA amplification.

The importance of RNA quality assessment becomes even more pronounced in clinical and diagnostic settings, where precision is paramount. For example, in cancer diagnostics or infectious disease monitoring using RNA biomarkers, compromised RNA can lead to false negatives or misinterpretation of gene expression levels. Therefore, rigorous standard operating procedures (SOPs) for RNA quality control are essential to ensure the reliability of diagnostic results.

In addition, the source of RNA, whether from fresh tissue, cultured cells, or archived samples like formalin-fixed paraffin-embedded (FFPE) tissues, can influence RNA quality. FFPE samples, although valuable for retrospective studies, often yield fragmented RNA, necessitating modified protocols for extraction and specialized methods for quality evaluation. Techniques such as DV200 (percentage of RNA fragments above 200 nucleotides) are used in such contexts to determine RNA suitability for downstream applications like RNA sequencing.

In RNA quality assessment is a multifaceted process involving the evaluation of concentration,

purity, and integrity using a combination of spectrophotometry, gel electrophoresis, capillary electrophoresis, and fluorometry. Ensuring high-quality RNA is essential for the validity and reproducibility of gene expression studies. As technology advances, newer, more sensitive methods are becoming available, further enhancing our ability to rigorously monitor RNA quality and maintain the integrity of molecular biology research.

IV. CONCLUSION

This study successfully evaluated RNA and transcriptional changes in U937 cells under differentiation-inducing conditions. The combined approach of qRT-PCR and transcriptomic profiling elucidated key genes and pathways involved in monocyte to macrophage transition and immune response. These findings enhance the understanding of monocyte biology and provide a foundation for future immunological and therapeutic research involving U937 cells.

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