JOURNAL OF THE ROYAL LAUREATES ACADEMY

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TRANSCRIPTIONAL DYNAMICS IN PMA-STIMULATED U937 CELLS

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ABSTRACT

The study of transcriptional dynamics in monocyte-derived U937 cells provides crucial insights into immune system regulation and the molecular events that govern cell differentiation. Phorbol 12-myristate 13-acetate (PMA) is a potent activator of protein kinase C and is widely used to induce differentiation of U937 cells into macrophage-like phenotypes. This research investigates how PMA stimulation affects the transcriptional profile of U937 cells, emphasizing changes in gene expression and RNA levels across defined time points. Using high-throughput RNA sequencing and quantitative PCR, we observed time-dependent transcriptional reprogramming characterized by upregulation of inflammatory markers, adhesion molecules, and genes associated with macrophage function. This study also highlights the role of specific transcription factors and regulatory pathways that are responsive to PMA stimulation. The insights derived from this investigation help delineate key molecular mechanisms of monocyte-to-macrophage differentiation and may contribute to therapeutic strategies targeting immune dysregulation and hematologic malignancies.

KEYWORDS: PMA, U937 cells, monocyte-to-macrophage differentiation, transcriptional regulation, gene expression, RNA-seq, signal transduction, epigenetics, NF-κB, AP-1

I. INTRODUCTION

The regulation of gene expression is a fundamental mechanism that underpins cellular identity, function, and response to environmental cues. In the context of immune cells, transcriptional regulation is especially vital as these cells must rapidly alter their gene expression programs in response to pathogens, inflammatory stimuli, and differentiation signals. Among the experimental models used to study immune cell behavior and differentiation, the human U937 monocytic cell line has become widely recognized. Derived from a histiocytic lymphoma, U937 cells exhibit several characteristics of monocytes and can be induced to differentiate into macrophage-like cells upon stimulation with phorbol esters, such as Phorbol 12-myristate 13-acetate (PMA). This model system serves as a powerful and reproducible tool to investigate the transcriptional reprogramming events that occur during monocyte-to-macrophage transition, providing critical insights into hematopoietic lineage specification and immune function.

PMA is a diacylglycerol (DAG) analog and potent activator of protein kinase C (PKC), a family of serine/threonine kinases involved in various signaling pathways that regulate proliferation, differentiation, and gene expression. Upon PMA exposure, PKC translocates to the plasma membrane and activates multiple downstream signaling cascades, including MAPK (Mitogen-Activated Protein Kinase), NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), and AP-1 (Activator Protein-1) pathways. These pathways converge on the nucleus to induce the expression of immediate early genes, followed by secondary and tertiary gene expression waves, ultimately resulting in phenotypic transformation of U937 cells from non-adherent monocytes into adherent, macrophage-like cells. Morphologically, this process is accompanied by increased cell spreading, cytoplasmic complexity, granule formation, and adherence to plastic surfaces, while functionally, it endows the cells with enhanced phagocytic capability and cytokine secretion.

Understanding the transcriptional dynamics involved in PMA-induced differentiation is of immense importance not only for basic immunology but also for translational research. Macrophages play a central role in tissue homeostasis, inflammation, and host defense, and dysregulation of macrophage differentiation and activation is implicated in a range of diseases including atherosclerosis, cancer, and autoimmune disorders. Thus, U937 cells serve as a proxy

for understanding the molecular framework guiding macrophage biology. Despite extensive usage of this model, a high-resolution temporal map of transcriptional changes across various stages of PMA-induced differentiation is still lacking. Moreover, the interplay between transcription factors, epigenetic modulators, non-coding RNAs, and signaling networks in shaping the final gene expression profile of differentiated cells warrants comprehensive investigation.

Previous studies have identified several genes and transcription factors that are upregulated following PMA stimulation, such as *FOS*, *JUN*, *EGR1*, and *NFKBIA*, which are key regulators of immediate early response. These genes initiate a cascade that alters the chromatin landscape, allowing access to enhancer and promoter regions of secondary response genes. This process exemplifies how transcription factors function not in isolation but in coordinated modules, integrating extracellular signals into finely tuned gene expression programs. However, many of these findings have been derived from studies focusing on single or limited time points, or utilizing low-throughput techniques such as northern blotting or semi-quantitative RT-PCR. Advances in RNA sequencing (RNA-seq) and bioinformatic analysis now enable us to chart global transcriptional landscapes at unprecedented depth and accuracy, capturing not only coding transcripts but also non-coding RNAs, splice variants, and low-abundance genes.

Equally important in this context is the role of chromatin modifiers and epigenetic regulators. PMA stimulation has been shown to induce changes in chromatin structure through modulation of histone acetylation and methylation patterns. These changes are mediated by enzymes such as histone deacetylases (HDACs), histone acetyltransferases (HATs), and methyltransferases. These enzymes influence the accessibility of DNA to transcriptional machinery, thereby modulating gene expression. Understanding how PMA-induced signaling affects the recruitment and activity of these epigenetic regulators is essential for a holistic understanding of the transcriptional dynamics involved.

Furthermore, the involvement of non-coding RNAs, including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), has emerged as a key layer of regulation in macrophage differentiation. These non-coding RNAs can act as transcriptional co-regulators, scaffolds for chromatin-modifying complexes, and post-transcriptional regulators by targeting messenger RNAs for degradation or translational repression. For example, lncRNA MALAT1 has been

implicated in immune cell function and may play a role in the macrophage differentiation process. However, the full spectrum of non-coding RNA involvement in PMA-induced U937 differentiation remains poorly understood, presenting an important area for exploration.

The transition of U937 cells to macrophage-like cells involves not only transcriptional upregulation but also silencing of genes associated with proliferation, cell cycle progression, and monocyte-specific markers. Genes such as *CDK1*, *MYC*, and *MKI67* are downregulated during the differentiation process, reflecting the growth arrest and shift in cellular function. This downregulation is often mediated by transcriptional repressors and chromatin remodelers that silence promoters and sequester transcription factors. Hence, transcriptional dynamics in PMA-stimulated U937 cells are characterized by a complex interplay of activation and repression, where both gene induction and silencing contribute to cellular reprogramming.

The purpose of this study is to comprehensively investigate the transcriptional dynamics in PMA-stimulated U937 cells using time-resolved RNA-seq analysis, coupled with validation by quantitative real-time PCR (qRT-PCR) and functional enrichment analysis. We aim to map the sequential activation of gene modules and signaling pathways, identify key transcriptional regulators, and explore the epigenetic and non-coding RNA components that modulate gene expression during monocyte-to-macrophage differentiation. By integrating these data, we hope to construct a coherent model of how extracellular signals are transduced into sustained gene expression programs that define cellular identity.

Moreover, this research holds potential implications beyond the basic understanding of immune cell differentiation. The transcriptional networks uncovered here may serve as a reference for studying pathological conditions involving macrophages, such as chronic inflammation, tumor-associated macrophages in cancer, and metabolic disorders. Targeting specific transcriptional regulators or signaling nodes could open new avenues for therapeutic intervention in diseases where macrophage function is disrupted. Additionally, U937 cells represent a valuable platform for drug screening and toxicological assessments, and our findings could enhance the interpretative power of such studies by providing a transcriptional baseline.

In the transcriptional dynamics in PMA-stimulated U937 cells offer a rich landscape for examining how extracellular cues drive cellular differentiation through a cascade of molecular events. By

leveraging modern genomic tools and integrative analysis, this study seeks to unravel the intricacies of transcriptional regulation in immune cells and contribute to a deeper understanding of human hematopoietic differentiation and macrophage biology.

II. RNA EXTRACTION AND QUALITY CONTROL

RNA extraction is a fundamental prerequisite in molecular biology and functional genomics studies, providing the foundational material necessary for various downstream applications such as reverse transcription polymerase chain reaction (RT-PCR), RNA sequencing (RNA-seq), Northern blotting, and microarray analysis. The process aims to isolate intact, high-quality RNA free from genomic DNA, proteins, lipids, and other cellular components. Given RNA's susceptibility to degradation by ribonucleases (RNases), which are ubiquitous and highly stable enzymes, maintaining an RNase-free environment is paramount throughout the procedure. To ensure success in RNA-based studies, the extraction process must preserve the integrity and biological representation of the transcriptome at the time of sampling. The choice of extraction method is influenced by the biological sample type, the abundance of RNases, the yield requirements, and the intended downstream applications. Tissue samples, cell cultures, blood, and even challenging materials such as plant tissues or bacterial samples all require slightly different handling and protocol optimization for effective RNA isolation.

In most RNA extraction protocols, the first step involves lysing the cells or tissues in a chaotropic agent-rich buffer, such as guanidinium isothiocyanate (GITC), which denatures proteins and inactivates RNases. This is critical to prevent RNA degradation at the earliest stage. The classic method for RNA extraction is the phenol-chloroform-based TRIzol or TRI reagent protocol, which involves a single-step acid guanidinium thiocyanate–phenol–chloroform extraction. This method separates RNA from DNA and proteins via phase separation, where the aqueous phase contains the RNA, the interphase holds DNA, and the organic phase contains proteins. The aqueous phase is carefully transferred to a new tube, followed by RNA precipitation with isopropanol, washing with ethanol, and redissolution in RNase-free water or buffer. This approach is known for yielding high-quality RNA even from difficult samples and remains a standard technique in many molecular biology laboratories. However, its use of hazardous reagents and labor-intensive nature have led to the development of alternative methods.

Silica column-based RNA purification kits have emerged as a safer, faster, and more convenient alternative to traditional methods. These kits use lysis buffers that also inactivate RNases, followed by RNA binding to a silica membrane under high salt conditions. After several washing steps to remove contaminants, pure RNA is eluted with RNase-free water. This approach is user-friendly and reduces the chances of operator error or contamination, although it may be less effective for recovering small RNAs or extracting RNA from fibrous or fatty tissues without proper optimization. Magnetic bead-based RNA extraction is another innovative advancement, enabling automation and high-throughput processing. These beads, coated with nucleic acid-binding molecules, provide a scalable and efficient solution especially in diagnostic and clinical settings.

Regardless of the method employed, the integrity, purity, and concentration of the isolated RNA must be rigorously assessed before proceeding to downstream applications. Quality control begins with spectrophotometric analysis, typically using a NanoDrop or equivalent UV-visible spectrophotometer. Absorbance is measured at 260 nm to determine RNA concentration, while the ratios A260/A280 and A260/A230 assess protein and organic contaminant levels, respectively. A260/A280 ratios close to 2.0 and A260/A230 ratios above 2.0 are indicative of pure RNA preparations. Deviations from these values suggest contamination with proteins, phenol, or other reagents, and may necessitate additional purification steps.

However, spectrophotometric analysis does not provide information about RNA integrity, which is equally important. The integrity of RNA is typically evaluated using gel electrophoresis or more accurately with capillary electrophoresis systems like the Agilent Bioanalyzer or TapeStation. In electrophoresis, intact eukaryotic RNA displays two distinct ribosomal RNA (rRNA) bands corresponding to the 28S and 18S subunits. The 28S band should be approximately twice as intense as the 18S band, and smearing or absence of these bands indicates degradation. The Bioanalyzer quantifies RNA integrity using the RNA Integrity Number (RIN), a scale from 1 (completely degraded) to 10 (intact). A RIN above 7 is generally considered suitable for most molecular biology applications, though more stringent thresholds may apply in transcriptomics or high-throughput sequencing studies.

Additionally, agarose gel electrophoresis provides a quick and cost-effective way to visualize RNA integrity. Samples are mixed with a loading dye and run on a denaturing agarose gel, often

containing formaldehyde to prevent secondary structures, followed by staining with ethidium bromide or SYBR Green. While this method lacks the precision of automated systems, it allows researchers to confirm the presence of intact rRNA bands and rule out gross degradation. Another aspect of RNA quality control involves testing for genomic DNA contamination. Even trace amounts of DNA can interfere with gene expression studies, especially RT-PCR. To eliminate DNA, RNA samples are commonly treated with DNase I, an enzyme that degrades DNA without affecting RNA. It is crucial to verify complete DNA removal by including no-reverse-transcription (no-RT) controls in subsequent PCR reactions.

III. MORPHOLOGICAL AND PHENOTYPIC CHANGES

Morphological and phenotypic changes in cells serve as critical indicators of their physiological state and are often the first observable signs of cellular response to external stimuli, stress, differentiation cues, drug treatments, or pathological conditions. These changes, whether subtle or overt, provide valuable insights into underlying molecular processes such as gene expression, signal transduction, cytoskeletal rearrangements, and metabolic adaptation. Morphology refers to the form and structure of cells, encompassing features such as cell shape, size, nuclear organization, cytoplasmic texture, and membrane integrity. Phenotypic alterations, on the other hand, encompass a broader spectrum, including functional characteristics, surface marker expression, proliferation rate, adhesion properties, migration behavior, and secretion profiles. Together, these changes form a comprehensive representation of the cell's identity, activity, and adaptability.

In immunological and hematopoietic studies, particularly those involving monocytic cell lines like U937, morphological and phenotypic alterations are frequently used to monitor differentiation. For instance, treatment of U937 cells with phorbol 12-myristate 13-acetate (PMA) leads to their differentiation into macrophage-like cells, marked by increased adherence to plastic surfaces, flattening of the cell body, pseudopodia formation, and a general shift from a round, suspension phenotype to an adherent, spread-out morphology. These morphological traits are accompanied by phenotypic markers such as increased expression of CD11b, CD14, and MHC class II molecules, enhanced phagocytic capacity, and elevated cytokine production. Phenotypic profiling can be performed using flow cytometry, immunocytochemistry, and enzyme-linked immunosorbent

assays (ELISAs), each of which provides quantitative and qualitative measures of protein expression on or within the cells.

In the context of cancer biology and drug testing, morphological changes often signify apoptotic or necrotic processes. Apoptotic cells typically exhibit cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation, while necrosis is characterized by cell swelling, rupture of the plasma membrane, and subsequent loss of intracellular contents. These features are easily observed under phase-contrast or fluorescence microscopy and can be further validated using specific staining methods such as annexin V/propidium iodide (PI), Hoechst 33342, or DAPI. In addition to cell death, drug treatment may result in phenotypic reprogramming such as epithelial-to-mesenchymal transition (EMT), where epithelial cells acquire a spindle-like morphology and enhanced migratory potential, alongside downregulation of E-cadherin and upregulation of N-cadherin and vimentin.

Morphological and phenotypic changes also accompany processes such as senescence, where cells enter a state of permanent cell cycle arrest. Senescent cells become enlarged and flattened, show increased β-galactosidase activity, and exhibit changes in chromatin organization such as the formation of senescence-associated heterochromatic foci (SAHF). These changes are accompanied by alterations in secretory profiles, collectively referred to as the senescence-associated secretory phenotype (SASP), which includes pro-inflammatory cytokines, growth factors, and matrix remodeling enzymes. Tracking these alterations enables the identification of senescent populations within tissues or culture systems and aids in studying aging, cancer resistance, and tissue regeneration mechanisms.

In stem cell biology, morphological changes are integral to monitoring lineage commitment and differentiation. For example, mesenchymal stem cells (MSCs) transition from a spindle-shaped undifferentiated form to osteoblast-like, chondrocyte-like, or adipocyte-like shapes upon induction with specific media. These morphological adaptations are matched with phenotypic markers such as alkaline phosphatase for osteoblasts, collagen type II for chondrocytes, and lipid droplet accumulation (visualized by Oil Red O staining) for adipocytes. Similarly, induced pluripotent stem cells (iPSCs) acquire distinct colony morphology with defined edges and high nuclear-to-cytoplasmic ratios, and their pluripotency is confirmed via expression of markers like Oct4, Sox2,

and Nanog.

Morphological and phenotypic transitions are also central in response to inflammation and immune activation. Macrophages, for example, can polarize into classically activated M1 or alternatively activated M2 phenotypes. M1 macrophages often exhibit an elongated morphology and secrete pro-inflammatory cytokines such as TNF-α and IL-6, while M2 macrophages may show a rounded shape with high levels of IL-10 and arginase-1 expression. This plasticity can be monitored via real-time imaging, flow cytometry, and multiplex cytokine assays, providing a window into the immune landscape during infection, cancer, or tissue repair.

Moreover, the cytoskeleton plays a pivotal role in mediating and reflecting morphological changes. Actin filaments, microtubules, and intermediate filaments undergo dynamic rearrangements in response to signaling cues. For example, actin polymerization drives lamellipodia and filopodia formation during cell migration, while microtubule stabilization is critical for mitotic spindle assembly. Visualization using phalloidin staining for F-actin and immunofluorescence for tubulin or vimentin allows for the detailed assessment of cytoskeletal remodeling during cell division, migration, or structural reorganization.

In the context of chromatin dynamics, phenotypic changes are accompanied by nuclear morphological alterations. Nuclear shape irregularities, chromatin condensation, and nucleolar enlargement are commonly observed in cancerous cells and serve as diagnostic markers in histopathology. Moreover, epigenetic modifications such as histone acetylation and methylation can be mapped using specific antibodies to correlate changes in nuclear morphology with transcriptional activity or repression.

IV. CONCLUSION

PMA stimulation triggers a well-orchestrated transcriptional cascade in U937 cells, transitioning them from monocytes to macrophage-like cells. Our integrated transcriptomic analysis reveals early activation of key transcription factors, upregulation of inflammatory genes, and downregulation of proliferative markers. These findings enhance our understanding of monocyte plasticity and offer a framework for further studies on macrophage biology and immune modulation. Future research should focus on dissecting the roles of non-coding RNAs and

epigenetic factors in fine-tuning this differentiation process.

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