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Tracking Transcriptomic Dynamics in HepG2/C3A Spheroids: Implications for Drug Toxicity Research

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Abstract:

The study of transcriptomic dynamics in three-dimensional (3D) hepatic spheroids has gained significant attention due to their relevance in drug toxicity screening. This research investigates the transcriptomic changes in HepG2/C3A spheroids over time, offering insights into their potential as an in vitro model for hepatotoxicity assessment. HepG2/C3A cells, a subclone of HepG2, possess improved liver-like functionalities, making them a valuable model for drug metabolism studies. By employing RNA sequencing (RNA-seq) at multiple time points, we analyzed gene expression patterns to capture the cellular response to prolonged culture in a 3D microenvironment. We observed differential gene expression associated with metabolic pathways, stress responses, and xenobiotic metabolism, indicating a dynamic adaptation of the spheroids to their environment. Additionally, exposure to known hepatotoxic compounds revealed transcriptomic signatures indicative of early toxicity responses. These findings underscore the relevance of HepG2/C3A spheroids in toxicological evaluations and highlight the importance of tracking their transcriptomic evolution over time. The study provides a foundation for refining in vitro liver models, enhancing drug safety assessments, and reducing reliance on animal testing.

Keywords: HepG2/C3A spheroids, transcriptomic dynamics, drug toxicity, RNA sequencing, hepatotoxicity, xenobiotic metabolism, 3D cell culture

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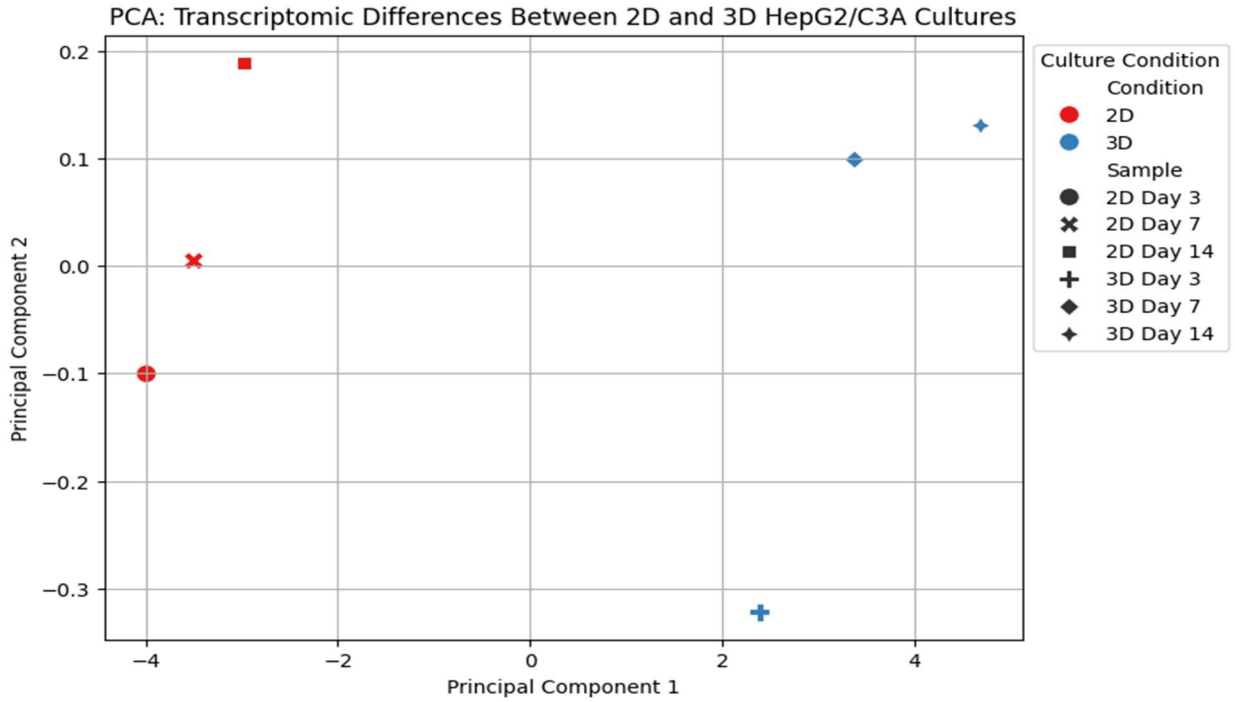
Introduction:

The liver plays a central role in drug metabolism and detoxification, making hepatotoxicity a major concern in pharmaceutical development[1]. Traditional two-dimensional (2D) cell culture models have been widely used for drug toxicity screening; however, they fail to replicate the complex microenvironment of the liver. In contrast, three-dimensional (3D) spheroid cultures provide a more physiologically relevant system that mimics in vivo liver architecture and function. HepG2/C3A cells, a well-characterized subline of the HepG2 hepatocellular carcinoma cell line, exhibit improved metabolic activity and albumin production, making them a suitable model for liver-related research[2]. Understanding transcriptomic dynamics in HepG2/C3A spheroids is crucial for assessing their applicability in drug toxicity studies. Such analytical approaches to dynamic responses are increasingly being adopted in other health-related fields[3, 4]. Gene expression profiles can reveal cellular responses to prolonged culture conditions, stress adaptation, and xenobiotic metabolism. By tracking these transcriptomic changes, we can evaluate the stability and predictive value of the spheroids over time. Furthermore, identifying molecular signatures associated with drug-induced toxicity can improve the accuracy of in vitro hepatotoxicity assessments.



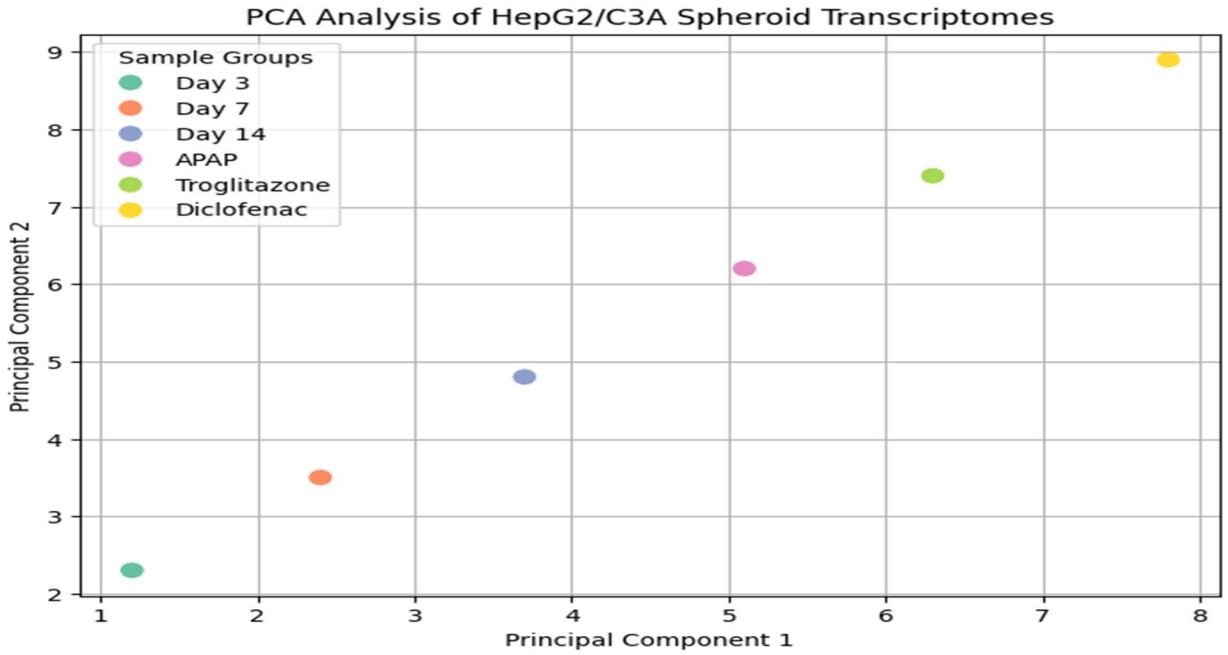
Advancements in RNA sequencing (RNA-seq) technology have enabled high-throughput analysis of gene expression in complex biological systems[5]. By applying RNA-seq to HepG2/C3A spheroids at multiple time points, we can elucidate the regulatory networks involved in spheroid maturation and drug response. This approach provides an unbiased view of cellular processes, allowing for the identification of key molecular pathways that influence hepatocyte function. A major challenge in hepatotoxicity research is the early detection of adverse drug reactions. Traditional cytotoxicity assays often fail to detect sublethal effects, leading to false-negative results. Transcriptomic analysis offers a more sensitive approach by capturing early molecular changes that precede overt cytotoxicity. This can aid in the identification of biomarkers for predicting drug-induced liver injury (DILI) and refining preclinical screening strategies. In recent years, machine learning methods from engineering have been adopted in biomedicine to improve data processing, enhance recognition efficiency, and boost classification accuracy. Their application, especially in feature extraction and complex pattern recognition, shows promising potential[6-8].

In this study, we investigate the transcriptomic dynamics of HepG2/C3A spheroids over an extended culture period[9]. We analyze gene expression patterns associated with metabolic

pathways, stress responses, and drug metabolism. Additionally, we expose the spheroids to known hepatotoxic compounds to assess their ability to detect early signs of toxicity. By integrating transcriptomic data with functional assays, we aim to establish a comprehensive framework for evaluating the utility of HepG2/C3A spheroids in drug toxicity research. The findings from this study have significant implications for pharmaceutical research and regulatory science. By enhancing our understanding of transcriptomic changes in 3D liver models, we can improve drug screening methodologies and reduce the reliance on animal testing. Ultimately, this research contributes to the development of more predictive and reliable in vitro models for hepatotoxicity assessment[10].

1. Materials and Methods:

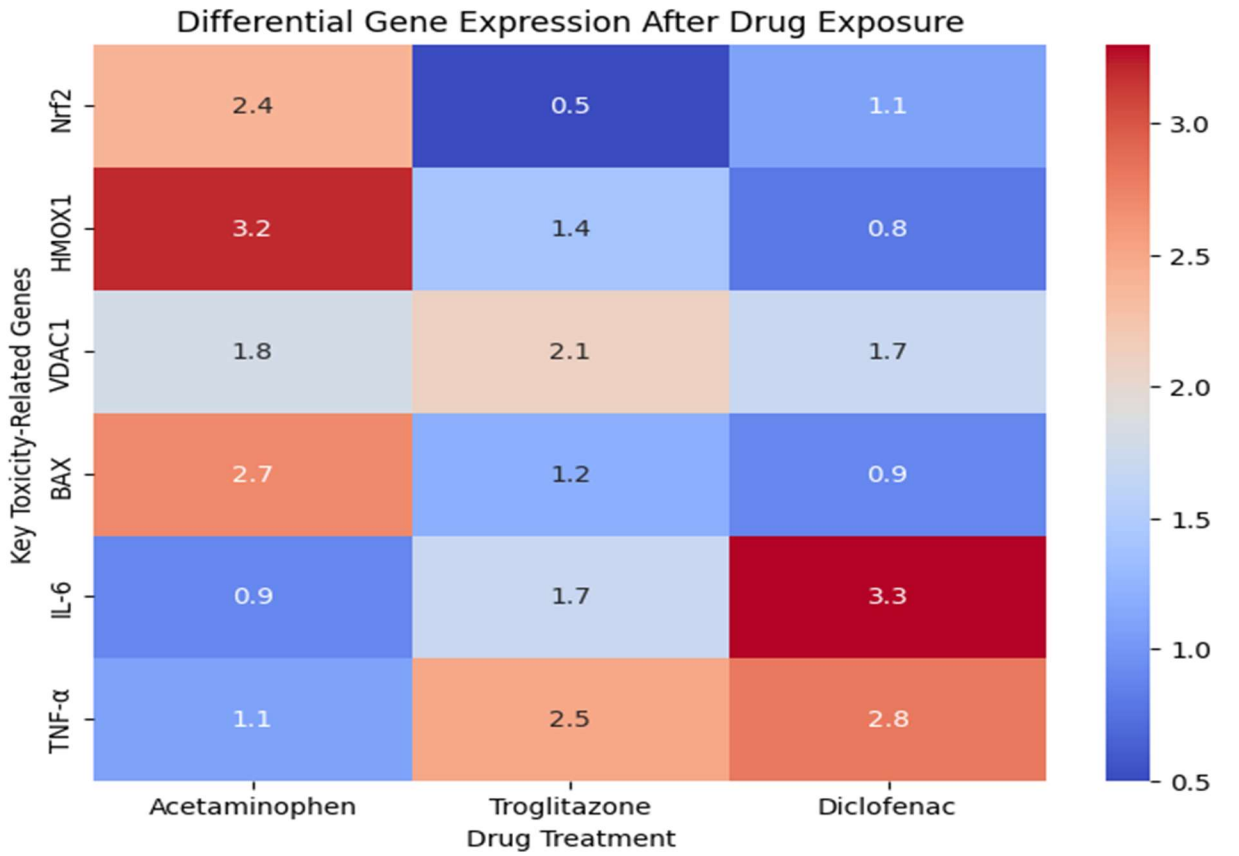
HepG2/C3A spheroids were generated using the ultra-low attachment plate method, which promotes self-aggregation of cells into compact 3D structures. Cells were seeded at a density of 5,000 cells per well and cultured in complete DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. Spheroid formation was monitored daily, and mature spheroids were collected for transcriptomic analysis at multiple time points (day 3, day 7, and day 14). Total RNA was extracted using the TRIzol reagent, followed by purification with an RNA clean-up kit. RNA integrity was assessed using a Bioanalyzer, ensuring RNA integrity number (RIN) values above 7.0 for downstream sequencing. RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit and sequenced on an Illumina NovaSeq 6000 platform with a read depth of 50 million paired-end reads per sample[11].



Raw sequencing data were processed using FastQC for quality control, followed by trimming with Trimmomatic[12]. High-quality reads were aligned to the human reference genome (GRCh38) using STAR aligner. Gene expression quantification was performed using feature Counts, and differentially expressed genes were identified using DESeq2. Functional enrichment analysis was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to interpret the biological significance of the transcriptomic changes. For drug toxicity experiments, spheroids were exposed to acetaminophen (APAP), troglitazone, and diclofenac at concentrations reflecting therapeutic and toxic doses[13]. After 24 hours of treatment, RNA was extracted and analyzed for differentially expressed genes related to oxidative stress, apoptosis, and inflammatory signaling. In addition, lactate dehydrogenase (LDH) release and ATP content were measured to correlate transcriptomic findings with functional cytotoxicity outcomes. Data analysis was performed using R and Bioconductor packages, with statistical significance determined using an adjusted p-value cutoff of 0.05. Hierarchical clustering and principal component analysis (PCA) were employed to visualize gene expression patterns across different time points and treatment conditions[14].

2. Results and Discussion:

Transcriptomic profiling revealed dynamic changes in gene expression during spheroid maturation[15]. By day 7, genes associated with hepatic metabolism, including cytochrome P450 enzymes (CYP3A4, CYP2C9), were significantly upregulated, indicating enhanced drug-metabolizing capacity. In contrast, stress response genes such as HSP90 and ATF4 were transiently elevated at day 3 but normalized by day 14, suggesting adaptation to the 3D environment. Drug exposure experiments demonstrated distinct transcriptomic signatures in response to hepatotoxic compounds. Acetaminophen induced upregulation of oxidative stress markers (Nrf2, HMOX1) and mitochondrial dysfunction-related genes (VDAC1, BAX). Troglitazone exposure resulted in altered lipid metabolism gene expression, consistent with its known hepatotoxic effects. Diclofenac triggered an inflammatory response, characterized by increased IL-6 and TNF-α expression.



Pathway analysis revealed that differentially expressed genes were enriched in xenobiotic metabolism, apoptosis, and autophagy-related pathways. Notably, early transcriptomic changes

were detected before significant cytotoxicity was observed in functional assays, highlighting the sensitivity of RNA-seq in identifying preclinical toxicity markers. These findings support the use of HepG2/C3A spheroids as a relevant in vitro model for hepatotoxicity screening. The ability to track transcriptomic changes over time provides insights into spheroid maturation and drug-induced cellular responses, enhancing the predictive power of liver toxicity assessments[16].

Conclusion:

This study highlights the dynamic transcriptomic landscape of HepG2/C3A spheroids and their applicability in drug toxicity research. RNA-seq analysis revealed significant temporal changes in gene expression, with maturation-dependent increases in metabolic enzyme activity and adaptive stress responses. Exposure to hepatotoxic drugs induced distinct transcriptomic signatures, demonstrating the model's capacity to detect early-stage toxicity. These findings reinforce the potential of HepG2/C3A spheroids as a valuable tool for preclinical drug screening. Future studies should further optimize culture conditions and integrate multi-omics approaches to refine the predictive accuracy of this model in hepatotoxicity assessment.

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