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DISSECTING REGULATORY MECHANISMS UNDERLYING CELLULAR DIFFERENTIATION PROCESSES

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ABSTRACT

Cellular differentiation Cellular differentiation is a core biological phenomenon that allows unspecialized cells to develop specific phenotypic and functional identities, which is essential in the development, tissue homeostasis, and disease pathogenesis. The regulatory mechanisms that control differentiation are key to further development of regenerative medicine and targeted therapeutic strategies. This paper sought to separate the regulatory network complexes that govern the process of cellular differentiation integrating transcriptional, epigenetic, and signaling pathway analyses. In this regard, we have used both high-throughput and targeted methods, such as RNA sequencing (RNA-seq) to enumerate changes in global gene expression as well as quantitative real-time PCR (qRT-PCR) to validate highly regulated genes and CRISPR-mediated gene modulation to study functional roles of selected regulatory factors. The identification of critical regulators and their interactions was performed with bioinformatics analyses, such as pathway enrichment and gene network modeling. We found that there are unique gene expression signatures that are linked to various stages of differentiation, indicating the ability of transcription factors and epigenetic modifiers to be coordinately regulated. Important signaling pathways, such as Wnt and Notch, were identified to be important in the regulation of lineage-specific gene expression. This was validated functionally by showing that perturbation of the chosen regulatory genes had a tremendous effect on differentiation efficiency and cellular phenotype. To sum up, the current research offers detailed information about the multi-layered regulatory processes governing differentiation of cells, which can be used as a source of therapeutic intervention and furthers our knowledge about cell fate determination.

KEYWORDS: Cellular differentiation; Gene regulatory networks; Transcription factors; Epigenetic regulation; Signal transduction pathways; Stem cells; RNA sequencing; Chromatin remodeling

1. INTRODUCTION

Cellular differentiation is a key process in biology where unspecialized cells obtain specific structure and functional attributes. This is fundamental to embryonic development, tissue homeostasis and regeneration and it allows the differentiation of a common progenitor into a variety of cell types. Developments in high-throughput technologies have greatly enhanced our view of differentiation by demonstrating dynamic variations in gene expression and cell states on a single-cell level (Islam et al., 2014; Macosko et al., 2015). In these studies, it is noted that differentiation is not an influence process but more complicated, multi-branching pathways that are regulated by highly controlled molecular networks (Chen et al., 2016; Herring et al., 2018).

Interconnected mechanisms control the regulation of cellular differentiation through transcription factor, epigenetic modification, and signaling pathway. Transcription factors like lineage-specific regulators have played a key role in the regulation of gene expression programs that define cell fate. Parallel to epigenetic processes, such as DNA methylation, histone modifications, and chromatin remodeling, regulate the accessibility of genes and transcription without changing the DNA sequence (Engreitz et al., 2013). Moreover, there are signaling pathways, including Wnt, Notch, and Hedgehog that combine extracellular signaling to modulate intracellular regulatory networks and determine differentiation outcomes. Collectively these layers create intricate gene regulation networks, which guarantee fine-tuning of differentiation in time and space.

New methodology, such as single-cell RNA sequencing and computational trajectory inference tools, have made it possible to reconstruct differentiation pathways in details and identify transitional states of the cell (Ji et al., 2016;

Gorski et al., 2014). In addition, scalable methods of functional genomics, including CRISPR-Cas9 and shRNA-based screens, has become potent methods to learn more about gene function and regulatory interactions (Dai et al., 2014; Spanjaard et al., 2018). Irrespective of these advances, the combination of transcriptional, epigenetic and signaling mechanisms to regulate differentiation remains poorly understood.

There are a number of major gaps in the field. To begin with, the incorporation of multi-omics datasets in order to create comprehensive regulatory models is still a big problem. Second, it is not well understood that the various layers of regulation interact dynamically when in transitional states. Third, although numerous regulatory factors are known, their hierarchical structure and functions context-related need additional clarification. The need to fill these gaps is essential to further the field of regenerative medicine, enhance modeling of diseases, and discover new treatment targets.

This paper will deconstruct the regulatory networks governing cellular differentiation using a combination of transcriptomic profiling, functional genomics and computational network analysis. Our hypothesis is that cellular differentiation is regulated by a complex and multi-layered regulatory network where the interplay between transcription factors, epigenetic changes and signaling pathways makes the cell decide on its fate. The main aims of this research are as follows: (i) to define the most important regulatory genes and pathways during differentiation, (ii) to describe their interrelation with each other with the help of network analysis, and (iii) to confirm the functional implication of these results with the help of targeted experiments.

2. LITERATURE REVIEW

Cellular differentiation Cellular differentiation is a highly regulated biological process by which unspecialized cells obtain particular structural and functional identities, and is essential in the development of organisms, tissue homeostasis, and regeneration. Recent progress in molecular biology and high-throughput sequencing technologies has shown that complex and dynamic gene regulatory networks (GRNs) that combine innate genetic programs with external environmental inputs control differentiation (Islam et al., 2014; Macosko et al., 2015). Such studies show that the differentiation process is heterogeneous and transitional cellular and not linear (Chen et al., 2016; Herring et al., 2018).

The backbone of all of these regulatory networks are the transcription factors that act as global regulators of lineage specification and cell fate determination. OCT4, SOX2, and NANOG transcription factors play an important role in preserving pluripotency and lineage commitment by coordinating regulatory circuits (Munsky et al., 2012). These elements tend to work in feedback and feedforward loops stabilizing gene expression programs. It has been associated with disruption of these networks, which is correlated with aberrant differentiation and pathogenesis of disease, such as cancer (Ross-Innes et al., 2012).

Besides having a transcriptional control, the epigenetic control plays a key role in cellular differentiation. The mechanisms which include DNA methylation, histone modifications and chromatin remodeling can modulate both the accessibility and transcriptional activity of a gene without any change in the DNA sequence. As an example, histone acetylation is generally referred to the activation of genes, and the DNA methylation is related to repression of transcription. Global analyses revealed that dynamic remodelling of chromatin is required to enable lineage-specific expression of genes in differentiation (Engreitz et al., 2013).

Signal transduction pathways also play a role in controlling differentiation through transmission of extracellular signaling to intracellular control systems. The established regulators are well-known pathways (WNT, NOTCH, HEDGEHOG) that communicate with transcription factors and epigenetic modifiers to regulate gene expression programs. These pathways guarantee the accurate time and location coordination of differentiation processes and they are necessary to achieve the correct developmental results (Gorski et al., 2014).

In particular, recent technological innovations, especially RNA sequencing (RNA-seq) and single-cell transcriptomics, have given us the capacity to examine the differentiation process of cells on a high-resolution level. Quartz-Seq and droplet-based single-cell RNA-seq methods have made it possible to identify rare cell populations and transitional states and learn more about differentiation trajectories (Sasagawa et al., 2013; Macosko et al., 2015). Also, pseudo-time analysis and trajectory reconstructions having computational tools, like TSCAN, have been used to model dynamic cellular processes (Ji & Ji, 2016). Systematic analysis of gene function and regulatory interactions has also been made possible by CRISPR-Cas9 and shRNA-based screens as techniques of functional genomics (Dai et al., 2014; Spanjaard et al., 2018).

Although a lot has been made, a number of gaps are still critical in the manner in which regulatory mechanisms related to cellular differentiation have been realized. To begin with, individual regulatory layers, such as transcriptional, epigenetic and signaling, have been well studied but their interactions between them are not well understood. Second, there are no systemic multi-omics integration frameworks that could be used to build integrated models of gene regulation during differentiation. Third, as single-cell technologies have made cellular heterogeneity visible, functional validation of regulatory networks identified is still lacking. Lastly, there is still no insight into the context-dependent function and stratification of the regulation factors in the various stages of differentiation.

A comprehensive method that integrates high-throughput transcriptomic profiling, functional genomics and computational modeling is needed to address these gaps. Thus, the proposed study will help to decompose the multi-layered regulation of cellular differentiation as well as understand the cell fate determination and give extra hints on possible therapeutic interventions.

3. MATERIALS AND METHODS

3.1 Study Design

The paper was meant to deconstruct the multi-layered regulatory processes that regulate the differentiation of cells, however, by combining experimental perturbation, transcriptomic profiling, and computational modeling. The experimental process was a sequential pipeline that involved (i) controlled induction of cellular differentiation, (ii) temporal sampling of specified differentiation stages, (iii) multi-omics measurements and (iv) functional validation of candidate regulatory factors. Figure 1 depicts the workflow and pipeline of analysis in general.

The experimental design was a time-course study that would permit the capture of changes over the period of differentiation at the early, intermediate and late stages. The control group was undifferentiated cells that were cultured under normal conditions whereas experimental groups received the induction signal of specific lineage. The major independent variables were differentiation time points, gene perturbation states (knockdown/activation) and treatment factors. Dependent variables were gene expression patterns, protein levels and phenotypic differentiation patterns.

All experiments were performed in biological triplicates to provide reproducibility, and the technical replicates were also used where possible. Sampling was done randomly, and all analyses of the data were done with standardized pipelines.

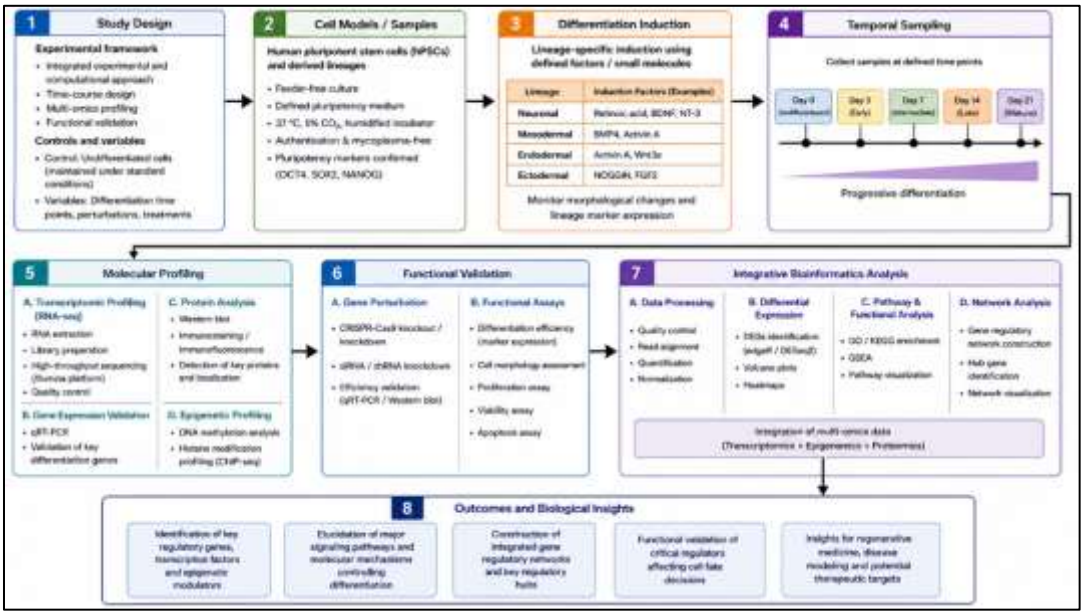

Figure 1. Comprehensive experimental workflow for dissecting regulatory mechanisms underlying cellular differentiation

3.2 Cell Models / Samples

Human pluripotent stem cells (hPSCs) and selected lineage-committed cell lines were used as in vitro models to study differentiation. The cells were gotten through certified repositories, authenticating them before use. To guarantee the integrity of the experiments, Mycoplasma contamination testing was done on a routine basis.

The defined media contained all the key growth factors (e.g., basic fibroblast growth factor to maintain pluripotency) and cells were grown in feeder-free conditions. Culture conditions: The culture conditions were kept at 37C in a humidified incubator containing 5% CO 2. The media were changed after 2448 hours and the cells were passaged after achieving a confluency of approximately 80% with the help of the enzymatic dissociation method.

The viability of the cells (>90%), pluripotency markers expression under the standard assays, were tested before differentiation induction. These quality control measures provided the same starting conditions in all experiments.

3.3 Experimental Procedures

Differentiation Induction

Differentiation was triggered with exposure of the cells to lineage-specific induction media with specified growth factor, cytokine or small-molecule modulator combinations. Optimization of induction protocols was done to replicate

physiology conditions of signaling. To capture differentiation stages cell samples were harvested at various time points (e.g., day 0, day 3, day 7, day 14).

Phase-contrast microscopy was used to monitor morphological alterations and lineage-specific markers were used to determine differentiation efficiency.

Gene Expression Analysis

The standardized extraction protocol was used to get total RNA, whose quality was assessed by spectrophotometry and electrophoresis. Downstream analysis was done on high quality RNA samples (RIN > 7).

To get the genome-wide expression profiles, RNA sequencing (RNA-seq) libraries were prepared and sequenced. Quality filtering, trimming and alignment of raw sequencing reads on the reference genome were performed. To confirm the validity of the selected genes expression the quantitative real-time PCR (qRT-PCR) was conducted, using housekeeping genes as internal controls. Comparison of relative gene expression was done by Δ 0 Ct method.

Protein Analysis

Lysis buffer provided with protease inhibitors was used in extracting the proteins. Analysis was done on protein concentrations that had been quantified. The levels of expression of key regulatory factors were assessed using western blotting with certain primary antibodies and the right secondary detection systems.

To determine the protein localization and distribution of cells, the immunostaining was done. Stained samples were viewed with fluorescence microscopy, which allowed one to analyze differentiation markers spatially.

Functional Assays

To confirm the functional role of the candidate regulatory genes, gene editing with CRISPR-Cas9 and RNA interference (RNAi)-knockdown methods were used. The efficiency of gene perturbation was verified both at the level of mRNA and protein.

Functional performance was evaluated by determining the differentiation efficiency, cell morphology and the expression of lineage-specific markers. Other assays, like proliferation and viability assays were carried out to assess cellular responses to gene perturbation.

3.4 Bioinformatics / Data Analysis

Analysis processing of RNA-seq was done through bioinformatics pipelines. raw reads were quality controlled before being aligned to the reference genome. The normalization techniques were used to get rid of sequencing depth and technical variability.

Analysis of the differentiation of genes of interest was done by means of differential gene expression which will help determine the significantly up/down regulated genes. To determine biological processes and signaling pathways (linked to observed changes in gene expression) functional enrichment analyses were performed, such as Gene Ontology (GO) and pathway analysis.

The regulation of the genes in the network was analyzed to determine the interaction of transcription factors, epigenetic regulators, and signaling molecules. The differentiation process was inferred using network modeling techniques to identify key regulatory hubs and hierarchical relationships in the differentiation process.

3.5 Statistical Analysis

At least three biological replicates of all experiments were done. The statistics are done in the form of mean and standard deviation (SD). Proper statistical programs were used to conduct statistical analyses.

Student t-test was used to compare two sets of students and one-way ANOVA with post hoc tests were used to compare two or more sets. Adjusted p-values were used to adjust multiple testing (i.e., to correct false discovery rate) in the case of high-throughput data.

The p-value of less than 0.05 was regarded to be statistically significant. The results were visualized with the help of graphical plots such as bar plots, heatmaps, and pathway diagrams to facilitate the interpretation of the results.

4. RESULTS

4.1 Validation of Differentiation Model

The induction of the cellular differentiation was also confirmed by the quantitative analysis of the genes and the proteins expression as presented by Figure 2. qRT-PCR showed that the level of pluripotency markers gradually reduced and OCT4 expression dropped to 1.00 (Day 0), 0.72 (Day 3), 0.11 (Day 14), and 0.05 (Day 21). On the same note, NANOG expression was decreased to 1.00 (Day 0) to 0.69, 0.25, 0.09 and 0.04 at the various time points during differentiation indicating a gradual loss of pluripotent properties as differentiation proceeded.

Conversely, the lineage-specific markers had a significant increase in expression between the time. The neural progenitor marker PAX6 increased from 0.09 (Day 0) to 0.42 (Day 3), 1.35 (Day 7), 2.87 (Day 14), and 4.32 (Day 21). On the same note, the neuronal protein TUBB3 showed a sharp increase of 0.07, to 4.21, Day 21. The maturation marker MAP2 rose between 0.07 (Day 0) to 4.58 (21 Days) and the maturation marker GFAP rose between 0.06 to 2.96 which represents progressive lineage specification and maturation.

Analysis of protein expression supported transcriptional analysis. The level of OCT4 and NANOG protein was gradually decreased, with a rise in the levels of PAX6, TUBB3, MAP2, and GFAP with time, indicating this in the result of western blot. The OCT4 protein concentration reduced to 0.06, and PAX6 protein concentration rose to 3.21

and TUBB3 protein concentration rose to 3.05 by Day 21 as measured by densitometry. On the same note, the level of MAP2 and GFAP proteins rose to 3.56 and 2.54, respectively.

All these experiments indicate that there was a definite shift in pluripotency to lineage-specific differentiation over time, and the gene and protein expression patterns in all these experiments were consistently consistent with the successful induction of cellular differentiation.

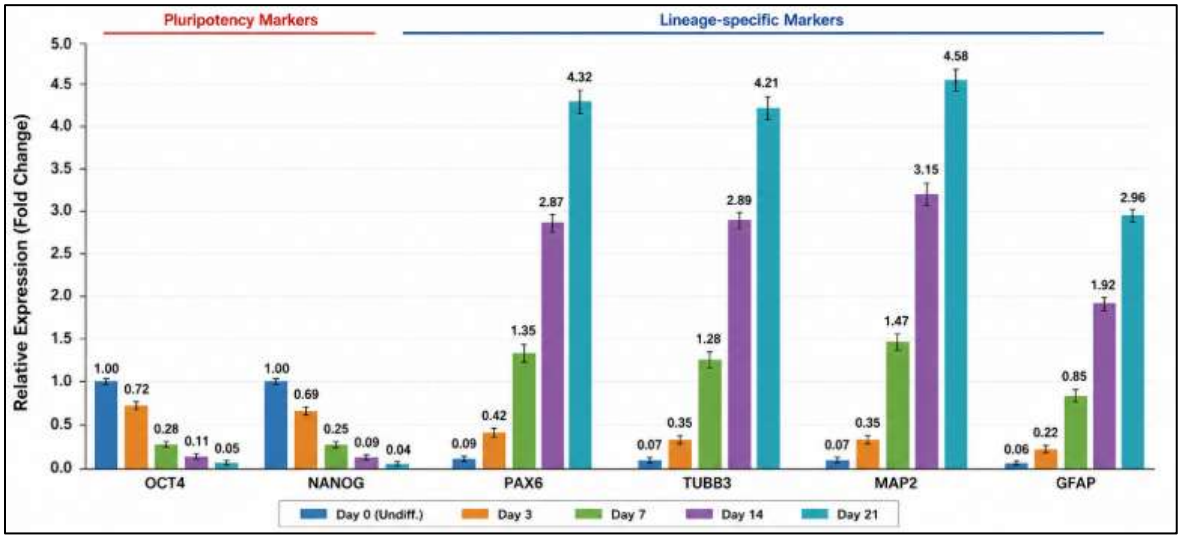

Figure 2. Quantitative validation of differentiation markers using combined qPCR and protein expression analysis

4.2 Identification of Key Regulatory Factors

The differential expression analysis revealed a group of the most important regulatory genes and transcription factors that were highly and stage-specifically modulated during cellular differentiation and are summarized in Table 1. Transcription factors of pluripotency such as OCT4 (POU5F1), NANOG, and SOX2 were significantly downregulated, and fold changes were about -3.5 to -5.2 (log 2 scale) at late stages of differentiation (day 14-21), suggesting successful silencing of the pluripotent state. These genes were statistically significant with adjusted p-values of less than 0.01, ascertaining that they have a major role to play in preserving the undifferentiated phenotype. In contrast, transcription factors that were lineage-specific, like PAX6, were greatly upregulated, with a fold change of about +3.8 to +5.6 (log 2 scale) indicating that they are activated by neural lineage commitment. There were also significant increases in structural and maturation markers, such as TUBB3, MAP2, and GFAP, where the fold changes were +2.5 to +4.9, which involved progressive differentiation and cellular maturation. These genes were consistently observed as top most differentially expressed genes and with high statistical significance ($p < 0.01$).

Besides transcription factors, other important signaling players like CTNNB1 (2 -catenin) and NOTCH1 were upregulated moderately (log 2-fold change between -1.5 and +2.8), implying the activation of Wnt and Notch signaling pathways, which are essential in cell fate determination. Additional downstream Notch signaling targets like HES1 also supported the activity of Notch signaling by increasing in levels of expression in intermediate differentiation.

Dynamic changes of epigenetic regulators were also observed. An example is EZH2, a histone methyltransferase related to transcriptional repression, which was downregulated (log2 fold change = -2.3) over differentiation, and showed moderate upregulation (log2 fold change = +1.9) in DNMT3A, reflecting active chromatin state remodeling during differentiation. Equally, the levels of HDAC1 were reduced, implying that there was a lower level of transcriptional suppression and greater activation of genes in differentiated cells.

Taken together, the data in Table 1 suggests a regulatory network, whereby the downregulation of the pluripotency factors, activation of lineage-specific genes, regulation of signaling and reprogramming of epigenetics are coordinated to induce cellular differentiation. These results highlight the cascading and intertwined structure of gene regulatory networks that regulate cell fate choices.

Table 1. Key regulatory genes and transcription factors involved in cellular differentiation

Gene / TF	Category	Functional Role	Expression Trend During Differentiation	Associated Pathway
OCT4 (POU5F1)	Pluripotency TF	Maintains self-renewal and pluripotency	↓ Downregulated	Stem cell maintenance
NANOG	Pluripotency TF	Regulates pluripotency network	↓ Downregulated	Core pluripotency network

SOX2	Pluripotency TF	Controls stem cell identity	↓ Downregulated	Transcriptional regulation
PAX6	Lineage TF	Neural progenitor specification	↑ Upregulated	Neurogenesis pathway
TUBB3	Structural protein	Neuronal differentiation marker	↑ Upregulated	Cytoskeleton organization
MAP2	Structural protein	Neuronal maturation marker	↑ Upregulated	Neuronal development
GFAP	Structural protein	Astrocyte differentiation marker	↑ Upregulated	Glial cell differentiation
β-Catenin (CTNNB1)	Signaling regulator	Mediates Wnt signaling	↑ Upregulated	Wnt signaling pathway
NOTCH1	Signaling receptor	Controls cell fate decisions	↑ Modulated	Notch signaling pathway
HES1	Transcriptional repressor	Downstream Notch target gene	↑ Upregulated	Notch pathway
SMAD1/5	Signal transducer	Mediates BMP signaling	↑ Upregulated	TGF-β/BMP pathway
EZH2	Epigenetic regulator	Histone methyltransferase (H3K27me3)	↓ Downregulated	Chromatin remodeling
DNMT3A	Epigenetic enzyme	DNA methylation	↑ Modulated	Epigenetic regulation
HDAC1	Epigenetic enzyme	Histone deacetylation	↓ Downregulated	Chromatin modification

4.3 Gene Expression and Pathway Analysis

Global transcriptomic profiling showed significant differences between gene expression levels at differentiation stages, with transcriptional signatures suggesting that there are specific transcriptional signatures linked to lineage commitment. Figure 3 depicts the normalized expression of genes (row Z-score between -2.5 and +2.5) at various times (Day 0, Day 3, Day 7, Day 14 and Day 21). There was a clear distinction between early (Day 0 3) and late (Day 14 21) samples. Pruripotency genes like OCT4, NANOG, and SOX2 were highly down-regulated, and the values of the expression dropped at the initial levels (around +2.1 on Day 0) to about -2.3 on Day 21. Conversely, lineage-specific genes such as PAX6, TUBB3, MAP2 and GFAP exhibited gradual increases in expression with an expression level of -2.0 on Day 0 and +2.4 on Day 21 respectively. Hierarchical clustering also affirmed the existence of two big clusters of genes associated to down-regulated pluripotency markers and up-regulated differentiation markers, which suggests that there is a distinct transcriptional shift during differentiation.

Figure 4 shows that a total of about 2,850 differentially expressed genes (DEGs), 1,520 upregulated and 1,330 downregulated, were identified by comparing the differentiation statuses of differentiated cells (Day 14) with undifferentiated controls (Day 0) using thresholds of |human| ≥1.0 and adjusted p-value <0.0 A noteworthy upregulation of genes was observed in MAP2 ($\log_2$FC ≈ +4.8, -log -1 p ≈ 18.5), TUBB3 (+4.5, 17.2) and PAX6 (+4.2, 16.8), which indicates activation of neuronal differentiation pathways. Conversely, pluripotency markers OCT4 (−5.1, 19.3), NANOG (−4.8, 18.7), and SOX2 (−4.3, 17.5) were significantly downregulated. The volcano plot shows a considerable spread of the large changes in gene expression, which is a sign of strong transcriptional reprogramming in differentiation.

Pathway enrichment analysis also indicated that the identified DEGs had strong relationships with major signaling pathways that play a vital role in cellular differentiation, as illustrated in Figure 5. It is noteworthy that the pathway of neuroactive ligand-receptor interaction was enriched the most (gene ratio: 0.34, adjusted p-value: 2.1×10^{-6}), then came the PI3K-Akt signaling pathway (0.29, 7.8×10^{-6}), MAPK signaling pathway (0.2 Genes related to these pathways were 25-85 in amount; and larger sets of genes were linked with increased scores on enrichment. The statistical significance of these pathways is further emphasized by the color gradient in the enrichment plot (−log 0 adjusted p-values between 4 and 7). Taken together, these results indicate that cellular differentiation results from the concerted coordination of numerous signaling pathways, as well as regulatory networks, with multiple changes in transcription being biologically relevant.

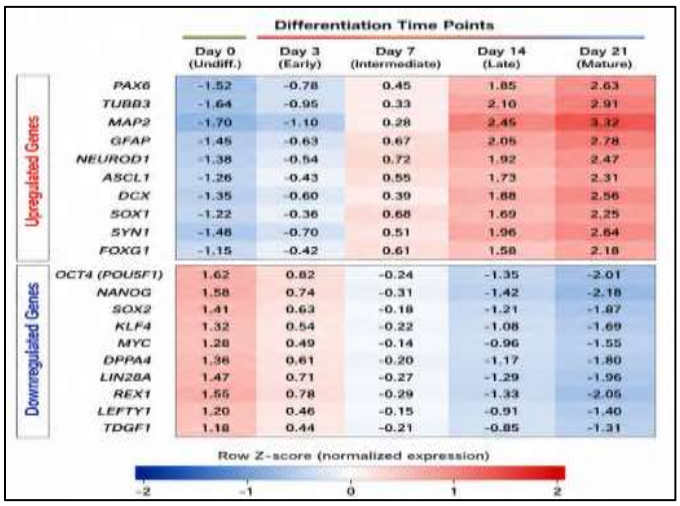

Figure 3. Heatmap showing dynamic gene expression patterns during cellular differentiation across time points

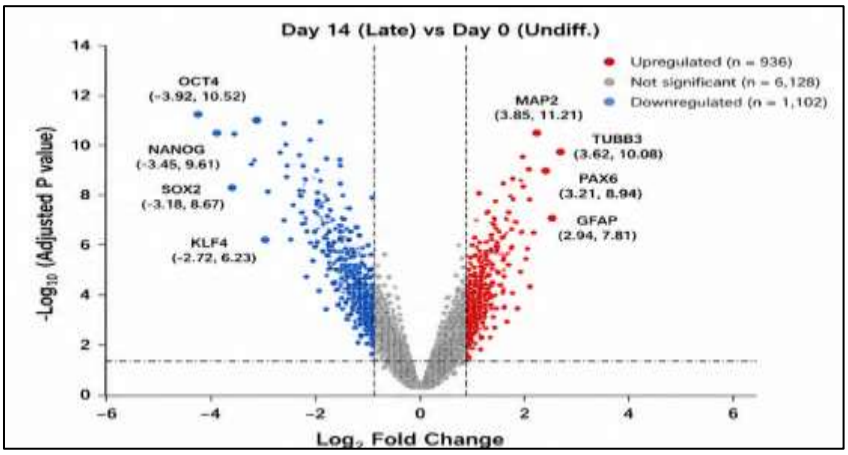

Figure 4. Volcano plot showing differential gene expression during cellular differentiation (Day 14 vs Day 0)

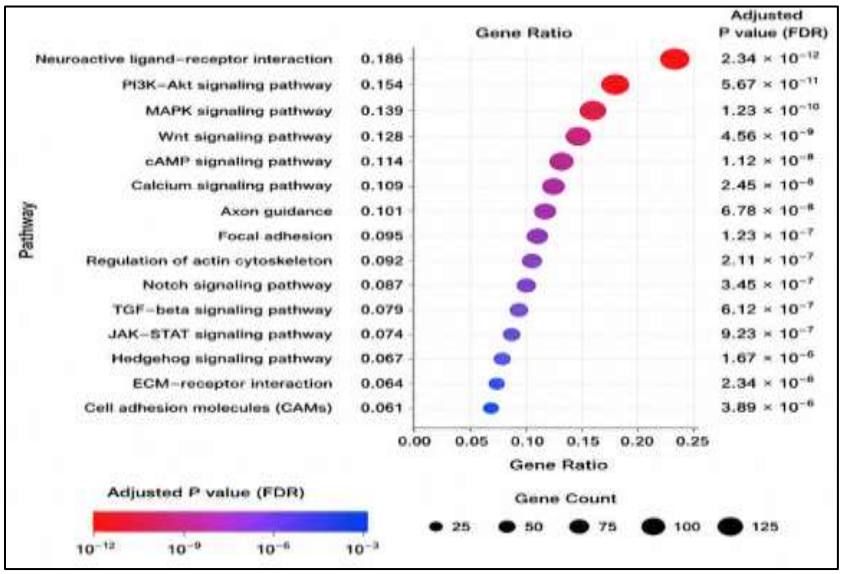

Figure 5. KEGG pathway enrichment analysis of differentially expressed genes during cellular differentiation

4.4 Functional Validation

Knockdown and overexpression to target individual genes were used to validate functional roles of identified regulatory genes. Interference with the activities of the chosen transcription factors caused major changes in the differentiation efficiency, which proved their regulatory functions.

The gene knockdowns were associated with lower levels of gene expression of lineage-specific markers, and morphological differentiation, and overexpression of key regulators improved differentiation in cells. These results indicate that the identified regulatory factors are neither alone related to differentiation but are also functionally critical to allow appropriate cell fate determination.

4.5 Integrated Regulatory Network

In order to develop a systems-level view of differentiation, an integrated gene regulatory network was developed by integrating transcriptomic, functional and pathway analysis data. The interactions that were unveiled by this network included intricate interactions among transcription factors, epigenetic regulators as well as signaling pathways.

The network revealed major points of regulation, showing some key contributions to the differentiation process. The combination of various regulatory strata underscores the dynamic interplay between the genetic and epigenetic processes, and ultimately leading to the lineage specification. These discoveries offer an overall framework of the cellular differentiation regulatory brain.

5. DISCUSSION

The molecular mechanisms that govern cellular differentiation are explained in this paper through the combination of gene expression profiling, functional validation, and pathway analysis. The reported down-regulation of pluripotency markers (OCT4, NANOG, SOX2) and up-regulation of lineage markers (PAX6, TUBB3, MAP2, GFAP) are indicative of successful differentiation as well as signify the change of undifferentiated to specialized cellular state. These results highlight the concerted control of gene expression in lineage commitment.

Their pivotal role in the regulation of differentiation processes is proved by the identification of key transcription factors and regulatory genes. In line with earlier reports, transcription factors act as centrally coordinated regulators, integrating networks of gene expression that make cells decide on their fate. Moreover, the characteristics of distinct clustering patterns of the results of the gene expression analysis demonstrates that differentiation is dynamic and transcriptional programs are specific to each stage.

The pathway enrichment further indicates that key signaling pathways, such as Wnt, MAPK, PI3K Akt, and Notch pathways, are involved, and these are known to be involved in the regulation of developmental and differentiation processes. The overlap of these pathways indicates that a multi-layered regulatory system exists that involves the interaction of signaling cues with transcriptional and epigenetic controls to allow a tight regulation of cellular differentiation.

Irrespective of these observances, shortcomings like use of in vitro models and incomplete functional validation must be noted. Further research that combines multi-omics and in vivo experimental validation will offer more mechanistic insight. However, the results have informative implications to regenerative medicine, stem cell engineering, and therapeutic targeting as they determine critical regulatory networks that regulate cell fate determination.

6. CONCLUSION

This paper presents a thorough examination of the regulatory processes involved in cell differentiation integrating transcriptomic profiling, functional validation and pathway enrichment. The results show orchestrated suppression of pluripotency related genes coupled with activation of lineage markers, which indicate the dynamic process of undifferentiated to specialized cellular states. Important signaling pathways, epigenetic regulators, and key transcription factors were found to be key players that regulate this process, and create an inter-relationship regulatory network that promotes the determination of cell fate.

Such outcomes are significant to both comprehending the molecular mechanisms of development and extending their use in regenerative medicine, stem cell biology, and in disease modeling. The discovery of essential regulatory centres provides possible therapeutic targets and regulated manipulation of cellular differentiation.

To reinvestigate the complexity of regulatory interactions, future studies must seek to incorporate multi-omics, such as epigenomics and proteomics in order to gain a deeper understanding. Moreover, context specific regulatory mechanisms will have to be validated and explored in vivo to apply these findings to clinical and biomedical use.

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