TIME-COURSE AND DOSE-DEPENDENT TRANSCRIPTOME PROFILING REVEAL KEY REGULATORS FOR NEURAL CONVERSION OF HUMAN iPSCs UNDER CHEMICALLY DEFINED CONDITIONS

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ABSTRACT

The systematic study of cell differentiation provides deeper insights into developmental pathways and how they control complex genetic programs. Neural induction of human pluripotent cells can be used as a model system to investigate the interplay between pathway manipulation and gene activation/silencing in chemically defined E6 medium. Small molecule based inhibition of bone morphogenetic protein (BMP) and transforming growth factor-beta (TGF-β) pathways (dual SMAD inhibition) is a widely used approach to convert pluripotent cells into neuroectoderm and neural crest. While previous studies characterized human neural induction in bulk cultures, high-resolution analysis should capture the dynamic molecular changes more comprehensively. Here, we performed 7-day time-course single-cell sequencing (scRNA-Seq) to reconstruct differentiation trajectories induced by blocking BMP and TGF-β pathways either separately or in combination. Using dual SMAD inhibitors at single concentrations (0.1 M LDN-193189, 2 M A83-01), we identified distinct transition stages characterized by transiently expressed genes (e.g. SIX3, HESX1, and LMO1), which led to expression of PAX6, DLK1, TPBG, TMSB15A, HES4, IGFBP5, FOXG1, SOX11. Next, to systematically investigate gene expression dynamics, RASL-Seq was used for gene expression profiling upon BMP inhibition and TGF-β inhibition alone or in combination across seven different small molecule concentrations. During BMP inhibition, FOXG1, SOX1, FZD5, ZIC4, HEXS1, SIX3, PTN, HES4 were strongly upregulated while PAX3, FOXD3, SOX10, SNAI2, S100B were antagonized in a dose-dependent manner. Conversely, TGF-β inhibition alone positively regulated PAX3 and SOX10, suggesting that this strategy favors the induction of neural crest. In summary, modulation of cell signaling pathways via high-throughput gene expression profiling and small molecule titration can control expression of transcription factors determining cell specification. This strategy should help to optimize cell differentiation protocols using precisely calibrated small molecule combinations to produce functional phenotypes for clinical therapies.

METHODS

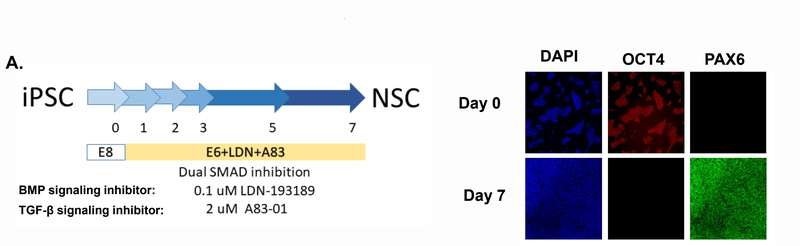

Figure 1. Neural conversion of iPSCs and characterization of compound effects by dose-response gene expression profiling. (A) Highly efficient neural conversion of iPSCs by dual SMAD inhibition: inhibition of TGF-β (A83-01) and BMP signaling (LDN-193189). Immunostaining for OCT4 (also known as POU5F1; red) and PAX6 (green) expression of cells at Day 0 and Day 7. Cells were collected at different time points during the differentiation process for transcriptome analysis using droplet-based single-cell RNA sequencing (scRNA-Seq) technology (ddSEQ and Illumina NextSeq 500). **(B)** High-throughput gene expression profiling using RASL-Seq method to identify genes with dose-dependent gene expression changes by automated curve fitting. Each assay can detect 384 samples with 358 gene expression profiles including 3 lineage markers, stress response genes, and the top 50 genes from dual SMAD inhibition. **(C)** Example plots illustrating the identification of compound combination effects. Concentration–response titration points for each compound at fixed modulator concentration were fitted to a four-parameter Hill equation using an iterative grid algorithm.

RESULTS

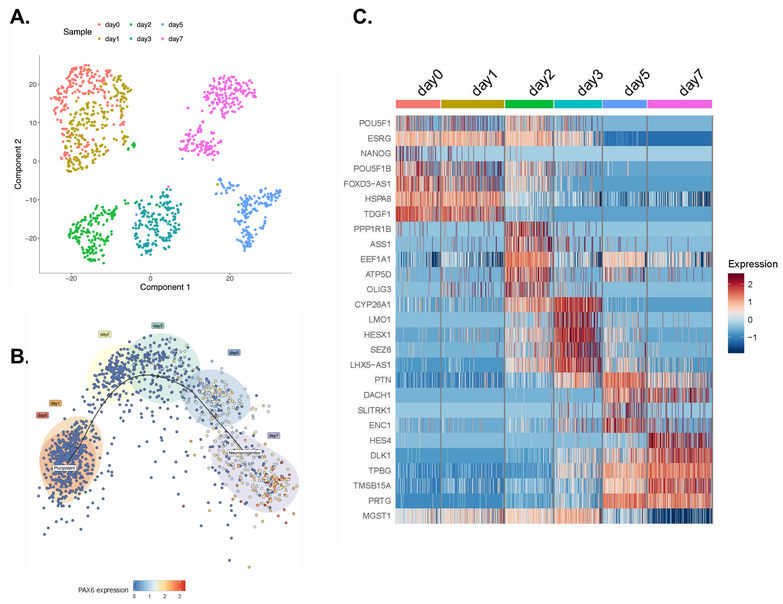

Figure 2. Time-course single-cell sequencing analysis of neural differentiation. (A) t-SNE plot (dimensionality reduction algorithm) reveals distinct subpopulations of iPSCs under chemically defined culture conditions at different stages of differentiation. Each dot represents a single cell and is colored according to treatment duration. **(B)** Identification of Day 3 as critical transition stage using scRNA-Seq data. The neural marker PAX6 was first detectable at day 3 and continuously increased until day 7. **(C)** Heatmap reveals the scaled expression of differentially expressed genes during differentiation (Day 0-7).

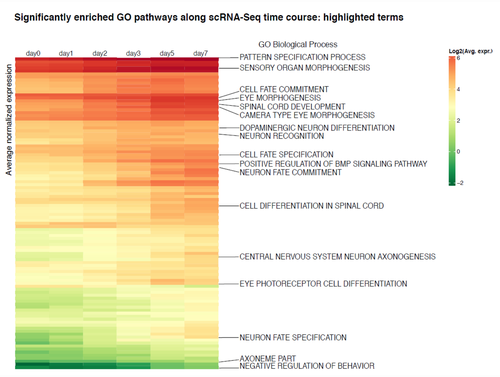

Figure 3. Identification of signaling pathways using single-cell transcriptome analysis. Pathway enrichment of single cell RNA-Seq data reveals neuronal cell fate commitment through dual SMAD inhibition. Normalized gene expression was averaged within each GO term and day. Key GO Biological Process terms are labeled.

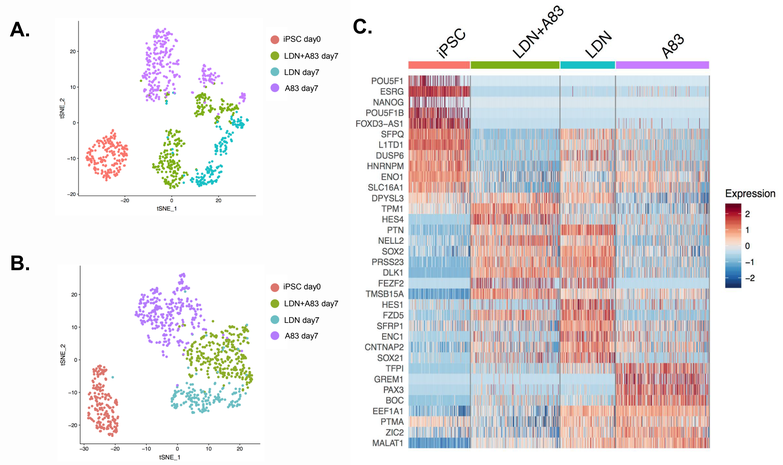

Figure 4. Characterization of single treatment and dual SMAD inhibition for neural induction. (A) LDN-193189 (BMP signaling inhibition), A83-01 (TGF-β signaling inhibition), and in combination (dual SMAD inhibition) show distinct populations upon 7-day treatment. **(B)** t-SNE plot illustrating that cell cycle status is a major factor resulting in different subpopulation within a treatment group. **(C)** Heatmap showing the top differentially expressed genes for different treatment groups. Cells treated by LDN-193189 resulted in similar gene expression pattern to dual SMAD treatment, while A83-01 alone generated a more distinct expression signature.

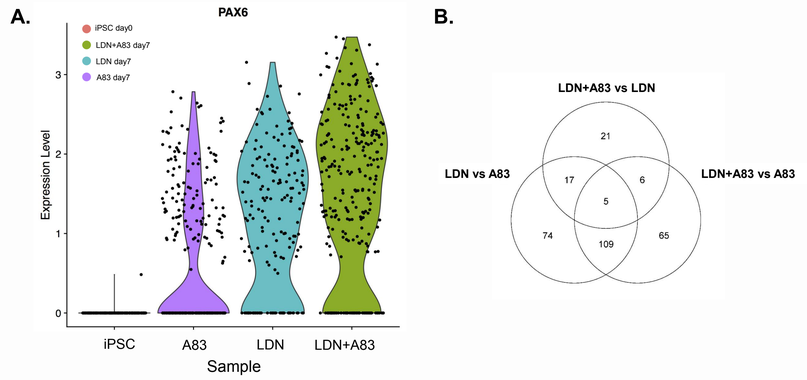

Figure 5. Dual SMAD inhibition and BMP inhibition leads to high PAX6 expression compared to TGF-β inhibition alone. (A) Violin plot reveals the expressional level and distribution of PAX6 expression at single-cell level. A83-01 treatment alone shows significantly lower PAX6 expression at Day 7. (**B)** A83-01 treatment shows large number of unique differential expression genes compared to LDN-193189 versus combination treatment.

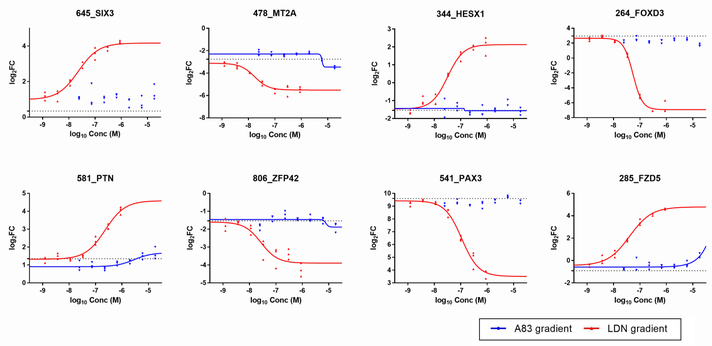

Figure 6. Dose-dependent gene expression profiling identify genes regulated by LDN-193189 but not by A83-01. Representative examples of LDN-dependent genes which failed to be induced by A83-01 even at higher concentrations (18 M as the highest concentration was 9-fold over the reference concentration).

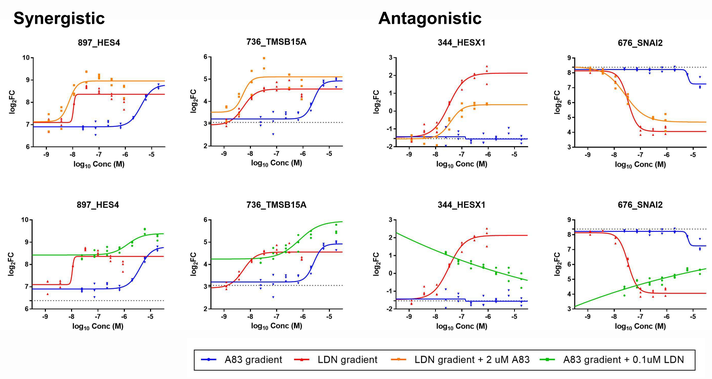

Figure 7. Combination effects can be examined by titration of one compound at fixed modulator concentration. The effect of A83-01 in combination (orange) was obtained by comparing the curve shift with LDN titration (red). The effect of LDN in combination (green) was obtained by comparing the curve shift with A83-01 titration (blue). The top significant DEG (HES4 and TMSB15A) can only be maximized in combination treatment. A key neural commitment transcription factor, HESX1, was identified to be negatively regulated by A83-01 in combination treatment. Neural crest marker, SNAI2, was strongly inhibited by LDN treatment while inducible when combined with A83-01 at higher concentrations. Systematic characterization of small molecules administered alone, in combination or in varied concentrations can help to better control the cell differentiation process and prevent unspecific off-target effects and toxicity.

SUMMARY

- The time-course scRNA-Seq experiment dissected the neural lineage entry process of human pluripotent cells at unprecedented resolution.
- Cell state transition analysis revealed important time windows during differentiation and identified new potential key regulators of neural fate commitment.
- Dose-response experiments identified compound-specific gene regulation enabling accurate control of specific transcription factors.
- Precise characterization of small molecule combinations may facilitate data-based selection of chemicals to better control gene expression programs and formulate improved cell differentiation protocols.

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