Cis-regulatory elements regulate alternative cell fate choice.

Introduction

The differentiation of the neural progenitor cells in the neural tube is controlled by the combined actions of spatial and temporal programmes of gene regulatory networks, giving rise to the massive diversity of the glia and neurons (Delás et al., 2023, Frith et al., 2024). Between spatial domains, the neural progenitors employ a differential binding strategy, where there is a shared set of CREs, and it is the binding of repressive transcription factors (TFs) that determines the cell (Delás et al., 2023). Over time these neural progenitors generated different subtypes of neurons and glial, and this temporal programme is regulated by altering the availability of the CREs at different time points (Zhang et al., 2024). Under the current model, the temporal program establishes sets of elements at each stage that rewire how the spatial TFs act to direct cell fate decisions.

This model predicts that elements that are accessible in multiple neural progenitors at a specific time point control gene expression only in a neural progenitor type. This project aims to show the time and cell type-specific activity of the CREs using a lenti-viral reporter system during stem cell differentiation. The long-term aim is to elucidate the gene regulatory network that determines the cell differentiation in the ventral neural tube.

Materials and Methods

Molecular Cloning

The backbone was built from individual component plasmids via a Golden Gate reaction using restriction enzyme Esp3I. The reaction was incubated at 37˚C for 1 hour.

Cell Culture

Mouse embryonic stem cells maintained in ES cell medium were first tested for pluripotency after 7 passages to ensure that the proportions of cells expressing the pluripotency markers are over 90%. The cells were then differentiated following two differentiation panel, the glial differentiation panel and the SAG concentration panel.

For the glial differentiation panel, the cells were maintained in N2B27 media supplemented with 10nM SAG and 100nM RA for eleven days and were collected at day 5 and day 11. For the SAG concentration panel, the cells were differentiated in N2B27 media supplemented with 100nM RA and either 0nM of SAG or 500nM of SAG for 6 days and were collected at day 5 and day 6.

Flow Cytometry

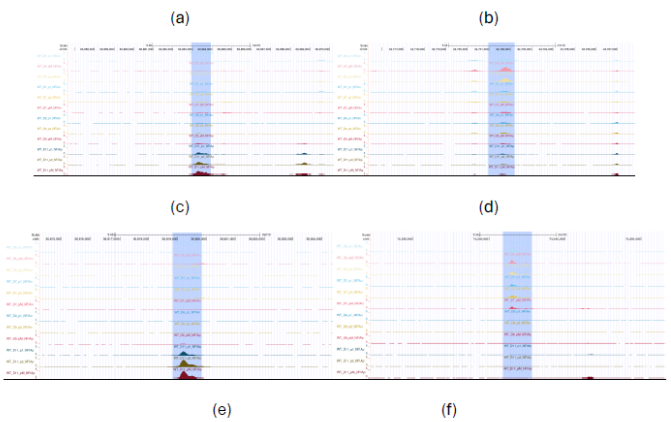
After cell collection, samples from the glial differentiation panel were stained with antibodies SOX2-V450, NKX6.1-PE, goat OLIG2 unconjugated followed by AF647, rabbit NFIA unconjugated followed by AF568, and LIVE/DEAD Cell Stain for further flow analysis, samples from the SAG concentration panel were stained with antibodies SOX2-V450, NKX2.2-PE, goat OLIG2 unconjugated followed by AF488, and LIVE/DEAD Cell Stain.

RT-qPCR

The cells were collected, and the RNA was extracted using the RNeasy Mini Kit following manufacturer's instructions. cDNA was synthesized from 1µg of RNA using Superscript III reverse transcriptase, and the following RT-qPCR analysis was performed with PowerUP SYBR Green Master Mix.

Results

1. In-silico identification of candidate CREs



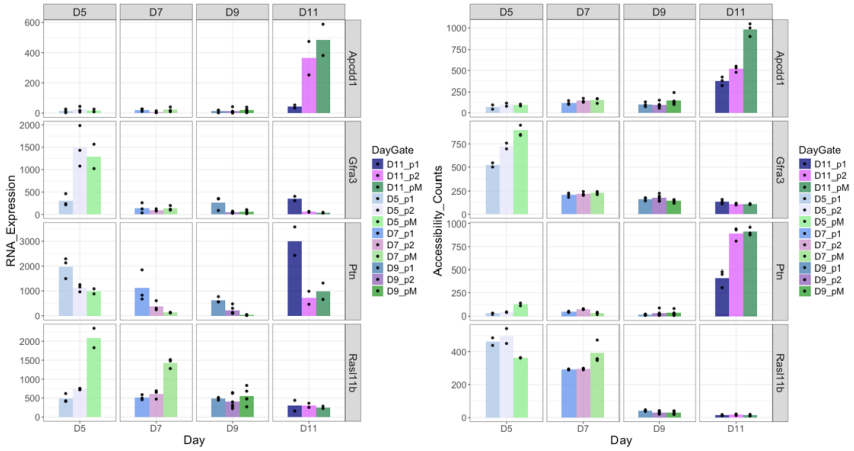


Figure 1. In-silico analysis of CRE *Apcdd1*, *Gfra3*, *Ptn* and *Rasl11b*

(a)-(d) The genome browser plot showing the chromatin accessibility of the different cell types across an eleven-day period for elements *Apcdd1*, *Gfra3*, *Ptn* and *Rasl11b* respectively

(e) The RNA expression of the CREs in different cell types at different day points

(f) The chromatin accessibility counts of the CREs in different cell types at different day points

In order to select the candidate elements to test in the reporter system, the chromatin accessibility of the CREs (Fig 1a-d) and the RNA expression (Fig 1e) of their targets were analyzed. The elements shown were selected because the accessibility of the CREs vary temporally, and their targets are expressed in a cell-type specific manner at the time points when the CREs are accessible.

2. Cloning of an optimized reporter system

The desired final backbone contains a minimal promoter (Shh-mP) driving the mGreenLantern genes that encode the fluorescent protein under the control of the CRE of choice. The FKBP fragment is also needed to ensure that the fluorescent protein is degradable.

The backbone was cloned by golden gate and the final colony sequence was verified (Fig 2). The candidate CREs from (1) were subsequently cloned into this backbone although all colonies analysed had errors.

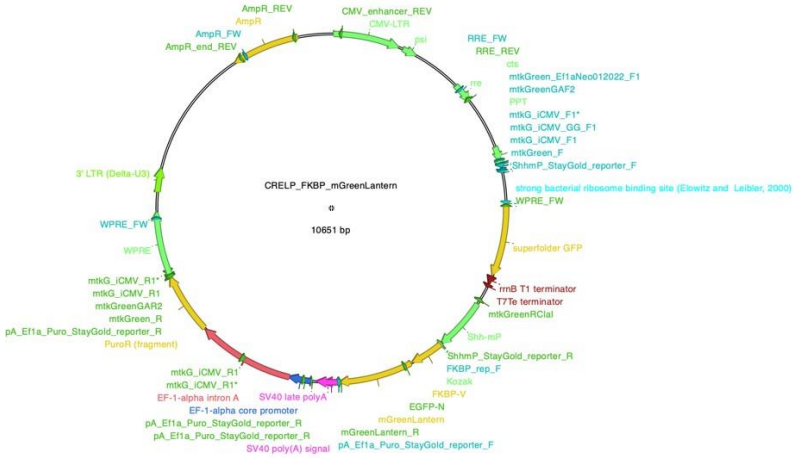


Figure 2. The plasmid map of the built backbone

3. Supplementation of SAG mimics the differentiation of ventral neural tube

To analyze the activities of the CREs in the gliogenesis process, single cell identity was assayed following a differentiation system that generates cells of various spatial- temporal properties. By supplementing SAG at different concentrations, the stem cells would differentiate into progenitors of different types, which were

then identified by different markers. Specifically, pMN, the most ventral cell type analyzed in this panel, is marked by the transcription factor OLIG2, and p2 is marked by NKX6.1. The late marker NFIA is also stained for the temporal activity.

Cell type proportions reveal that at higher concentrations of SAG or at later timepoints, the proportions of the ventral cell types pMN and p2 are much higher, matching the ventralizing activity of SAG (Fig 3a). Also, the number of cells expressing the late neural progenitor marker NFIA drastically increased at D11, indicating that this panel can be used to investigate the activity of the temporal programme as well.

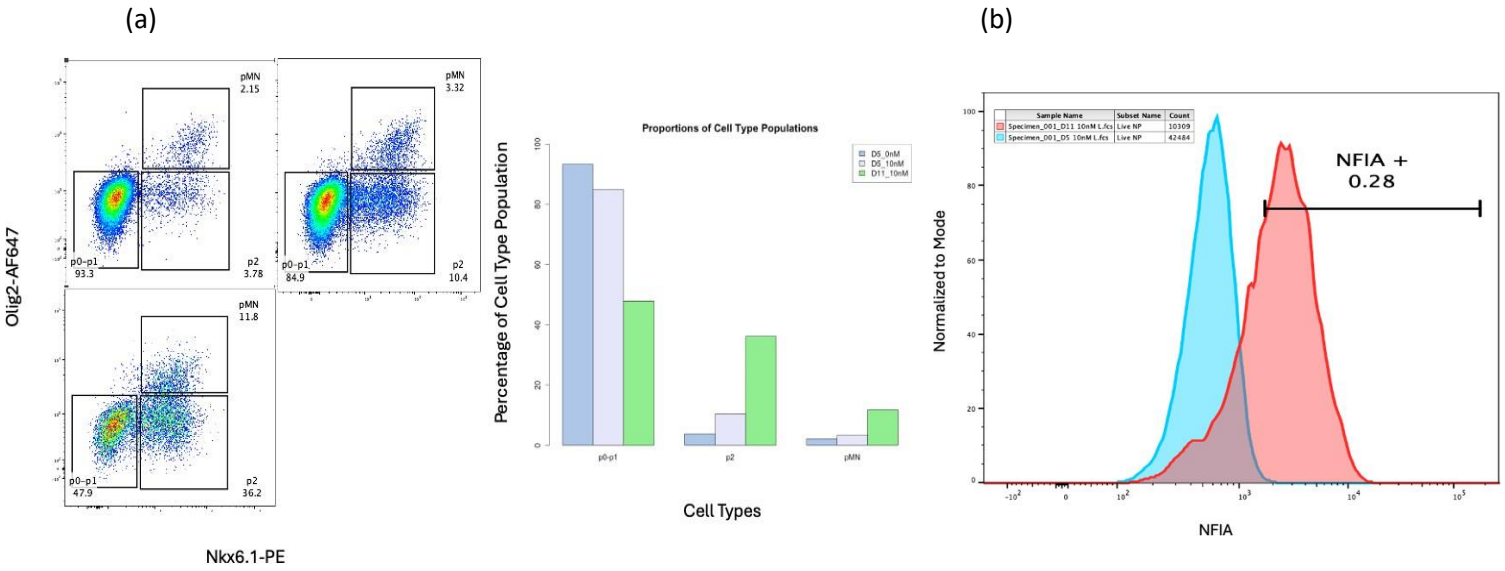


Figure 3. (a) Proportions of the cell type populations under the glial differentiation panel
(b) Histogram showing the NFIA+ populations at 10nM of SAG concentrations at D5 (blue) and D11 (red)

With the addition of 500nM of SAG, the cells were additionally differentiated to be more ventral identities, such as p3 that is marked by NKX2.2, as assessed by flow cytometry. At 500nM of SAG, the proportions of pMN and p3 drastically increase compared with 0nM of SAG, confirming previous results.

4. High SAG concentrations coincide with mRNA expression of Shh signaling targets

To investigate the expression level of the genes that are associated with the vertebrate neural tube development, RT-qPCR was performed with genes *Nkx2.2*, *Nkx6.1*, *Olig2*, *Gli1*, *Ptch1*, and *Shh* as targets. *Nkx2.2*, *Nkx6.1*, and *Olig2* are both highly expressed at 500nM of SAG, matching the flow cytometry results. *Gli1*and *Ptch1* are both target genes of the Shh pathway during ventral neural tube patterning, which also exhibit a higher mRNA expression level at 500nM of SAG (Andrews et al., 2019). Therefore, the results confirm that SAG supplementation would mimic the Shh signaling.

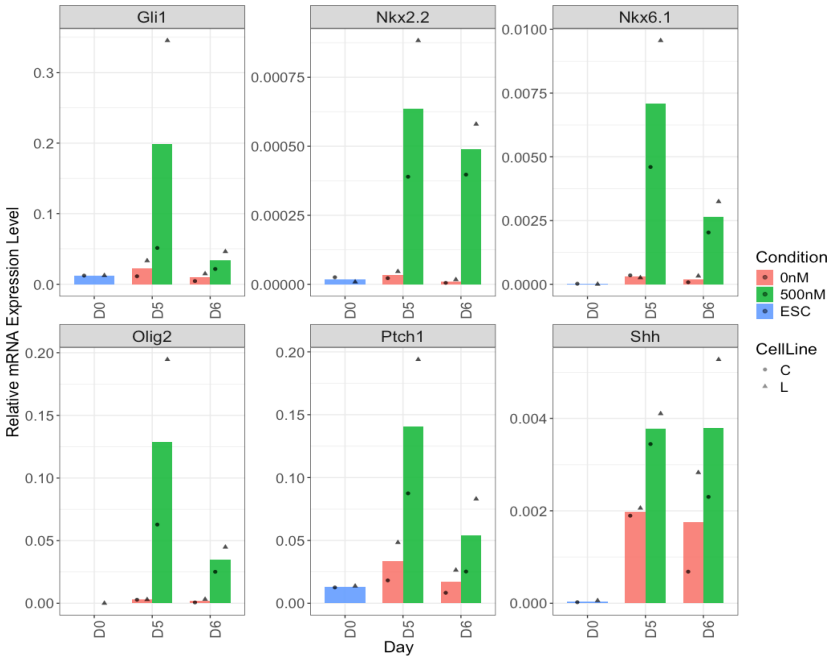


Figure 5. The Relative mRNA Expression Level Quantified by RT-qPCR of Genes Associated with the Differentiation of the Vertebrate Neural Tube.

Conclusions and Future Directions

This project has successfully built a backbone that can be used to investigate the activities of the CREs and tested the differentiation panels that can be repeated in future studies with the CRE-vector transfected into the cells. Future studies can focus on the CREs identified in this project and induce mutations on the transcription factor binding sites to assay their effects on the differentiation of the neural tube. Also, more in-silico analysis such as single-cell ATAC-seq can be used to predict the activities of the CREs, aiding the understanding of the gene regulatory networks that regulate the neural tube differentiation.

Acknowledgement

I really appreciate the Biochemical Society for providing this studentship that allows me to explore my areas of interest via wet lab research. I am also very grateful to Dr. Delas for supervising me and teaching me both the new dry lab and wet lab techniques.

This studentship has provided me the opportunities to learn and practice cell culturing and molecular cloning techniques such as Golden Gate cloning and flow cytometry, which allows me to improve my manual lab skills. Using R to analyze ATAC-seq and RT- qPCR data, I also improved my coding skills. By attending joint lab meetings either with the Chubb Lab or the Fernandes Lab, I had the chance to improve my critical thinking skills. The seminars at UCL LMCB where Delas Lab is located at also expose me to a large range of biology-related topics. All these experiences have deepened my passion for doing research in the field of molecular and cell biology and would be of great value to my final year of undergraduate and future post-graduate studies.

Reference

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