Identification of Regulators in Neuronal Differentiation and Induction through CRISPR Gene Activator Screening

Mark Youssef, Ruiqi Hu, Linda Lee, Nan Yang
Department of Development, Regeneration, and Stem Cells & Neuroscience

Icahn School of Medicine at Mount Sinai

ABSTRACT

Rationale: 2 main issues arise when inducing cells to neurons. Typically, these neurons are immature (*inaccurate models of human diseases*), and it takes a long time to produce sufficient neurons. Recent advances to CRISPR-CAS9 models can help alleviate some of these challenges.

Background: Expression of ASCL1 and DLX2 to exclusively induces GABAergic neurons, which are useful models for diseases affected by inhibitory synaptic transmission. Utilization of a tripartite activator, VP64-p65-rta (VPR) fused to Cas9 would increase ASCL1 and DLX2 expression which would cause neuronal induction.

This study sought identified that RNA guided activation of CRISPR would increase induction of neurons from human pluripotent stem cells.

Post selection, cells were exhibiting morphological changes showing. We also saw MAP2 and Beta III Tubulin expression under immunofluorescence staining. qPCR indicated that combination of both DLX2 and ASCL1 was efficient in producing neuronal markers. This confirms that CRISPR activation using transcription factors does indeed induce human neurons.
In the future, neurons produced from using these gRNAs can then be used for CRISPRa screening of other gRNAs for specific GABAergic neuronal subtypes.

BACKGROUND

CRISPRa using dCAS9 tripartite activator Mechanism

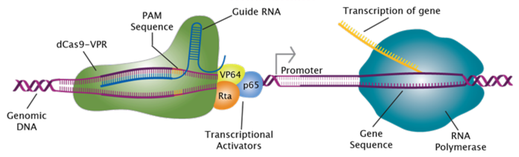


Figure 1. CRISPRa relies on the deactivation of normal Cas9 DNA cutting functionality, which then enables binding to transcriptional factors (VP65, RTA, p65). This complex is bound to a guide RNA which is used to target promoter region, This leads to increases in transcription factors. (Transomic Technologies).

CRISPR tripartite activator, increasing RNA expression

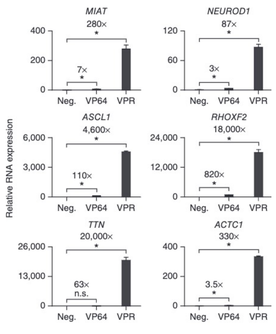


Figure 2. CRISPR activation using tripartite activator (VP65, RTA, p65) is consistently shown to increase transfection in HEK293T compared to utilization of only VP64 (Chavez, 2015).

ASCL1 and DLX2 gRNAs inducing neurons as marked by immunofluorescent staining

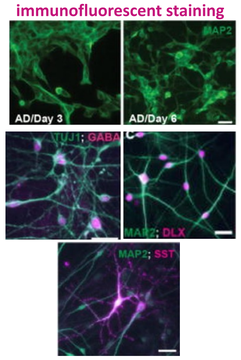


Figure 3. Ascl1 and Dlx2 without Mytl1 explicitly is shown to produce GABAergic inhibitory neurons from ES cells. ASCL1 and Dlx2 also exhibit neuronal markers and changes in neuronal morphometry (Yang, 2017).

METHODS

Golden Gate Assembly and Bacterial Cloning of guide RNA's in CRISPR Plasmid

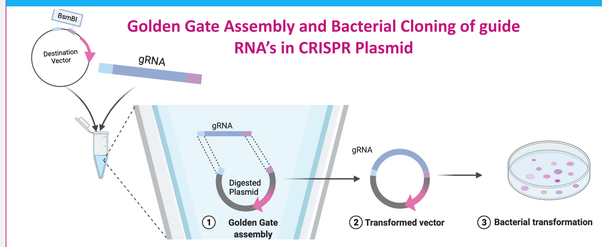


Figure 4. Ascl1 and Dlx2 gRNAs inserted into CRISPR dCAS9 VPR plasmid into BSMBI Digested area using Golden Gate Assembly. This assembly is a one step as the restriction enzyme only cuts outside of the recognition site.

Experimental Design: Lentivirus Transduction, Embryonic Stem Cells Differentiation, Quantification of Neuronal Markers

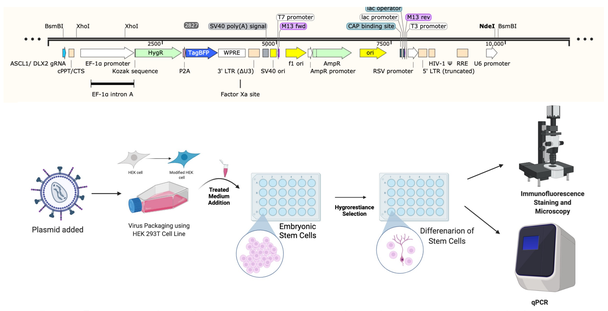


Figure 5. During viral transduction, viral RNA is inserted into the cell, reverse transcribed into DNA, and integrated into the cell's genome. After transduction an antibiotic is used for the selection of ASCL1/DLX2-expressing cells. The medium which is secreted by HEK cells is used for induction of human ES cells to neurons. This is followed by immunocytochemistry to detect Beta III Tubulin, and MAP2.

Neuron Development from Human Stem Cells

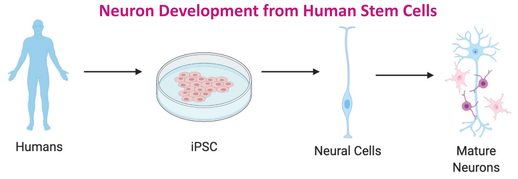


Figure 6. Human primary cells are collected followed by culturing and differentiation to produce induced pluripotent stem cells. The stem cells are redifferinated to produce unspecific neural cells. These cells are for the generation of specific mature neurons (Adapted from Youssef, 2020).

HYPOTHESIS / PREDICTIONS

Hypothesis: We hypothesize that there exist novel transcription factor (TF) combinations sufficient to produce homogenous or enriched populations of inhibitory neuron subtypes, such as somatostatin (SST)- and parvalbumin (PV)-expressing neurons. In this proof-of-principle work, we tested if CRISPRa using sgRNAs for ASCL1 and DLX2 is sufficient to induce neuronal cells.

Prediction: Utilization of the dCAS9-VPR would cause transfection of HEK293T (observed by blue fluorescent expression). Medium collected would contain the transcription factors (ASCL1, DLX2) guide RNAs (Figure x). Neuronal morphometry would be observed a few days after these viruses (would observe Td-Tomato SST). Post antibiotic selection, only transfected cells should remain and can be used for qPCR and immunofluorescent staining. Immunofluorescent staining for neuronal markers (MAP2 and Beta III Tubulin) would confirm neuronal morphometry. qPCR would be used to validate the result observed in Immunofluroscence and quantify the amount of the neuronal markers found in the cells.

RESULTS

Transfection of HEK 293T Cell After 6 Hours

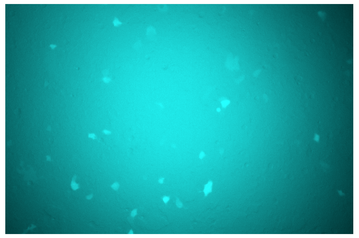


Figure 7. 24 hours after plating cells, viral DNA is added to medium. After 6 hours, BFP expression was observed in HEK cells, indicating that cells contain viruses and are secreting viral DNA into medium.

Neuronal Morphometry Found Post-Hygromycin Selection

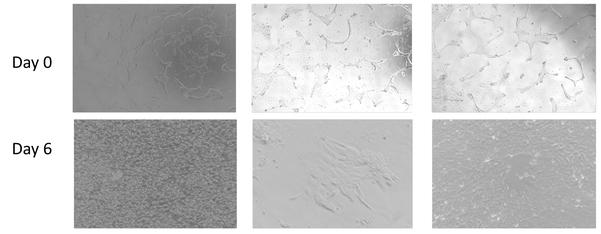
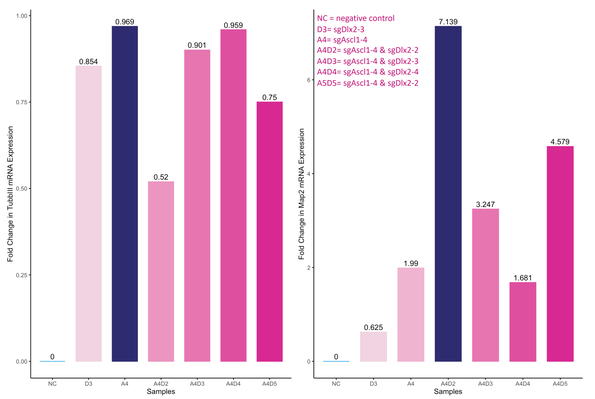


Figure 8. Morphological changes observed in ES cells after addition of sgASCL1-4. Neuronal cell bodies are identified within and are observed in both sgAscl1 and positive control, while the Negative Control shows only dead cells.

Neuronal Markers Found Through Immunofluorescence

Figure 9. Early neuronal markers observed in cells for both positive control line and sgAscl1-4. Markers TUJ1 stain for cell bodies and processes, while marker MAP2 identifies microtubules which are in the processes. In the non-targeting control, we observe only DAPI staining indicating that there are no neurons.

Relative Quantity of Neuronal Markers Found in Culture Increases via Co-Expression of ASCL1 and DLX2 gRNAs



Relative Quantity of ASCL1 and DLX2 RNA Found in Culture

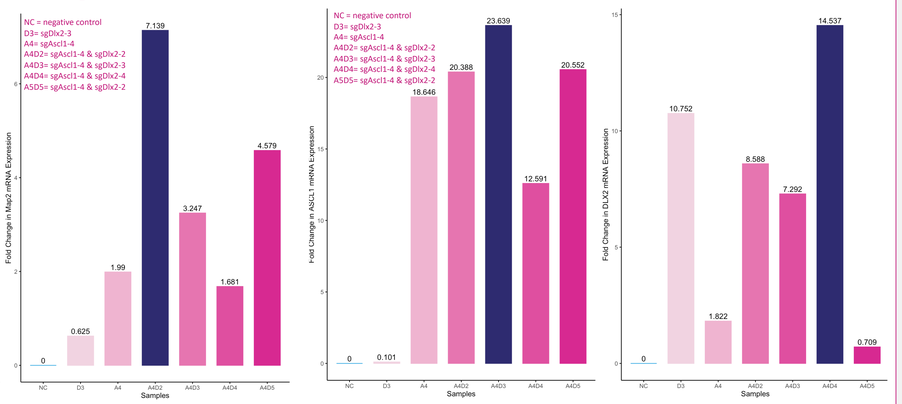


Figure 10 & 11. Results from qPCR indicate that TubilinIII and Map2 RNA is observed increasing after addition of gRNAs compared to the Negative Control. Ascl1 and Dlx2 RNA expression increased significantly indicating that CRISPRa did increase transcription.

CONCLUSIONS

- sgRNA for ASCL1 and DLX2 are sufficient in inducing neurons after 5 days post addition of viruses
- Combinations of both gRNAs for ASCL1 and DLX2 show increase expression in neuronal markers
- CRISPR activation did increase transduction of ASCL1 and DLX2 as observed by significant increase.

FUTURE DIRECTIONS

- Observing if additional time would increase neurons in culture
- Observing maturity of neurons along time post addition of sgRNAs
- Observing if increasing virus titer would increase neuronal induction
- Screening other sgRNAs to see if specific GABAergic neuronal subtypes can be formed

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