BMSIP Final Report

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Examining Changes in Senescence and Maturation across Neural Cell Types in a Down Syndrome Model

Abstract

Down Syndrome is a chromosomal disorder with an array of clinical impacts affecting many biological systems. Most notably dysregulated is the brain and central nervous system, with alterations in neural development and maturation. This work addresses several different mechanisms by which neurological systems are impacted in Down Syndrome, spanning from neural maturation to premature senescence. In this work we identify a putative driver and mechanism for impaired neurogenesis in the triploid model. We additionally identify a proposed mechanism for altered cell proliferation, autophagy, and apoptosis in the triploid model via the downstream impacts of overexpression of an X chromosome inactivating gene. We expand upon recent analysis investigating the expression of senescence associated genes in triploid neurons and identify differential patterns of expression which vary with cell type. We investigate the expression of genes associated with mature, developed neurons and identify differences between our triploid and euploid model systems. In this work we demonstrate aberrant patterns of neural cell evolution at all stages of development, from proliferation, to maturation, and finally to senescence and cell death.

Introduction

Down Syndrome is the most common chromosomal disorder observed in infants (Mai et al, 2019). Down Syndrome is caused by the partial or complete triplication of human chromosome 21, resulting in a spectrum of clinical manifestations most notably intellectual disability and congenital heart defects. While quality of life for individuals diagnosed with Down Syndrome has improved drastically over the past fifty years, individuals with Down Syndrome are still at higher risk for a wide variety of health issues, ranging from eye disease and impaired vision to hearing loss.

This project implemented an induced pluripotent stem cell(IPSC) derived organoid model to analyze differences in gene expression in Down Syndrome, with a specific focus on genes related to maturation and senescence. The IPSC model allows for study of Down Syndrome and associated genetic and epigenetic changes in a human system without the ethical concerns associated with research in live human brain tissue or the genetic dissimilarities associated with the mouse model. Furthermore, the use in this study of isogenic pairs removes confounding variables which arise when comparing samples of different genetic backgrounds (Klein and Haydar, 2022, Jakel, Schneider and Svendsen, 2004). Previous analysis of this model system identified single cell transcriptomic changes as well as differentially expressed genes (Li et al, 2022). In this study we both validate these results and expand upon these findings.

Methods

Data

The data in this study is derived from a pair of isogenic cell lines derived from a female Downs Syndrome patient. These cell lines were cultured, and their growth was directed into cortical organoids composed of progenitor cells, neurons, astrocytes, and oligodendrocytes. These cortical organoids were cultured for 130 days then submitted for single cell RNA sequencing (scRNA-seq). Samples were prepared according to the 10X Genomics Chromium® protocol. Sequencing was performed on the NovaSeq 6000 at the Single Cell Sequencing Core at Boston University School of Medicine.

Quality Control and Normalization

Paired-End reads were aligned to the GRCh38 build of the Human Genome with 10x Genomics CellRanger package count functionality using default parameters. Samples were aggregated with CellRanger aggr with default parameters. Quality control was performed with fastQC (Andrews, 2010) and MultiQC (Ewels et al, 2019). Cells expressing mitochondrial genes at over 25% were pruned and removed from downstream analysis. Additional more stringent removal of all mitochondrial genes was performed. Data was normalized and scaled with NormalizeData and ScaleData functionalities of Seurat with default parameters (Stuart, Butler et al. 2019).

Clustering and Dimension Reduction

The top 2000 variable genes were identified with the FindVariableGenes functionality of Seurat using the variance stabilizing transformation selection method. Jackstraw analysis was performed with the Seurat Jackstraw package, with a hundred replicates of all fifty originally identified principal component groups. Principal component groups were then assessed against a significance threshold of 0.01, and groupings which did not meet this threshold were not considered in downstream analysis . Clustering was performed with the FindNeighbors and FindClusters functionality of Seurat (Stuart, Butler et al. 2019). Uniform Manifold Approximation and Projection (UMAP) and t-distributed stochastic neighbor embedding (tSNE) dimension reduction analysis was performed in Seurat with increasing levels of granularity until appropriate resolution was achieved. This identified thirteen distinct clusters.

Cluster Annotation

The thirteen distinct clusters identified in the dimension reduction analysis were manually annotated based on the expression of canonical marker genes. The Seurat FindAllMarkers functionality was implemented to identify markers differentially expressed between clusters, iterating through clusters using only genes detected in 25% of either cluster and differentially expressed by 25% in each cluster being compared. Top marker genes for each cluster were entered into DAVID for gene ontology analysis. To facilitate cluster annotation the expression of known canonical marker genes was plotted against the UMAP projection. Clusters were manually annotated based on the expression of these canonical marker genes.

Differential Gene Expression Analysis

To identify differentially expressed genes between the Triploid and Euploid samples, the Seurat FindMarkers function was used to identify all genes expressed with a minimum absolute value of the log2foldchange > 0.25. This was performed across all cell types and for each individual cluster. Genes were assessed to be upregulated if the log2foldchange > 0.6 between Triploid and Euploid. Genes were assessed to be downregulated if the log2foldchange was < -0.6 between Triploid and Euploid samples.

Gene Set Enrichment Analysis

To identify differentially expressed genes for use in gene set enrichment analysis, the Seurat FindMarkers function was used to identify all genes expressed with a minimum absolute value log2foldchange of .25 across all cell types and for each cluster. Gene Set Enrichment analysis of gene sets associated with Senescence was performed with the fgsea package on these differentially expressed genes (Korotkevich, Sukhov and Sergushichev, 2019). This analysis was additionally performed on the The Molecular Signatures Database (MSigDB) hallmark gene set collection, a well validated dataset which spans a variety of biological processes(Liberzon et al. 2015).

Differential Expression of Maturation Associated Genes:

To identify differentially expressed genes across cell types in Triploid and Euploid Models the Seurat FindMarkers function was used to identify all genes expressed with a minimum absolute value log2foldchange of .25 across all cell types and for each cluster. A previous study demonstrated downregulation of cell surface ion channel genes in a triploid stem cell model, resulting in impaired development and differentiation of neural progenitor cells(Haim-Abadi, Guy et al. 2023). The expression of sets of channel associated genes relevant to neural cell maturation was evaluated with a Bonferroni corrected p-value on a cluster by cluster level.

Results

Cluster Annotation

Assessing the expression of known canonical marker genes identified seven distinct neural cell types, including Excitatory Neurons(ExN), Inhibitory Neurons(InN), Intermediate Progenitor Cells(IPC), Oligodendrocyte Precursor Cells(OPC), Astrocytes(Ast), Radial Glial Cells(RGC) and Basal Radial Glial Cells(bRGC).

Excitatory Neuron Clusters

Excitatory neurons were identified based on the expression of canonical ExN marker genes CUX2, BCL11B, NEUROD6, RORB, Slc17a7, and Slc17a6. Based on expression of these genes, clusters zero through five and cluster eleven were annotated as ExN.

Astrocyte Cluster

Cluster six was identified as an astrocyte cluster based on the expression of ALDH1L1 and GFAP. However cluster six also had higher than expected levels of expression of oligodendrocyte associated genes, including OLIG1 and PLP1. Reclustering with increased granularity was performed to resolve this, but plots of

these oligodendrocyte associated genes across the reclustered UMAP continued to show expression in this region. To address this, cluster six was isolated and clustering analysis was performed only on the subset of the data included in cluster six. However, oligodendrocyte associated genes continued to be observed at low levels across clusters. To ensure downstream analysis and observations about this cluster reflected the annotation as Astrocyte, the decision was made to prune cells expressing these oligodendrocyte genes from the cluster.

Radial Glial Cell Clusters

Clusters seven and eight were identified as Radial Glial based on the expression of radial glial markers SOX2, PAX6, and GLI3. Cluster seven was further identified to be basal radial glial based on the expression of the HOPX and SLC1A3 genes.

Intermediate Progenitor Cell Cluster

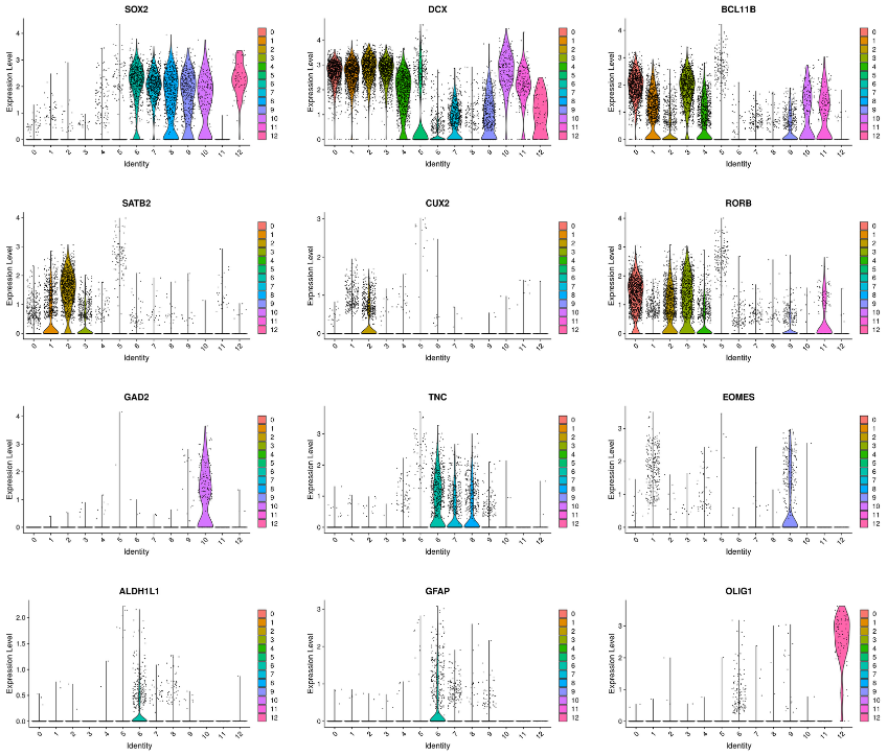
Cluster nine was identified to be an intermediate progenitor cell cluster based off of a strong expression signal for the EOMES marker gene.

Inhibitory Neuron Cluster

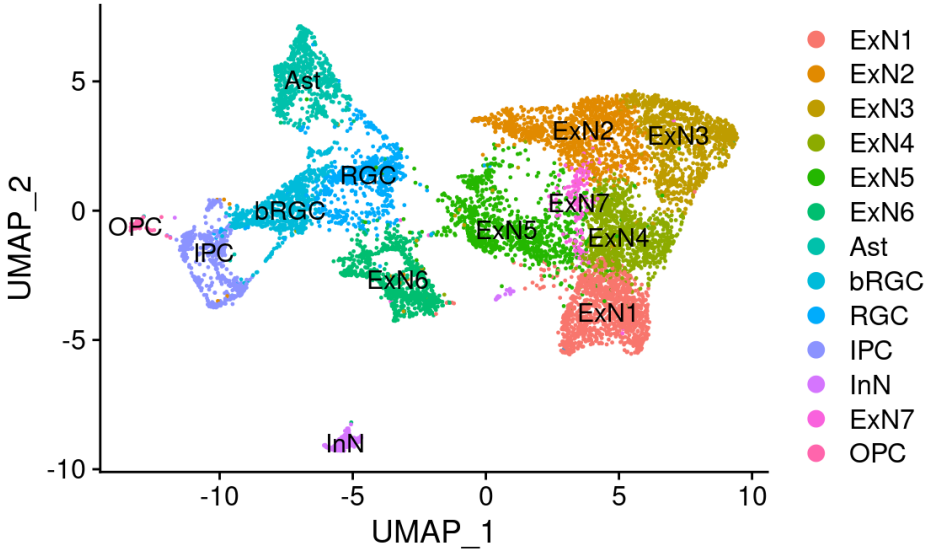
Cluster ten was annotated as an inhibitory neuron cluster based off of the strong expression signal for the GAD2 marker gene.

Oligodendrocyte Precursor Cell Cluster

Cluster 12 was initially identified as an oligodendrocyte cluster on the basis of the expression of OLIG1 and OLIG2. Further investigation into the expression of genes associated with different oligodendrocyte cell stages specified this annotation to an oligodendrocyte precursor cell cluster. This distinction was made because cluster twelve expressed both PDGFRA and CSPG4, genes associated with the OPC cell stage and did not express markers such as PLP1 or MAL which are associated with more mature oligodendrocyte lineage cells.



Canonical Marker Gene expression across all clusters.



UMAP projection of clusters colored by cluster, with cluster annotations.

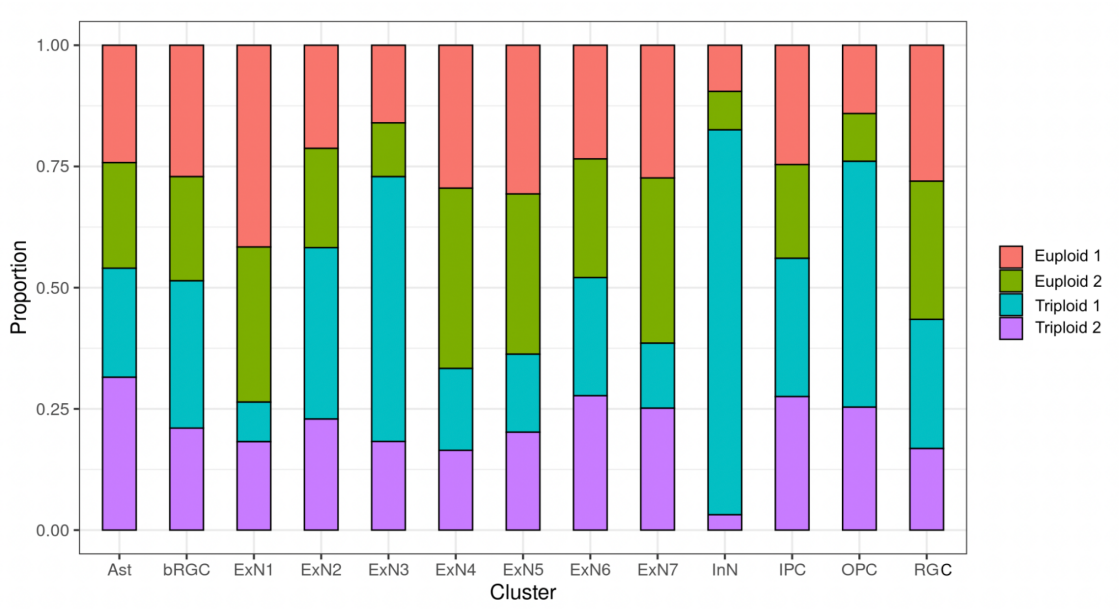


Figure demonstrating sample participation across all clusters.

Differential Gene Expression

Differential Gene Expression analysis across all clusters identified ___ upregulated and ___ downregulated genes in our Triploid samples relative to the Euploid samples. An array of the most significant genes identified in this analysis have biological significance relevant to this study.

Overexpression of Human Chromosome 21 genes

In our study we observe expected gene dosage effects in our triploid model, namely the increased expression of genes present on or positively regulated by human chromosome 21 which is present in triplicate in Down Syndrome.

Overexpression of a neural cell adhesion molecule

This analysis identified that CHL1, cell adhesion molecule L1 like, a member of the L1 gene family of neural cell adhesion molecules was upregulated in our Triploid samples. CHL1 overexpression inhibits hedgehog signaling, and disruption of hedgehog signaling is associated with a wide variety of health issues. A recent paper on the Sonic hedgehog mammalian homolog of Hedgehog pathway identified that disruption of this pathway is associated with decline in neurogenesis(Wang et al. 2022).

Overexpression of an X inactivation gene

XIST upregulated in Triploid samples. Brain-expressed X-linked (BEX) genes including BEX2 3 and 4 are downregulated in our Triploid samples. The overexpression of the X-inactivation gene XIST has been shown to result in decreased expression of X associated genes(Yu, Bingfei et al. 2019). BEX3 deficiency has been shown to lead to aberrant mTOR signaling in the brain. mTOR signaling has been shown to regulate cell proliferation,

autophagy, and apoptosis (Dowling et al. 2010). Additionally BEX genes have been shown to be differentially expressed in many neurological disorders(Alvarez et al. 2005).

Reduced Expression of a synaptogenesis gene in Astrocytes

Expression of SPARCL1 was downregulated in triploid astrocytes. SPARCL1 controls excitatory synaptogenesis in the central nervous system (Gan and Südhof, 2020). Synaptogenesis has been shown to be abnormal in Down Syndrome, with deficiencies identified both in fetuses (Becker, Laurence, et al. 1991) as well as in mature adults(Takashima, Sachio, et al. 1994).

Gene Set Enrichment Analysis Hallmark Pathways

Gene Set Enrichment Analysis of MSigDb Hallmark pathways identified no pathways significantly differentially expressed across all cell types(Bonferroni corrected p-value <0.1), but identified several pathways differentially expressed between Triploid and Euploid samples within cell type clusters.

Within the OPC cluster the E2F Targets, MYC Targets V1, Spermatogenesis and MTORC1 Signaling pathways were statistically significant with a positive normalized enrichment score, and the UV Response DN with a negative normalized enrichment score. Within the InN cluster no positively enriched pathways were statistically significant, however five negatively enriched pathways did; the Glycolysis, Estrogen Response Late, Fatty Acid Metabolism, MTORC1 signaling and Oxidative Phosphorylation pathways. Within the RGC cluster one positively enriched pathway was statistically significant, the MYC Targets V1 pathway. Three negatively enriched pathways were statistically significant, the Reactive Oxygen Species, Apoptosis, and Xenobiotic Metabolism pathways. Within the bRGC cluster the Fatty Acid Metabolism pathway was statistically significant with a positive enrichment score, and no pathways with a negative enrichment score achieved statistical significance.

Gene Set Enrichment Analysis of Senescence Associated Genes

Gene Set Enrichment Analysis of senescence associated gene sets identified no gene sets significantly differentially expressed across all cell types after the Bonferroni correction was applied. However several gene sets were significantly differentially expressed between Triploid and Euploid samples within Excitatory Neuron clusters. This aligns with a previous study murine model by Rusu et al., and our expectation of overexpression of senescence associated genes within mature neural cell types(Rusu et al. 2023).

Five excitatory neuron clusters expressed statistically significant levels of senescence associated gene sets. The ExN2 cluster overexpressed the SASP gene set with a normalized enrichment score of 2.80. The ExN4 cluster overexpressed the SenMayo gene set with a normalized enrichment score of 2.41. The ExN5 cluster overexpressed the SASP gene set with a normalized enrichment score of 3.02. Additionally the ExN5 cluster overexpressed two gene sets identified in a recent publication as enriched in a trisomic murine model, one corresponding to interferon-stimulated genes was expressed with a normalized enrichment score of 2.75 and another broadly related to senescence with a normalized enrichment score of 2.32 (Jin, Mengmeng et al. 2022).

The ExN6 and 7 clusters additionally overexpressed the interferon-stimulated gene set with normalized enrichment scores of 2.46 and 2.33.

Differential Expression Analysis of Maturation Associated Genes

Differential expression analysis of 5 sets of channel genes associated with mature neuronal cells identified patterns of differential expression between Triploid and Euploid samples. These differences were more clearly observed on a cell type level, with immature cell type clusters showing the most marked differences. Namely, IPC and OPC cell clusters exhibited the highest range of differential expression, particularly with regard to expression of calcium and potassium channel genes.

Discussion

In our analysis we demonstrate that senescence associated genes are differentially expressed across cell types. We observe overexpression of senescence associated gene sets in excitatory neurons. Previous analysis of this data set as reported in Li et al. identified that excitatory neuron populations were most affected by transcriptomic changes between the triploid and euploid model(Li et. al. 2022). Overexpression of these senescence associated gene sets within this population is an important additional component to consider when evaluating the impact of trisomy on neurons.

Within identified oligodendrocyte precursor cells we observe increased differences in expression levels of maturation associated channel genes between the Triploid and Euploid model. Oligodendrocyte precursor cells are an immature cell stage of the Oligodendrocyte Lineage. Mature oligodendrocyte cells form the myelin sheaths which encase central nervous system axons and facilitate the rapid transmission of neuronal signals (Butt et al, 2019, Hill et al, 2003). Loss of this myelin sheath has been shown to result in axonal and neuronal degeneration (Stassart et al, 2018) and abnormal myelination in Down Syndrome can contribute to deficient connectivity, resulting in intellectual deficits. Impaired maturation of these oligodendrocyte precursor cells could therefore result in impaired myelination and associated impaired connectivity and intellectual deficits in Down Syndrome.

In this study we demonstrate overexpression of a neural cell adhesion molecule CHL1 across all examined neural cell types in the Triploid model. The role of CHL1 inhibiting hedgehog signaling and the work by Wang et al. identifying the causality between impaired sonic hedgehog signaling and decline in neurogenesis indicates that CHL1 should be further investigated as a putative driver of reduced neurogenesis in Down Syndrome.

We additionally observe overexpression of XIST and subsequent reduced expression of BEX genes. We additionally observe negative aberrant expression of the mTOR signaling hallmark pathway, with significant overexpression in one cell type cluster and significant under expression in another. As BEX3 deficiency has been shown to cause aberrant mTOR signaling in the brain, we propose that the mechanism for aberrant mTOR expression in triploid neurons is the overexpression of XIST, which subsequently impairs BEX gene expression leading to the observed altered mTOR expression in our model.

This study demonstrates the dysregulation of neural cell development at all stages of development, from the creation of neurons to their maturation, to senescence. We show reduced expression of a gene critical to

synaptogenesis in our triploid astrocyte population, as well as a putative genetic driver of reduced neurogenesis across all cell types in our model system. We proceed to demonstrate alterations in genes associated with cellular maturation across cell types critical to cognition and central nervous system function. We additionally demonstrate premature expression of senescence related genes across a population of neural cells in our triploid model.

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