The Insurgents beyond the Wire: Cancer stem cell mystery deepens as lineage-related neural progenitors are spotted in the subventricular zone of glioma patients

Single-cell transcriptomics–driven cellular census of the human adult subventricular zone captured adult neural stem cell diversity and revealed neurodevelopment progenitors in glioma patients

Department of Neurology and Neurosurgery

McGill University

December 2022

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Dedicated to

This thesis is dedicated to all those who have lost their lives in their battle against Glioblastoma, and to those who continue to fight with their unwavering, enduring courage and resilience.

To the families and friends of these warriors, who have been left bereft by the departure of their loved ones and must carry this sorrowful void for the rest of their lives, never to be filled again. Nothing in this world could hold a candle to your fortitude and bravery that you display in the face of this adversity.

Most importantly, to our unsung heroes of the Islamic Golden Age for reviving Greek and Roman philosophical science with their sheer intellect and wisdom that led to the remarkable foundation of experimental science.

Al-Khwarizmi

Al-Biruni

Al-Razi

Ibn-Sina (a.k.a Avicenna)

Al-Tusi

Ibn Al-Haytham

nanos gigantum humeris insidentes – Bernard of Chartres

Abstract

Brain cancers, including Glioblastoma (a lethal grade IV brain cancer) are the most complex and challenging of all cancers in terms of treatment, morbidity, and mortality. These tumors exhibit genomic heterogeneity and are nurtured by a complex ecosystem of various cell types. From a genomic perspective, these tumors are constituted by genetically diverse populations of abnormal cells and this genomic complexity has remained a bottleneck in our understanding of the disease and, therefore, hindered any therapeutic attempts. However, recent transcriptomic studies have exposed a cellular order to these tumors. These studies have not only revealed parallels between neurodevelopment and gliomagenesis but also hinted at potential involvement of evolutionarily conserved neurodevelopmental programs. As per this theory, these tumors follow fundamental neurodevelopmental framework, a cellular hierarchy that originates with the stem cells and ends with differentiated/specialized cell types. Cancer stem cell (CSC) hypothesis lies at the core of this theory which is rooted in the functional similarities between cancer and non-neoplastic stem cells, making adult neural stem cells (NSCs) ideal candidates for cancer stem cell. Nevertheless, this notion is compounded by controversies surrounding the existence of adult neural stem cells in the human adult neurogenic zone, subventricular zone (SVZ). In pursuing the role of adult human NSCs in the origin and maintenance of GBM, we explored the existence and identity of human adult NSCs by performing comprehensive cellular census of the human adult SVZ using single-cell RNA-sequencing (scRNAseq) technology and charted the cellular constituents of the adult SVZ in glioma patients. By using well-annotated fetal progenitor populations as our framework, we uncovered lineage-related gene signature bearing adult NSC subpopulations – neuronal (nNSC), astrocytic (aNSC) and oligodendrocytic (oNSC). Moreover, an early radial glia-like (eRG) subpopulation - early NSC (eNSC) – that shared transcriptomic features with fetal eRG was

also discovered. Additional molecular processes/pathway analysis further confirmed lineage-specific identity of adult NSC sub-populations and revealed neurodegenerative, brain repair and injury response-associated programs and migratory nature of nNSCs and oNSCs only. Contrary to the abundance of these lineage-specific progenitors in glioma patients, these adult NSC progenitors were rarely observed in normal autopsy adult brain SVZ. Computational lineage inference approach further revealed dynamic relationship between adult subpopulations where eNSC preceded all other progenitor populations on the trajectory scale. Copy number variation (CNV) analysis subsequently revealed glioma related CNV signature in adult NSC progenitors only. Taken together, this study has brought to light the status of human adult neural stem cells (NSCs) in the SVZ and the likelihood of these NSCs to give rise to and maintain the tumor. More importantly, presence of lineage-specific progenitors and injury response programs in glioma patients further reinforces the role of developmental programs in gliomagenesis and necessitates the need to re-examine the significance of a perpetual role of SVZ in gliomagenesis for early intervention and targeted therapy.

Résumé

Les cancers du cerveau, ainsi que le glioblastome (un cancer du cerveau mortel de grade IV) sont les plus complexes et les plus difficiles parmi tous les cancers en termes de traitement, de morbidité et de mortalité. Ces tumeurs présentent une hétérogénéité génomique et sont nourries par un écosystème complexe de divers types cellulaires. D'un point de vue génomique, ces tumeurs sont constituées de populations génétiquement diverses de cellules anormales et cette complexité génomique est restée un goulot d'étranglement dans notre compréhension de la maladie et, par conséquent, a entravé toute tentative thérapeutique. Cependant, des études transcriptomiques récentes ont mis en évidence un ordre cellulaire dans ces tumeurs. Ces études ont non seulement révélé des parallèles entre le neurodéveloppement et la gliomagenèse, mais ont également fait allusion à une implication potentielle de programmes de développement neurologique conservés au cours de l'évolution. Selon cette théorie, ces tumeurs suivent un cadre neurodéveloppemental fondamental, une hiérarchie cellulaire, qui prend naissance avec des cellules souches et se termine avec des types de cellules différenciées/spécialisées. L'hypothèse des cellules souches cancéreuses (CSC) est au cœur de cette théorie qui est enracinée dans les similitudes fonctionnelles entre les cellules souches cancéreuses et non néoplasiques, faisant des cellules souches neurales (CSN) adultes des candidats idéaux pour les cellules souches cancéreuses. Néanmoins, cette notion est aggravée par les controverses entourant l'existence de CSN adultes dans la zone sous-ventriculaire (ZSV). En poursuivant le rôle des CSN humains adultes dans l'origine et le maintien du GBM, nous avons exploré l'existence et l'identité des CSN adultes humains en effectuant un recensement cellulaire complet de la ZSV adulte humaine à l'aide de la technologie de séquençage d'ARN unicellulaire (scARNseq). Échantillons et cartographié les constituants cellulaires de la ZSV adulte chez les patients atteints de gliome. En utilisant des populations de progéniteurs fœtaux bien

annotées comme cadre, nous avons découvert une signature génétique liée à la lignée portant des sous-populations CSN adultes - neuronales (nCSN), astrocytaires (aCSN) et oligodendrocytaires (oCSN). De plus, une sous-population de type glie radiale précoce (eGR) - précoce CSN (eCSN) - qui partageait des caractéristiques transcriptomiques avec l'eGR fœtal a également été découverte. Une analyse supplémentaire des processus / voies moléculaires a confirmé l'identité spécifique à la lignée des sous-populations NSC adultes et a révélé des programmes neurodégénératifs, de réparation cérébrale et de réponse aux blessures et la nature migratoire des nCSN et des oCSN uniquement. Contrairement à l'abondance de ces progéniteurs spécifiques à la lignée chez les patients atteints de gliome, ces progéniteurs NSC adultes ont rarement été observés dans les SVZ cérébrales adultes normales à l'autopsie. L'approche d'inférence de lignée informatique a en outre révélé une relation dynamique entre les sous-populations d'adultes où l'eNSC a précédé toutes les autres populations de progéniteurs sur l'échelle de la trajectoire. L'analyse de la variation du nombre de copies (VNC) a ensuite révélé la signature de la VNC liée au gliome chez les progéniteurs CSN adultes uniquement. Dans l'ensemble, cette étude a mis en lumière le statut des cellules souches neurales humaines adultes (CSN) dans la ZSV et la probabilité que ces CSN provoquent et entretiennent la tumeur. Plus important encore, la présence de progéniteurs spécifiques à la lignée et de programmes de réponse aux blessures chez les patients atteints de gliome renforce encore le rôle des programmes de développement dans la gliomagenèse et nécessite la nécessité de réexaminer l'importance d'un rôle perpétuel de ZSV dans la gliomagenèse pour une intervention précoce et une thérapie ciblée.

Acknowledgement

This study could not have been possible without the participation of the patients who underwent neurosurgery at the Montreal Neurological Institute under the care of Dr. Kevin Petrecca and contributed to the subventricular zone samples utilized in this study.

To my advisor, Dr. Kevin Petrecca, a skilled neurosurgeon, this study would not have been possible without you. I am grateful to you for your guidance and support throughout my research career. Thank you for giving me this opportunity in your lab to contribute to the field of neuroscience. Most importantly, for allowing the pursuit of a wild hypothesis and encouraging research independency. Your understanding and thoughtfulness made my scientific quest possible.

To my lab mates (especially Redouane Allache, Phuong Uyen Le & Maryam Safisamghabadi), committee members and other invaluable researchers at the Neuro (especially Dr. Jack Antel and Dr. Stefano Stifani), I am eternally grateful for your advice, guidance, and unwavering support.

To Shukrullah Baig (my father), Darren Matthews (my love and my best friend) and Gerry Matthews, nothing in this world holds a candle to our philosophical, poetic, and scientific discussions; thank you for your philosophy and wisdom which has guided my research. To my mother, Liza Baig, sisters, Hina Baig and Sara Baig, and brother Wasim Baig, thank you for your support, motivation, courage, and good laughs.

To my daughter, Tesira Rosemund, my beacon of hope. I wouldn't be here without you.

Contribution to original knowledge

- Addressed the long-standing controversy concerning the existence of human adult neural stem cells in the neurogenic zone (subventricular zone), by generating the first single-cell transcriptomic cellular chart of the human adult subventricular zone from fresh tissue.
- Carried out extensive characterization of these transcriptomic neurogenic zone cell types *in situ by* performing immunohistochemistry in human adult brain tissue.
- Produced significant evidence, thus further, of the adult human subventricular being potentially involved in the origin and maintenance of glioblastoma tumor.
- Captured adult neural stem cell-like populations in the human adult subventricular zone of glioma patients.
- Revealed presence of neurodegenerative, brain repair and injury response pathways in the neurogenic zone (SVZ) of glioma patients.
- Established the cellular identity of adult human neural progenitors of the subventricular zone for isolation and manipulation.
- Established presence of active neurodevelopmental programs in adult human brain SVZ tissue from glioma patients.

Publication & Contribution of Authors

Chapter 2 & 3: Findings from chapter 2 were used in the published manuscript *(Couturier et al. 2020)* and parts of chapter 3 were used in the published manuscript "Glioblastoma scRNAseq shows treatment-induced, immune-dependent rise in mesenchymal cancer cells, and structural variants in distal neural stem cells" *(Neuro-Oncology 2022; 24(9), 1494–1508)*. Author contribution is as follows: Salma Baig conceptualized and designed the study with assistance and supervision from Kevin Petrecca. Kevin Petrecca harvested the tissue. Salma Baig collected and processed the adult human SVZ tissue with assistance from Jack Antel lab and collected the scRNAseq data. Salma Baig analysed and interpreted the scRNAseq data with assistance. Javad Nadaf designed and implemented the scRNAseq data analysis pipeline and assisted in data analysis. Salma Baig performed all the biological experiments, including multiplexed immunohistochemistry and pathway analysis. Kevin Petrecca participated in data interpretation and supervised the study.

Chapter 3 & 4: Findings from chapter 4 & 5 have been used in a manuscript currently under preparation for submission "Single cell atlas of the adult human subventricular zone identifies neural stem cell diversity". Author contribution is as follows: Salma Baig conceptualized and designed the study with assistance and supervision from Kevin Petrecca. Kevin Petrecca harvested the tissue. Salma Baig collected and processed the adult human SVZ tissue with assistance from Redouane Allache and Jack Antel lab and collected the scRNAseq data. Salma Baig analysed and interpreted the scRNAseq data with assistance from Javad Nadaf. Javad Nadaf designed and implemented the scRNAseq data analysis pipeline, performed data analysis with assistance from Salma Baig, and performed CNV & Velocity analysis. Salma Baig performed SVZ cell culture, immunocytochemistry, and immunohistochemistry, and differentiation experiments with

assistance from Redouane Allache. Salma Baig performed the pathway analysis. Alexander Prat from Centre Hospitalier Universite de Montreal through Dr. Jack Antel department of Neurology and Neurosurgery. Kevin Petrecca participated in data interpretation and supervised the study. Figures and schematics were prepared by Phuong Ue Le and Salma Baig. Salma Baig and Kevin Petrecca co-wrote the paper with input from other authors.

i. Abstract

ii. Resume

iii. Acknowledgement

iv. Contribution to original knowledge

v. Contribution of authors

1. Chapter 1 – INTRODUCTION 1

1.1 Gliomas: a notorious family of brain tumors 4

1.2 Glioblastoma – The black sheep of the family 7

1.2.1 Landscape of genomic alterations in Glioblastoma 9

1.2.2 The Cancer Genome Atlas (TCGA) subtypes 11

1.3 Therapeutic bottlenecks and decades of frustration 14

1.3.1 Resistance and recurrence – The Two "Rs" 15

1.3.2 Tumor heterogeneity and therapeutic resistance 17

1.4 Brain tumors: a complex system 20

1.4.1 Are cancer stem cells the seeds of the tumor? Opening Pandora's box 23

1.4.2 Cancer: an organized crime or genomic chaos? Digging deeper 25

1.5 Tumor mafia – Where do the dirty secrets lie? Homing in on the crime boss 27

1.5.1 The adult neurogenic niche: an accomplice to the crime? 27

1.5.2 Adult neural stem cells (NSCs) – The most elusive of all 28

1.6 Introduction to thesis 32

1.6.1 Tumor mass: An allegedly siloed, non-native and a self-sustaining system 32

1.6.2 Cancer stem cells – Retracing the roots 33

1.6.3 Harnessing brain development to understand brain tumors 34

1.6.4 Beyond the bounds of tumor mass 35

1.6.5 Aims and objectives of the study 36

2. Chapter 2 – CELLULAR LANDSCAPE OF THE DEVELOPING HUMAN FOETAL BRAIN – DECONVOLUTING CELL TYPES/STATES AND RECONSTRUCTING DEVELOPMENTAL TRAJECTORIES 37

2.1 Preamble 38

2.2 Aim and objectives 44

2.3 Results 45

2.3.1 Single-cell RNA-sequencing (scRNAseq) reveals major transcriptomic cell types and states in the developing foetal brain 45

2.3.2 Identifying cell types/states in the human fetal brain 47

2.3.3 Spatio-temporal significance of radial glia (RG): Molecular characterisation of fetal RG in human fetal germinal zone during corticogenesis 50

2.3.4 Fetal neural cultures demonstrated sphere-forming capacity and radial

glial identity 53

2.4 Summary 57

3. Chapter 3 – scRNAseq-DRIVEN CELLULAR CHART OF THE HUMAN ADULT SUBVENTRICULAR ZONE IDENTIFIED A NORMAL AND NEOPLASTIC NEURAL STEM CELL-LIKE CELL POPULATION 58

3.1 Preamble 59

3.2 Aim and objectives 68

3.3 Results 69

3.3.1 Acquisition of intraoperative subventricular zone (SVZ) samples for scRNAseq 69

3.3.2 Cellular clustering revealed diverse populations in the human adult subventricular zone 72

3.3.3 Gene expression analysis further reveals a variety of human adult subventricular zone cell types 74

3.3.4 *In situ* mapping of human adult subventricular zone populations further validated transcriptome-derived cell types 77

3.3.5 Abundance of microglia observed in the human adult subventricular Zone 80

3.3.6 Gene expression signature of NSC-like cluster reveals heterogeneous nature of the cluster 83

3.3.7 Pathway analysis reveals enrichment of comprehensive neurodevelopmental process in the NSC-like cluster 85

3.3.8 Copy number variation (CNV) analysis revealed CNV-carrying cells

in the adult SVZ 88

3.3.9 CNV-carrying cells were traced back to the NSC-like cluster 89

3.4 Summary 91

4. Chapter 4 – INTEGRATING FETAL PROGENITOR POPULATIONS WITH ADULT SVZ NSC-LIKE CELLS DISENTANGLED UNIQUE PROGENITORS WITH NEUROGENIC AND GLIOGENIC PROPERTIES AND FEATURES OF BRAIN REPAIR AND REGENERATION 95

4.1 Preamble 96

4.2 Aim and objectives 102

4.3 Results 103

4.3.1 Integrating high-content fetal progenitor transcriptome datasets 103

4.3.2 Conflation of adult and fetal progenitor populations resolved adult NSC-like cluster into sub clusters 105

4.3.3 Gene expression analysis reveals sub cluster-specific expression patterns of neurodevelopment-related genes 109

4.3.4 Gene-set enrichment analysis (GSEA) revealed enrichment of unique fetal cell type signatures for each of the subclusters 113

4.3.5 Neuronal signature bearing subcluster (C4-nNSC) revealed enrichment for neuron developmental processes 115

4.3.6 Astrocytic subcluster (C7-aNSC) was highly enriched for niche regulating factors, gliogenesis and wound healing processes 121

4.3.7 Adult oligodendrocyte progenitor-like subcluster (C9-oNSC) demonstrated remyelinating and brain repair signature 125
4.3.8 Oligodendrocyte progenitor subcluster (C9-oNSC) demonstrated immunogenic and inflammatory profile 130
4.3.9 Key quiescence features were revealed in subcluster C5 (early NSC) 132
4.3.10 Summary of biological processes and pathway analysis across the subclusters 137
4.3.11 Mapping adult SVZ NSC-like subclusters revealed the presence of eNSC and aNSC, and scarcity of nNSC and oNSC in the adult human SVZ 140
4.3.12 C5-eNSC shared transcriptomic features with fetal early radial glia 144
4.3.13 C5-eNsc preceded neurogenic nNSC and gliogenic oNSC subclusters on the lineage trajectory 145
4.3.14 Cycling cells identified in C5-eNSC and C4 nNSC 148
4.3.15 Human adult SVZ-derived cells demonstrated sphere-forming capacity and multi-lineage differentiation potential 149
4.4 Summary 151

5. Chapter 5 – DISCUSSION 155
6. Chapter 6 – CONCLUSION 171

Methods 174

Abbreviations 185

References 188

Table of Figures

Figure 1.1	Histological assessment integrated with molecular diagnosis	5
Figure 1.2	Possible pathways of gliomagenesis based IDH mutation status	11
Figure 1.3	Gene expression data identify four gene expression sub-types	14
Figure 1.4	Timeline of Glioblastoma therapy	15
Figure 1.5	Actors and factors implicated in cancer resistance to treatment	16
Figure 1.6	Distinguishing linear from branched tumor evolution	19
Figure 1.7	Model of clonal evolution in neoplasia	21
Figure 1.8	Models of tumor growth	22
Figure 1.9	Hierarchical organization of tumor constituency in IDH-mutant gliomas	26
Figure 1.10	Neurogenesis across the mammalian lifespan	29
Figure 1.11	Neural stem cells (NSCs) and brain tumor stem cells	31
Figure 1.12	Adult neurogenesis – from quiescent NSCs to neurons	33
Figure 2.1	Neural stem cells (NSCs) reside in specialized niches	39
Figure 2.2	Lineage tree of neurogenesis	40

Figure 2.3	Origin of radial glia	42
Figure 2.4	Landscape of a developing human fetal brain	46
Figure 2.5	Delineating cell-types and states in the human fetal brain dataset and comparison with Nowakowski et al. 2018	49
Figure 2.6	Immunohistochemistry in human fetal brain	52
Figure 2.7	Cell-cycle status in the human fetal brain dataset and tissue	55
Figure 3.1	Development of the adult mammalian SVZ from embryonic epithelium and associated corticogenesis	60
Figure 3.2	Consequences of species differences during neurogenesis	62
Figure 3.3	Diffusion plot of the projection of selected fetal cell types onto the roadmap	64
Figure 3.4	Sampling details and the workflow	69
Figure 3.5	Patients and sampling details	70
Figure 3.6	Removal of abnormal cells carrying 7 amplification/10 deletion chromosomal anomaly	71
Figure 3.7	Identification of cell types in the adult human subventricular zone (SVZ) dataset	72

Figure 3.8 Cell-type annotation in the adult human subventricular dataset 74

Figure 3.9 Visualisation of CNV-carrying cells and contribution of each cluster to the dataset 76

Figure 3.10 *In situ* mapping of SVZ cell types in the adult human subventricular zone and the adjacent parenchyma in normal adult human brain tissue 77

Figure 3.11 *In situ* status of microglia in the adult human subventricular and adjacent parenchyma in normal autopsy brain tissue 81

Figure 3.12 Feature plot visualisation of neural progenitor markers in the SVZ dataset 84

Figure 3.13 Pathway analysis of the SVZ NSC-like cluster 86

Figure 3.14 Cell-type analysis of the SVZ NSC-like cluster 87

Figure 3.15 Identification of copy number CNV-carrying cells in the adult human SVZ dataset 89

Figure 3.16 Identification of copy-number CNV-carrying cells in the NSC-like cluster 90

Figure 4.1 PCA projection of human glutamatergic neuron differentiation 97

Figure 4.2 Radial glia nature of embryonic and adult neural stem cells:

	the role of imprinted genes	100
Figure 4.3	Conflation of high-content foetal progenitor datasets with human adult SVZ NSC-like cluster	103
Figure 4.4	Human adult NSC-like cluster comprised of sub-populations	105
Figure 4.5	Cell type signatures of each of the clusters in the merged dataset	107
Figure 4.6	mRNA expression levels of key progenitor markers in the merged dataset	109
Figure 4.7	Lineage-specific expression patterns amongst adult SVZ subclusters	111
Figure 4.8	Differential expression and gene-set enrichment analysis of adult SVZ subclusters	113
Figure 4.9	Pathway analysis of C4-nNSC	115
Figure 4.10	Biological processes enriched in C4-nNSC	118
Figure 4.11	Pathway analysis of C7-aNSC	121
Figure 4.12	Biological processes enriched in C7-aNSC	124
Figure 4.13	Pathway analysis of C9-oNSC	125
Figure 4.14	Biological processes enriched in C9-oNSC	128
Figure 4.15	Summary of enrichment analysis for cell type signatures in C9	129
Figure 4.16	Schematic presentation of core C9-oNSC function as informed by	

pathway analysis 130

Figure 4.17 Pathway analysis of C5-eNSC (early NSC) 132

Figure 4.18 Biological processes enriched in C5-eNSC 135

Figure 4.19 Protein-protein interaction and MCODE components identified for C5-eNSC 136

Figure 4.20 Enrichment of lineage-related developmental programs across the four subclusters (eNSC, aNSC, nNSC ad oNSC) 137

Figure 4.21 Delineating adult SVZ NSC-like subclusters *in situ* 140

Figure 4.22 C5-eNSC and C7-aNSc are relatively abundant in the adult human SVZ 143

Figure 4.23 Transcriptome size and ribosomal protein content in C5-eNSC and fetal early RG (eRG) 144

Figure 4.24 Velocity analysis revealed C7-aNSC and C5-eNSC as independent points of origin 145

Figure 4.24 (b) Velocity analysis 147

Figure 4.25 Cycling cells identified in C5-eNSC and C4-nNSC 148

Figure 4.26 Sphere-forming capacity and multilineage differentiation potential of adult SVZ-derived cells *in vitro* 15

Figure 4.27 Schematic representation of the summary of adult SVZ NSC-like subclusters as profiled by extensive pathway analysis 151

Figure 4.28 Neurodevelopmental roots of gliomagenesis in both the origin and maintenance of the tumor 165

CHAPTER 1

INTRODUCTION

Humankind has long been plagued with illnesses. In fact, the tale of the two is as old as *homo sapiens*. Ailments have been with us since our hunter-gatherer days, persisted throughout the shift to agrarian life (roughly 10,000 years ago), and continue to accompany despite the great advances in modern medical science. As a matter of fact, diseases predate animal life, let alone *homo sapiens*, and remain one of the biggest foes of mankind.

From the Black Death to SARSCOV2, mankind has demonstrated extraordinary resilience to survive whatever nature has thrown at it for thousands of years. In keeping with this motto "survival of the fittest", humans have made such remarkable leaps, particularly during the last 500 years that most of the current diseases are manageable and curable – except for cancer. So, how is cancer different from other maladies? And why has it remained unbeaten for so long?

Cancer: The mysterious and the deadliest of all:

Although there has been a massive increase in the prevalence of cancers in recent decades, cancer has haunted mankind for centuries. In fact, a case of bone cancer recently found in fossilized leg of a 240-million-year-old turtle from the middle Triassic period reveals that cancer, considered to be a modern-day disease, has its roots deep in evolutionary time (Haridy et al., 2019).

Cell-division lies at the heart of life; it forms, perpetuates, and sustains life. However, it is a double-edged sword. We all started out with the fusion of sperm and egg, turning into a ball of about 100 cells within a matter of days. By the time we reach our adulthood, those cells have divided so many times that the exact number of cells, thought to be in the trillions, has not been agreed upon by scientists. The ability of cells to divide and form new cells takes the center stage in developmental biology and is an evolutionarily conserved and strictly regulated process.

The process discussed above is central to cancer; cancer is a by-product of uncontrolled cell division, formed because of a cell(s) gone awry. Like any other cancer, intracranial tumors, also called brain tumors, are abnormal masses of tissue that are formed owing to unwanted and uncontrolled growth of cells of the brain and its surrounding tissue. These are the abnormal cells that seemingly go unchecked by the mechanisms responsible for controlled division and growth of cells and go rogue with this inevitable cell division and growth. This disruption of the finely tuned balance of cellular birth and death lies at the heart of the remains the subject of scientific research.

There are two major types of brain tumors, primary and metastatic; the former being the tumor that originates in the tissue of the brain and the latter originates elsewhere in the body and invades the brain, usually through the bloodstream. Gliomas are primary brain tumors that originate in the brain.

In this chapter, we will review introduction to gliomas and its types, particularly glioblastoma (GBM) and will see how far we have come in this fight against glioblastoma.

1.1 GLIOMAS - A NOTORIOUS FAMILY OF BRAIN TUMORS

The most common malignant primary brain tumors are gliomas. These tumors account for about 75% of malignant primary brain tumors and have been, for the past century, classified based on their histological appearance, reflecting degrees of malignancy. Adult gliomas have been classified into grades I – IV, based on the concepts of histogenesis and their microscopic similarities with their putative cell of origin. This classification scheme considers the cell morphology, neovascularization, nuclear atypia, necrosis and follows the guidelines of the International Classification of Disease for Oncology (ICD-O) for topography and histology (Komori, Sasaki, & Yoshida, 2016). However, this classical morphology-based diagnostic of the tumor has been challenged by the progress made in the field.

The last two decades have witnessed a rapid change in our understanding of gliomas, largely owing to the advent of high-throughput sequencing technologies, giving us this enormous opportunity to view tumors from a genetic standpoint that has significantly contributed to the classification of these tumors, especially adult diffuse gliomas that constitute ~ 25 – 30% of the primary intracranial tumors. Subtypes of diffuse gliomas include astrocytomas, oligodendrocytomas and diffuse midline glioma, H3 K27M-mutant (Ferris, Hofmann, Solomon, & Perry, 2017).

To facilitate more precise diagnosis, the 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (2016 CNS WHO) breaks with the principle of "integrated diagnosis": the diagnosis should be layered with histological classification, WHO grade and molecular information (Louis et al., 2016). This new integrated

classification system incorporates genotypic and phenotypic information to classify tumors (Figure 1.1).

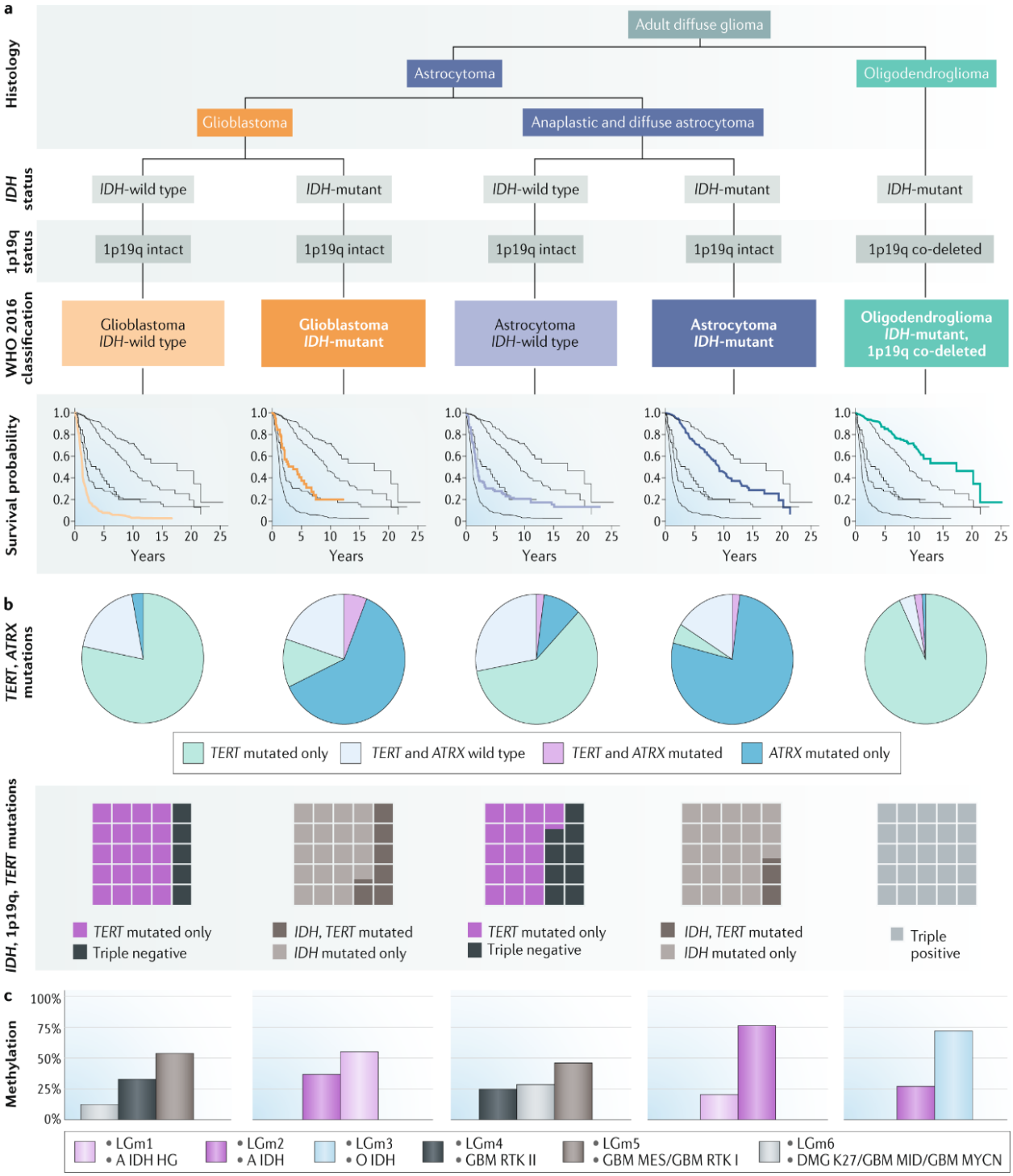


Figure 1.1 Histological assessment integrated with molecular diagnosis

a. Histological assessment integrated with molecular diagnosis (based on *IDH* mutation and 1p19q co-deletion status) defines the five principal subtypes of adult diffuse glioma included in the WHO 2016 classification. Kaplan–Meier survival curves for each subtype are based on data from 1989 to 2012, b. For each disease subtype, mutation frequencies are depicted in pie charts showing the proportion of tumors with mutations in *TERT* and/or *ATRX* and in waffle plots showing the relative proportions of *IDH*-mutant, *TERT*-mutant and 1p19q co-deleted tumors in each WHO 2016 disease subtype. c. Graphs showing methylation classifications for each WHO 2016 subtype. A IDH, *IDH*-mutant astrocytoma; A IDH HG, *IDH*-mutant high-grade astrocytoma; DMG K27, H3 K27-mutant diffuse midline glioma; GLIOBLASTOMA MES, *IDH*-wild type glioblastoma, mesenchymal subclass; GLIOBLASTOMA MID, *IDH*-wild type glioblastoma, midline subclass; GLIOBLASTOMA MYCN, *IDH*-wild type glioblastoma, *MYCN*-amplified subclass; GLIOBLASTOMA RTK I, *IDH*-wild type glioblastoma, receptor tyrosine kinase I subclass; GLIOBLASTOMA RTK II, *IDH*-wild type glioblastoma, receptor tyrosine kinase II subclass; O IDH, *IDH*-mutant oligodendroglioma.
Figure adapted from (Molinaro, Taylor, Wiencke, & Wrensch, 2019)

This classification system includes five primary designations of adult diffuse glioma (Louis et al., 2016):

1. Diffuse or anaplastic astrocytomas (IDH-wild type)
2. Diffuse or anaplastic astrocytomas (IDH-mutant)
3. Oligodendroglioma or anaplastic oligodendroglioma (IDH-mutant and 1p19q co-deleted)
4. Glioblastoma (IDH-wild type)

The upcoming sections will take a closer look at various aspects of Glioblastoma (WHO grade IV astrocytoma), including disease prognosis, existing modalities for treatment & management and the landscape of somatic genomic alterations of Glioblastoma.

1.2 GLIOBLASTOMA – THE BLACKSHEEP OF THE FAMILY

Approximately 75% of all gliomas are astrocytomas, with the most common being the highest-grade subtype, glioblastoma. Glioblastoma is the nastiest of all gliomas with a bleak prognosis. Sadly, there are no curative treatment options for the glioblastoma patients, and despite extensive and rigorous therapeutic research survival rate of patients diagnosed with glioblastoma remain dismal with median overall survival of 15-23 months (5-year survival is less than 6%).

Therapeutic Management:

Patients diagnosed with gliomas (grade II – IV) receive similar treatment regimens with standard of care being surgery, followed by radiation therapy and chemotherapy (Chinot et al., 2014; Gilbert et al., 2014; Stupp, Mason, et al., 2005).

- ***Surgical resection***

  Surgery is the initial therapeutic approach for glioblastoma. Although strong predictors of good outcomes vary greatly from patient-to-patient, the most important predictor is extent of resection (Wolbers, 2014). Such a resection is associated with longer life expectancy in patients who undergo gross total resection followed by radiation and chemotherapy (Sanai, Polley, McDermott, Parsa, & Berger, 2011). The extent of resection (EOR) is an active area of research that has resulted in several publications on the relationship between gross total resection (GTR), residual volume (RV) and survival (K. L. Chaichana et al., 2014; Pichlmeier, Bink, Schackert, & Stummer, 2008; Walter Stummer & Kamp, 2009; W Stummer et al.; W Stummer et al., 2008).

In the majority of patients, maximal safe resection (MSR) is the desired approach for management of the disease (Metcalfe, 2000; Sawaya, 1999; Shapiro, 1982). The primary objective of gross total resection (GTR) is to maximize tumor resection and minimize morbidity. The value of maximal resection has been repeatedly confirmed to improve survival in patients with glioblastoma. With more extensive resections providing added advantages.

A meta-analysis (systematic review on relevant papers from 1996 to 2015) on association of the extent of resection with survival in glioblastoma was carried out by Brown et al, revealing that GTR perhaps increases the likelihood of 1-year survival compared to subtotal resection (STR) by about 61%, 2-tear survival by about 19%, and progression-free survival at 12 months by 51% (Brown et al., 2016).

- ***Radio and chemotherapy***

Following surgery, adjuvant radiotherapy, with or without chemotherapy (temozolomide), is considered the standard of care for patients with newly diagnosed glioblastoma and has been demonstrated to improve survival after surgical resection (Hochberg & Pruitt, 1980; Wallner, Galicich, Krol, Arbit, & Malkin, 1989). Such adjuvant treatments have been shown to increase progression free survival (PGS) and overall survival (OS) of patients (Stupp, Mason, et al., 2005) - IDH mutated patients show the best treatment response, though (Houillier et al., 2010).

Temozolomide (TMZ) is the standard of care and the most used drug for chemotherapy in glioblastoma treatment regime. Temozolomide, an imidazotetrazine derivative of the alkylating agent dacarbazine, exhibits antitumor activity by targeting DNA replication. It has demonstrated activity against both newly diagnosed and recurrent glioma. A recently published meta-analysis

(Feng, Sui, Wang, & Sun, 2017) revealed a median OS (13.4 – 19.0 months) in the combination treatment group, as opposed to radiotherapy alone group (7.7-17.1 months).

One of the most important biomarkers for glioblastomas is the methylation status of *MGMT*, which predicts sensitivity to temozolomide (Esteller et al., 2000; Hegi et al., 2005). Newly diagnosed glioblastomas with *MGMT* methylation respond well to treatment with alkylating agents.

Despite such multimodal, aggressive treatment regimen, prognosis and management of glioblastoma remains grim, hinting at more complex resistance and repair mechanisms being at play.

1.2.1 Landscape of Genomic Alterations in Glioblastoma:

Numerous alterations, genetic and epigenetic, have been identified to underpin gliomagenesis in adults. Frequently observed genetic alterations in astrocytomas include isocitrate dehydrogenase 1 & 2 mutations, TP53 and TERT promoter (pTERT); EGFR amplification and pRB deletion (Bralten & French, 2011; Richterová & Kolarovszki, 2016). Importantly, mutations observed in primary glioblastomas are EGFR amplification or mutation, PTEN deletion or mutation, CDKN2A-p16^{INK4A} deletion, whereas those specific to secondary glioblastomas are TP53 mutations, IDH 1 & 2 mutations and Platelet-derived growth factor receptor (PDGFRA) mutations (Crespo et al., 2015).

- ***The discovery of IDH mutations***

One of the most clinically relevant observations was the discovery that lower and higher-grade gliomas harbored mutations in the genes isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2). IDH mutations were first identified by Parsons et al (Parsons et al., 2008) when they

sequenced over 20,000 genes in 22 glioblastomas and identified a common point mutation in the metabolic gene IDH1 in 12% of the samples analyzed. This landmark discovery in the history of gliomas is used as a good diagnostic and prognostic biomarker in gliomas and has led to the accumulation of wealth of data. Mutations in IDH2 have also been identified in gliomas, although they are less common and mutually exclusive with mutations in IDH1 (Hartmann et al., 2009; Yan, Parsons, et al., 2009) – IDH mutations are rare in primary GBM.

All mutations identified to date have been a single amino acid missense mutation in IDH1 at arginine 132 (R132) or the analogous residue in IDH2 (R172). Almost all mutations in IDH1 appear to affect amino acid residue 132, with a vast majority containing a heterozygous missense mutation of arginine to histidine (R132H) (Watanabe, Vital, Nobusawa, Kleihues, & Ohgaki, 2009). Although IDH1 mutations often occur with a TP53 mutation in astrocytic tumors, IDH mutation is observed in almost all oligodendrogliomas, with 1p/19q co-deletion, with p53 mutation rarely found (Labussiere et al., 2010; Watanabe, Vital, et al., 2009; Yan, Parsons, et al., 2009).

Considering these findings, IDH mutation has been suggested to be, perhaps, an early event in gliomagenesis and formation of gliomas, prominently in low-grade tumors of astrocytic and oligodendroglial lineage. In fact, several studies suggest that IDH mutations precede TP53 mutations in 63% of diffuse astrocytomas and 80% of glioblastomas (Watanabe, Nobusawa, Kleihues, & Ohgaki, 2009; Yan, Bigner, Velculescu, & Parsons, 2009). However, IDH mutation is rarely observed with genetic alterations frequently observed in primary GBMs (e.g., EGFR), dismissing the role of IDH mutations in gliomagenesis. Thus, a three-pathway model was suggested to underlie the development of glioma (Fig 1.2).

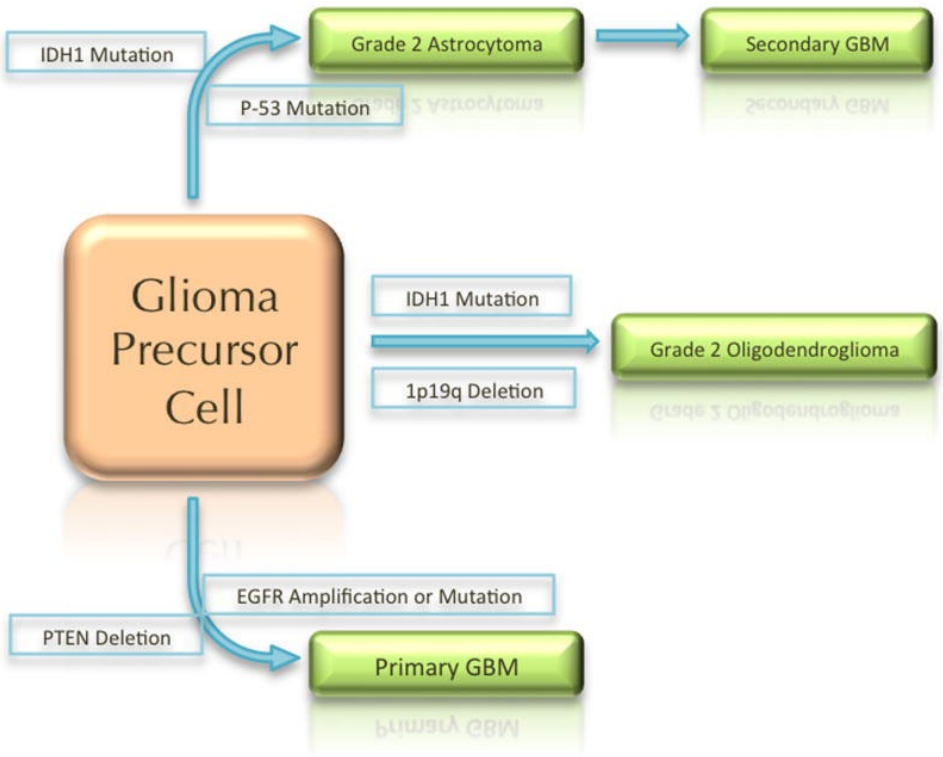


Figure 1.2 Possible pathways of gliomagenesis based on IDH mutation status.
Figure adapted from (Cohen, Holmen, & Colman, 2013)

1.2.2 The Cancer Genome Atlas (TCGA) subtypes:

Based on molecular profiles, gliomas have been subtyped by at least three studies (McLendon et al., 2008; Phillips et al., 2006; Q. Wang et al., 2017). Of these three studies, The Cancer Genome Atlas (TCGA) pilot project published a large-scale multi-dimensional analysis to disentangle molecular basis of cancer, reporting integrative analysis of DNA copy number, gene expression and DNA methylation aberrations in 206 glioblastomas, and nucleotide sequence aberrations in 91of these 206 glioblastomas (McLendon et al., 2008).

In this effort to catalogue and discover major cancer-causing genome alterations in large cohorts of human tumors, the team provided insights into the roles of ERBB2, NF1 and TP53,

uncovering frequent mutations of the phosphatidylinositol-3-OH kinase regulatory subunit gene *PIK3R* and pinpointed deregulation of RB, p53 and RTK/RAS/PI(3)K pathways as obligatory events in virtually all glioblastoma tumors.

In 2010, TCGA performed an integrated genomic analysis and described ***"a robust gene expression-based molecular classification of GBM into Proneural, Neural, Classical and Mesenchymal subtypes"*** (Verhaak et al., 2010) (Fig 1.3). These genomic profile-defined four subtypes of tumors were chosen based in prior meaning and the expression of signature genes: Proneural, Neural, Classical and Mesenchymal (Fig 1.3).

Classical subtypes were defined by chromosome 7 amplification and ten deletions in all the Classical subtypes, a highly frequent event in GBMs. Moreover, higher levels of EGFR amplification were observed in 97% of the Classical subtypes ($p<0.01$, adjusted two-sided Fisher's Exact test). Twelve of twenty-two Classical sub-types carried a point or vIII EGFR mutation (Verhaak et al., 2010). Along with high rates of EGFR alteration, a lack of TP53 mutation in the subset of Classical samples sequenced was also observed ($p+0.04$, adjusted two-sided Fisher's Exact test).

Mesenchymal subtype was predominantly defined by focal hemizygous deletions of a region at 17q11.2, containing the gene NF1 ($p<0.01$, adjusted two-sided Fisher's Exact test). Most samples had lower NF1 expression levels ($p<0.01$, two-sided Student's t-test); no methylation probes found in or around the NF1 locus (Verhaak et al., 2010). CO-mutations of NF1 and PTEN were observed in the Mesenchymal subtype. In addition, members of the tumor necrosis factor family, including TRADD, RELB, and TNFRSF1A were highly expressed in Mesenchymal subtype.

Pro-neural subtype exhibited 2 major alterations: alterations of PDGFRA and point mutation in IDH1. Higher levels of PDGFRA gene expression was reported to be almost exclusive in this tumor subtype ($p<0.01$, two-sided Student's t-test). Interestingly, 11 out of 12 mutations in the IDH1 gene were found in this subtype ($p<0.01$, adjusted two-sided Student's t-test) without any PDGFRA abnormality. Moreover, Proneural subtype also displayed higher expression of oligodendrocytic lineage-associated genes, including PDGFRA, NKX2-2 and OLIG2 (Noble, Pröschel, & Mayer-Pröschel, 2004; Verhaak et al., 2010).

The Neural subtype exhibited typical neuronal markers, including MEFL, GABRA1, SYT1 and SLC12A5.

The study also inferred biological meaning by correlating these subtypes with the gene signatures of major cell lineages of the brain: neurons, astrocytes, cultured astroglial cells and oligodendrocytes (Barbie et al., 2009; Verhaak et al., 2010). *"The Pro-neural class was highly enriched with the oligodendrocytic signature but not the astrocytic signature while the Classical group is strongly associated with the murine astrocytic signature. The Neural class shows association with oligodendrocytic and astrocytic differentiation but additionally had a strong enrichment for genes differentially expressed by neurons. The Mesenchymal class was strongly associated with the cultured astroglial signature"* (Verhaak et al., 2010).

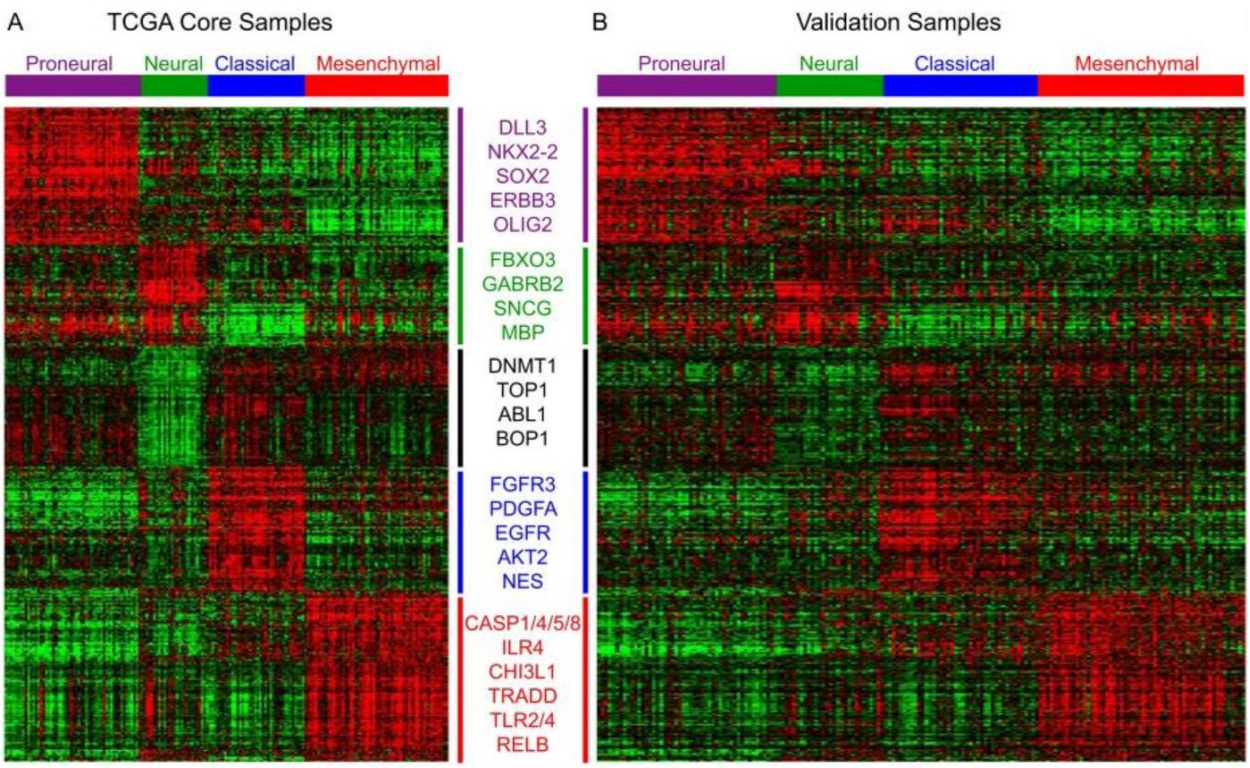


Figure 1.3 Gene expression data identify four gene expression sub-types
(A) Using the predictive 840 gene list, samples were ordered based on subtype predictions and genes were clustered using the core set of 173 TCGA GBM samples. (B) Gene order from the TCGA samples was maintained in the validation dataset (n=260), which is comprised of GBMs from four previously published datasets. (C) Ordered gene expression for 24 xenograft samples. Samples are ordered based on their predicted identity using the 840 gene list. Selected genes are displayed for each gene expression subtype.
Figure adapted from (Verhaak et al., 2010)

In the next section, we will review how our efforts in identifying and understanding the disease from a therapeutic perspective have been limited, and that how we have not succeeded or progressed much in achieving effective treatments for glioma patients.

1.3 THERAPEUTIC BOTTLENECKS AND DECADES OF FRUSTRATION

The absence of a breakthrough in the treatment of GBM is, in part, due to our poor understanding of the disease, including the tumor biology and the mechanisms underlying treatment resistance in recurrent GBMs. A brief glance at the timeline of GBM therapy reveals

that we have not progressed much in improving treatment options for the patients with GBM (Fig 1.4). We still utilise the same treatment regime that we did decades ago: Surgery followed by radiotherapy and chemotherapy.

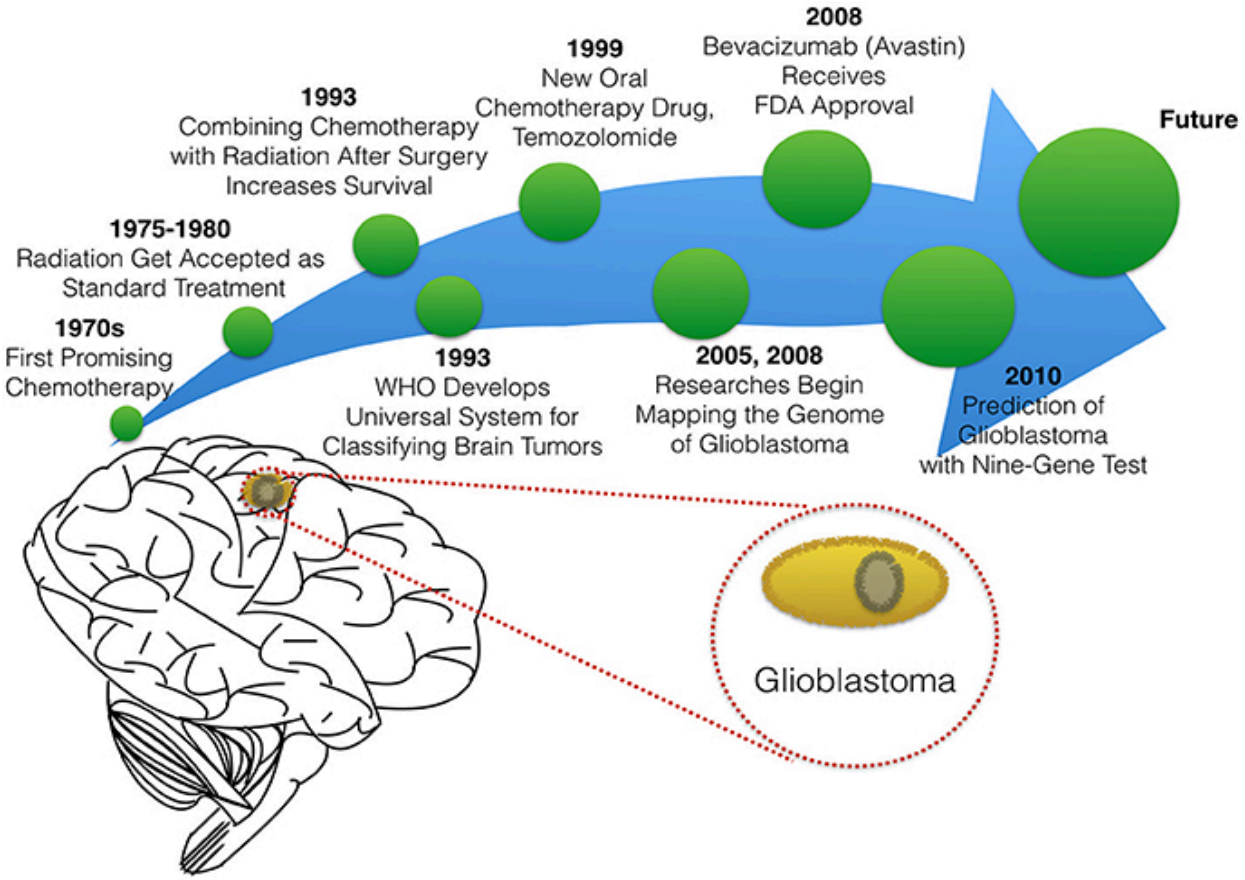


Figure 1.4 Timeline of Glioblastoma therapy
Brief timeline of glioblastoma therapy (from 1970s – 2010)
From (Ozdemir-Kaynak, Qutub, & Yesil-Celiktas, 2018)

1.3.1 Resistance and recurrence – The two "Rs"

Curative cancer therapy remains a major challenge, particularly in cancers displaying multidrug resistance. Treatment resistance has vexed the scientists for centuries. Drug resistance limits the therapeutic efficacy in cancers, leading to tumor recurrence through both intrinsic and adaptive mechanisms (Fig 1.5). Investigations into understanding the mechanisms that regulate

resistance lie at the heart of management of the disease and, sadly, remains the hardest nut to crack. Cancers have been shown to achieve resistance by various cellular resistance mechanisms, such as overcoming apoptosis, chromatin or metabolic remodelling, TMZ-specific resistance, tumor microenvironment-associated resistance etc. (Al-Nedawi et al., 2008; Challagundla et al., 2015; Corcoran et al., 2012; Mao et al., 2016).

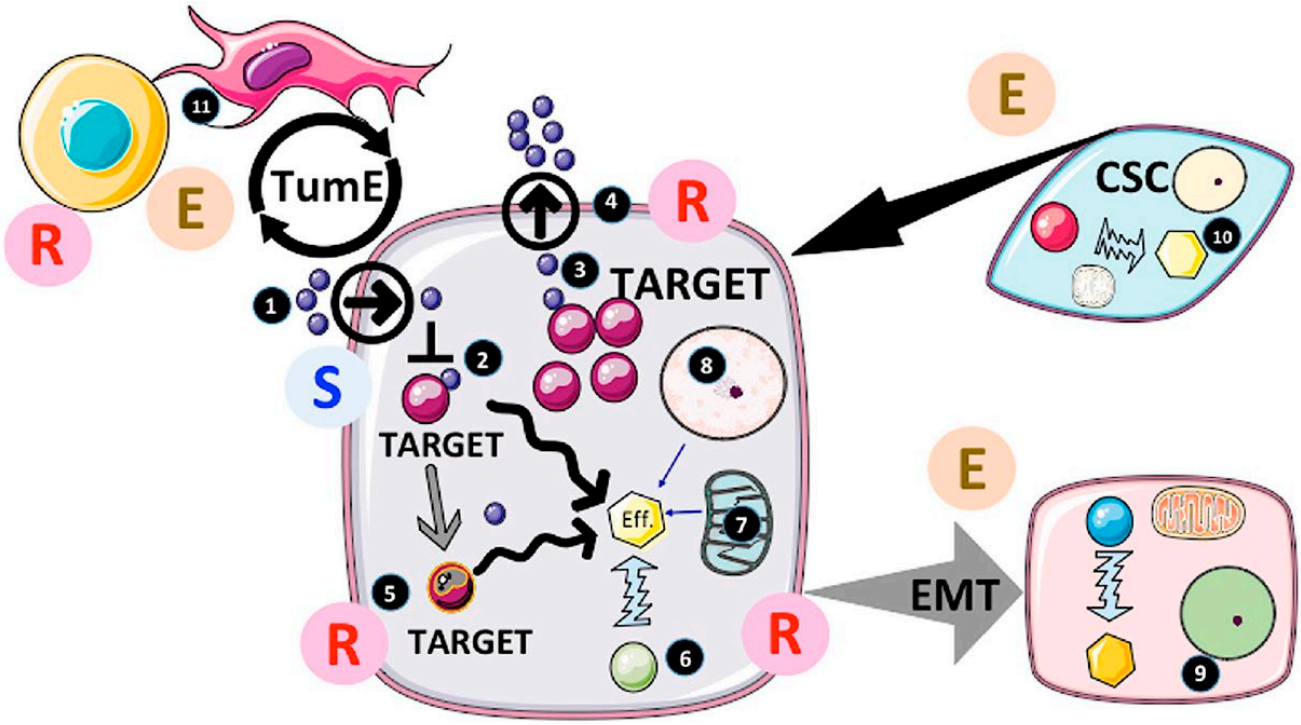


Figure 1.5 Actors and factors implicated in cancer resistance to treatments
Figure adapted from (Oliver et al., 2020)

In GBM, both chemotherapy and radiotherapy resistance have been shown to limit effectiveness of the treatment. Particularly in chemotherapy, the resistance to TMZ treatment is modulated by DNA repair systems and the expression of O^6-methylguanine-DNA methyl transferase (MGMT) (Hegi et al., 2005; Stupp, van den Bent, & Hegi, 2005). The expression of MGMT is silenced by promoter methylation in ~50% of GBM tumors. Furthermore, clinical studies have demonstrated elevated MGMT protein levels or lack of MGMT promoter

methylation to be associated with TMZ resistance in GBM (Esteller et al., 2000; Hegi et al., 2008). However, Bocangel et al. showed found no correlation between MGMT activity and response to TMZ in their study of 7 human glial tumor cell lines, indicating that a non-functional p53 response to DNA damage was associated more with TMZ resistance than with MGMT activity (Bocangel et al., 2002).

In addition to chemotherapy resistance, recurrence of GBM, characterised by radioresistance, remains inevitable (Hou, Veeravagu, Hsu, & Victor, 2006; Wong et al., 1999). There is very little data available regarding the radiation effects on global gene expression in radioresistant GBM models. Ma et al. demonstrated inactivation of early pro-apoptotic genes and late activation of anti-apoptotic genes at transcriptomic level following radiotherapy (H. Ma et al., 2013). Another study performed on complete RNA sequencing (RNAseq) data of control tissue and radioresistant U87-based GBM model demonstrated aberrant gene expression in irradiated U87-10gy, particularly, genes involved in enhancing tumor malignancy and invasion, upregulation of anti-apoptotic genes, promoting epithelial to mesenchymal transition and those involved with metalloendopeptidase activity (Doan et al., 2017; Doan et al., 2018; Nguyen, Shabani, Awad, Kaushal, & Doan, 2018).

1.3.2 Tumor heterogeneity and therapeutic resistance

Tumor heterogeneity has broadly been divided into intertumoral and intratumoral heterogeneity: the former refers to heterogeneity amongst patients harbouring tumors of the same histological type and the latter as spatial heterogeneity characterised of uneven distribution of genetically diverse tumor subpopulations. Intratumoral heterogeneity takes the center stage in therapy resistance. However, such heterogeneity is thought to arise and maintain because of increased levels genomic stability, leading to clonal diversity (Andor et al., 2016; Jamal-Hanjani

et al., 2017). Various models have been propounded addressing clonal diversity and maintenance.

Two mechanisms have been propounded to contribute to treatment resistance: The selective pressure perpetrated by therapy and the cancer-stem-cell model. The evolution of tumor cells under therapy has been viewed as a Darwinian process. The process engenders replacement of sensitive clones by resistant clones (Merlo, Pepper, Reid, & Maley, 2006). It is, perhaps, a disease of clonal evolution within the body with profound implications for cancer progression, prevention and therapy. However, this model is propped up by the contention that tumors are composed of many clones and that owing to the selection pressure, the treatment results in the change of the course of cancer evolution as dominant clones at diagnosis are replaced by others present within the cancer colony/mass.

Nevertheless, on the other hand, the cancer-stem-cell premise implicates a small population of cancer, within a hierarchically organised cancer mass, in tumorigenicity and treatment resistance (Lamprecht et al., 2017). The proponents of cancer-stem-cell (CSC) model regard heterogeneity as the fuel for resistance and have insisted on dissecting the complex clonal architecture of cancers, with non-uniform distribution of genetically distinct tumor-cell populations, to develop more-effective personalised therapies. They counterpose the evolutionary theory of cancer progression and therapy resistance with the argument that the stochastic nature of cancer initiation strengthens the idea that the development and progression of cancer does not follow a fixed course (Dagogo-Jack & Shaw, 2018; Hanahan & Weinberg, 2011). The proponents of the CSC model maintain that the cancer mass exhibits intratumoral heterogeneity and that chemotherapy successfully kills all the members of the cancer mass

except for the small population of cancer stem cells which is resistant to therapy and is, therefore, responsible for repopulation of the cancer mass and recurrence (Fig 1.6).

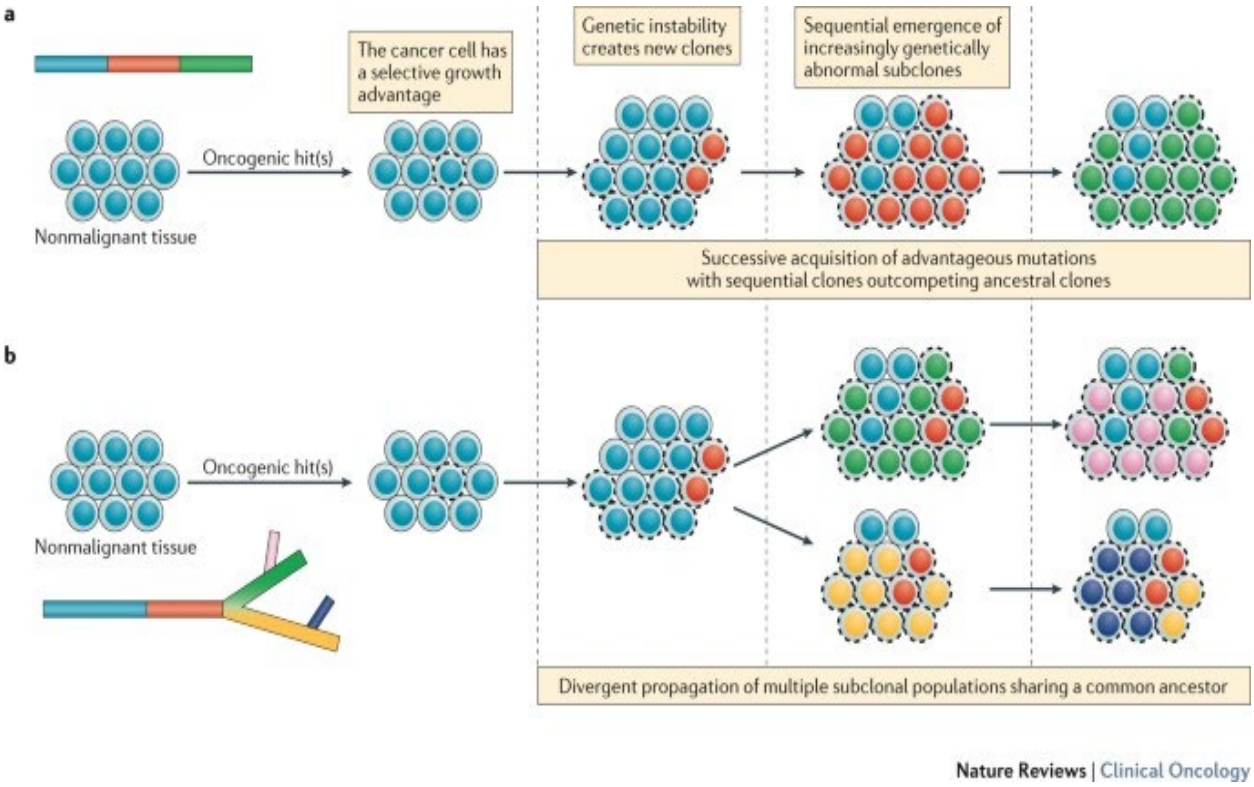


Figure 1.6 Distinguishing linear from branched tumor evolution
Figure adapted from (Dagogo-Jack & Shaw, 2018)

The upcoming section will take an evolutionary perspective to demonstrate ways in which cancer is thought to initiate and evolve, and the significance of fundamental framework of biological systems and its consequences in the field of cancer biology & evolution. Furthermore, it will also shed some light on how cancer-stem-cell model has fared in the field of glioblastoma research and what we have learned, in the context of GBM disease management, from this revelation.

1.4 BRAIN TUMORS: A COMPLEX SYSTEM

How is cancer initiated, maintained till its host supports it and refuses to lay the king down in this battle of survival between cancers and humans? Cancer has a mysterious past with unknown beginnings, and a convoluted present, maintained by highly complex biological processes. But what do we know about these peculiar beginnings? The idea that cancers originate from a single cell and follow a clonal growth pattern (cancer-stem-cell model) has gained wide recognition and remains the most favoured model as opposed to the clonal evolution of tumor cell populations: the sequential selection by an evolutionary process (the stochastic model). These two models that were briefly discussed in the previous section, in the context of treatment resistance, laid the foundation of cancer biology and is the very basis on which decades of our research is built on. Nevertheless, as much as these models have shaped our understanding of how cancers behave, it also has left the scientific community divided.

Almost five decades ago, Peter Nowell rattled the field of cancer biology by proposing the ground-breaking clonal evolution/selection model: *"Acquired genetic liability permits stepwise selection of variant sublines and underlies tumor progression"* (Nowell, 1976). This was in defiance of the evidence that in many primary tumors, all cells show the same abnormal karyotype, suggesting a unicellular origin (Sandberg & Hossfeld, 1970; Yosida, 1975), and that the immunoglobulin produced by plasma cell tumors has in almost every case the homogeneity characteristic of a single clone (Linder & Gartler, 1965; Milstein, Frangione, & Pink, 1967). Nowell fundamentally aligned his hypothesis with somatic Darwinian evolution and submitted that over time, in tumor cell populations, there is a well-defined sequential selection by an evolutionary process of sublines which are increasingly abnormal, both genetically and biologically, and that the initiation of this process (conversion of a normal cell into a neoplastic

cell) provides it with a selective growth advantage over surrounding normal cells. Nowell further notes that this "neoplastic" transformation is followed by proliferation, either immediately or after a latent period, resulting in a population of mutant progeny with an increase in genetic instability over generations (Fig 1.7).

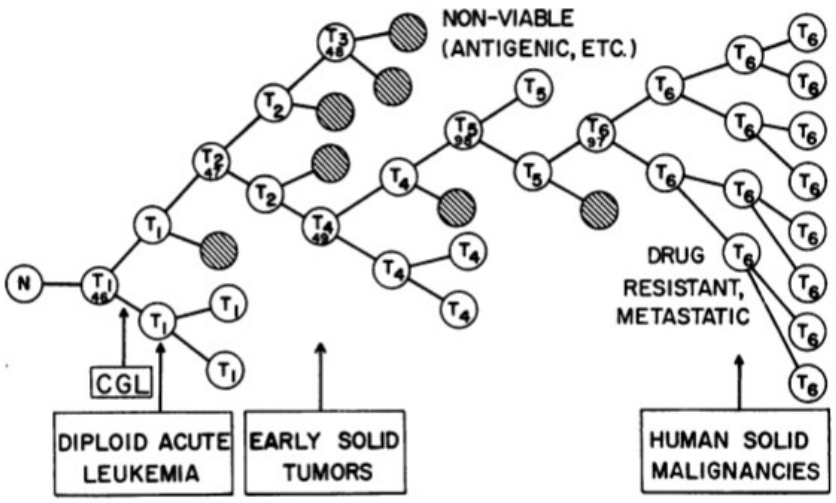


Figure 1.7 Model of clonal evolution in neoplasia
Figure adapted from (Nowell, 1976)

In Nowell's model of tumor evolution (Fig 1.7) carcinogen-induced transformation in progenitor normal cell (N) produces a diploid tumor cell (T_1, 46 chromosomes) with the beginning of clonal expansion. Genetic instability in T_1 cell leads to production of more abnormal variants (T_2 to T_6). During this clonal expansion, most variants die due to metabolic or immunologic disadvantage (hatched circles) with the ones with the selective advantage (T_2, 47 chromosomes) becoming the predominant subpopulation until an even more favourable variant appears (T_4). However, this stepwise sequence differs from tumor-to-tumor (intertumoral heterogeneity) determined, partially, by environmental pressures on selection (Nowell, 1976).

While Nowell described tumor development as a process that obeys the evolutionary framework of biological systems, modern tumor biology research bangs the drum for the cancer-

stem-cell (CSC) model. During the last decade, a stem-cell-like subset of cancer cells has been identified in many malignancies. This subset takes the center stage in tumor biology as the clonogenic core of the tumor responsible for the initiation, maintenance, and perpetuation of hierarchically organised tumors (Vermeulen, e Melo, Richel, & Medema, 2012) (Fig 1.8).

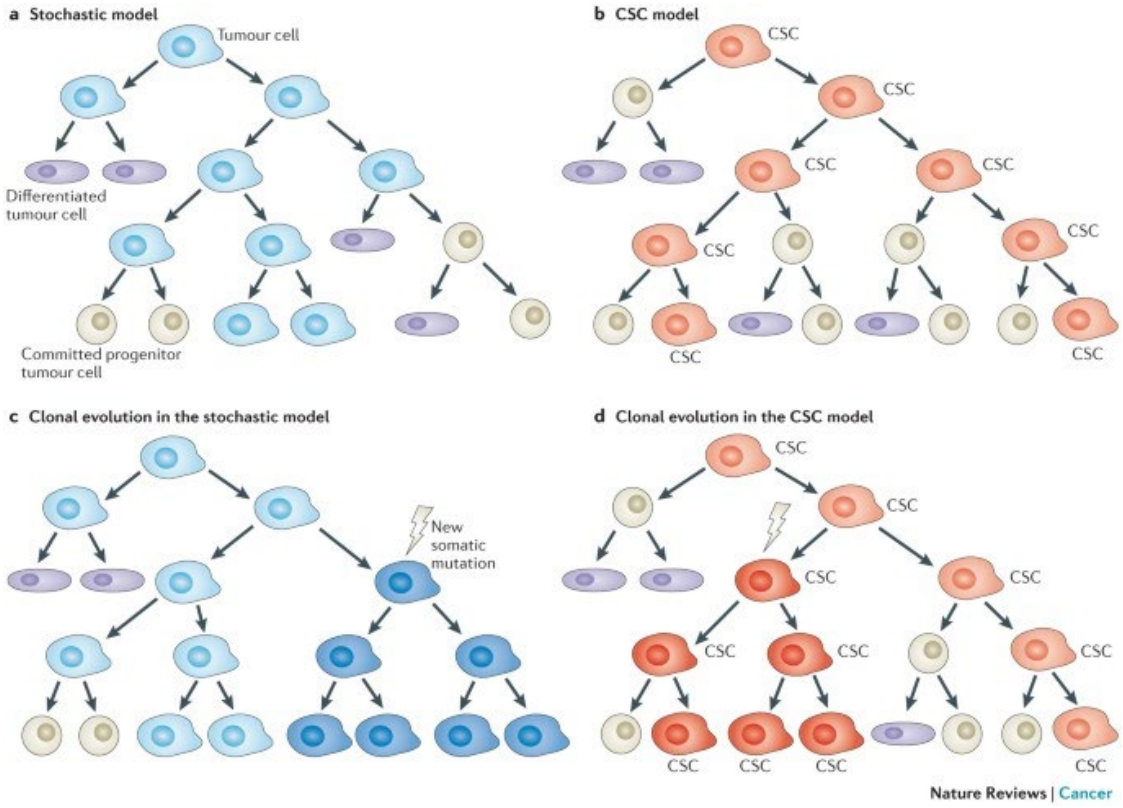


Figure 1.8 Models of tumor growth
Figure adapted from (Beck & Blanpain, 2013)

So, are tumors Nowell's chaotic, haphazard, and messy biological entities or well-defined, delineated and hierarchically organised systems, as depicted by the cancer-stem-cell model? What are CSCs? Let us look at how the haematopoietic stem cell (HSCs) epiphany laid the foundation of CSC model in the field of modern tumor biology.

1.4.1 Are cancer stem cells the seeds of the tumor? Opening Pandora's box

The concept of CSCs was introduced by the landmark studies published by Dick and colleagues, in 1994 and 1997 (Bonnet & Dick, 1997; Lapidot et al., 1994): *"A cell initiating human acute myeloid leukemia after transplantation into SCID mice"*. Dick and colleagues achieved this by engrafting fractioned different subpopulations of bone marrow cells from patients with acute myeloid leukemia (AML) and demonstrated that only a minor population of AML cells expressing markers of normal haematopoietic stem cells (HSCs) have the potential to propagate the leukaemia in immunodeficient mice, naming this subset "SCID leukemia-initiating cell" – based on its ability to establish human leukemia in SCID mice.

Following this development by Dick and colleagues in the late 20th century, cancer biologists scurried to test the same in other cancers with the hope of achieving the same readout and welcomed the 21st century with an avalanche of publications demonstrating what Dick and colleagues did, in breast cancer (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003), colorectal cancer (O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007; Vermeulen et al., 2008), skin squamous cell carcinoma (Malanchi et al., 2008), head and neck cancer (Prince et al., 2007), lung cancer (Eramo et al., 2008), pancreatic cancer (C. Li et al., 2007), prostate cancer (Collins, Berry, Hyde, Stower, & Maitland, 2005), ovarian cancer (Curley et al., 2009; S. Zhang et al., 2008) and brain tumors (Singh et al., 2004).

In the context of GBM, Singh and colleagues reported development of a xenograft assay that identified human brain tumor initiating cells that initiated tumors *in vivo* (Singh et al., 2004). This was achieved by performing enrichment of prospective CSCs using the haematopoietic stem cell antigen "cluster of differentiation 133" (CD133) from 3 medulloblastoma (from children), 3 glioblastomas (from adults) and 1 childhood glioblastoma. The team was able to initiate tumor

formation in 16 out of 19 mice with as low as 100 CD133+ cells within the space of 12-24 weeks, whereas, as high as 100,000 CD133- did not yield tumors in the NOD-SCID brain.

Although this was the very first evidence, in the field of brain tumors, that proved the existence of the subtype "tumor-initiate cells" within the tumor mass, other markers have also been used, including CD44 and CD15, to isolate glioma initiating cells (GICs) from glioblastomas.

While the CSC model stood its ground in the face of Nowell's "messy tumor" hypothesis, the prospect of tumors being faithful to the laws of Darwinian's evolutionary model started to fade away. These discoveries of "tumor-initiating cells" across various types of cancers were widely recognised by the scientific community and viewed as a breakthrough in the field of cancer and stem cell biology, however, there is a catch.

If you recall the tenets of the CSC model, tumors are:

1. ***initiated and maintained by a subset of cells within the tumor mass called cancer stem cell.***
2. ***hierarchically organised tissues along the stem-differentiation axis***

While these ground-breaking studies satisfy the first principle, hence aptly calling the subset "tumor-initiating" cells, none of the reports demonstrate the cellular architecture and hierarchical organisation in these tumors. In other words, the readouts of these studies do reveal tumor-initiating cells, but not, in any way, describe these as CSCs.

Before this realisation could dampen the spirit of the scientific community, the second decade of the 21st century witnessed a second wave of discoveries, enabled by the dawn of high-

throughput technologies, such as single-cell RNA-sequencing, successfully satisfying the CSC model to the letter.

1.4.2 Cancer: an organized crime or genomic chaos? Digging deeper

With the emergence of sequencing technologies, particularly single-cell RNA-sequencing, tumor biologists as well as developmental biologists have gained extraordinary insights into complex biological systems, revealing the tissue architecture and its cellular make-up. Some of the earliest studies that marked the dawn of single-cell RNA-sequencing (scRNAseq) were not only limited to understanding normal tissue arrangement and cellular diversity (Shalek et al., 2014; Treutlein et al., 2014; Zeisel et al., 2015) but this new technology was also applied to various cancers, including melanomas (Tirosh, Izar, et al., 2016), gliomas (Filbin et al., 2018), primary breast cancers (Chung et al., 2017), oligodendrogliomas (Tirosh, Venteicher, et al., 2016) and primary glioblastomas (Patel et al., 2014).

These studies, however, could not reach the full potential of the technology, owing to the lack of computational tools. With the emergence of refined and innovative computational tools, subsequent scRNAseq studies are beginning to provide unprecedented insights into tumor biology, particularly in gliomas (Venteicher et al., 2017) (Fig 1.9).

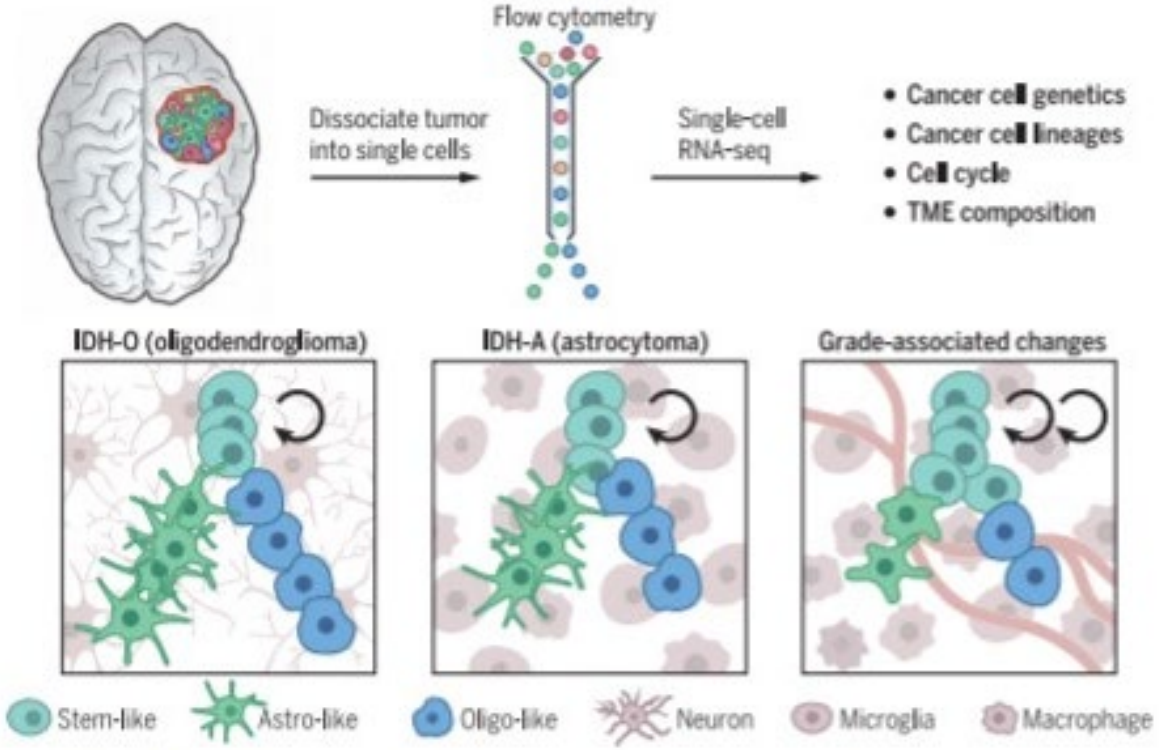


Figure 1.9 Hierarchical organization of tumor constituency in IDH-mutant gliomas
Figure adapted from (Venteicher et al., 2017)

Interestingly, our team recently demonstrated that glioblastomas, too, are hierarchically organised, in such a way that the landscape of these tumors mimic that of a developing brain (C. P. Couturier et al., 2020). Cells that reside at the top of this hierarchy are fast-cycling progenitors that bear resemblance to human fetal progenitors. These extraordinary insights have propped up the long-standing conjecture that cancers fundamentally arise from normal stem cells. While many of you maybe nodding your heads in agreement, the concept of adult stem cells is a highly controversial subject. If a normal adult stem cell/(s), indeed, are the perpetrators of the crime that not only initiate but also ensure survival of this criminality, then perhaps we ought to investigate their existence.

In the next section we will see what we know of adult neural stem cells and the record of the accused. We will also uncover the spatial significance of normal neural stem cells and the importance of stem cell niche for the survival and maintenance of stem cells.

1.5 TUMOR MAFIA - WHERE DO THE DIRTY SECRETS LIE? HOMING IN ON THE CRIME BOSS

Tumors are no less than an organized crime, such as mafia. These are highly coordinated and well-governed entities with a vast network and a strong hold over the territories they govern. If we are to disrupt this network, we need to understand where the commands are coming from and where does the boss operate from, in other words, if CSCs are normal stem cells gone awry, then what and where are normal adult neural stem cells?

1.5.1 The adult neurogenic niche: an accomplice to the crime?

Adult neural stem cells have been the highly sought-after cell type across various disciplines, including developmental neuroscience, neurodegenerative diseases, and cancer. While human brain is viewed as a post-mitotic organ, Eriksson et al. published evidence asserting otherwise. Studies in songbirds demonstrated newly formed cells as neurons (Burd & Nottebohm, 1985; Paton & Nottebohm, 1984) with both electrophysiological and functional evidence. Subsequently, the presence of long-term self-renewing NSCs/primary progenitors was suggested by isolation and *in vitro* propagation of cells with stem cell properties (Gage et al., 1995; Reynolds & Weiss, 1992; Richards, Kilpatrick, & Bartlett, 1992), and has been propped up by various studies (Gonçalves, Schafer, & Gage, 2016; Lim & Alvarez-Buylla, 2016; Song, Olsen, Sun, Ming, & Song, 2016).

1.5.2 Adult neural stem cells (NSCs) - The most elusive of all

In the adult mammalian brain, the vast majority of NSCs are found along the lateral walls of the lateral ventricles. These progenitors, particularly in mice, have been thoroughly demonstrated to generate young neurons that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they are thought to contribute to fine odour-discrimination and odour-reward association (Grelat et al., 2018; W. L. Li et al., 2018; Lledo & Saghatelyan, 2005). However, NSCs are also found in the subgranular zone (SGZ) of the hippocampus, where they generate new neurons for the dentate gyrus (DG) (Ming & Song, 2011) (Fig1.9).

Nevertheless, the story of adult human NSCs is not as clear and profound as it is in mice. In fact, it has remained one of the most contested and controversial topics in the field of neuroscience. It comes as a surprise that such a fundamental question in the field of neuroscience has gone unanswered for centuries; but why? The answer lies in the challenges associated with the acquisition of tissue from neuropathologically normal adult humans. While this may not be true in the case of fetal brain tissue, methodology that has largely been employed to inquiry the existence of adult human neural stem cells entirely relies on immunohistochemical studies of the post-mortem human brain tissue by using a series of well-known markers.

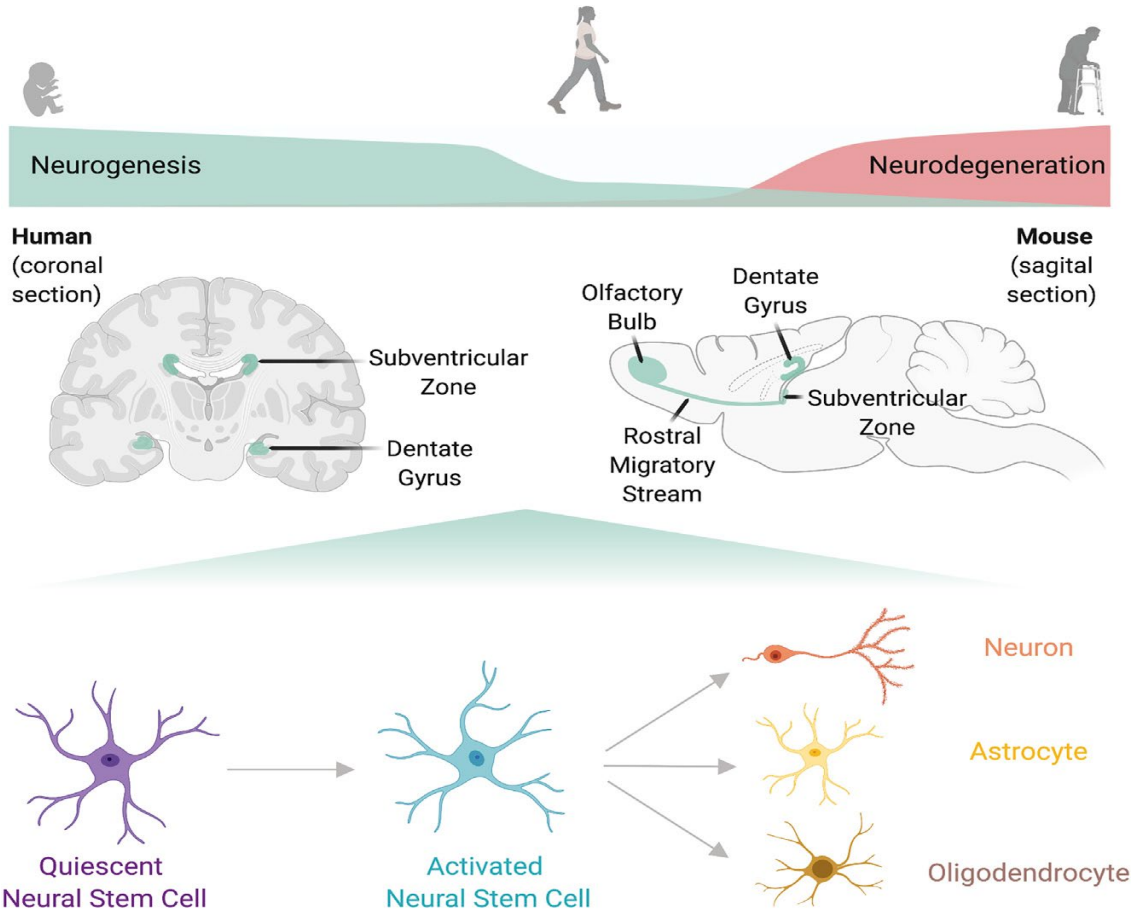


Figure 1.10 Neurogenesis across the mammalian lifespan
The ability of NSCs to proliferate and produce new neurons declines sharply after development and continues to decline during aging, whereas the incidence of neurodegeneration and age-related diseases increases (the diagram shows conceptual trajectories for neurogenesis and neurodegeneration). The adult mammalian brain contains two reservoirs of regenerative neural stem cells (NSCs): the DG of the hippocampus and the SVZ of the lateral ventricles (teal green). These niches contain qNSCs (quiescent NSCs) that can be activated to produce actively proliferating NSCs, aNSCs (activated NSCs). aNSCs have the potential to differentiate into neurons, oligodendrocytes, or astrocytes.
Figure adapted from (Negredo, Yeo, & Brunet, 2020)

In 2018, (Sorrells et al., 2018), employed the same histochemical approach and concluded that ***"neurogenesis in the dentate gyrus does not continue, or is extremely rare, in adult humans".*** This inference was based on neurosurgical specimens from medically resistant intractable epilepsy patients, embryonic cases, and healthy adult humans that were probed for the presence of DCX and PSA-NCAM, two key proteins that mark immature neurons. However,

given the continued presence of adult neurogenesis in other species, the study was received with certain level of hesitation and uncertainty. While the scientific community was still mulling over these findings, (Boldrini et al., 2018) the field was rattled with opposite findings in a paper published only two weeks later, demonstrating ***"presence of human neuronal precursors throughout life, while acknowledging the decline in markers of stem cells and angiogenesis".*** These two contrasting findings left the experts in the field scratching their heads, mainly because, both studies utilised very similar approaches on comparable tissues, reaching opposing observations and conclusions, triggering significant debate around methodological variables that may have contributed to such discrepancies, including fixation time, post-mortem delay and agonal state, among others.

Nearly a few months later, persistence of post-natal neurogenesis was further attested by two other independent groups (Moreno-Jiménez et al., 2019; Tobin et al., 2019).

While there may be a beacon of hope in the case of hippocampal neurogenesis, SVZ neurogenesis has not had quite the same attention as the hippocampus, that too when SVZ relishes a vigorous germinal past. But what happens to this germinal zone post development and its significance in the context of maintenance and repair remains a mystery. Though anatomically well-characterised (Doetsch, Garcıa-Verdugo, & Alvarez-Buylla, 1997; Peretto, Merighi, Fasolo, & Bonfanti, 1997; Quiñones-Hinojosa et al., 2006), very little is known about the cellular architecture and topography, and the functional significance of this region. Existing data, indicating the existence of NSCs in the SVZ is bolstered by both *in vitro* and *in vivo* studies, including neurosphere assay, self-renewal capacity and multipotency (K. Chaichana, Zamora-Berridi, Camara-Quintana, & Quiñones-Hinojosa, 2006; Eriksson et al., 1998; Reynolds

& Rietze, 2005; Reynolds & Weiss, 1992). Nevertheless, none of these studies definitively prove that the human brain possesses NSCs.

Adult SVZ has been a hot target in the field of both neuroscience and neuro-oncology; it is believed to retain neural stem cells by neurodevelopmental biologists (Sanai, Tramontin, Quiñones-Hinojosa, et al., 2004; van Strien, van den Berge, & Hol, 2011) and is accused of being the site of tumor initiation by brain tumor biologists (Galli et al., 2004; Hemmati et al., 2003; Ignatova et al., 2002; J. Lee et al., 2006; Singh et al., 2004; Taylor et al., 2005), owing to the similarities between the NSCs and the brain tumor stem cells (BTSCs) (covered thoroughly in the previous section) (Fig 1.10).

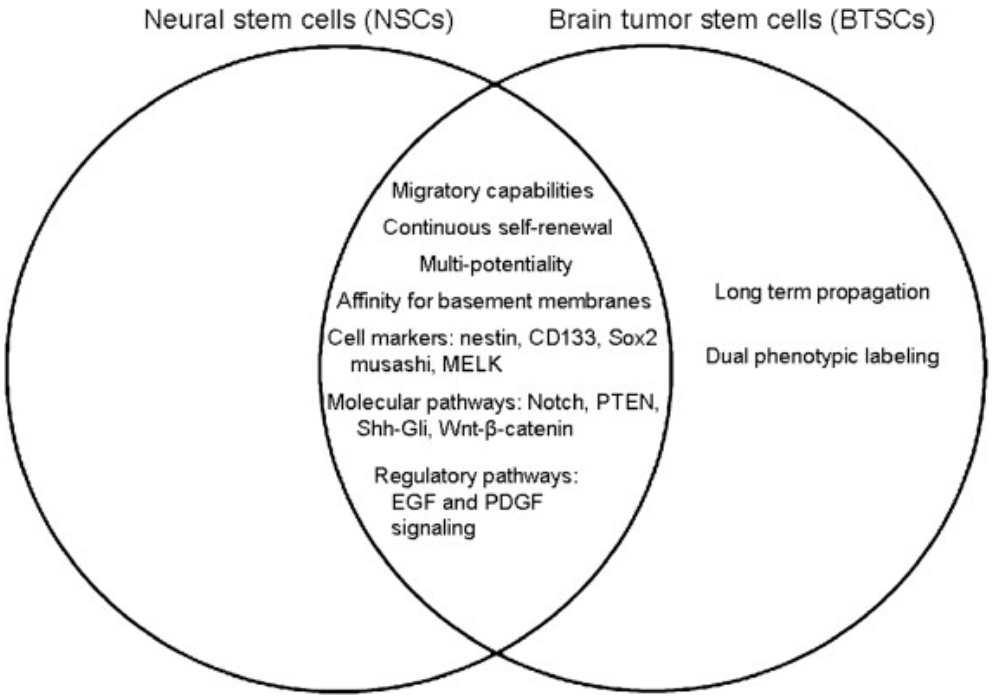


Figure 1.11 Neural stem cells (NSCs) and brain tumor stem cells.

Venn diagram depicting the similarities and differences between NSCs and brain tumor stem cells.

Figure adapted from (Quiñones-Hinojosa & Chaichana, 2007)

1.6 INTRODUCTION TO THESIS

Genetic architecture and analyses have shaped much of our understanding of cancer. However, it is becoming clearer that cancer cells display features of normal neural tissue with a population of cancer stem cell-like cells driving tumor growth. Understanding the relationship between cancer stem cells and neural stem cells has been at the heart of this work especially in the light of the processes that not only relate to oncogenesis, events resulting in transformation of normal cells into cancer leading to the initiation of tumorigenesis, but also to the mechanisms that potentially maintain the tumor. It remains to be seen whether gliomagenesis could be explained by either the cancer stem cell model or the genetic model, however, in the light on recent studies supporting the former, we explore the possibility of gliomagenesis following the cancer stem cell model by integrating elements of neurodevelopment.

1.6.1 Tumor mass: An allegedly siloed, nonnative, and a self-sustaining system

Glioblastoma cancer mass has remained the center of attention for decades, and why it should not be; after all, it is where the disease manifests and all the action take place. At face value it is a dynamic and forceful system, so much so that it was thought to be a self-sustaining, chaotic mass of abnormal cells, strong enough to thrive and survive in an opposing environment. Nevertheless, we now have seen that how recent investigations into the nature and heterogeneity of glioblastoma have corrected our misconceptions regarding the constituents of the tumor, revealing an order to this abnormal system, which was previously thought to be an extra-terrestrial, disorganized cluster of abnormal cells that somehow claimed its place in the brain. These studies have also disclosed that this mass not only mimics normal developmental

organization but also harbors neural cell types, including neurons, astrocytes, and oligodendrocytes. So how can a system that looks and almost behaves like a normal neural system be treated as a siloed system and where is the root of this hierarchy?

1.6.2 Cancer stem cells – Retracing the roots

Normal neurodevelopmental hierarchy originates with quiescent stem cells at the apex of the hierarchy that progresses through multiple cell states and types and culminates with terminally differentiated cell types at the bottom. This aerial perspective of developmental hierarchy has been known to us for decades, however, the molecular complexities within this hierarchy have started to emerge only lately (Fig 1.11).

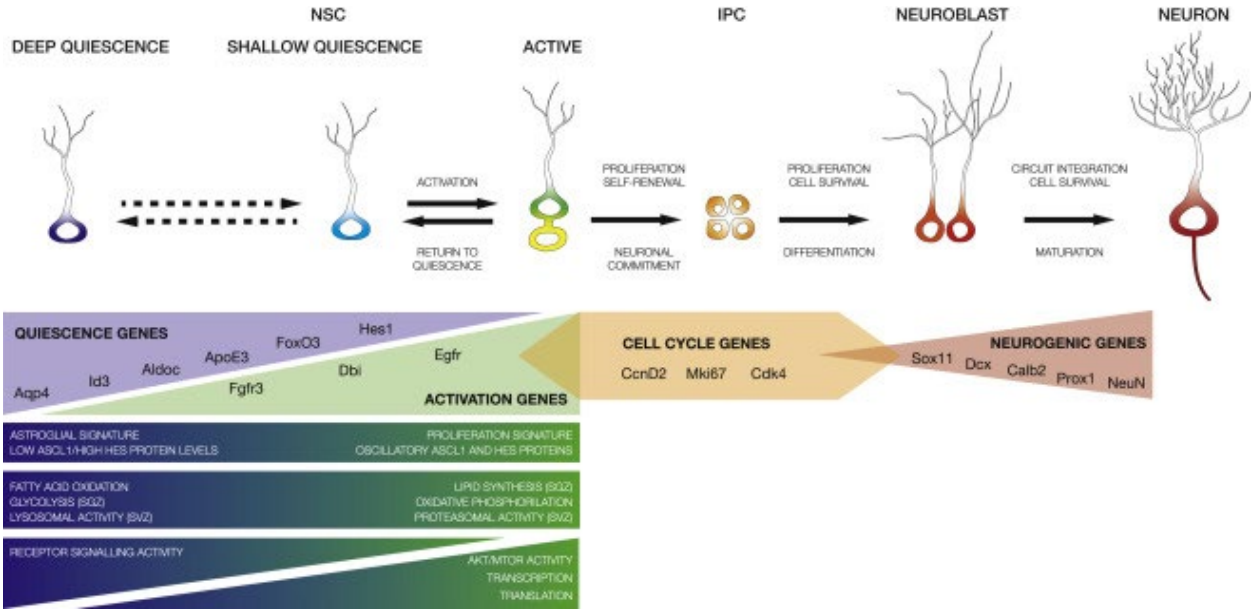


Figure 1.12 Adult Neurogenesis - from quiescent NSCs to neurons

Figure reproduced from (Urbán, Blomfield, & Guillemot, 2019)

Increasing evidence suggests existence of this hierarchy in tumor mass, nevertheless, it is worth noticing that this hierarchy has only been traced up to the progenitor/cycling population.

This absence of a more quiescent-like stem cell pool within the tumor hierarchy opens several questions and compels us to turn to the mystery of adult human neural hierarchy which has remained an unresolved question for decades.

1.6.3 *Harnessing brain development to understand brain tumors*

If we are to investigate the existence of neural stem cells, and perhaps the hierarchy, in adult human brain, then we certainly can take inspiration from early brain development mechanisms that laid the foundation of brain development, and perhaps maintain the adult human brain during adulthood.

To understand the spatial significance of neural cell types, let us look at how this hierarchy is distributed across the developing brain. During development, neural stem cells reside in the subventricular zone (SVZ) as radial glia and spin off progenitors that migrate and take on a neuronal fate, ultimately colonising the cortical plate. This spatial distinction in the distribution of cell types, with stem cells restricted to the germinal zone (SVZ) and migration of more committed cell types out of the germinal zone suggests dedicated brain regions for occupancy.

The germinal zone is crucial for maintenance of neural stem cells both in the human fetal and adult brain. This specialized environment looks after the stem cells with its unique microenvironment composition. Why should cancer be any different? The composition of tumor mass appears to mimic that of normal neural hierarchy – cycling cancer progenitors sit at the apex of the hierarchy and the differentiated at the bottom, however, if the root of the normal neural hierarchy, the normal neural stem cells, and lies in the SVZ then where can the root of the cancer hierarchy be? Within the bounds of fundamental biological laws, it makes sense for an

oncogenic/cancer cell to escape growth restrictions, but it seems very unlikely for it to go rogue beyond the principles and regulations of evolutionarily conserved mechanisms.

1.6.4 Beyond the bounds of tumor mass

Neural stem cells have long been accused of being the origin of glioblastoma, and our lack of knowledge and understanding of adult human neural stem cells has made it challenging to address and test this hypothesis for decades. As much as the cell of origin is vital in understanding oncogenesis and tumor initiation, identification of the apex of this tumor hierarchy will play a crucial role in treating this disease.

For this study we took inspiration from normal developmental hierarchy and hypothesised that developmental hierarchies not only lay the foundation of the human brain, but perhaps, also utilise this developmental framework for brain repair and maintenance during adulthood. And that glioblastoma is a consequence of anomalies accumulated by the normal neural stem cells that sit at the apex of this developmental hierarchy, that not only initiate but also maintain glioblastomas by operating from the subventricular zone via these developmental trajectories.

1.6.5 Aims and objectives of the study

This study took advantage of the unique opportunity to analyze samples from the subventricular zone (SVZ) of adult glioma patients excised at surgery to address key questions concerning neural stem cells in SVZ on the origin and maintenance of this cancer.

To achieve this aim, we set out to comb through the adult human SVZ with the objective of exploring the cell types of the germinal zone to uncover adult neural stem cells, and, perhaps, finding the apex of the cancer hierarchy. Moreover, we also utilised fetal neural stem cells to unravel brain development hierarchy, using which, we endeavoured to define adult human neural stem cells and reconstruct adult neural stem trajectory.

The objectives of the study are:

1. To uncover cell types of human fetal brain development and rebuild developmental trajectories to assemble a fetal brain development chart using single-cell RNA-sequencing technology.
2. To chart cellular identities of the adult human subventricular zone using single-cell RNA-sequencing technology and map these populations *in situ.*
3. To establish adult neural stem cell identity by using fetal progenitors as our developmental framework.

CHAPTER 2

CELLULAR LANDSCAPE OF THE DEVELOPING HUMAN FETAL BRAIN – DECONVOLUTING CELL TYPES/STATES AND RECONSTRUCTING DEVELOPMENTAL TRAJECTORIES

Data from this chapter has been used in the following research article:

Single-cell RNA-seq reveals that glioblastoma recapitulates a normal neurodevelopmental hierarchy (Nature Communication 2020)

Charles P. Couturier, Shamini Ayyadhury, Phuong U. Le, Javad Nadaf, Jean Monlong, Gabriele Riva, Redouane Allache, Salma Baig, Xiaohua Yan, Mathieu Bourgey, Changseok Lee, Yu Chang David Wang, V. Wee Yong, Marie-Christine Guiot, Hamed Najafabadi, Bratislav Misic, Jack Antel, Guillaume Bourque, Jiannis Ragoussis and Kevin Petrecca

2.1 Preamble

Our understanding of neural stem cells, including their origin, function and spatiotemporal significance has tremendously evolved over the last decade. However, this evolution of our understanding has only revealed how little we know of the major cell type of the central nervous system (CNS) that may play a crucial role in the "making and maintaining" of the mammalian brain, and that the extraordinary process of how mammalian brain is designed and made is of great intricacy and complexity.

During the development of the CNS, the major stem cell type that gives rise to all the neurons of the CNS and the glial cell types, including astrocytes and oligodendrocytes (Alvarez-Buylla, García-Verdugo, & Tramontin, 2001; Doetsch, García-Verdugo, & Alvarez-Buylla, 1999) plays a critical role in development and maintenance of the mammalian brain. However, such cells with self-renewal and multilineage differentiation capacities appear to be heterogeneous and ubiquitous in the brain. On the contrary, NSCs have also been shown to strictly reside in two neurogenic zones, the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (Fig 2a).

In a developing fetal brain, the birth and migration of newborn neurons are understood to be facilitated by glial-like cells that extend the processes radially with their cell bodies rested in the SVZ. Although, thought to be only involved in neuronal migration of newborn neurons to the cortex, these glial-like cells, better known as radial glial cells or radial glia (RG) have come to be recognized as the fundamental drivers of neurogenesis that not only act as scaffolds to support tangential placement of neurons across cortical layers, but now take center stage in the field of developmental neuroscience.

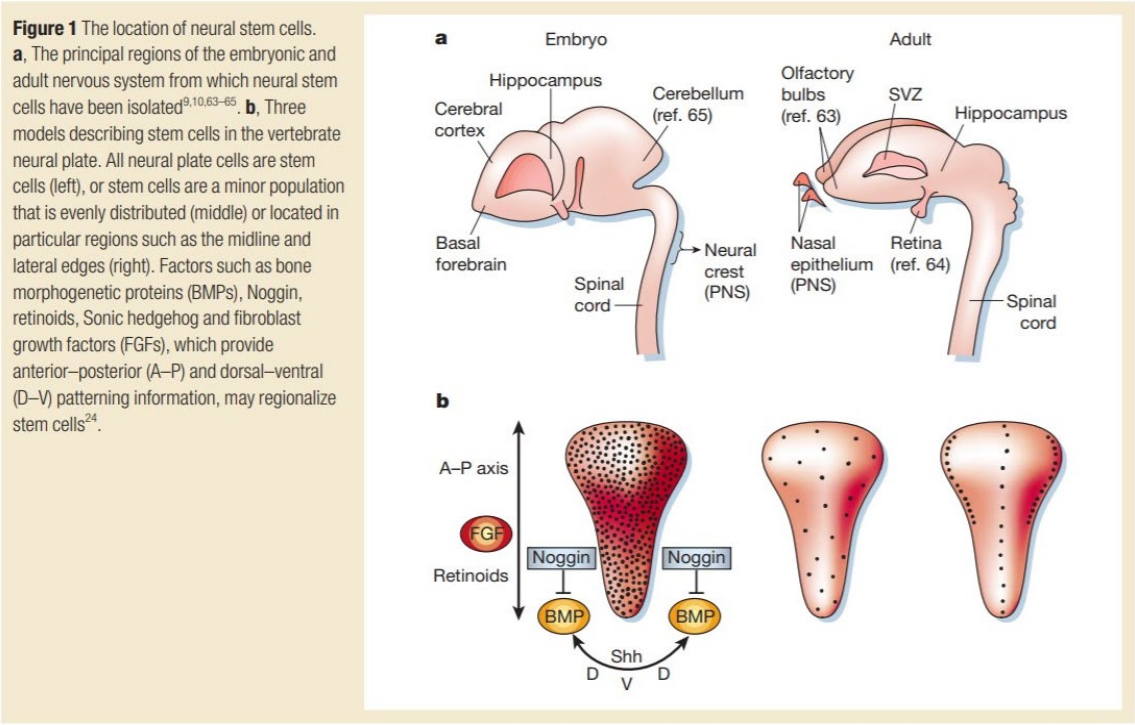


Figure 1 The location of neural stem cells. **a**, The principal regions of the embryonic and adult nervous system from which neural stem cells have been isolated[9,10,63–65]. **b**, Three models describing stem cells in the vertebrate neural plate. All neural plate cells are stem cells (left), or stem cells are a minor population that is evenly distributed (middle) or located in particular regions such as the midline and lateral edges (right). Factors such as bone morphogenetic proteins (BMPs), Noggin, retinoids, Sonic hedgehog and fibroblast growth factors (FGFs), which provide anterior–posterior (A–P) and dorsal–ventral (D–V) patterning information, may regionalize stem cells[24].

Figure 2.1 Neural stem cells (NSCs) reside in specialized niches
Figure reproduced from (Temple, 2001)

In the coming paragraphs, we will take a brief tour of the developing fetal brain with an emphasis on the radial glial cells (RG), including their origin, heterogeneity, and spatiotemporal significance. In the ensuing passages, we will see how this chapter aims to take on the challenge of addressing the fundamental question regarding the NSCs identity and studies heterogeneity in extraordinary detail, thereby, defining the NSC pools beyond their morphology and phenotypic characteristics.

Radial glial cells: The derivates of neuroepithelial cells:

The fundamental issue in developmental neuroscience remains to be that of the origin of neural stem cells (NSC). The earliest stem cells in the neural tube are the neuroepithelial cells that expand through symmetric division. During the time leading up to neurogenesis, neural plate

and neural tube are composed of a single layer of cells, called epithelial cells, that exhibit typical epithelial features. These neuroepithelial cells undergo symmetric divisions, followed by many asymmetric, self-renewing divisions, generating progenitors or a neuron (Fig 2b).

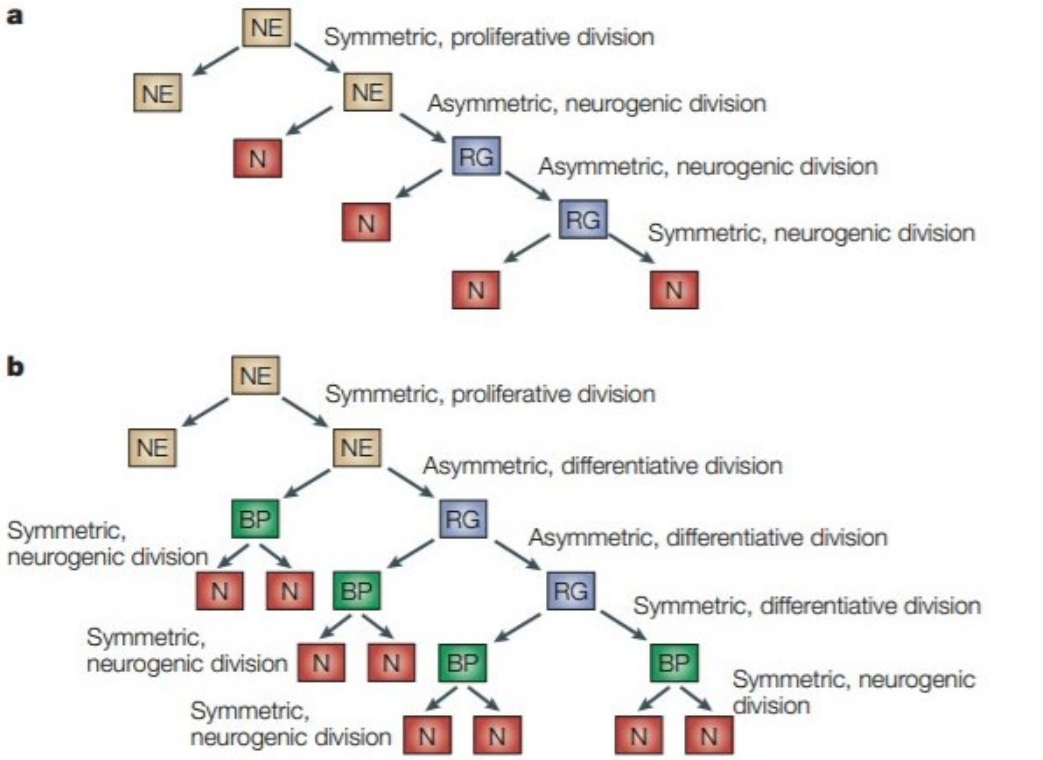


Figure 1 | **Lineage trees of neurogenesis.** The lineage trees shown provide a simplified view of the relationship between neuroepithelial cells (NE), radial glial cells (RG) and neurons (N), without (**a**) and with (**b**) basal progenitors (BP) as cellular intermediates in the generation of neurons. They also show the types of cell division involved.

Figure 2.2 Lineage tree of neurogenesis.
From (Götz & Huttner, 2005)

Following the transition from epithelial to radial glial cells comes the onset of neurogenesis (birth of neurons) where this neuroepithelium transforms into a layered tissue. The layer that lines the ventricle is referred to as the ventricular zone - the cell layer abundant in progenitor cell bodies. This epithelial-to-radial glial transition, a defining event during the

development of CNS, is of one great significance. This shift not only marks the birth of the cell type that goes on to "build" the central nervous system, but also lays the foundation of neurogenesis.

Neurogenesis – The post-transition drama:

Once considered to serve as the ladder for the newborn neuron to migrate up to the basement membrane at the pial surface, radial glial cells or RG now take the center stage in the field of developmental neuroscience. With cellular and molecular characteristics of astroglia, these cells express glutamate transporter GLAST, S100β, glutamine synthase (GS), vimentin (VIM), tenascin-C (TNC), and GFAP (Götz & Barde, 2005). Therefore, RGs are truly distinct from neuroepithelial cells in their glial nature but are as "astrocytic" as can be assessed by molecular standards.

Neurogenesis, a highly sophisticated, evolutionarily conserved, and tightly regulated process is orchestrated by RGs. Developmental processes may vary from species to species in various aspects, nevertheless, general principles of NSC lineages are very likely to be preserved. At the onset of cortical neurogenesis (around E9- E10 in mice), RGs appear to serve as neural precursors (Fig 2c). During the peak of neurogenesis (GW12-GW17 in humans) these RG continue to spin off intermediate progenitors (IPs) which produce newborn neurons, filling the cortical tissue in an inside-out fashion. Additionally, with the waning of neurogenesis and waxing of gliogenesis, RG also serve as the precursors of astrocytes and oligodendrocyte precursor cells (OPC) which in turn yield mature oligodendrocytes. However, it remains to be discovered if intermediate progenitors for astrocytes exist to initiate and support the generation of astrocytes (Fig 2c).

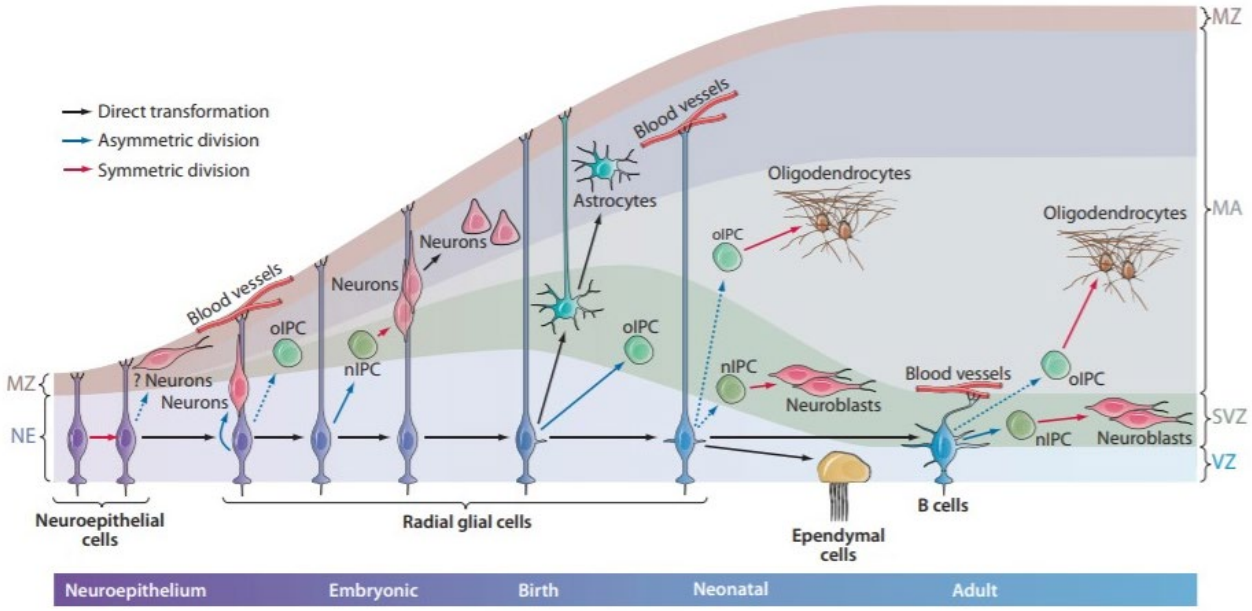


Figure 1

Glial nature of neural stem cells (NSCs) in development and in the adult. Neuroepithelial cells in early development divide symmetrically to generate more neuroepithelial cells. Some neuroepithelial cells likely generate early neurons. As the developing brain epithelium thickens, neuroepithelial cells elongate and convert into radial glial (RG) cells. RG divide asymmetrically to generate neurons directly or indirectly through intermediate progenitor cells (nIPCs). Oligodendrocytes are also derived from RG through intermediate progenitor cells that generate oligodendrocytes (oIPCs). As the progeny from RG and IPCs move into the mantel for differentiation, the brain thickness, further elongating RG cells. Radial glia have apical-basal polarity: apically (down), RG contact the ventricle, where they project a single primary cilium; basally (up), RG contact the meninges, basal lamina, and blood vessels. At the end of embryonic development, most RG begin to detach from the apical side and convert into astrocytes while oIPC production continues. Production of astrocytes may also include some IPCs (see **Figure 2**) not illustrated here. A subpopulation of RG retain apical contact and continue functioning as NSCs in the neonate. These neonatal RG continue to generate neurons and oligodendrocytes through nIPCs and oIPCS; some convert into ependymal cells, whereas others convert into adult SVZ astrocytes (type B cells) that continue to function as NSCs in the adult. B cells maintain an epithelial organization with apical contact at the ventricle and basal endings in blood vessels. B cells continue to generate neurons and oligodendrocytes through (n and o) IPCs. This illustration depicts some of what is known for the developing and adult rodent brain. Timing and number of divisions likely vary from one species to another, but the general principles of NSC identity and lineages are likely to be preserved. Solid arrows are supported by experimental evidence; dashed arrows are hypothetical. Colors depict symmetric, asymmetric, or direct transformation. IPC, intermediate progenitor cell; MA, mantle; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic progenitor cell; oIPC, oligodendrocytic progenitor cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone.

Figure 2.3 Origin of radial glia
From (Kriegstein & Alvarez-Buylla, 2009)

Despite what we know of the neural developmental programs and the entities that orchestrate these processes, many fundamental questions continue to bother us like a splinter in the flesh. Are mature cell types, including neurons, solely derived from the RGs? Is every cell in the SVZ with apical processes, extended to the pial surface, an NSC? Do these RGs undergo extensive proliferation, to the point of exhaustion, or exist in multiple states? Is neurogenesis a

once-in-a-lifetime process or does it continue to support and maintain the mammalian brain throughout the lifespan? What are NSC beyond their morphological features?

These fundamental questions continue to cast a shadow on our understanding in the field of developmental neuroscience and warrant investigations at unparalleled depths and resolutions. Certainly, these are some extraordinary and challenging questions, or perhaps a little overambitious, but this knot of frustration, once untied, will revolutionise the way we perceive one of the critical and historically significant processes implicated in various aspects of health and disease, including cancer.

We set out to probe the human fetal brain (n=3) at various developmental stages [gestational weeks (GW)], particularly the neurogenic phase, including GW13, GW17 and GW19 by using the state-of-the-art technology in the field, commonly known as single-cell RNA-sequencing (scRNAseq). In biological settings, the scRNAseq findings were corroborated in human fetal brain tissue sections and cells derived from human fetal brain samples.

2.2 Aim and objectives

To extricate fetal neural stem cell populations, this chapter discusses experiments and observations utilized to uncover the cell types, molecular profile, and function of NSCs at an unprecedented resolution and seeks to build a map of neurodevelopmental programs and reconstruct lineage trajectories to reveal intermediate cellular states that are masked when investigated using conventional tools and techniques.

The objectives of this chapter are:

1. To reveal the cellular architecture of the developing human brain.
2. To capture and characterize RGs and reconstruct the developmental trajectory.
3. To spatially validate the RGs and catch a glimpse of their temporal dynamics.

2.3 Results

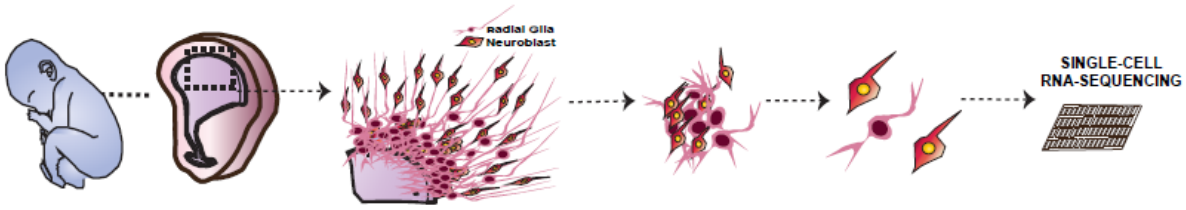
2.3.1 single-cell RNA-sequencing (scRNAseq) reveals major transcriptomic cell types and states in the developing fetal brain

To reveal the cellular landscape of the developing human fetal brain, we performed single-cell RNA-sequencing on fetal brain samples (n=3) at three different developmental time points: gestational week (GW) 13, 17 and 19 (Fig 1a) a generated sequencing library. A total of 3500 cells were sequenced (Fig 1a and c).

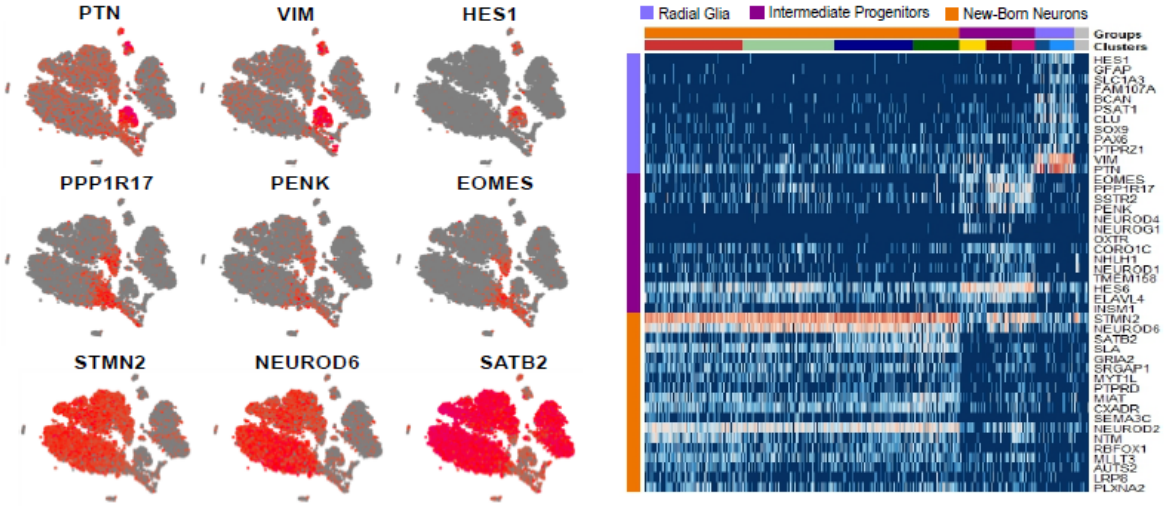
To classify the major cell types in the developing fetal brain, we used t-distributed stochastic neighboring embedding (*t*-SNE), a dimension reduction approach, to visualize and cluster cells. We identified three major cell types: radial glia (RG), intermediate progenitors (IPs) and newborn neurons (Fig 1b) and 14 clusters in total. These cell types were characterized by the expression of the classical cell type-defining markers: RG cell type was marked by HES1, vimentin (VIM) and pleiotrophin (PTN); IPs cluster was highlighted by EOMES, PENK and PPP1R17 expression, and newborn neurons expressed STMN2, NEUROD4 and SATB2 (Fig 1b). These cell type-specific genes were amongst the top differentially expressed genes for each cluster as seen in the heatmap (Fig1 b).

Cells from all three fetal samples (n=3) were visualized together as a conflated dataset (Fig 1b). RG cell type was composed of 2 clusters, IPs 3 clusters and newborn neurons consisted of 6 clusters. The remaining 3 clusters represented microglia, endothelial and excitatory late-born neurons.

a. Work flow of the foetal brain sample processing for single-cell RNA-sequencing



b. Visualisation of neurogenesis-associated genes in the human foetal brain dataset in t-SNE plot and the heatmap



c. List of patients and their diagnosis for the samples utilised in this study

	Sample	Gender	Age	Location	Diagnosis
Main Cohort - Adult SVZ	SVZ 1	male	53	SVZ	anaplastic astrocytoma
	SVZ 2	male	51	SVZ	glioblastoma multiforme
	SVZ 3	male	51	SVZ	high grade glioma
	SVZ 4	male	62	SVZ	glioblastoma multiforme
	SVZ 5	female	70	SVZ	metastatic adenocarcinoma
	SVZ 6	male	55	SVZ	glioblastoma multiforme
	SVZ 7	male	38	SVZ	recurrent astrocytoma
	SVZ 8	male	63	SVZ	recurrent glioblastoma multiforme
	SVZ 9	male	62	SVZ	glioblastoma multiforme
	SVZ 10	female	72	SVZ	glioblastoma multiforme
	SVZ 11	female	63	SVZ	glioblastoma multiforme
Fetal	Fetal 1	N/A	GW 13	Brain	N/A
	Fetal 2	N/A	GW 17	Brain	N/A
	Fetal 3	N/A	GW 19	Brain	N/A

Figure 2.4 Landscape of a developing human fetal brain

a. Schematic representation of the work flow. Samples from 3 human foetal brains at three different gestational weeks (GW13, GW17 and GW19) were processed to capture single cells for single-cell RNA-sequencing.

b. Left panel: Visualtion of key cell types/states associated with neurogeneis, including radial glia (RG)/progenitor cluster: pleiotrophin (PTN), vimentin (VIM) and HES 1; Intermediate Progenitor (IP) cluster: EOMES, PENK and PPP1R17; New-born Neurons: stathmin 2 (STMN2), NEUROD6 and SATB2. Colour represents expression levels of the signature: high (red) to low (blue). Right panel: Heatmap visualisation of genes associated with the three main cell types/states of neurogensis. Colour represents expression levels of the signature: high (red) to low (blue).

c. List and details of patients samples were derived from for this study. Only foetal samples were utilised for chapter 2.

2.3.2 Identifying cell types/states in the human fetal brain

To reveal the sub-clusters or the transient cellular states in the conflated fetal dataset, we took an unbiased approach to not only define the embedded cellular states but to validate our major clusters described earlier (Fig 1b). This unbiased approach included conducting comparisons with fetal datasets available.

Two key studies have addressed various aspects of fetal neurodevelopment; Nowakowski et al. investigated spatiotemporal gene expression trajectories revealing developmental hierarchies (Tomasz J. Nowakowski et al., 2017), whereas Zhong et al. explored the developmental landscape of prefrontal cortex (Zhong, Zhang, Fan, Wu, Yan, Dong, Zhang, Li, Sun, Pan, et al., 2018). Owing to the descriptive nature and anatomical significance of Pollen et al. dataset, we equated our 14 clusters to the signatures defined by Nowakowski et al. (Fig 2.4b). This comparison exposed neuronal populations masked, partly, by the more dominant, global neuronal gene signatures within our newborn neuron cell type. This enabled us to extract and define anatomically distinct neuronal subtypes, including excitatory and inhibitory neurons. Both early and late excitatory neurons of prefrontal cortex (PFC) and V1 region were found in our dataset (Fig 2.4a). Newborn neurons of medial ganglionic eminence (MGE) and inhibitory neurons of lateral ganglionic eminence (LGE) were also identified (Fig 2.4 a and b).

Intermediate progenitors are transit amplifying cells (TACs) that specifically express TBR2 (EOMES) and are understood to play a critical role in migration of neural stem cells (Nelson et al., 2020). We observed the presence of two IP clusters (highlighted in lilac and orange) (Fig 2.4a). The signatures of these two clusters corresponded to two key IP molecular

signatures defined by Nowakowski et al., dividing IPs (IP-Div2), shown in lilac, and early neuron-like (IP-EN-like), shown in orange (Fig 2.4a).

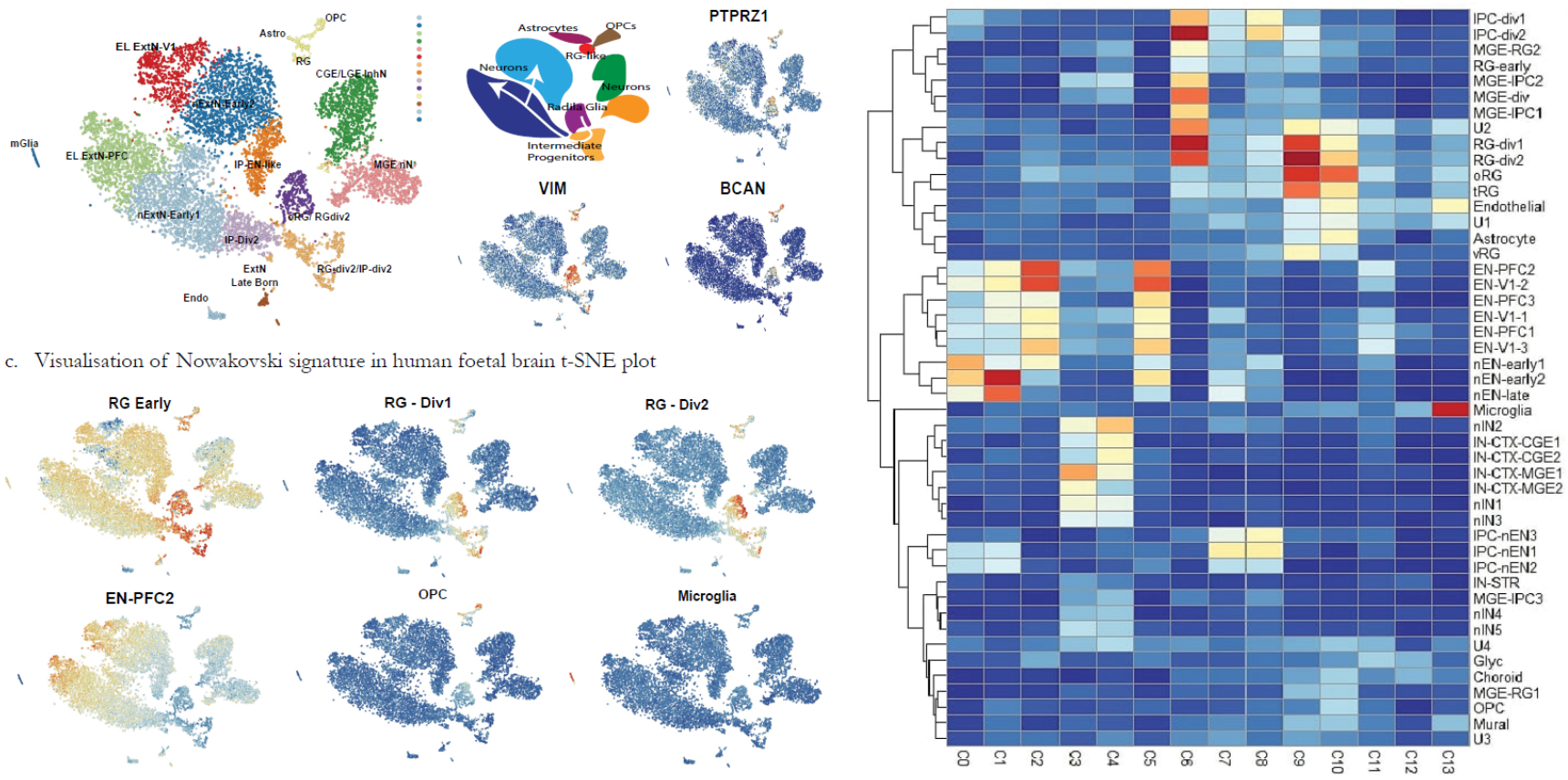


Figure 2.5 Delineating cell-types and states in the human fetal brain dataset (n=3) and comparison with Nowakowski et al. 2018

with major cell types labelled. Expression of key radial glial genes, including vimentin (VIM), brevican (BCAN) and PTPRZ1. Colour represents expression levels of the genes: high (red) to low (blue).

b. Heatmap visualisation of Nowakovski signatures in human foetal brain dataset. To further confirm the cell types in our foetal dataset, signatures defined by Nowakovski et al. were used to define cell types in our dataset. Horizontal lines highlight the Nowakovski signatures/cell types and vertical lines highlight our foetal clusters. Cycling NSCs/progenitors (RG) and quiescent NSCs/progenitors (RG) are highlighted by vertical lines. Colour represents expression levels of the signature: high (red) to low (blue).

c. Visualisation of Nowakovski signatures in the human foetal dataset, including radial glia Early (RG early), dividing radial glia 1 (RG - Div 1), dividing radial glia 2 (RG - Div 2), excitatory neurons of prefrontal cortex (EN - PFC 2), oligodendrocyte progenitor cells (OPCs) and microglia. Colour represents expression levels of the signature: high (red) to low (blue).

To better delineate the molecular nature and character of NSCs, also called radial glia (RG), we set out to tease out the molecular identity of 2 RG-like clusters (clusters 9 and 10) in our dataset and noticed that our RG clusters bore striking resemblance to multiple RG-related signatures, including early radial glia (RG early), dividing radial glia (RG-div1 and RG-div2), outer radial glia (oRG) and truncated radial glia (tRG) (Fig 2.5 b and c). However, what caught our attention was the observation that of all these 4 signatures cluster 9 (highlighted in purple) exhibited the strongest correlation with RG-div1 and div 2, including oRG and tRG, whereas cluster 10 (highlighted in gold) showed stronger correlation score for oRG only with a weaker correlation score for dividing RG signature (Fig 2.5 b and c). Both RG-like clusters expressed stem cell markers, including VIM, BCAN, SOX9, HES1 and GFAP.

Having captured cells during the peak of neurogenesis (GW12 – GW20), we were able to reconstruct developmental lineage and hierarchy (Fig 2.5a). To further validate these cell types in the germinal zone during human cortical neurogenesis, we performed immunolabelling in fetal brain tissue sections across 3 developmental time points (GW16, GW17 and GW21).

2.3.3 Spatio-temporal significance of radial glia (RG): Molecular characterization of fetal RG in the human fetal germinal zone during corticogenesis

Transcriptional heterogeneity amongst radial glia is predominantly driven by cell cycle status and spatial significance; RGs have been shown to exist in distinct molecular states, such as oRG, tRG and ventricular radial glia (vRG). Outer radial glia (oRG) have recently been shown to play vital role in directly supporting the niche, enabling the developmental and evolutionary

expansion of the human neocortex (Alex A. Pollen et al., 2015). To uncover the spatial significance of our RG-like clusters (C9 and C10), we performed immunolabelling of key RG markers, including SOX9, VIM, GFAP, PAX6, FAM107A and GFAP-delta. To assess the impact of cycling status on the heterogeneity of RG, we also incorporated MKI67 in our immunohistochemistry panel.

As expected, PAX6, SOX9 and FAM107A was predominantly in the SVZ and the outer subventricular zone (oSVZ) across all three developmental time points (Fig 2.6a). While PAX6 is a classical RG marker, it has been shown to persist in new-born neurons during neurogenesis and cortical plate expansion (Thakurela et al., 2016). Consistently, expression of PAX6 was observed across the width of the neocortex, highlighted in blue (Fig 2.6a). Whereas MKI67 and SOX9 expression was restricted to the SVZ, typical of RG population. To mark the birth of new-born neurons in the SVZ, that ultimately travel along the radial processes of the RG to the cortical plate, we labelled new-born neurons (red) with doublecortin (DCX) and found these cells negative for GFAP (green) in the SVZ (Fig 2.6b). Expectedly, co-localisation of SOX9 (magenta) and GFAP (green) was also observed in the SVZ (Fig 2.6b).

a. Expression profile of NSC/RG in the subventricular zone and the cortical plate.

b. Expression of stem cell/RG markers in the subventricular zone (SVZ) and the birth of new neurons.

c. Spatiotemporal changes over time in human foetal brain during neurogenesis

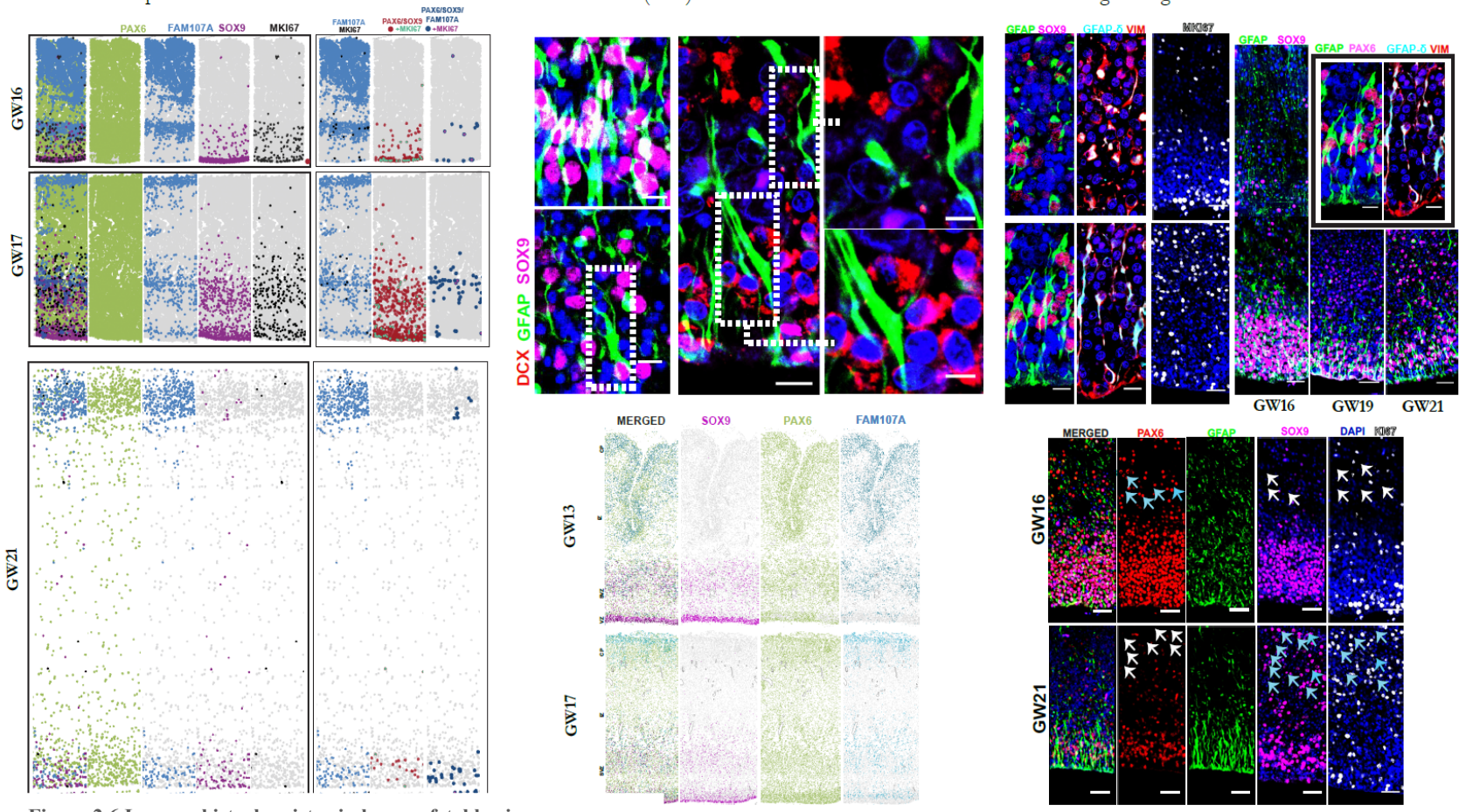


Figure 2.6 Immunohistochemistry in human fetal brain

a. Immunohistochemistry in human foetal brain sections. Coronal sections across three ages (GW16, GW17 and GW21) exposing the width of the cortex (SVZ to the pial surface) were stained for major NSC/RG markers, including PAX6, FAM107A, SOX9 and a cell-cycle marker MKI67. Heavy presence of this panel was observed along the SVZ.
b. Top panel: Expression of SOX9 and GFAP in the SVZ. SOX9 positive cells and GFAP positive processes extending radially could be seen. DCX positive but SOX(and GFAP negative new-born neurons can also be observed in the SVZ. Bottom panel: PAX6 was observed to be expressed by neronal lineage-committed progenitors, with persistent experessions in new-born neurons. Significant decrease in SOX9 pool along the SVZ and translocation of these cells to the outer SVZ (oSVZ) was observed .
c. To observe cellular density along the SVZ and the fate of NSC/progenitors, immunohistochemitry was performed in coronal human foetal brain sections. Sharp decline in SOX9 + and MKI67 (cycling cells) could be seen over time, inlcuding PAX6+ cells, indicating culmination of neurogenesis.

But what do we know about the fate of RG? Do they disappear post-neurogenesis? What is their role in defining germinal niche? To address this, we compared immunolabelling across three developmental time points GW16, GW19 and GW21 (Fig 2.6 a and c). We made several observations, including the formation of the outer subventricular zone (oSVZ) by translocation of SOX9-positive RG and depletion of SOX9-positive RG in the ventricular zone over time. Interestingly, cycling MKI67-positive cells (white) translocated from the SVZ to the oSVZ alongside SOX9-positive cells by GW21 suggesting culmination of the neurogenic function of the RG in the SVZ (Fig 2.6 c - bottom). Such topographic changes accompanied by subsidence of neurogenesis, delamination of the SVZ, translocation of MKI67/SOX9+ cells mark a historical moment in brain development that commemorates not just the end of neurogenesis, but the dawn of a specialised germinal zone that carries the secrets of the neural stem cells that are crucial for post-neurogenesis and post-natal era.

2.3.4 Fetal neural cultures demonstrated sphere-forming capacity and radial glial identity

Neural stem cells can proliferate and self-renew, differentiate into astrocytes, oligodendrocytes and neurons, and form neurospheres in restricted media. To establish the presence of neural stem cells in fetal neural cultures, we investigated these properties *in vitro.*

One of the hallmarks of stem cells is their ability to undergo self-renewal and maintain multilineage differential potential. Owing to the dynamic nature of a developing fetal brain, one would expect to see cells undergoing extensive cell division to meet the demands of the developing system and fulfill the needs of corticogenesis. To validate the cycling status and sphere-forming capacity of fetal neural stem cells, we performed cell-cycle analysis by assigning

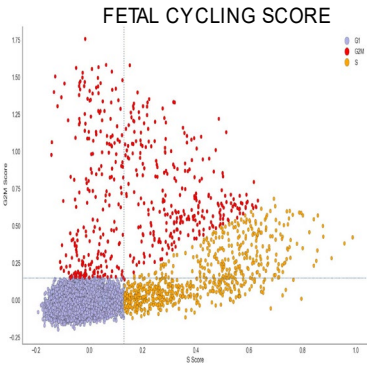
every cell a cell-cycle score to reveal the cell-cycle cycling cells in different cell-cycle phases (Fig 2.7a).

This revealed a massive number of cells in the human fetal dataset as cycling cells in the G1 phase (purple), G2M phase (red) and S phase (gold) (Fig 2.7a). This observation is line with the dynamic nature of the developing fetal brain as it meets the need of this developing system by undergoing extensive cell division and spinning off new neurons. This was consistent with the neurospheres observed *in vitro* and the radial morphology in adherent cultures (Fig 2.7b).

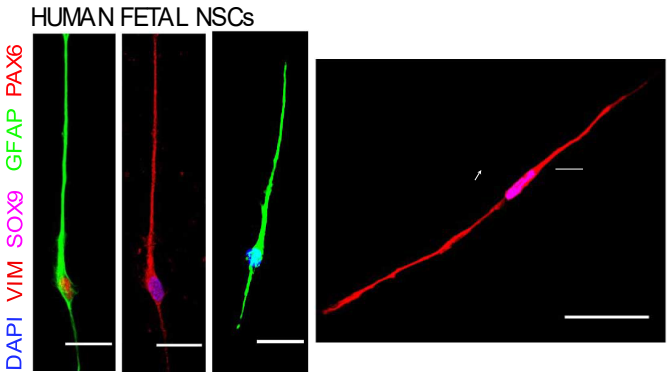
To further confirm the identity of radial glia in our adherent fetal neural cultures, we tested the same radial glia marker panel that we utilized for immunohistochemical investigations for our adherent cultures and found that pan radial glia markers, including VIMENTIN, SOX9 and GFAP were expressed by cells exhibiting radial morphology in our neural cultures (Fig 2.7c).

Cell cycle status is a widely used measure to assess the state of stem cells; it not only facilitates the identification of stem cells or progenitors but also reveals key developmental milestones. To explore the topographical and anatomical developmental changes, we tracked stem cells by targeting cycling cells in the human fetal brain. This immunohistochemical approach was sort of a longitudinal investigation as we examined these changes over multiple developmental time points (Fig 2.7c) to document the topographical and regional anatomical changes.

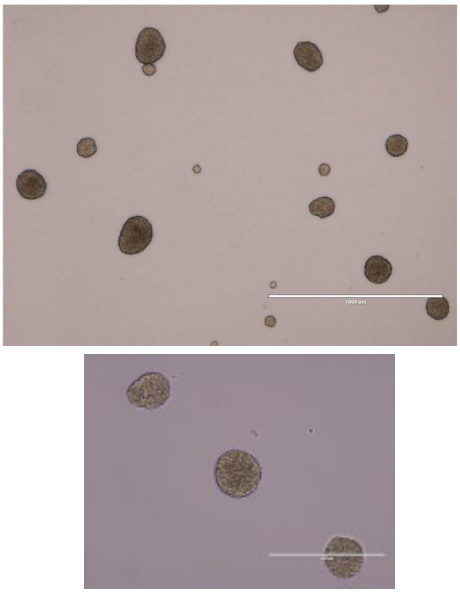
a. Cell-cycle status of cells in the human foetal dataset (n=3)



b. Expression profile of NSC/RG in thuman foetal neur sphere-derived cells.



c. Spheres derived from human foetal brain tissue



d. Cycling cell in the human foetal SVZ

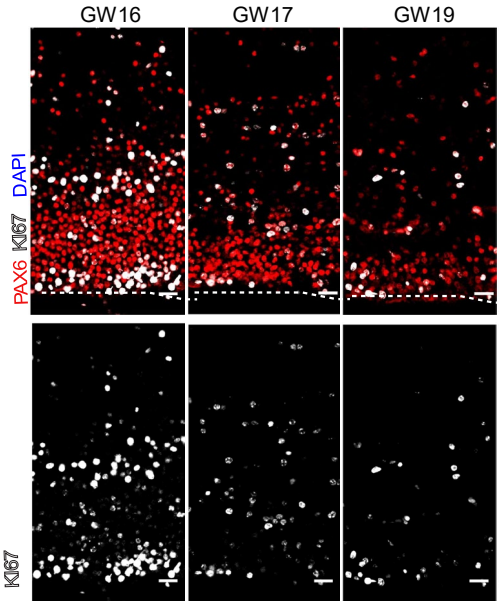


Figure 2.7 Cell-cycle status in the human fetal brain dataset and tissue

a. Cell-cyclestatus was assessed for human foetal SVZ dataset. Signatures for differentphases of cell-cyclewere utilisedto assign cellsto each of the cell-cyclephase. Differentcell-cyclephases are colourcodedG1-phase (blue), S-phase (yellow) and G2M-phase (red). Left panel: Human foetal cellswere scored for cell-cyclephases with a great numberof cellsactive cycling in S-phase and G2M-phase (reflective of highly active nature of development).

b. Immunocytochemistrywas performedon human foetal cells. Radial morphologytypical of RG cells was observed. Sphere-derivedcellswere grown on laminin in restrictedmedia; expressionof NSC markers, includingVIM (red), SOX9 (magenta), GFAP (green) and PAX6 (red) was observed. Scale: 10 um.

c. Spheres dervied from foetal brain tissue.

d. To validate the cyclingcell pattern and depletionof cyclingcells over time, immunohistochemistrywas performedin human foetal brain tissue sections. Expression patterns of MKI67, a cyclingcell marker, along with PAX6 (red) was observedat differenttime pointsduringfoetal development(GW16, GW17, GW19). This observationhintedat a decline in the number of cycling cells over time.

To achieve this, we immunolabelled human fetal brain sections from gestational weeks s16, 17 and 19. Given the heterogeneous nature of radial glia, we used a cell-cycle marker (MKI67) and a newborn neuron marker (PAX6) to visualize the dynamics and movement of cycling cells/ NSCs over time. The cycling cell population was restricted to the ventricular zone where PAX6+ newborn neurons were also observed (Fig 2.7c). We observed a sharp decline in cycling cell population by GW21, marked by MKI67 expression along with a diminishing newborn neuron population in the ventricular zone (Fig 2.7c). The newborn neurons, irrespective of the developmental stage, migrate along the fibers from the ventricular zone to the cortical plate. During the earlier stages of neurogenesis, RGs spin-off neurons in massive numbers in the ventricular zone; the newly formed neurons then migrate along the radial fibers out of the ventricular zone and towards the cortical plate. This dynamic production of neurons through extensive neurogenesis abates by GW19, a moment that, perhaps, marks the decline in neurogenesis (Fig 2.7c and d). This immunochemical investigation into cycling status in the ventricular zone coupled with newborn neurons revealed not only the neurogenic dynamics of the fetal radial glia but also the cellular dynamics of the germinal zone.

2.4 Summary

Fetal brain development is a remarkable feast celebrated by a single cell type that, miraculously, constructs one of the most complex and mysterious organs of the body by taking different shapes and forms. Yet, they all work in concert to get the job done by acting not only as the source of new cell types but also transport nascent neurons to their destination. It is awe-inspiring that though restricted to the ventricular and the subventricular zone, these cells have an extraordinary ability to coordinate with their distant counterpart – spatially heterogeneous radial glia – and put together this mysterious organ.

Neural stem cells have inspired scientists by their exceptional ability to produce progenies that are fundamental to the existence and the function of the brain. However, more astonishing is their ability to lay the framework via which they execute long-distance communication efficiently. These pathways not only hold key to understanding this connectivity, but also, may give us the power over controlling their function as and when required.

In this chapter, we have put forward our observations of a developing fetal brain, particularly radial glia, cell-by-cell to develop a roadmap of developmental trajectories and full spectrum of the neural stem cell hierarchy, which we would then use to identify adult human neural stem cells and explore their existence and functional significance. For our second objective, we conducted some fundamental exploratory work vis-à-vis the adult human germinal niche ventricular-subventricular zone (V-SVZ). Using the single-cell RNA-seq, we sought to reveal the cellular contents of the V-SVZ and translated our transcriptomics observations using immunohistochemical examinations to uncover the cellular topography of the adult human V-SVZ.

CHAPTER 3

scRNAseq-DRIVEN CELLULAR CHART OF THE HUMAN ADULT SUBVENTRICULAR ZONE IDENTIFIED A NORMAL AND NEOPLASTIC NEURAL STEM CELL-LIKE CELL POPULATION

Data/figures from this chapter have been used in the following research article:

Glioblastoma scRNA-seq shows treatment-induced, immune-dependent increase in mesenchymal cancer cells and structural variants in distal neural stem cells (Neuro-Oncology 2022)

Charles P Couturier, Javad Nadaf, Zhaorong Li, Salma Baig, Gabriele Riva, Phuong Le, Daan J Kloosterman, Jean Monlong, Andriniaina Nkili Meyong, Redouane Allache, Theresa Degenhard, Mariam Al-Rashid, Marie-Christine Guiot, Guillaume Bourque, Jiannis Ragoussis, Leila Akkari, Francisco J Quintana, Kevin Petrecca

3.1 Preamble

Adult Neural Stem Cells

Most adult organs retain a population of somatic cells with the ability to produce new cells for tissue homeostasis or repair, however, the brain was long considered an exception. This dogma was challenged in the 1960s, by Joseph Altman, when he and his team demonstrated that the addition of new neurons could take place in multiple regions of the adult mammalian brain, including the cortex and hippocampus (Altman, 1962; Altman & Das, 1965). Following this revelation, the laboratory of Fernando Nottebohm demonstrated neurogenesis in songbirds based not only on their electrophysiological properties and functional integration into the song-control nuclei (Burd & Nottebohm, 1985; Paton & Nottebohm, 1984). These findings established the foundation of primary progenitors/neural stem cells (NSCs).

In the adult mammalian brain, the majority of NSCs are found within the ventricular-subventricular zone (V-SVZ) along the walls of the lateral ventricles (Fig 3.1). These progenitors give rise to newborn neurons that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB) (Grelat et al., 2018; W. L. Li et al., 2018). Likewise, NSCs have also been shown to exist in the subgranular zone (SGZ) of the hippocampus where they generate excitatory neurons for the dentate gyrus (DG) (Ming & Song, 2011). Therefore, these two neurogenic niches are widely considered to be the primary reservoirs of regenerative NSCs.

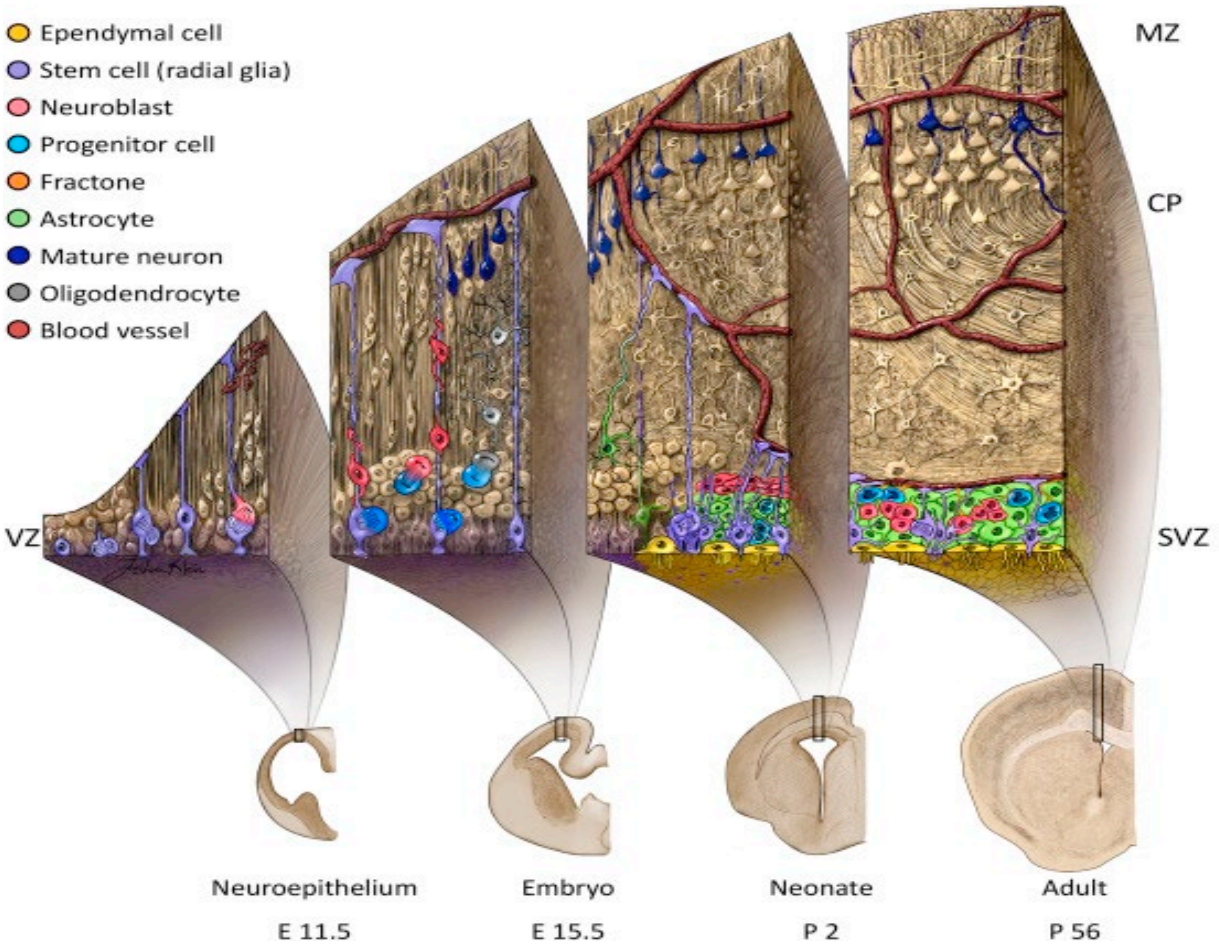


Figure 3.1 Development of the adult rodent V-SVZ from an embryonic neuroepithelium, and associated corticogenesis.
Figure adapted from (Conover & Todd, 2017)

A comparable germinal region in humans – SVZ NSCs:

The "gold standard" hippocampal neurogenesis method for identification and quantification of neurogenesis (birth dating studies with BrdU/IdU/14C) established in animal studies was applied to the human hippocampus by Eriksson in 1998, concluding that adult hippocampus in the same location and numbers as expected based on work in rats (Kempermann et al., 2018). Following this, stem cells with neurogenic potential were isolated from the adult hippocampus, including several studies reporting the detection of cell proliferation markers using

immunohistochemistry (Dennis, Suh, Rodriguez, Kril, & Sutherland, 2016; YWJ Liu et al., 2008; Palmer et al., 2001).

A glimpse into the adult subventricular zone and evidence of multipotent progenitors capable of multilineage differentiation potential in the adult human SVZ was provided by Sanai and team in 2004 (Sanai, Tramontin, Quiñones-Hinojosa, et al., 2004). They described a subventricular zone organization in the adult human brain that differed considerably from that of other vertebrates that had been studied. Although the study could not locate the precise location or identity of these progenitors, the authors concluded that adult human SVZ contains self-renewing, multipotent, astrocyte-like progenitors that can give rise to neurons in the absence of exogenous growth factors. They further stated that *"considering the size of the human lateral ventricular system, our work suggests that a substantial number of neural stem cells exist in the adult brain throughout life"* (Sanai, Tramontin, Quiñones-Hinojosa, et al., 2004).

Considering this, it is fair to say that our understanding of adult human neural stem cells, including their existence and identity is nebulous, to say the least. Despite the evidence discussed above, NSCs are yet to be given a definitive identity as they are confusingly referred to as astrocyte-like progenitors/ NSCs/ radial glia-like; but what are adult SVZ NSCs after all?

Identity Crisis:

While the adult human SVZ remains poorly understood, two recently published studies have renewed the adult neurogenesis debate: Sorrells and team made the case that hippocampal neurogenesis is not seen beyond childhood in humans (Sorrells et al., 2018), and Boldrini and colleagues reported the persistence of neurogenesis throughout aging (Boldrini et al., 2018). A

recently published study reported seeing newborn neurons and a modest decline in neurogenesis with age by probing post-mortem brain tissue from healthy adults (Fig 3.2).

But why is this idea hotly debated? Studies into adult neurogenesis have largely relied on immunohistochemical techniques, and given the rarity of well-preserved samples, techniques to identity newborn neurons vary greatly, and access to such precious tissue is very limited, if not impossible.

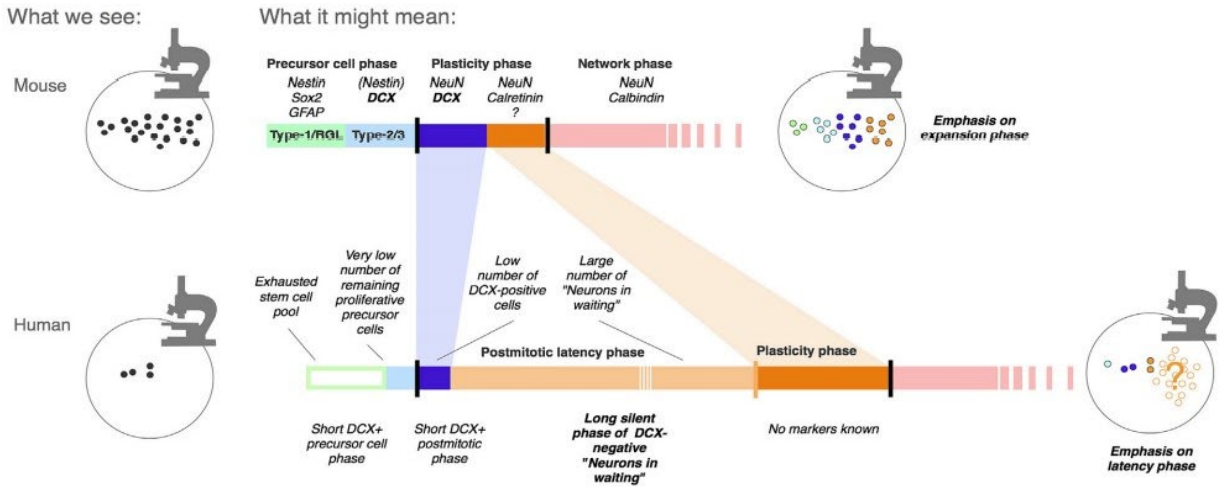


Figure 3.2 Consequences of species differences during neurogenesis
Besides methodological considerations, a hypothetical concept of a temporal decoupling of the stages of adult neurogenesis and species differences in marker expression, although largely speculative at this time, might explain part of the discrepancies between rodent and human data. The point is that alternative hypotheses that are consistent with the available data are possible.
Figure adapted from (Kempermann et al., 2018)

Glioma cancer stem cells:

The chaotic genome or the phenotypic state of cancer cells?

The discovery of brain tumor initiating cells (BTICs) in 2004 significantly shifted our focus away from the genomic investigations and prompted us to take a phenotypic view of the disease. Although some tumor cells were demonstrated to exhibit stem cell properties *in vitro* this was the first *in vivo* evidence that not only was robust evidence attesting the existence of

cancer stem cells in the tumor mass but also served as an impetus to entertain and pursue the possibility of glioblastoma to mimic the normal developmental trajectories. Following this discovery, coupled with the advent of single-cell transcriptomic technology, stout evidence started to flow in at a pace never witnessed before, bolstering the existence of developmental hierarchies. Unfortunately, since the discovery of CD133+ brain tumor initiating cells, we have not been able to make any significant progress vis-à-vis development of new therapies – but why is that?

The hunt for cancer stem cells and the demonstration of their tumorigenic potential *in vivo*, is indeed a significant finding but not without certain limitations. For instance, (Singh et al., 2004) compared the tumorigenic potential of CD133+ v/s CD133- tumor cells and observed the formation of tumors in NOD-SCID mice implanted with CD133+ cells but not in mice implanted with CD133- cells. Although this does underscore the tumorigenic potential of CD133+ cells, it does not demonstrate CD133+ cells being the cancer stem cells (the origin of the stem cell hierarchy).

Although this study supported the hierarchical cancer cell model, solid evidence demonstrating the existence of developmental hierarchy in glioblastoma tumor mass came to light only last year (C. P. Couturier et al., 2020), where the full spectrum of cellular hierarchy was uncovered that remarkably resembled neurodevelopmental hierarchy (Fig 4.1).

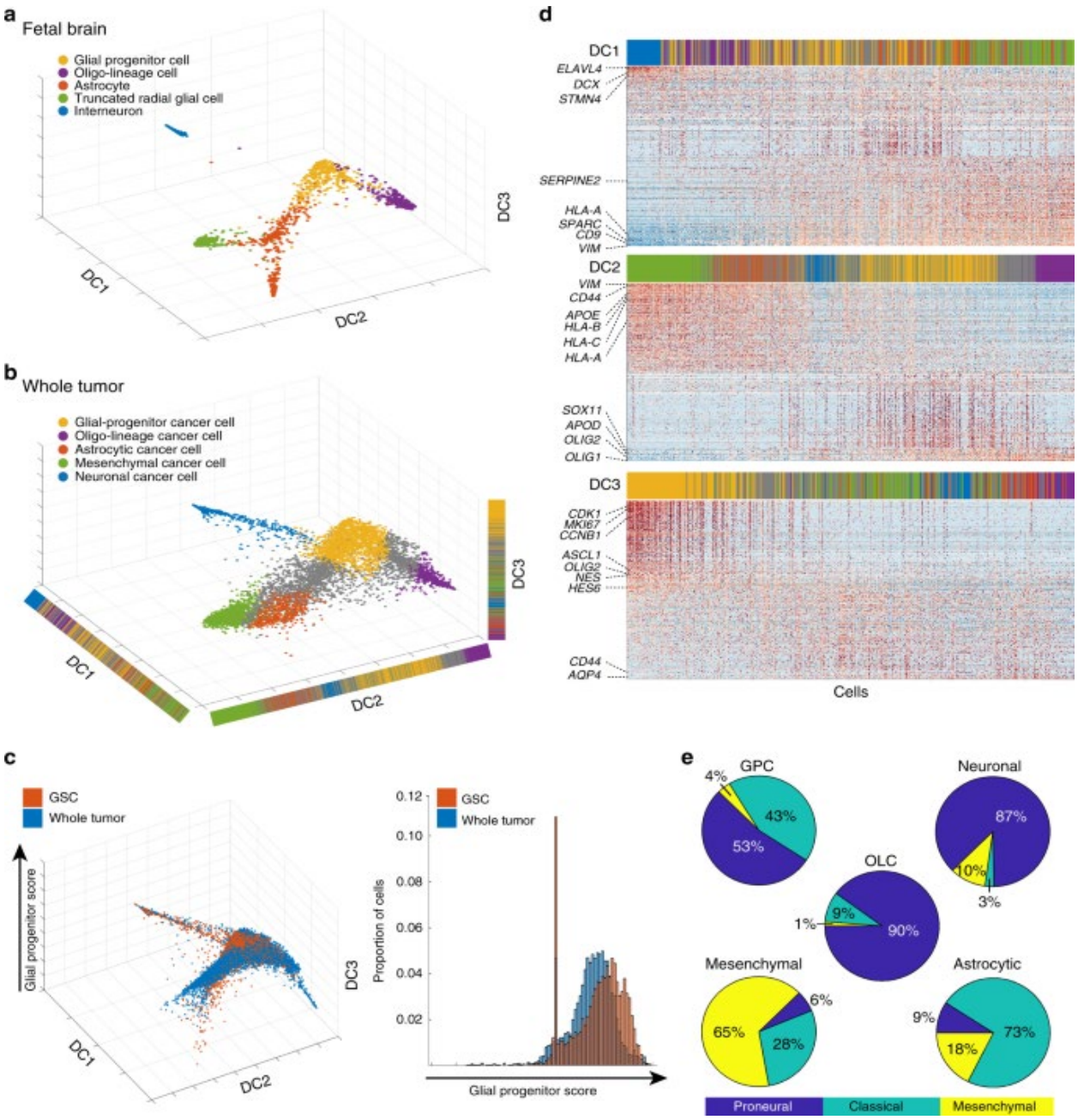


Figure 3.3 Diffusion plot of the projection of selected fetal cell types onto the roadmap.
Cells are colored by the cell type **b** Diffusion plot of the projection of an equal number of whole-tumor cancer cells from each patient onto the roadmap. Cells are colored based on their classification by linear discriminant analysis (LDA). Unclassified cells were colored gray. **c** Diffusion plot showing the location of glioma stem cells (GSCs) relative to whole-tumor cells (left) and histogram of glial progenitor score for GSCs and whole-tumor cells (right). An increase in proportion of cells with higher glial progenitor scores is seen in GSCs ($p < 1e\text{-}21$, two-sample Kolmogorov–Smirnov test). Only samples with paired GSC and whole-tumor data were used here. **d** Heatmaps showing relative gene expression (raw data) for cells ordered by each of the diffusion components of the roadmap. Genes are ordered from most correlated to least correlated with the diffusion component. The 200 most and 200 least correlated genes are shown. The top color bar indicates cell type classification from the LDA. Each color corresponds to the same classification as in **b**. **e** Pie chart for TCGA subtype by cell type for a subset of 1000 cells. Cell types are based on the LDA classification for all whole-tumor cells. and TCGA subtype was obtained using Gliovis.

Figure adapted from (C. Couturier et al., 2020)

This unification of the cancer stem cell model and neurodevelopment model revealed an unappreciated range of cellular hierarchy which, provided concrete evidence that not only attested the presence of a hierarchy in the tumor but strongly suggested a possible role of normal neural stem cells in initiating tumorigenesis. However, if the cancer mass were to adhere to the neurodevelopmental programs, cancer hierarchy must originate with quiescent stem cells and end with terminally differentiated cells, nevertheless, the cancer hierarchy observed here opened with fast-cycling cancer cells and closed with differentiated neural and glial progeny. So where is the quiescent head of the snake?

The cell-of-origin

Cancer stem cells and the cell-of-origin are viewed as two functionally different cell types, the former propagates the tumor and the latter initiates tumorigenesis. Normal neural stem cells that reside in the subventricular zone have carried the blame for being the glioma-initiating cells (cell that has accumulated genetic aberrations) for decades now. To test this, scientists have utilised Cre recombinase-expressing adenovirus injected into the SVZ of mutant mice with conditional *Tp53*, *Pten*, and *Nf1* or *Rb* knockout, subsequently leading to the formation of GBM; introduction of glioma-associated oncogenic events into NSCs or committed precursors, including glial precursors, oligodendrocyte precursors (OPC) and even matured astrocytes, for that matter, has resulted in glioma formation (Kim, Park, & Lee, 2021). These findings were further bolstered by Lee and colleagues (J. H. Lee et al., 2018) when they performed deep whole-exome sequencing of glioma-related genes and deep targeted sequencing of two hotspot mutations of telomerase reverse transcriptase (TERT) promoter of the SVZ which attested the

presence of glioma-related low-driver mutations in the SVZ of glioma patients. Lee et al. and colleagues further showed that the introduction of these driver mutations in the SVZ NSCs of a mouse led to the development of high-grade malignant gliomas. However, the demonstration of gliomagenesis in this mouse model displayed migration and formation of glioma away from the SVZ, whereas the SVZ samples from the glioma patients still harbored mutation-carrying cells. However, if gliomagenesis requires migration of mutated cells away from the SVZ to form gliomas, then why do they continue to exist in the SVZ of the glioma patients.

Although novel, this study has neither demonstrated the presence of adult NSCs in the SVZ nor has it established the identity of cell-of-origin. It does, however, strengthen the idea that the SVZ potentially harbors transformed NSCs that lead to tumorigenesis. The existence of NSCs in the adult human brain is highly controversial, and the mystery surrounding the cellular constituents of the adult SVZ and their potential role both in brain repair and neurological diseases, which remains unknown, is critical not just for the development of glioma therapies but also for early diagnostic strategies.

The cell-of-origin or the cancer stem cells

But what good is the past of a tumor? If the NSCs in the subventricular zone undergo an oncogenic transformation, and the tumor mass is propagated by a cancer stem cell population, what is the significance of the cell-of-origin in the present and the future of the tumor growth and development? and how are cancer stem cells different from the cell-of-origin? Perhaps, there is a crucial link or relationship between the two? But one is tempted to ask how there can be a significant link between two anatomically distant entities?

Tracing the roots of glioblastoma/developmental hierarchy to the subventricular zone

Perhaps there is more to it than meets the eye. Let us review all the evidence in chronological order that we have at hand:

1. We know that tumors are sustained and propagated by cancer stem cells that reside in the tumor (Singh et al., 2004).
2. We now know that glioblastoma is a hierarchically organised tissue that not only harbors cancer stem cells but also mimics neurodevelopmental trajectories (C. P. Couturier et al., 2020).
3. We also know that the SVZ of glioma patients potentially harbors cells with glioma-related mutations (J. H. Lee et al., 2018).

Considering all the evidence and findings, we go a step further and set out to uncover the cellular and molecular identity of the possible cell-of-origin and explore the possibility of gliomagenesis following the normal developmental framework; we achieve this by taking inspiration from the neurodevelopmental programs.

3.2 Aim and objectives

In the face of this renewed debate and existing evidence (Fig 3.2), we sought to resolve the cellular landscape of the adult human subventricular zone from glioma patients using the single-cell RNA-seq technology, thereby revealing the NSC population.

3.2.1 Objectives

The objectives of this chapter are:

1. To perform scRNAseq on human adult SVZ tissue, build a cellular chart and identify the adult neural stem population.
2. To map these transcriptomic-revealed cell types *in situ* in non-glioma adult human brain tissue.
3. To explore copy number variations (CNVs) in the human adult SVZ dataset to hunt down glioma cancer stem cells.

3.3 Results

3.3.1 Acquisition of intraoperative subventricular zone (SVZ) samples for scRNAseq

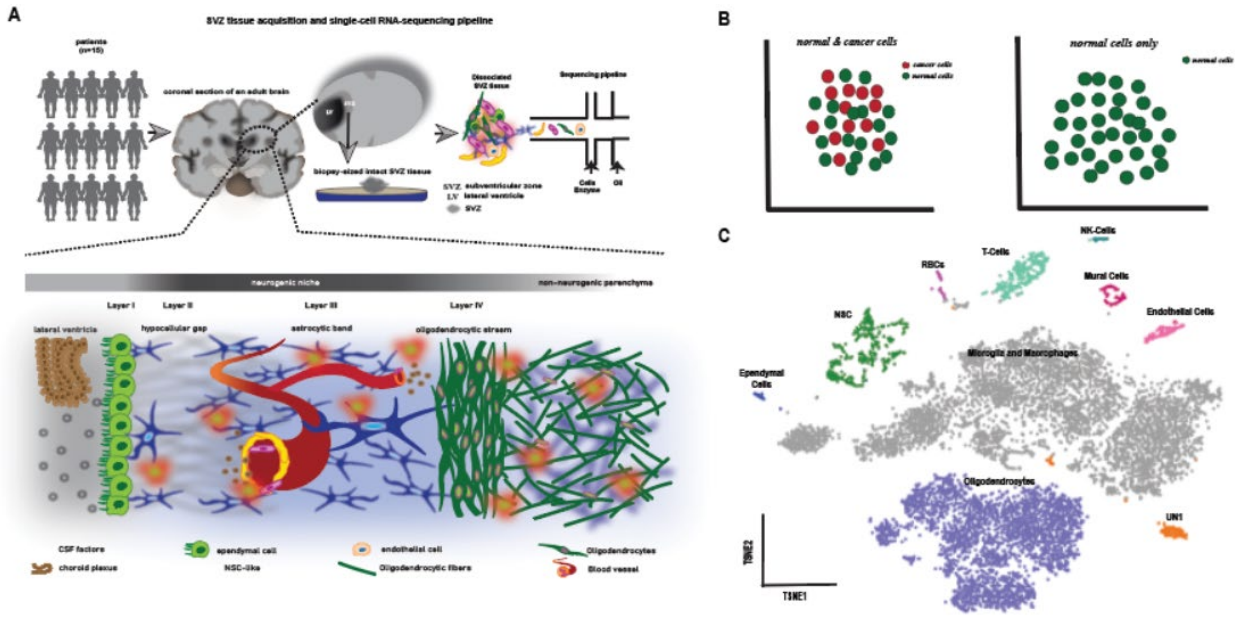
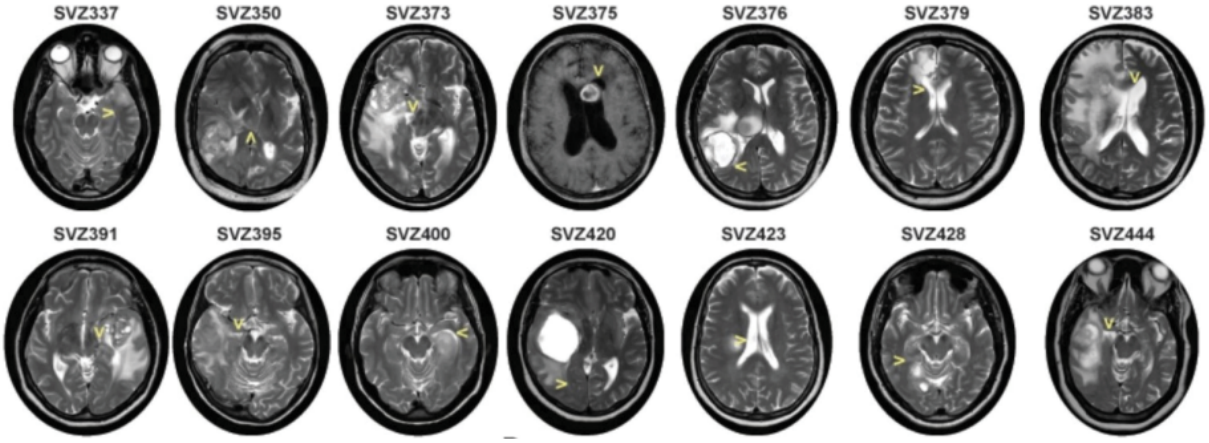


Figure 3.4 Sampling details and the workflow

A. Top panel: Schematic representation of the workflow. Adult human subventricular zone (SVZ) as sampled from 15 patients undergoing surgery. SVZ tissue was dissociated for single-cell mRNA sequencing. Bottom panel: Schematic representation of the adult subventricular zone. All four layers (Layer I to IV) of the SVZ have been shown. From left to right: ependymal layer (layer I), hypocellular gap (layer II), astrocytic band (layer III) and oligodendrocytic band (layer IV).
B. Schematic representation of exclusion of CNV-carrying cells prior to dimensionality reduction analysis; only cells without any CNV (9amplification/10deletion) were considered for downstream analysis.
C. tSNE visualization of adult SVZ cells colored by cell types. TSNE: t-distributed Stochastic Neighbor Embedding (n=15)

To perform single-cell RNA-sequencing (scRNAseq) on adult human subventricular zone (SVZ) tissue, SVZ was harvested from glioma patients undergoing surgery. A total of 15 patients were utilised in this study; a detailed workflow is presented in Fig 3.4. SVZ from these patients was harvested while making ventricular entry during tumor resection. SVZ regions that did not show pathological MRI signals that were to be removed during the tumor resection were chosen for sampling (Fig 3.5 A). A detailed description of the patients, including the age and diagnosis, utilised for this study are highlighted in Fig 3.5 B.

A



B

SAMPLE I.D	AGE	GENDER	LOCATION	DIAGNOSIS
SVZ337	53	Male	lateral ventricular wall	anaplastic astrocytoma
SVZ350	51	Male	lateral ventricular wall	glioblastoma multiforme
SVZ359	51	Male	lateral ventricular wall	high grade glioma
SVZ373	62	Male	lateral ventricular wall	glioblastoma multiforme
SVZ375	70	Female	lateral ventricular wall	metastatic adenocarcinoma
SVZ376	55	Male	lateral ventricular wall	glioblastoma multiforme
SVZ379	38	Male	lateral ventricular wall	recurrent astrocytoma
SVZ383	63	Male	lateral ventricular wall	recurrent glioblastoma
SVZ391	62	Male	lateral ventricular wall	large cell carcinoma
SVZ395	72	Female	lateral ventricular wall	glioblastoma multiforme
SVZ400	72	Female	lateral ventricular wall	glioblastoma multiforme
SVZ420	57	Female	lateral ventricular wall	glioblastoma multiforme
SVZ423	64	Female	lateral ventricular wall	glioblastoma multiforme
SVZ428	45	Male	lateral ventricular wall	glioblastoma multiforme
SVZ444	71	Female	lateral ventricular wall	Glioblastoma multiforme

Figure 3.5 Patients and sampling details

A. Magnetic Resonance Imaging (MRI) images of patients undergoing surgery; sampling target regions (lateral ventricular wall) are highlighted by arrows in yellow for all the patients.
B. Detailed list of all the patients utilised in this study, including age, sex and diagnosis.

Post harvest, SVZ tissue was subjected to digestion and dissociated to achieve single-cell suspension for sequencing. Single cells were captured and sequenced thereafter. A total of 10,834 cells from 15 patients were analyzed for downstream analysis that passed quality control checks. Considering the proximity of each tumor to the SVZ, we removed cells containing cancer-specific copy number aberrations from further analysis (Fig 3.4 B and 3.6). The total transcriptome of sequenced cells was visualised by a dimensionality reduction approach using the *t-distributed* stochastic neighbor embedding (tSNE) technique in two dimensions (Fig 3.4 C).

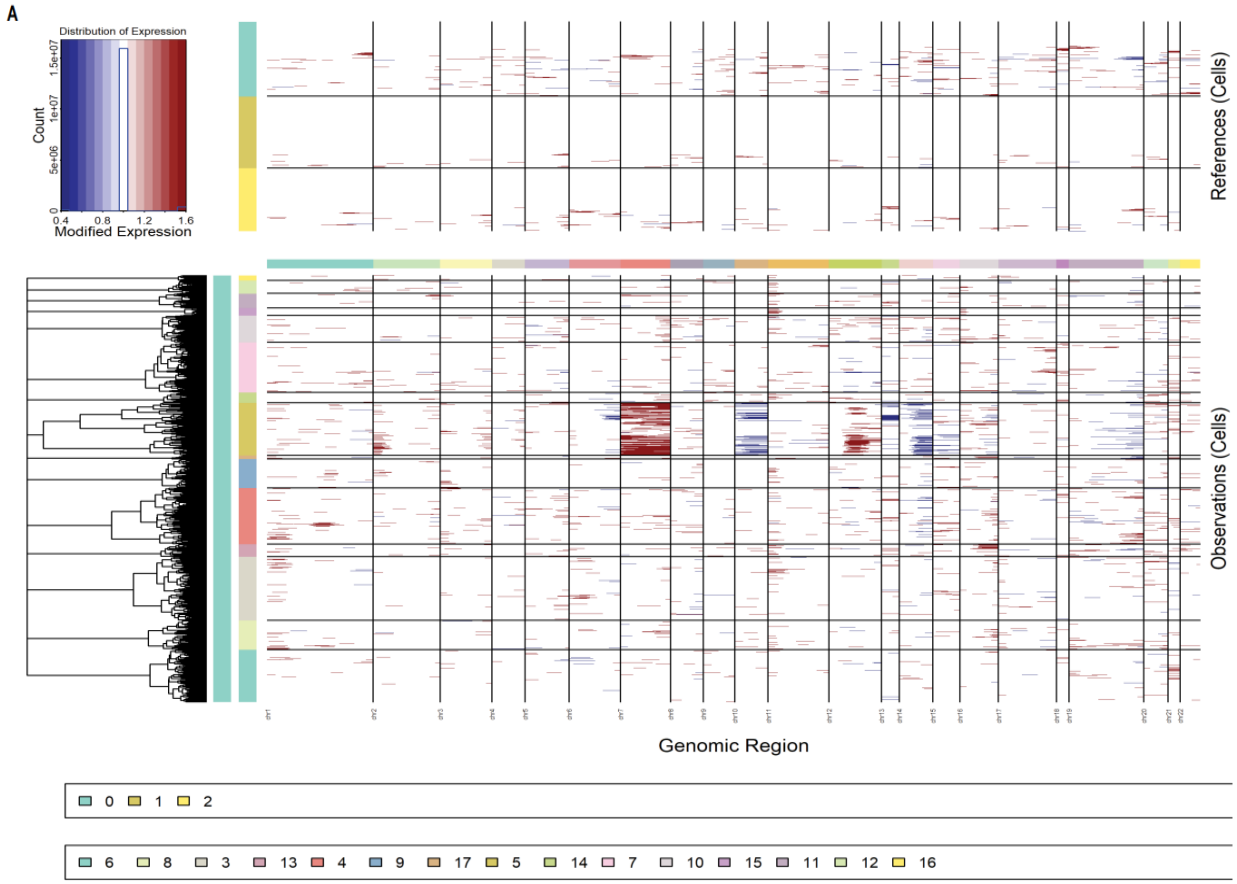


Figure 3.6 Removal of abnormal cells carrying 7 amplification/10 deletion chromosomal anomaly

A. CNA analysis of all SVZ cells were conducted, as a first step, to exclude the cancer cells from our downstream analysis. The analysis detected genomic aberrations that are common in GBM (e.g. amplification of Chr7 and deletion of chr10). No such aberrations detected in cells from non-GBM samples (n=3).

3.3.2 Cellular clustering revealed diverse populations in the human adult subventricular zone

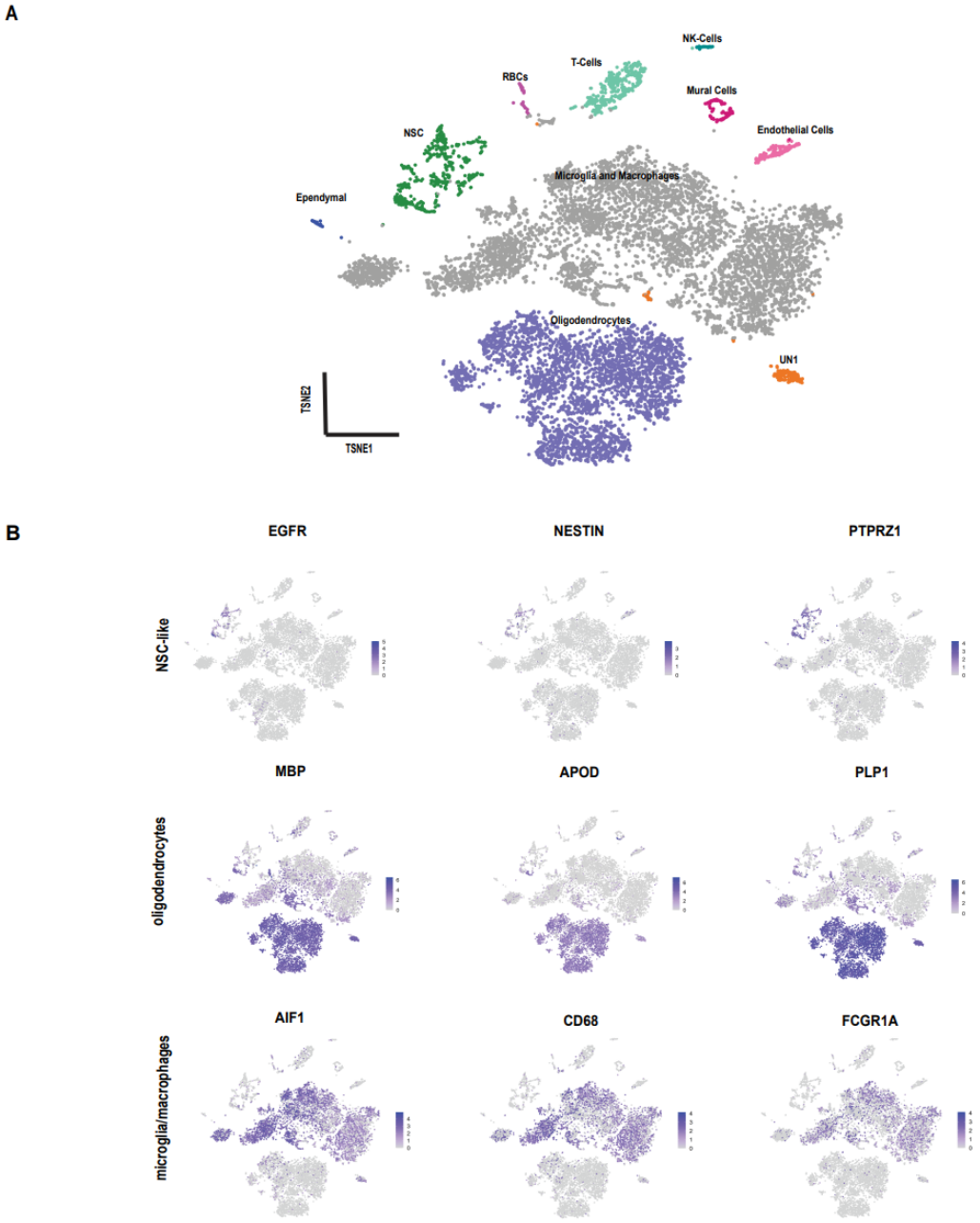


Figure 3.7 Identification of cell types in the human adult subventricular zone (SVZ) dataset

A. tSNE visualization of adult SVZ cells colored by cell types. TSNE: t-distributed Stochastic Neighbor Embedding
B. Expression of markers for major cell types in the dataset, including NSC-like, oligodendrocytes and microglia/macrophages.

t-SNE visualization revealed distinct clusters of cells; each dot represents a cell that is placed in the tSNE space based on the total transcriptome expression profile (cells with similar transcriptome cluster together) (Fig 3.7 A). To delineate the cell type of each cluster we utilized classical markers and visualised them in tSNE (Fig 3.7) which revealed 10 cell types, including oligodendrocytes; macrophage and microglia; neural stem-cells (NSC)-like; T cells; endothelial cells (ECs); mural cells; red blood cells (RBCs); natural killer cells (NK-cells); ependymal cells; and an unidentified cell cluster (Fig3.7 A).

Oligodendrocytes maintained expression of terminally differentiated oligodendrocyte markers, including PLP1, MAF and MOBP; macrophage/microglia expressed AIF1, APOC1 and HLA-DRB1; T-cells expressed NKG7, CCL5 and CST7; endothelial cells were defined by SLC2A1, IFI27 and CLDN5; mural cells expressed BGN, FRZB and TAGLN2; red blood cells maintained expression of HBD, HBE1 and HBA1; natural killer cells expressed KLRF1, MYOM2 and SPON2; ependymal cells were expressed DYDC2, CAPS and C1orf194. Lastly, NSC-like cluster expressed classical neural stem cell (NSC) markers, including GFAP, BCAN and EGFR. While cell types were successfully assigned to almost all the clusters only one cluster remained unidentified and was hence labeled as unidentified (UN1) (Fig 3.7).

Of all the cell types, two cell types (oligodendrocytes and microglia/macrophages) largely dominated our dataset (Fig 3.7 B).

3.3.3 Gene expression analysis further reveals a variety of human adult subventricular zone cell types

Figure 3.8 Cell-type annotation in the adult human subventricular zone dataset

A. Feature plots showing expression of classical markers utilised to annotate human adult subventrricular zone cell types.

To estimate the proportion of each cell type in the adult SVZ, we computed percentages of each cell type and found that the most abundant cell types (oligodendrocytes and microglia/macrophages) together accounted for approximately 86% of the cells in our dataset with 56% of microglia/macrophages and 30% of oligodendrocytes. Of the remaining 14%, NSC-like cluster accounted for 4.3% of the cells; the remaining 10% was constituted by endothelial cells, ependymal cells, NK-cells, T-cells, mural cells, red blood cells and the unidentified cluster (Fig 3.8 A and 3.9).

The astonishingly higher number of microglia/macrophages in the adult SVZ was a little unexpected as the current anatomical cellular description of the adult SVZ, which has been limited to immunohistochemical investigations in post-mortem human adult brain tissue, did not include neuroglia. Since the SVZ tissue was harvested from glioma patients, we attributed this to the pathological nature of the tissue and believed this heavy presence of neuroglia and macrophages to be a pathology-associated phenomenon.

In addition to defining these clusters based on classical markers, we probed entire gene expression profiles of these clusters and generated full-length signatures for each cell types, further strengthening the identity of each cell type. We achieved this by performing differential expression analysis amongst all the clusters and generated heatmap of top 100 genes differentially expressed in each of the cell types.

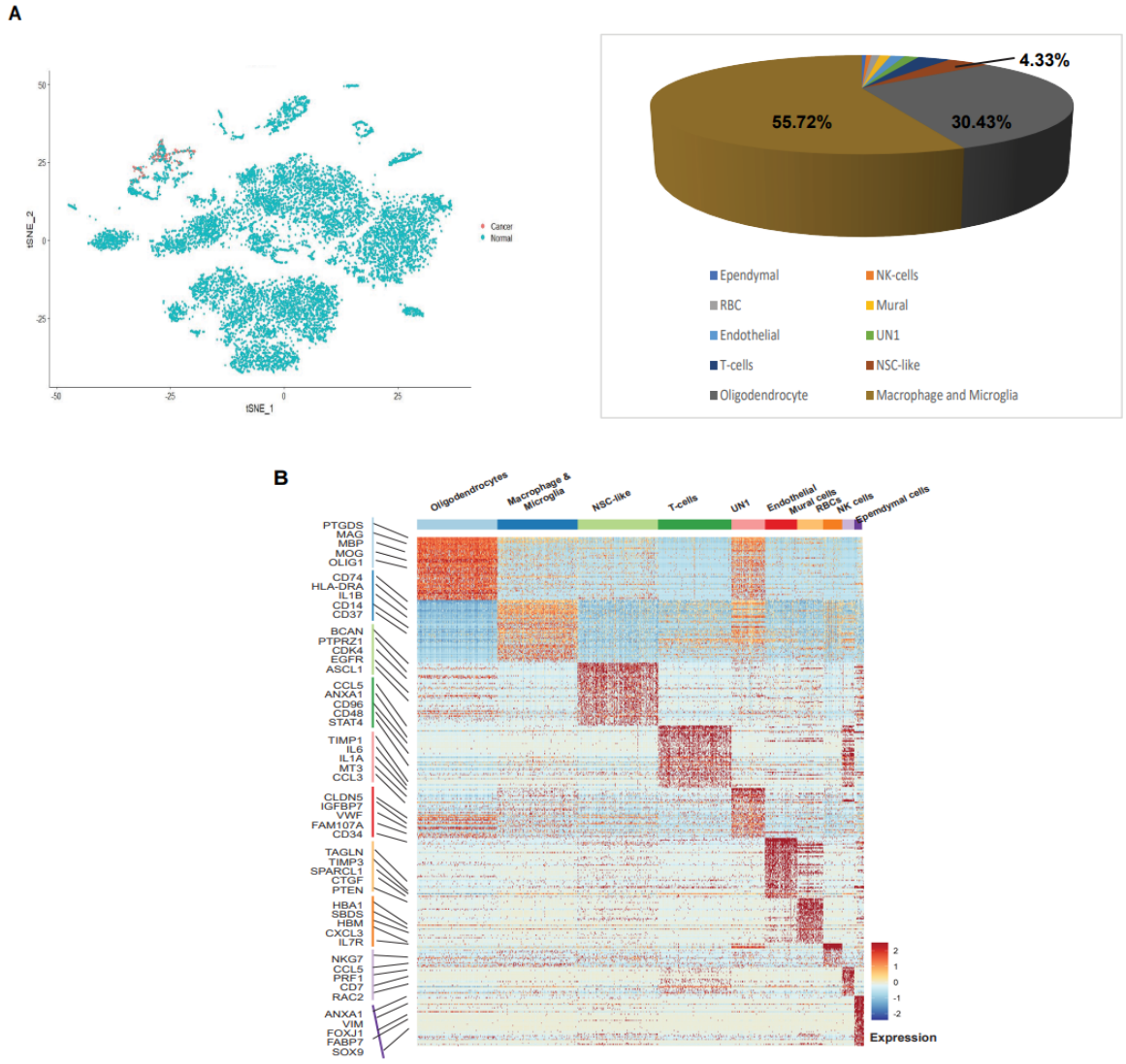


Figure 3.9 Visualisation of CNV-carrying cells and contribution of each cluster to the dataset

A. Visualisation of all the SVZ cells from all the patients (n=15) in t-distributed stocastic neighbour embedding (t-SNE) space. Neoplastic cells, defined by 7amp/10del, are highlighted in orange in the t-SNE plot (left panel). t-SNE plot showing cell type annotation for all the cells from 15 patients. C. Piechart representing percentage of cells in each of the clusters.
B. Heatmap of gene expression for each of the SVZ cell type using a maximum of top 100 genes for each cell type.

After characterizing transcriptomics defined adult SVZ cell types, we then sought to examine these cell types *in situ* in the neuropathologically normal autopsy human adult brain tissue. We achieved this by translating our transcriptomics-defined cell types to proteomics and performed immunohistochemical mapping of these cell types to validate these specialized populations in non-glioma autopsy brain sections.

3.3.4 *In situ* mapping of human adult subventricular zone populations further validate transcriptome-derived cell types

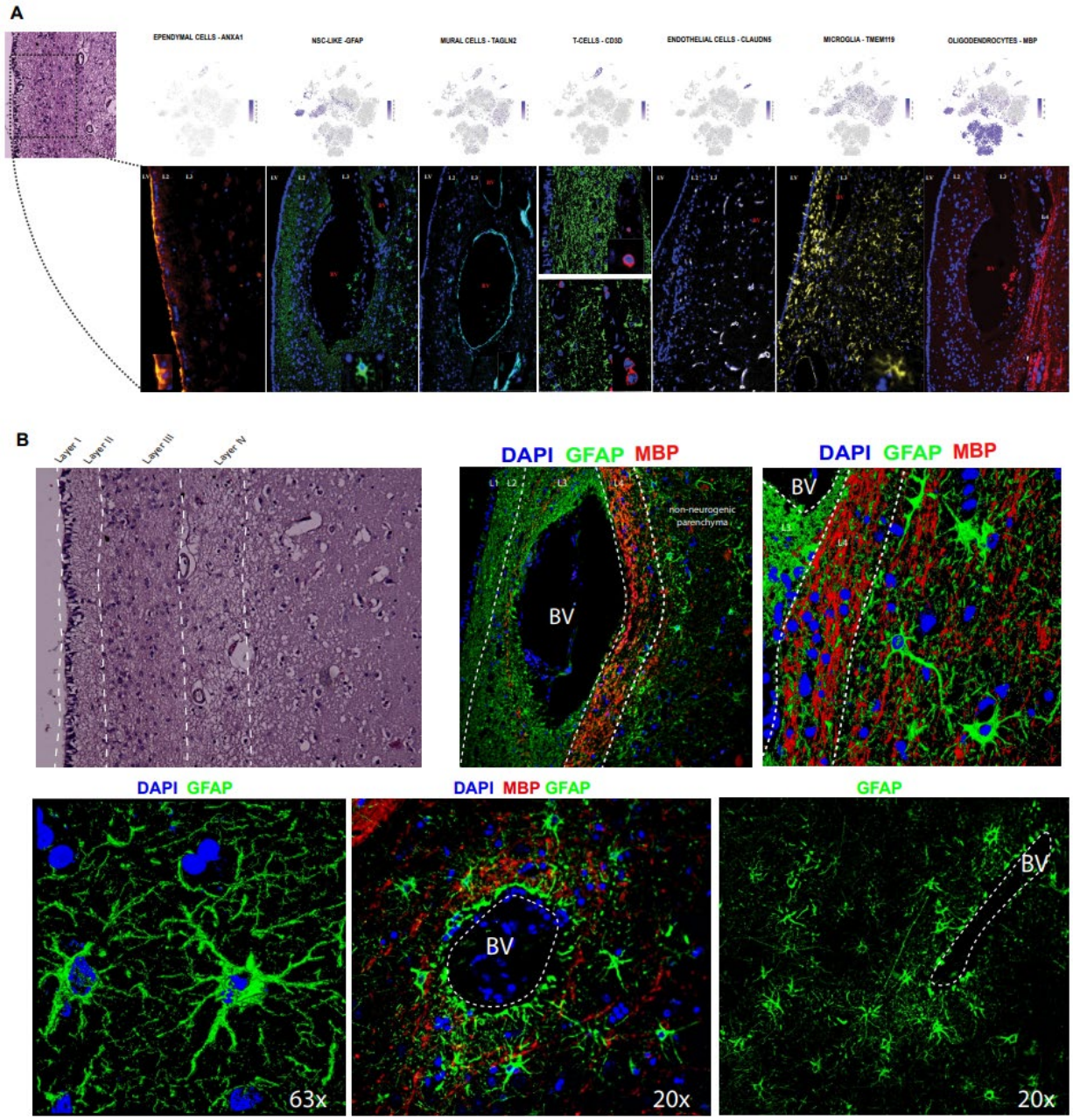


Figure 3.10 *In situ* mapping of SVZ cell types in the adult human subventricular zone and the adjacent parenchyma in normal adult human brain sections (A) Hematoxylin and Eosin (H&E) staining of the adult human SVZ. H&E staining was performed on neuropathologically normal adult human brain section, demonstrating cellular cytoarchitecture of the adult human SVZ. Right panel: Feature plots showing expression of key cell type markers. Immunohistochemical validation and distribution of the respective cell types identified in adult SVZ scRNA-sq dataset across the adult SVZ; left to right: ANXA1 for ependymal cells; GFAP (astrocytic band); TAGLN2 (mural cells); CD3D (T-cells); CLAUDIN 5 (endothelial cells); TMEM119 (microglia) and MBP (oligodendrocytes). Scale bar 100um. **B.** Hematoxylin and Eosin (H&E) staining of the adult human SVZ. Immunohistochemistry performed in adult brain tissue to visualise GFAP-positive cells both in the SVZ and the non-neurogenic parenchyma.

Our sequencing data revealed a variety of specialized cell types in the adult SVZ of glioma patients but corroboration of these populations in non-glioma SVZ was necessary to extricate any glioma-associated cell types and generate a clean cell atlas of the adult SVZ by mapping these populations in non-glioma SVZ. To achieve this, we utilized adult brain sections from adult human autopsy brain samples (n=3); diagnosis/disease status of all the autopsy brain samples was non-neuropathological.

Human adult SVZ has been grossly characterized to comprise four layers that are laid out adjacent to the lateral ventricles and covers an area of approximately a few hundred microns. Before proceeding with our immunohistochemical analysis, we established these four cellular in the subventricular zone: Ependymal layer (Layer I), hypocellular gap (Layer II), the astrocytic band (Layer III) and oligodendrocyte stream (Layer IV), via hematoxylin & eosin (H&E) assay demonstrated in Fig 3.10A (left panel; H&E image). While the ependymal layer is explicitly marked by a string of cells that run along the dorsal-ventral axis of the lateral ventricles, hypocellular gap is a nearly empty thin band populated by sparsely distributed cell bodies. Adjacent to it is the astrocytic band, the most prominent of all the layers, constituted by the highest number of cells. Owing to the presence of dense network of GFAP+ fibers, a well-known marker of astrocytes, this layer has been shown to contain GFAP+ astrocytes. Finally, a longitudinal stream of oligodendrocytes (Layer IV) serves as the junction between the SVZ and the non-neurogenic parenchyma.

Using the molecular signatures which we used to establish cell types in our scRNAseq dataset, we corroborated almost all the major annotated cell types *in situ.* Panel A in Fig 3.10 highlights all the main cell types. From left to right, Annexin A1, expressed in ependymal cells, clearly decorated the ependymal layer; TAGLN2, a marker of smooth muscle cells, highlighted

the mural cells; very few (~3) T-cells, marked with CD3D, were observed; TMEM119+ microglia were heavily present across the 4 layers of the SVZ and MBP, a classical marker of oligodendrocytes exclusively marked the oligodendrocyte layer of the SVZ. Unlike our scRNAseq dataset, no significant NK-cells and T-cells were observed in our autopsy brain sections. We attributed this to the pathological nature of the SVZ (whether traumatic or intrinsic disease-associated) as our scRNAseq SVZ samples were harvested from glioma patients. We used classical adult NSC marker GFAP to spatially corroborate the NSC-like cluster. While some cells in the scRNAseq NSC-like cluster maintained the expression of GFAP, it was very challenging to assign the GFAP+ process to a cell body with immunolabelling in autopsy brain SVZ. This is because GFAP is predominantly present as a fibrous network covering both the astrocytic band and the hypocellular gap.

Conventionally, adult NSCs of the SVZ as thought to be GFAP+ astrocytes, however, both morphologically and expression-related, this could not be established in the SVZ. Interestingly, in the non-neurogenic parenchyma (just outside the SVZ) clear GFAP+ astrocytes could be seen distinctly (Fig 3.10 B). The classical star shaped GFAP positive cells were seen evenly distributed across the tissue (except for the SVZ). Densely packed GFAP+ astrocytes were also observed around the blood vessels with GFAP+ processes extended to unsheathe the vessels (Fig 3.10 B).

3.3.5 Abundance of microglia observed in the human adult subventricular zone

Of all the cell types identified in our dataset, microglia & macrophages along with oligodendrocytes largely dominated our dataset. With oligodendrocytes constituting 30.4% of our dataset (3297 cells), microglia & macrophages contributed to 55.7% (6037 cells) of the dataset. Abundance of oligodendrocytes could be explained by the presence of oligodendrocytes, the layer IV of the subventricular zone, however, the number of microglia and macrophages, representing more than 50% of our dataset was intriguing.

The role of microglia in disease, including neuroinflammation and neurodegeneration, needs no introduction (Muzio, Viotti, & Martino, 2021). In brain tumors, tumor-associated microglia orchestrate the tumor microenvironment (Keane, Cheray, Blomgren, & Joseph, 2021). Because the SVZ dataset was generated from the subventricular zone samples harvested from glioma patients (although a few centimetres away from the tumor and MRI-normal SVZ), we reasoned that this could be a consequence of pathological nature of our samples and expected to see relatively resting state in our normal autopsy sections.

We used the classical microglia/macrophage marker transmembrane protein 119 (TMEM119) and performed immunohistochemistry in normal autopsy sections. Interestingly, remarkable abundance of microglia was observed; more excitingly, the neurogenic zone (subventricular zone) and the adjacent non-neurogenic parenchyma were almost anatomically delineated by two morphologically distinct microglia/macrophage populations (Fig 3.11).

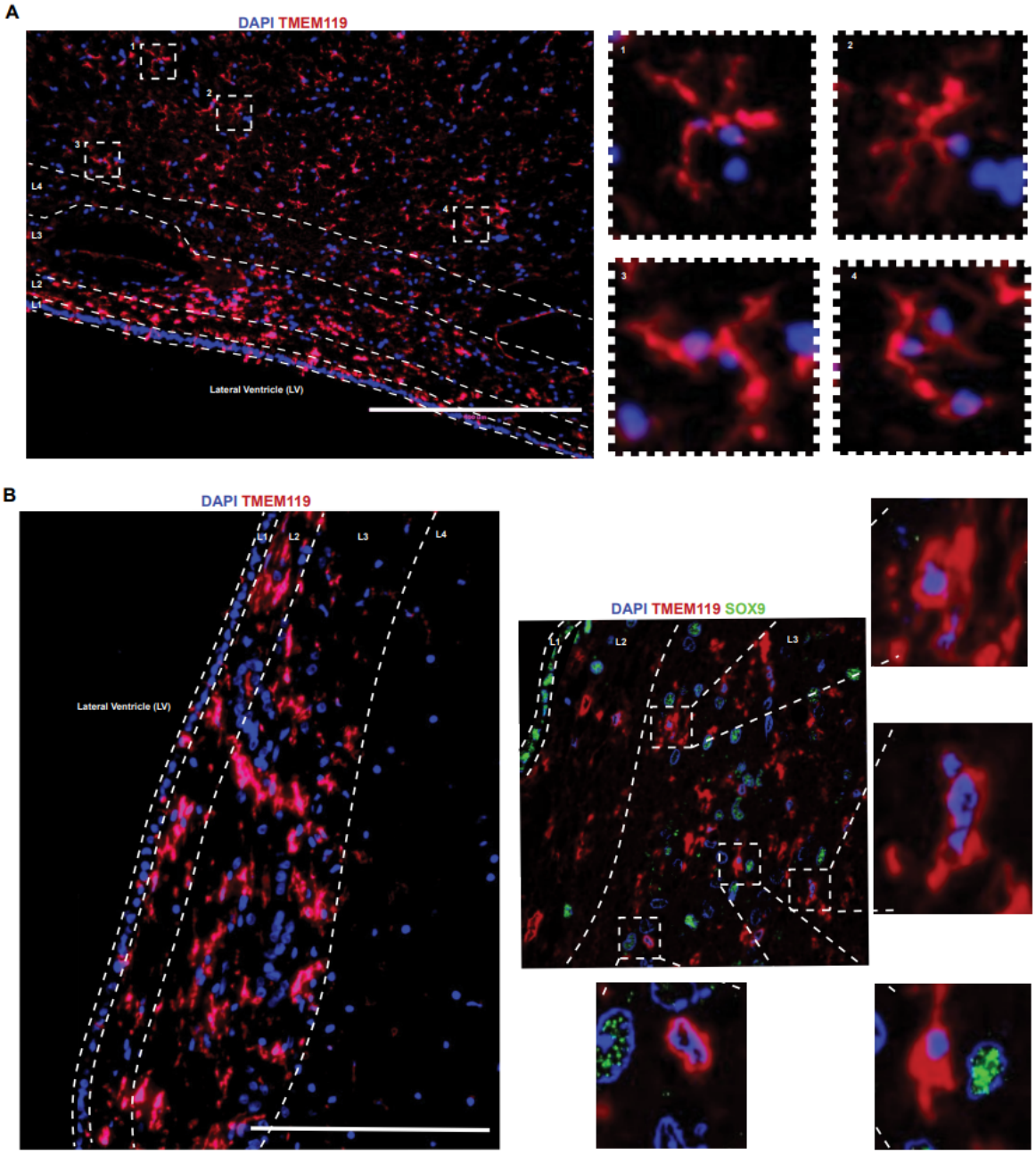


Figure 3.11 ***In situ*** **status of microglia in the adult human subventricular zone and adjacent parenchyma in normal autopsy brain tissue** **(A) (Left panel)** Immunolabelling of transmembrane protein 119 (TMEM119) of a coronal section of the adult human brain tissue section marking microglia. Dotted lines represents anatomical sub-sections (layers) of the adult SVZ (L1 - L4). Scale bar 400um. **(Right panel)** Boxed panels represent highlighted region in left panel (boxes are numbered respectively) in the non-neurogenic parenchyma adjacent to the neurogenic SVZ. Ramified morphology of the microglia indicates resting/surveillant microglia. **(B) (Left panel)** Immunolabelling of transmembrane protein 119 (TMEM119) in a coronal section of the adult human brain tissue marking microglia. Dotted lines represents anatomical sub-sections (layers) of the adult SVZ (L1 - L4). Amoeboid morphology was demonstrated by microglia in the SVZ niche. Scale bar 200um. **(Right panel)** Coronal section of the adult SVZ labelled for TMEM119 and SOX9. Boxed panels represent highlighted microglia with amoeboid morphology iin the non-neurogenic parenchyma adjacent to the neurogenic SVZ. Ramified morphology of the microglia indicates resting/surveillant microglia.

While the non-neurogenic parenchyma harbored microglia demonstrating typical star-like microglial morphology (Fig 3.11; panel A), microglia across all four layers of the subventricular exhibited amoeboid morphology (Fig 3.11; panel B). Furthermore, microglial processes of microglia close to the ependymal layer were also seen to protrude through the ependymal wall, contacting the ventricle (Fig 3.11; panel A). Consistent with our observations, microglia have been shown to undergo dynamic morphological rearrangements depending on their function and tissue status (Woodburn, Bollinger, & Wohleb, 2021). Surveilling/ resting microglia, also called homeostatic microglia, maintain branched morphology with complex ramifications, however, on the other hand, activated microglia undergo a morphological shift and take on an amoeboid or collapsed morphology (Kierdorf & Prinz, 2013; Ransohoff & Perry, 2009).

From development to adulthood, the versatile role of microglia in the development and maintenance of central nervous system (CNS) homeostasis is fundamental to normal functioning of the brain (Q. Li & Barres, 2018). As a brain-resident surveillance team, these interact with various other cell types and ensure the successful execution of various biological processes, including synapse engulfment and pruning, microglial migration and blocks neuronal excitotoxicity (Casali & Reed-Geaghan, 2021). More importantly, their role in adult neurogenesis alongside their role in neuroinflammation is only starting to emerge (Pérez-Rodríguez, Blanco-Luquin, & Mendioroz, 2021).

The presence of activated microglia in a neurogenic zone is a fascinating observation but whether these cells support any potential neurogenic process in the SVZ or use the neurogenic zone to support their self-renewal remains a mystery.

3.3.6 Gene expression signature of NSC-like cluster reveals heterogeneous nature of the cluster

So far, we have analyzed the transcriptomic clusters and using classical, well-known markers of known cell types, we assigned cell types to these clusters. To ensure the existence of these cell types in the normal SVZ, we further mapped these human SVZ identities *in situ* using immunohistochemical approaches.

Of all the cell types, the identity of the NSC-like cluster could not be established using the classical neural stem cell markers, including nestin (NES), glial fibrillary acidic protein (GFAP), epidermal growth factor receptor (EGFR), SRY-Box transcription factor 9 (SOX9), achaete-scute homolog 1 (ASCL1), also known as MASH1, SRY-Box transcription factor 2 (SOX2) and fatty acid binding protein 7 (FABP7) (Fig 3.12). While all the above-mentioned classical stem cell markers are widely used to define neural stem cells, each of these reflects a certain stem cell state. For example, NES, GFAP and SOX9 are used as pan-human adult NSC markers (Doetsch, Caille, Lim, García-Verdugo, & Alvarez-Buylla, 1999; Sanai, Tramontin, Quinones-Hinojosa, et al., 2004; Van Den Berge et al., 2010; C. Wang et al., 2011), whereas EGFR and ASCL1 expressing activated neural stem cells have been observed largely in adult mice brain (Blomfield et al., 2019; Chen et al., 2019; Cochard et al., 2021; Daynac et al., 2013; Morizur et al., 2018; Urbán et al., 2016). We also, surprisingly, observed decent levels of transcripts of new-born neurons and cycling cell markers, including CDK4, SOX2, SOX4 and SOX11 (Fig 3.12). Having assessed the expression of stem cell markers in our SVZ dataset, no single marker was globally expressed that defined the identity of the NSC-like cluster, rather, markers including those of activated cells, neurogenesis and neurodevelopment were unevenly expressed across the NSC-like cluster (Fig 3.12).

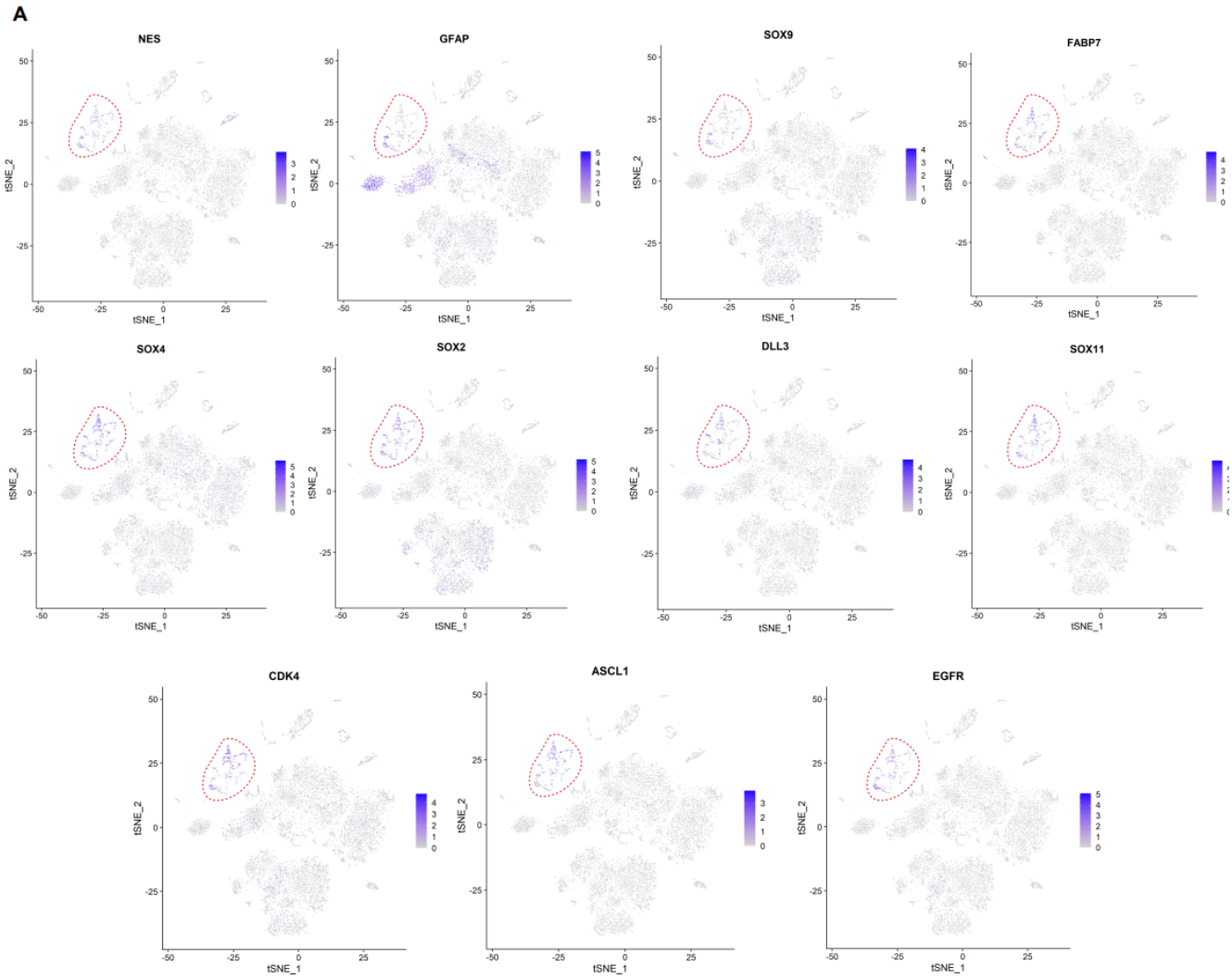


Figure 3.12 Feature plot visualisation of neural progenitor markers in the SVZ dataset
A. NSC-like cluster retained expression of neural stem cells (NSCs) as as lineage-related progenitor markers, including nestin (NES), glial fibrillary acidic protein (GFAP), SRY-box 9 (SOX9), fatty acid binding protein (FABP7), SRY-box4 (SOX4), SRY-box 2 (SOX2), delta-like ligand 3 (DLL3), SRY-box 11 (SOX11), cyclin dependent kinase (CDK4), achaete-scute homolog 1 (ASCL1) and epidermal growth factor receptor (EGFR)

Unable to conclude the identity of the NSC-like cluster, we further dug into the molecular nature of this cluster by performing pathway analysis (Fig 3.13).

3.3.7 Pathway analysis reveals enrichment of comprehensive neurodevelopmental processes in the NSC-like cluster

To gain insights into the biological pathways enriched in the NSC-like cluster we performed pathway analysis (Fig 3.13). The top 20 significantly enriched pathways are presented in panel A (Fig 3.13). Processes associated with neurodevelopment, including nervous system development (R-HAS-3560783), head development (GO: 0060322) and regulation of growth (GO: 0007423) were enriched. Interestingly, fundamental cellular programs associated with neurodevelopment, including regulation of neurogenesis (GO:0050767) and gliogenesis (GO: 0042063) were among the enriched terms (Fig 3.13). Enrichment of the GO term: regulation of neuron differentiation (GO: 0045664) and axon (GO: 0030424) was the most exciting one as the birth of newborn neurons in an adult brain is controversial. Given that the multilineage potential is one of the major features of neural stem cells (Gil-Perotín et al., 2013) enrichment of these cellular developmental programs was a crucial observation. Another key property of stem cells is their ability to undergo the cell cycle. Consistent with this, enrichment of cell cycle process (R-HSA-9675108) was also observed, indicating the mitotic potential of the NSC-like cluster.

To explore the relationship between these processes, we next visualised the network of these enriched terms (Fig 3.13; panels B & C). As expected, all the enriched pathways associated with development came together by demonstrating strong interaction reflected by the proximity of the hubs made up of similar enriched terms (Fig 3.13; panel C). Major developmental programs, including regulation of neurogenesis, regulation of growth, gliogenesis, cell part morphogenesis, transcription factor binding, regulation of neuron differentiation and head development showed the strongest significance (Fig 3.13; panel B and C).

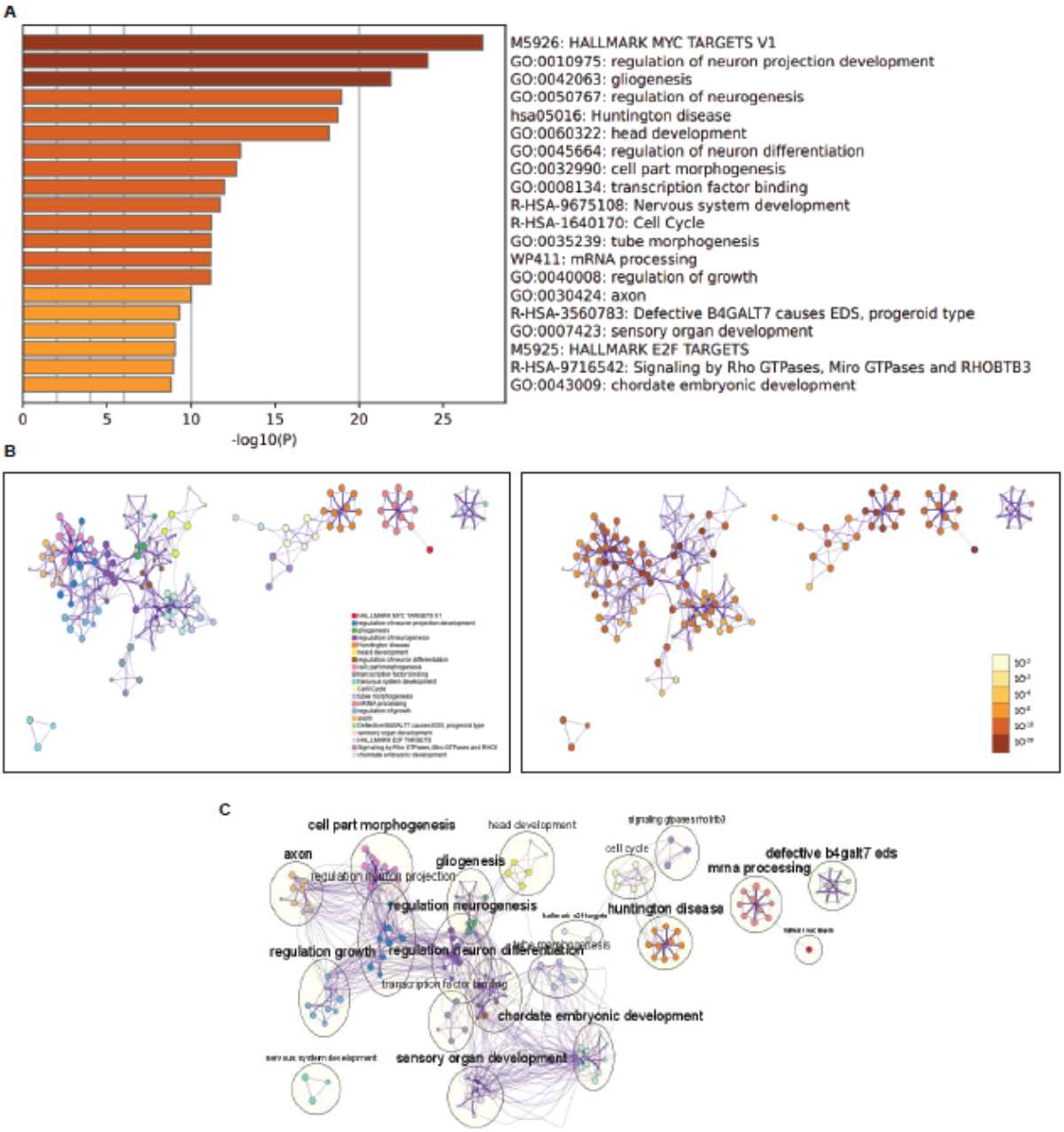


Figure 3.13 Pathway analysis of the SVZ NSC-like cluster

A. Bar graph of enriched terms across input gene lists, colored by p-values. Top 500 differentially expressed genes in the NSC-like cluster were used to perform the analysis. Top 20 clusters with their representative enriched terms (one per cluster). "Log10(P)" is the p-value in log base 10. "Log10(q)" is the multi-test adjusted p-value in log base 10.
B. Network of enriched terms: Left panel: Colored by cluster ID, where nodes that share the same cluster ID are typically close to each other; Right panel: Colored by p-value, where terms containing more genes tend to have a more significant p-value.
C. Network of enriched terms. Each hub is labelled by the respective enriched term and their proximity/interaction with other processes.

The enrichment of cellular and biological processes predominantly associated with neurodevelopment and the absence of processes associated with specialized cell function in our

NSC-like cluster further encouraged us to delve into the cellular nature of the NSC-like cluster. Using the same gene set of the NSC-like cluster, we assessed the enrichment of well-annotated cell type signatures (gene set enrichment analysis) established by published single-cell datasets (Fig 3.14).

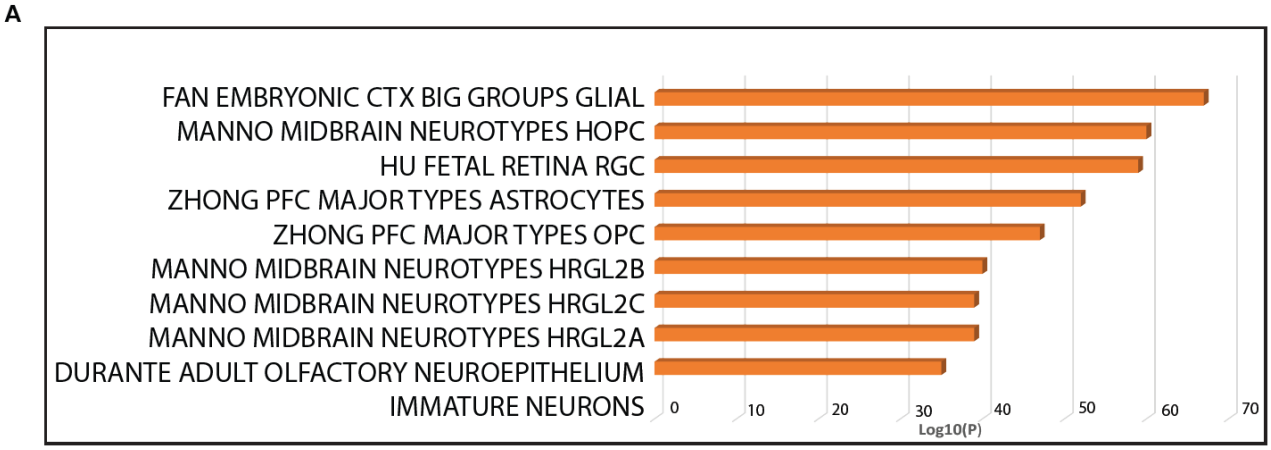


Figure 3.14 Cell-type analysis of the SVZ NSC-like cluster

A. Gene list enrichments are identified in the following ontology categories: Cell_Type_Signatures. All genes in the genome have been used as the enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. The top few enriched clusters (one term per cluster) are shown. The algorithm used here is the same as that is used for pathway and process enrichment analysis.

Consistent with the pathway analysis, neural stem cell signatures, including the diverse fetal radial glial populations, fetal immature neurons, embryonic glial cells and fetal OPCs were amongst the most similar signatures that correlated with our gene set for the NSC-like cluster (Fig 3.14). For example, strong enrichment for fetal radial glial cell type from Manno et al. and was achieved, both for retinal radial glial cells (RGC) (Hu et al., 2019) and the diverse population of radial glia, including human radial glia 2 (HRGL2) A, B and C (La Manno et al., 2016). More interestingly, enrichment for both fetal glial (Fan et al., 2018; Zhong, Zhang, Fan, Wu, Yan, Dong, Zhang, Li, Sun, & Pan, 2018), fetal oligodendrocyte progenitors (La Manno et al., 2016; Zhong, Zhang, Fan, Wu, Yan, Dong, Zhang, Li, Sun, & Pan, 2018) and neuronal

progenitors (Durante et al., 2020) was also observed. Unexpectedly, enrichment for immature neurons signature, particularly adult olfactory neurogenesis, and differentiation, was striking.

3.3.8 Copy number variation (CNV) analysis revealed CNV-carrying cells in the adult SVZ

As revealed by our cellular atlas of the SVZ, resident cell types revealed by scRNAseq covered various aspects of the neurogenic niche. However, no foreign cell cluster was documented in our cellular atlas, as we expected to spot a distinct cluster of cancer cells as an individual cell type.

To verify the absence of cancer stem cells in the SVZ, we explored copy number variations (CNV) in all the SVZ-derived cells. SVZ of patients with glioblastoma (n = 11), IDH-mutant glioma (n = 1), and metastatic brain tumors (n = 2) was utilized to call CNVs using CNV calling method (please see methods).

CNV analysis of all the SVZ-derived cells detected anomalies in a proportion of SVZ cells (Fig 3.15). Major CNVs observed in these cells included amplification of chromosome 7 and deletion of chromosome 10; in addition to other irregular CNVs, including amplification of chromosome 12 and deletion of chromosome 14 in some cells. Remarkably, CNV-carrying represented a portion of cells from the NSC-like cluster.

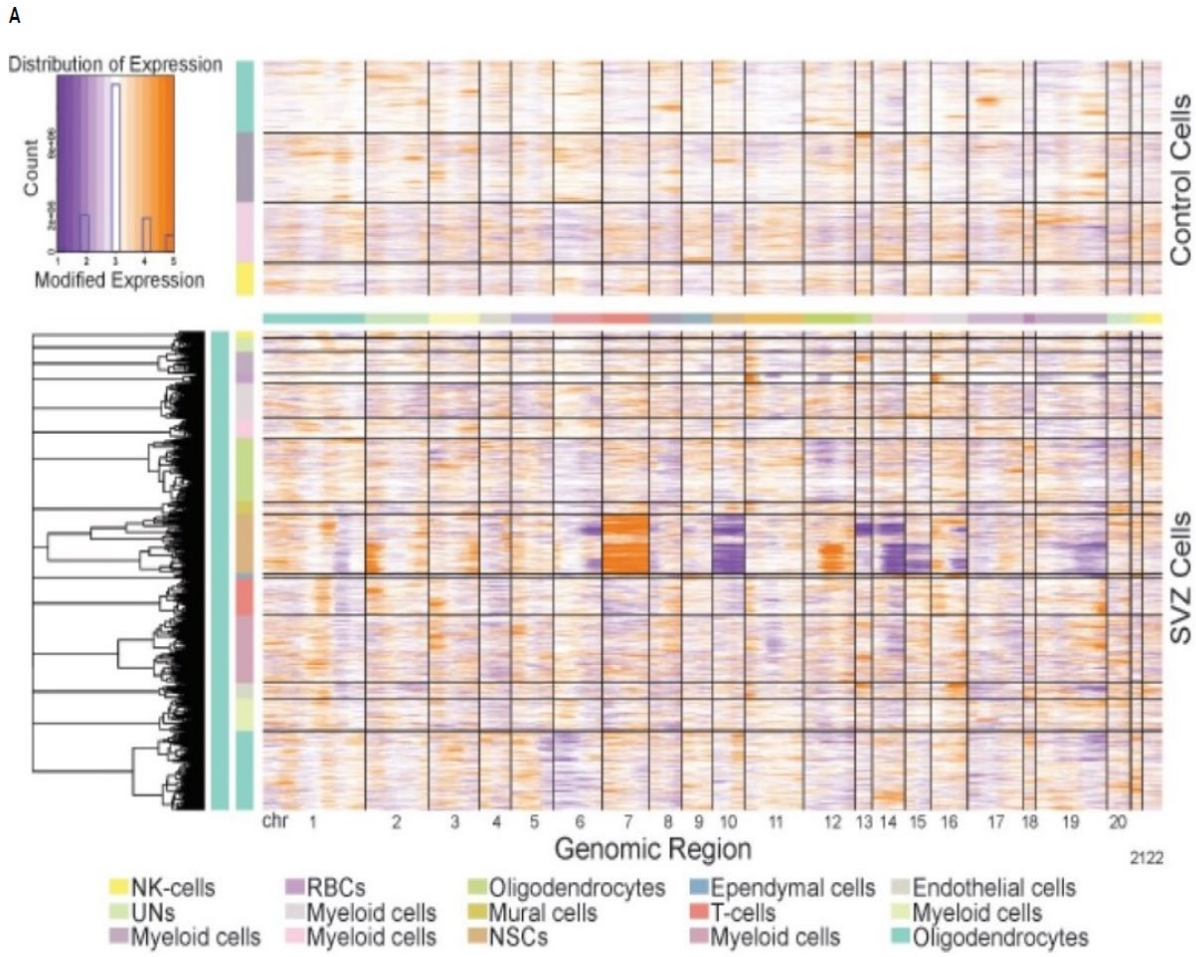


Figure 3.15 Identification of CNV-carrying cells in the human adult SVZ dataset

A. Heatmap visualisation of CNV in the adult human SVZ dataset inferrred at the RNA level.

3.3.9 CNV-carrying cells were traced back to the NSC-like cluster

One hundred and eighty-three of 578 cells in the NSC-like cluster contained statistically significant chromosome 7 amplification and chromosome 10 deletion (NSC-Chr7amp/Chr10del); interestingly; no other cell type harbored these anomalies (Fig 3.16; panel A and B). These CNV-carrying cells were only derived from the SVZ of patients with IDH wildtype glioblastoma. Normal NSCs (NSC-like cells) and NSC-Chr7amp/Chr10del cells clustered together with a high degree of similarity (Fig 3.16). The overall NSC-like cell type signature and

the NSC-Chr7amp/Chr10del signature were more like the NSC-like normal signature than any of the cancer meta-programs (data not shown).

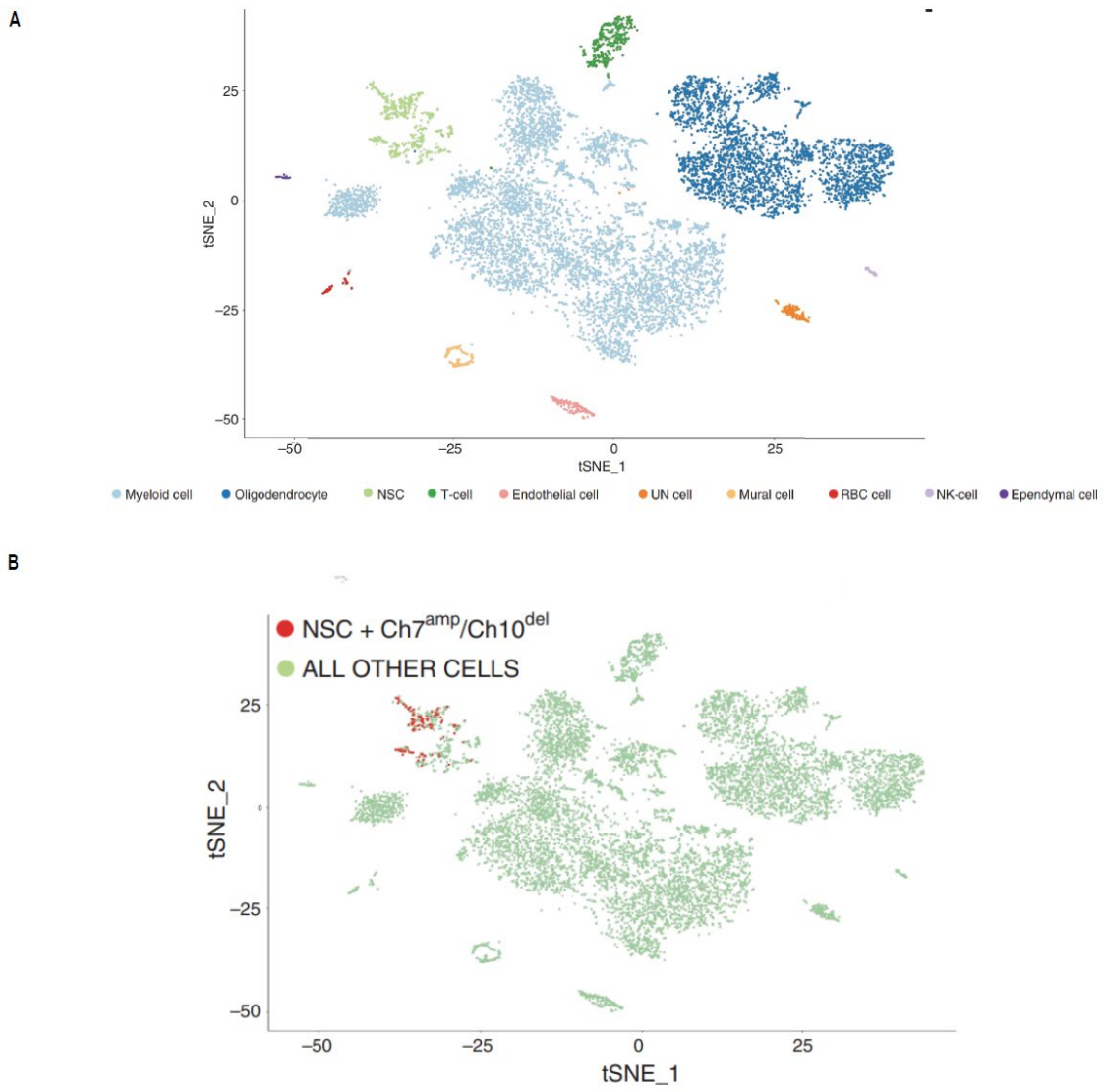


Figure 3.16 Identification of CNV-carrying cells in the NSC-like cluster

A. Visualisation of adult SVZ dataset with cancer cells.
B. Visualisation of CNV-carrying cells in the adult human SVZ dataset (tSNE); contribution of each patient to every cell type.

3.4 Summary

In this chapter, we achieved our second objective of building transcriptomics-driven cellular taxonomy of the human adult neurogenic niche (the subventricular zone) and, thereafter, charted these cellular identities *in situ*. By reconstructing the cellular cartograph of the human adult neurogenic zone, we characterized cell types by defining complete gene signatures but also by spatially mapping these cellular identities in normal human adult brain tissue.

We have achieved cellular clarity of the human adult neurogenic niche, a niche that has long remained a mysterious zone for developmental neuroscientists, neurobiologists, and cancer biologists (brain cancers) alike. We have revealed how the human adult subventricular zone becomes a transformed region from what it was during fetal development. Heavily populated by diverse radial glial populations during corticogenesis, the adult subventricular zone is largely dominated by specialized cell types in adulthood. We further corroborated these cell types *in situ* in autopsy brain tissue from individuals with no cancer or known neurological pathology.

In combing through the subventricular zone, cell-by-cell, our chief objective was to investigate the presence of adult neural stem cells and establish an elaborate transcriptomic signature of this cell type, if any. Using classical, well-known markers of neural progenitors, we identified a putative population that exhibited an NSC-like molecular profile. By further leveraging biological processes-related information embedded within the transcriptome of NSC-like cluster and reconstructing biological pathways by using the differentially expressed genes, we learned that the NSC-like population was a cluster of cells that exhibited various stem cell-related properties, including maintenance of NSC and lineage-specific progenitor markers expression, multilineage differentiation potential. Unlike the rest of the SVZ cell types, we could

not reliably identify NSC-like clusters in normal brain tissue. This was mainly due to two reasons:

1. NSC-like cluster displayed a comprehensive stem-like transcriptome, including markers associated with cell cycle, NSC progenitors, neuronal progenitors, glial cells, and oligodendrocyte progenitors; the expression of these markers greatly varied within the NSC-like cluster.
2. The classical marker used to identify adult SVZ NSCs is GFAP, however, given the fibrous nature of this protein, the entire astrocytic band was covered by GFAP-positive processes, making it challenging to assign it to a specific cell.

Owing to the disparate nature of fully matured different cell types, it is much easier to distinguish specialized cell types. For example, the transcriptome of a neuron is entirely different from a transcriptome of a glial cell. However, stem cell pools are composed of non-specialized, unrefined/undifferentiated cells that share core stemness programs but retain subtle molecular differences and only exist in transient states. Therefore, it is much easier to distinguish differentiated, specialized cells from undifferentiated/progenitor cells.

Another advantage of building a cellular atlas of the adult SVZ was the opportunity to be able to call neoplastic cells with a cell type. Previous bulk DNA/exome sequencing of the SVZ, which only one study has been able to achieve (J. H. Lee et al., 2018) did uncover cells with glioma-related genetic anomalies, both amplification and deletions, however, were unable to show these anomalies specific to any cell type, thus leaving the question of the cell-of-origin unanswered. Nevertheless, full characterization of the cell-of-origin/earliest cancer stem cell heavily relies on accurate profiling of adult neural stem cell pool and robust, concise description

of *bona fide* neural stem cells. It is worth noting that the cells carrying CNVs did not cluster as an independent cell type, nor did they bear any transcriptomic similarity to any other specialized type in our SVZ dataset – except for the NSC-like cluster. Rather, these neoplastic cells carrying 7amp/10del were traced back to the NSC-like cluster with robust similarity to the NSC-like cluster. At this point the both CNV-carrying and normal cells in the NSC-like cluster could not be described beyond progenitor-like/NSC-like cells.

The discovery of NSC-like cell cluster and novel biological heterogeneity thereof was extremely encouraging; however, the identity of this cluster, beyond enrichment of NSC-related programs, could not be conclusively confirmed in our NSC-like cluster. The current understanding of neurodevelopmental hierarchy, mainly informed by scRNAseq studies, both from human fetal development (Darmanis et al., 2015; Fan et al., 2018; La Manno, Soldatov, Zeisel, Braun, Hochgerner, Petukhov, Lidschreiber, Kastriti, Lönnerberg, & Furlan, 2018; Zhong, Zhang, Fan, Wu, Yan, Dong, Zhang, Li, Sun, & Pan, 2018) and mouse subventricular zone (Llorens-Bobadilla et al., 2015; Mizrak et al., 2019; Shin et al., 2015), would dictate that the neural stem cell pool is a linear chain of cellular states with subtle molecular differences that proceed in a sequential manner. Of main importance in this pool is the cell type that sits at the origin of this chain. However, our NSC-like cluster not only bore classical NSC signature but also demonstrated local lineage-related progenitor signatures that greatly varied locally within our NSC-like cluster. Given this local molecular variation in expression patterns within the NSC-like cluster we reasoned that the presence of more differentiated and specialized cell types in our dataset forced all the progenitors into a single cluster burying a rather molecularly diverse population.

Genomic heterogeneity, further complicated by therapy-induced selective pressure, in glioblastoma has largely been seen as a therapeutic bottleneck. However, recent longitudinal study in de novo-recurrent matched human samples have revealed phenotype plasticity rather than genetic evolution potentially explain therapy failure (L. Wang et al., 2022). Similarly, in the context of transcriptomic heterogeneity, Wang et al. demonstrated that the phenotypes of proliferating glioblastoma cell reside on a single axis of variation (L. Wang et al., 2019). Reflecting a common progenitor cell type as the source of tumor heterogeneity, Wang et al. reconstructed gliomagenic trajectories and identified a persistent NSC-like population at all stages of tumorigenesis (X. Wang et al., 2021). In light of these data and in the context of the CNV-carrying SVZ cells from our dataset, conservation of early molecular events driving tumorigenesis and conservation of the transformed cell type has crucial implications in gliomagenesis and developmental programs.

Although recent single-cell atlases have enabled identification of cell types and states remarkably effortless, scarcity of human adult NSC single-cell datasets made it challenging to conclusively verify and reliably identify adult NSCs. Enrichment of neuronal signatures and glial signatures in the NSC-like cluster inspired us to carry out in-depth characterization of the NSC-like cluster. To achieve this, we drew inspiration from developing human fetal brain with a focus on cell populations that participate in brain development and orchestrate corticogenesis, including radial glia.

In the next chapter, we dig deeper into the molecular details of the NSC-like cluster. Using neurodevelopmental programs as our guiding tool, we use fetal progenitors and developmental trajectories as our framework to disentangle NSC-like cluster and extract any crucial cell states embedded within the NSC-like cluster by resolving subtle molecular details.

CHAPTER 4

Integrating fetal progenitor populations with adult SVZ NSC-like cells disentangled unique progenitors with neurogenic and gliogenic properties and features of brain repair and regeneration

Data/figures from this chapter have been used for a research article currently being prepared for submission.

4.1 Preamble

Stem cell classification – a hierarchically organized system

A fundamental question in development and stem cell biology has revolved around the nature of stem cells and the mechanisms that are utilized by these cells to execute their function. Our understanding of stem cell behaviour has largely been informed by their ability to generate specialized diverse cell types by undergoing self-renewal (Clermont & Leblond, 1953; Fuchs & Chen, 2013; Leblond & Clermont, 1952; Leblond & Stevens, 1948; Watt, Hogan, & LM, 2000). This has been demonstrated by lineage-tracing methods *in vivo* and sphere-forming capacity *in vitro* transformed over the decades.

Classically, stem cell populations (particularly neural stem cells), have classically been considered to exist as discrete homogeneous population of cells. Typically, the definition of neural stem cells has largely relied on *in vivo* and *in vitro* observations. For example, identification and enrichment of neurosphere-forming cells coupled with asymmetric division and multi-lineage differentiation potential are used as key features to identify neural stem cells (discussed exhaustively in chapter 1).

Single-cell transcriptomics has revolutionized our understanding of adult neural stem cells and revealed these cells as a pool of molecularly heterogeneous populations. The use of scRNAseq technology to resolve neural stem cell heterogeneity has been applied to both developing and adult brain tissue; let's have a look at how this diverse cell type retains both spatial and temporal changes and operate along a highly dynamic trajectory.

Neurodevelopmental trajectory

As discussed in chapter 2, radial glia (the fetal neural stem cell population) were once considered a homogeneous neural stem cell population in the human fetal brain that was merely defined by their radial morphology. Cell-by-cell investigations into the transcriptome of radial glia derived from the subventricular zone of the developing fetal brain have revealed diverse populations of radial glia (Gertz, Lui, LaMonica, Wang, & Kriegstein, 2014; Kelly, Raudales, Moissidis, Kim, & Huang, 2019; D. R. Lee et al., 2022; Tomasz J Nowakowski et al., 2017; A. A. Pollen et al., 2015). Moreover, it has not only demonstrated local neurogenic niche diversity of radial glia but also revealed spatio-temporal molecular differences orchestrating diverse specialized cell lineages across the developing cortex. Reconstruction of these cell states/cell types along the lineage trajectory has revealed how these molecular states line up in a sequential manner (Fig. 4.1)

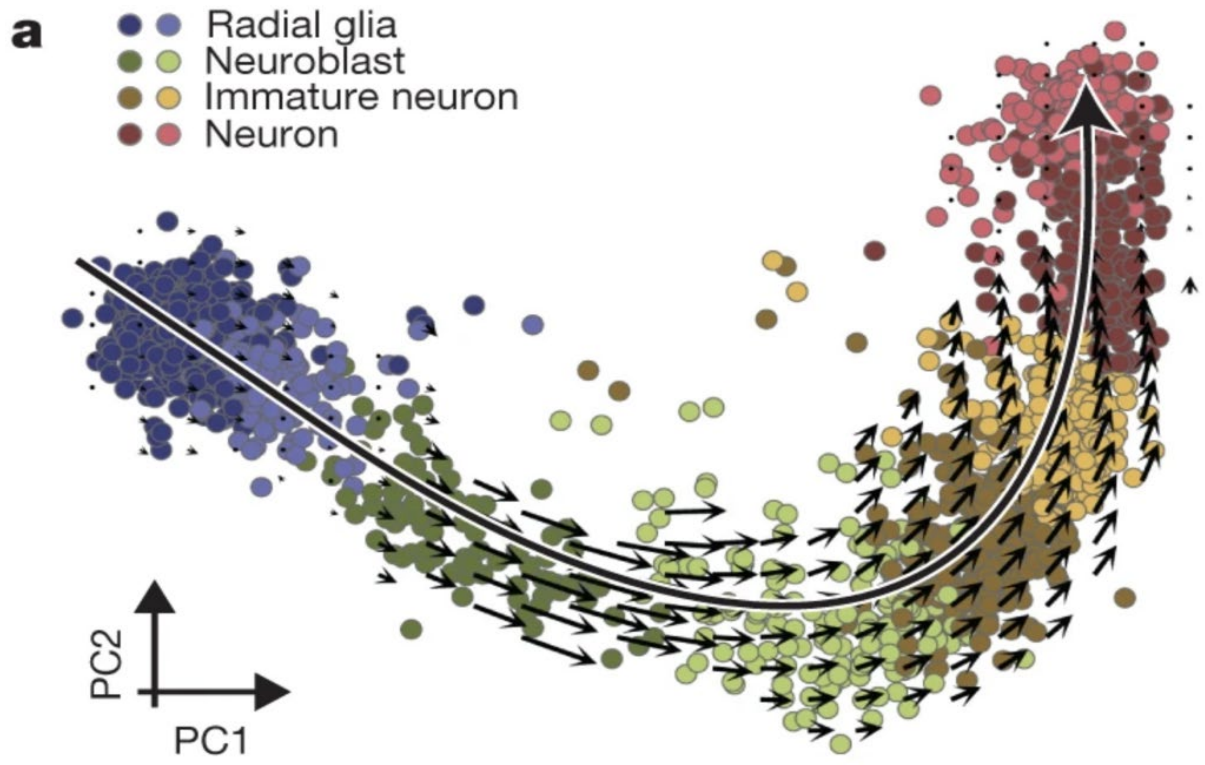


Figure 4.1 PCA projection of human glutamatergic neuron differentiation

Projection of (n = 1,720 cells) at post-conception week 10, shown with velocity field. Colours indicate cell types and intermediate states. A corresponding principal curve is shown in bold.

Figure adapted from ***(La Manno, Soldatov, Zeisel, Braun, Hochgerner, Petukhov, Lidschreiber, Kastriti, Lönnerberg, Furlan, et al., 2018)***

Adult brain repair and regeneration

Owing to the dynamic nature of a developing system, such as developing fetal brain, capturing cells in various transcriptomic states during corticogenesis has been less challenging. For example, active radial glia constantly undergoes proliferation to spin off new-born neurons by transitioning through various molecular stages as shown in Fig 4.1. This stem cell kinetic and reconstruction of neuronal lineage is clearly captured as stem cells remain the dominant population, hence the ratio of stem to differentiated cells is higher during development, However, identifying new-born cell types or activated neural stem cells in an adult brain has been challenging; given the postmitotic nature of the adult brain.

Interestingly, activated neural stem cells followed by a subsequent molecular chain of states has been captured in adult mouse brain (Borrett et al., 2020; Dulken, Leeman, Boutet, Hebestreit, & Brunet, 2017; Llorens-Bobadilla et al., 2015; Luo et al., 2015; Shu et al., 2022). This potential of neural stem cells was, intriguingly unleased following the introduction of brain injury, suggesting a latent but hidden potential of adult neural stem cells for brain repair and regeneration. Curiously, the molecular cascade observed in the adult neural stem cell hierarchy fundamentally followed the neurodevelopmental trajectory as witnessed in fetal brain development (Cebrian-Silla et al., 2021; Zywitza, Misios, Bunatyan, Willnow, & Rajewsky, 2018) (Fig 4.1). These conserved developmental programs have long tempted neurobiologists to speculate the embryonic origin of adult neural stem cells.

Embryonic roots of adult neural stem cells

In the adult mammalian brain, B1 cells residing in the SVZ of adult mice are the earliest quiescent known adult NSCs that are known to generate at least 6 different types of olfactory bulb interneurons (Tong & Alvarez-Buylla, 2014). B1 cells retain the apical-basal polarity, a hallmark of their antecedents or fetal counterparts (radial glia). Moreover, these B1 cells contact the ventricle through small, specialized apical processes that contain a single primary cilium and retain long basal processes with specialized endings contacting blood vessels. Cell-by-cell transcriptomic census of the neurogenic zones in the adult mouse SVZ has consistently revealed conserved developmental programs across fetal and adult stages (Fuentealba et al., 2015; Hochgerner, Zeisel, Lönnerberg, & Linnarsson, 2018; Tepe et al., 2018; Yuzwa et al., 2017). In addition, this continuum of germinal activity that was conserved across the lifespan of the mammalian brain has been shown to be regulated by genomic imprinting via epigenetic mechanisms, including DNA methylation and post translational modification (Lozano-Ureña, Montalbán-Loro, Ferguson-Smith, & Ferrón, 2017).

Evidence of adult neurogenesis in humans has been scarce, and therefore, the origin and heterogeneity of human adult neural stem cells remains elusive. To date, only two studies have been published concerning human adult SVZ neurogenesis: (Durante et al., 2020) identified neural stem cell and neural progenitor cell pools and neuron in the olfactory neuroepithelium and provided evidence for continued production of neurons in humans. In the second study reported by (Donega et al., 2022), single-cell was performed on CD271+ cells from the adult human SVZ and OPC-like progenitors in addition to quiescent NSCs were identified.

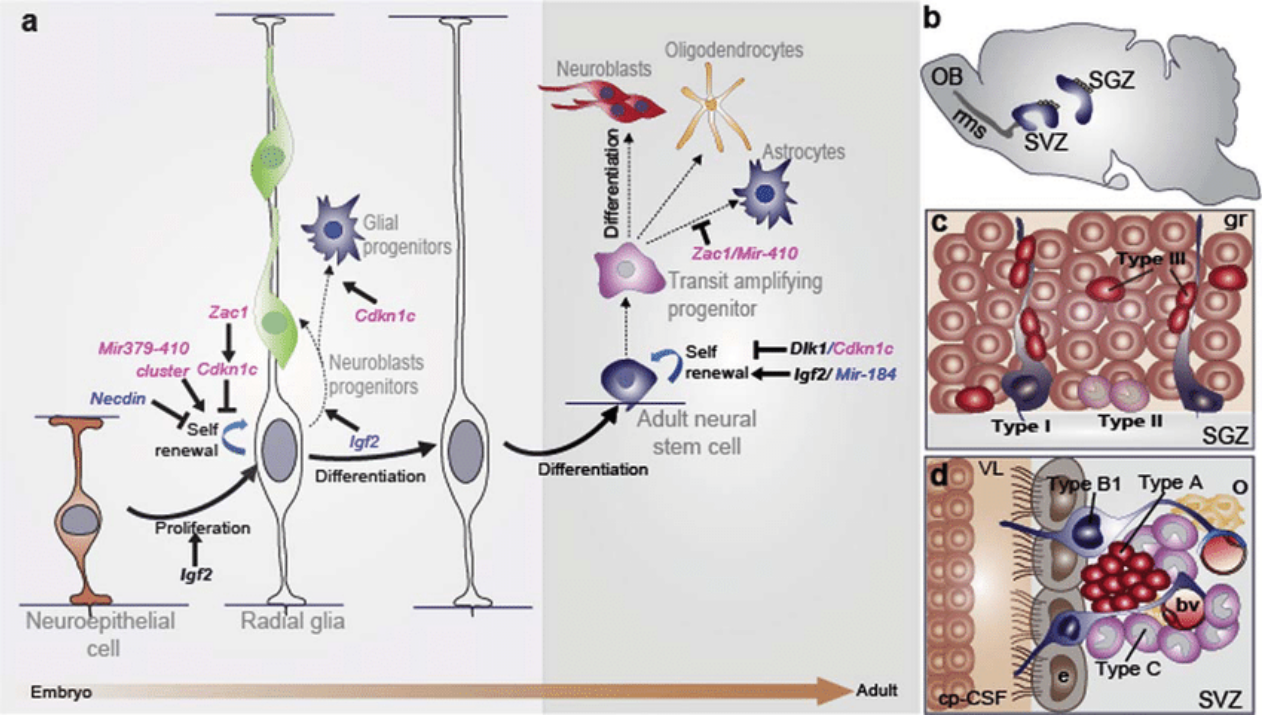


Figure 4.2 Radial glia nature of embryonic and adult neural stem cells: the role of imprinted genes.
(a) There is a continuum of germinal activity that links neuroepithelial stem cells to radial glia and ultimately to the astrocytes that are stem cells in the adult brain. Radial glia in the neocortex produce several major brain cell classes, including neurons and astrocytes, via several rounds of proliferation and differentiation. The roles of the imprinted genes are indicated. Maternally and paternally expressed genes appear in pink and blue respectively. Imprinted genes that show biallelic expression are in black and bold. (b) Sagittal view showing the SVZ and the SGZ neurogenic niches in the adult mouse brain. In the SVZ, neuroblasts reach the olfactory bulb (OB) through the rostral migratory stream (rms). (c) Enlarged view of the SVZ: type B1 stem cells (blue) contact the ventricle with a thin process extending between the ependymal cells (e; grey); transit-amplifying progenitors (TAP) or type C cells (purple) give rise to type A cells (red) and oligodendrocytes progenitors (yellow). Dividing stem cells and their TAP progeny are tightly apposed to blood vessels (bv); the choroid plexus-cerebrospinal fluid system (cp-CSF) is shown. (d) Enlarged view of the SGZ: Type I stem cells (blue) show a radial single prolongation through the granular layer; type II precursors (purple) give rise to neuronal lineage-restricted progenitor type III cells (red) that differentiate into neurons, which in turn integrate into the granular layer (gr).
From (Lozano-Ureña et al., 2017)

Developmental hierarchy and cell-of-origin in gliomas

Glioblastoma is notoriously understood to harbour heterogeneous populations at the genomic level, thus, resulting in sparse and inadequate treatment effects. And because the genomic landscape of glioblastoma is so diverse and chaotic, it has been awfully challenging to target every single cancer cell of the tumor mass since therapies that are developed for the treatment of glioblastoma target genomic anomalies. The genomic instabilities that underlie

tumor development and progression can range in magnitude from a single-base substitution to whole-genome doublings (Carter, Eklund, Kohane, Harris, & Szallasi, 2006; Hanahan & Weinberg, 2011). In some tumors, including brain tumors, such genomic instability arises because of chromosome-level changes. (J. H. Lee et al., 2018) demonstrated the presence of glioma mutation-carrying cells in the SVZ of glioma patients that were able to give rise to gliomas elsewhere in the brain.

Scientists have investigated and assessed tumor mass through the genomic lens for decades - the most recent effort being The Cancer Genome Atlas (TCGA) project, however, given the complex nature of genomic anomalies, insights into genomic lesions may have contributed to our understanding of these lesions in glioblastoma, but have not been useful in guiding our therapies. Therefore, if cancer imitates neurodevelopmental hierarchy, then identification of cancer stem cells in the quiescent adult NSC pool will help us understand cancer hierarchy better for therapeutic interventions.

In this chapter we seek to dig deeper into the NSC-cluster to extricate any possible stem cell populations that may have cluttered together due to the presence of differentiated cell types in our adult SVZ-dataset, thus masking key cell states. To unravel this, we used fetal developmental trajectory as our cellular framework. We utilized published fetal dataset and integrating these well-annotated cell types with our adult NSC-like cluster cells.

4.2 Aim and objectives

The aim of this chapter is to investigate the nature of the NSC-like cluster and disentangle the embedded molecular heterogeneity to divulge crucial cellular states/types.

To achieve this aim, the objectives of this chapter are:

- To integrate high-content fetal progenitor populations with adult NSC-like cluster to uncover embedded molecular subclusters.
- To comprehensively characterize human adult SVZ NSC subclusters by revealing biological processes and mechanism
- To extricate and profile the adult early neural stem cell (eNSC) subcluster

4.3 Results

4.3.1 Integrating high-content fetal progenitor transcriptome datasets

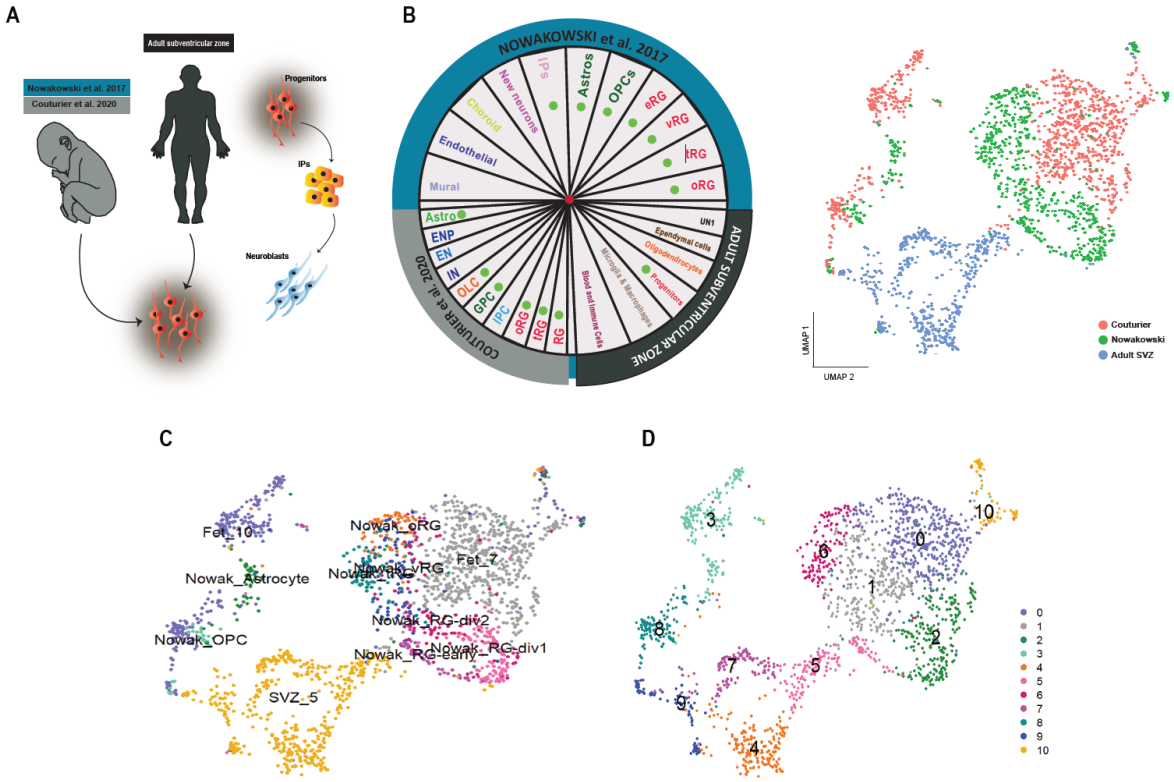


Figure 4.3 Conflation of high-content foetal progenitor datasets with human adult SVZ NSC-like cluster
A. Schematic diagram representing consolidation of foetal progenitor populations from two studies (Nowakowski et al. 2017 and Couturier et al. 2020) with adult SVZ progenitor cluster.
B. Detailed description of all the human foetal from annotated cell-types from Nowakowski et al. 2017 and Couturier et al. 2020 inflated with human adult NSC cluster only. Of all the annotated cell types, only the progenitors (marked with a green dot) were conflated. Uniform Manifold Approximation and Projection (UMAP) visualisation of conflated dataset with each colour representing the dataset utilised.
C-D UMAP visualization of conflated cell-types coloured by original cell type annotations (C) and by clusters (D).

Owing to the scarcity of adult SVZ single-cell datasets, we turned to fetal neurodevelopmental hierarchy and used it as our framework to dissect any viable molecular diversity within the NSC-like cluster and potential cellular hierarchy (Fig 4; panel A). To achieve this, we utilized studies that reported well-annotated fetal neurodevelopment cell types/states and their respective trajectories (Tomasz J Nowakowski et al., 2017). In addition to

Nowakowski et al. dataset, we also utilised the fetal cell types/states characterized in chapter 2 of this thesis; fetal dataset built in this thesis has also been published (C. P. Couturier et al., 2020) (Fig 4.1; panel A).

Both Nowakowski et al. and Couturier et al. datasets included diverse radial glial population, including oRG, truncated radial glia (tRG) and early radial glia (eRG), along with intermediate progenitors (IPs) and oligodendrocyte progenitor cells (OPCs). In addition to various new-born neuronal populations, other specialised populations, including endothelial cells, mural cells and astrocytes were also established by both the studies (Fig 4.1; panel B). In staying faithful to the hypothesis that the presence of differentiated cells in the dataset masks subtle molecular diversity by forcing raw and malleable progenitor diversity into one cluster, we pooled only the progenitors from both the published datasets (Nowakowski et al. and Couturier et al.) by excluding differentiated/ specialised cells. Only the cell types/states marked with a green dot were conflated with the NSC-like cluster from our adult SVZ dataset (Fig 4.1; panel B). Each dataset has been highlighted by unique colours: Couturier et al. (red), Nowakowski et al. (green) and adult SVZ NSC-like cluster (blue) (Fig 4.1; panel B).

Applying clustering algorithm to this unified dataset resulted in 10 clusters (Fig 4.1; panel D). Notice that the radial glial and the OPCs from both the published datasets demonstrated proximity in the transcriptomic space (Fig 4.1; panel C). However, while a few cells showed transcriptomic proximity to the fetal cells, adult SVZ NSC-like cluster remained as in independent cluster. However, adult SVZ NSC-like cluster showed pronounced transcriptomic diversity in the transcriptomic space, which was at odds with the singularity of the adult SVZ NSC-like cluster that we saw in the presence of differentiated cells in the SVZ dataset (chapter 3) (Fig 4.1; panel C and D).

4.3.2 Conflation of adult and fetal progenitor populations resolved adult NSC-like cluster into subclusters

Even before applying any clustering algorithm, it was clearly noticeable that conflating progenitor populations by removing differentiated specialised cells strongly hinted at transcriptomic diversity within the adult SVZ NSC-like cluster (Fig 4.3; panel C). Indeed, applying clustering algorithm declassified the adult SVZ NSC-like cluster into four subclusters: cluster 5 (C5), cluster 4 (C4), cluster 7 (C7) and cluster 9 (C9) (Fig 4.4).

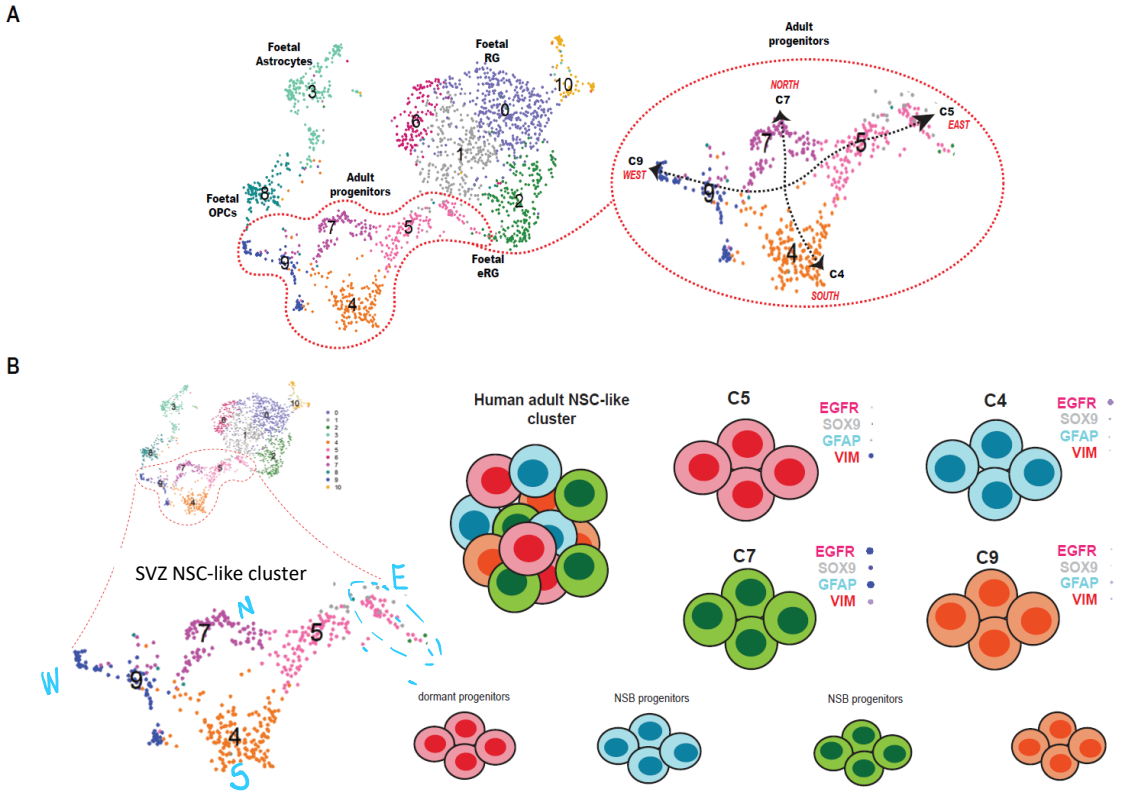


Figure 4.4 Human adult NSC-like cluster comprised of subpopulations
A. UMAP of conflated foetal and adult progenitors. Conflation resulted in reduction of the adult progenitor cluster into four subcluster (C5, C4, C7 and C9).
B. Magnified image of 4 adult SVZ clusters of progenitor cells shown in UMAP (A). Schematic representation of adult NSC sub-clusters and key differentially expressed genes. Colour scale represents average gene expression levels for each gene shown and dot size represents percentage of cells expressing the respective genes.

In keeping with our objective to characterise the adult SVZ NSC-like cluster, we focused on these four subclusters. While the adult SVZ NSC-like cluster showed no profound transcriptomic proximity to any of the fetal radial glia. Nevertheless, notice that some cells at the east end of the subcluster C5 exhibited strong transcriptomic contiguity to some fetal radial glial cells (Fig 4.4; panel A). Upon examining the precise radial glial subtype that sat at the eastern border of the adult SVZ NSC-like subcluster C5, the streak of cells at the east of C5 were identified as early radial glia from Nowakowski et al. dataset (Fig 4.3; panel C) that belonged to the adult SVZ NSC-like subcluster C5 (circled in blue) (Fig 4.4; panel B). Similar proximity at the western end of the subcluster C9 was observed, but with the fetal OPCs from both the studies (Fig 4.4; panel A and B). So, C5 subcluster contained fetal radial glial that were identified as early radial glia at the east end, however fetal OPCs did not cluster with C9 but only showed transcriptomic proximity.

To inquire into the molecular nature of the adult SVZ NSC-like subclusters, we set out to visualise the expression pattern of classical adult NSC markers, including epidermal growth factor receptor (EGFR), glial fibrillary acidic protein (GFAP), vimentin (VIM) and SOX9 (Fig 4.2; panel B). Intriguingly, subcluster-specific expression pattern was noticed; relative to the rest of the three subclusters, C5 maintained strongest expression of VIM and weakest expression of EGFR and SOX9. Whereas C7 exhibited the opposite; strong EFGR and weak VIM (Fig 4.4; panel B). Interestingly, all four markers were either nearly absent in C9 and C4, except for EGFR which was expressed weakly in C4 (Fig 4.4; panel B). Specificity of EGFR to C7 was an interesting observation as EGFR has been shown to play a crucial role in dormant to active NSC transition as a key focal point regulator (Chaker, Codega, & Doetsch, 2016). This unique

expression of classical NSC markers prompted us to dig deeper into the molecular nature of adult SVZ subclusters by exploring the transcriptome of each of adult SVZ subclusters (Fig 4.5).

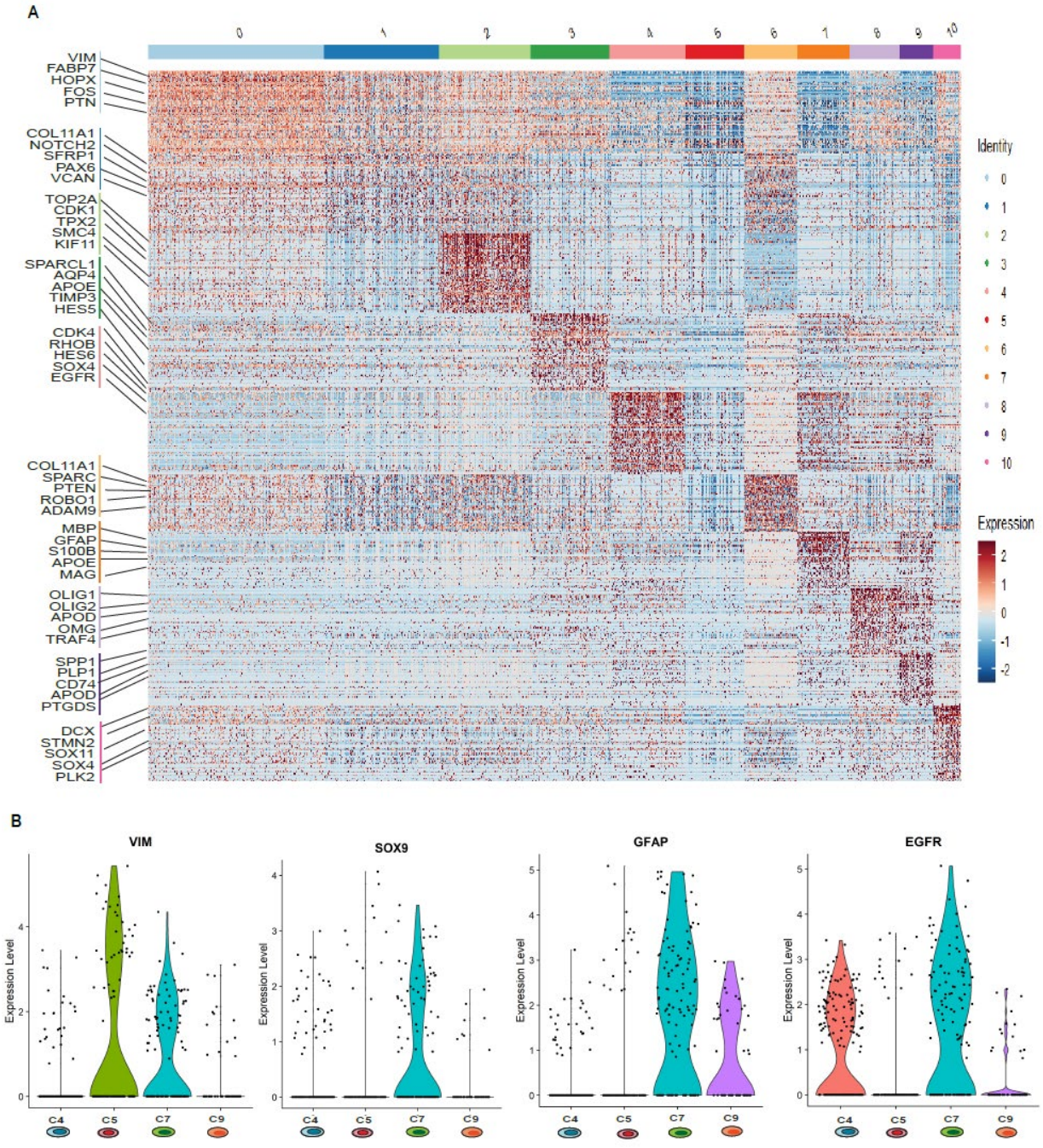


Figure 4.5 Cell type signatures of each of the clusters in the merged dataset
A. Heatmap of gene expression for the 11 clusters in the merge datasets of fetal (n dataset=2) progenitor cells (RG, APC- and OPC- like cells) and SVZ NSC-like cluster using a maximum of top 100 genes for each cell type (cluster 5 does not have any significant marker)
B. Violin plots showing expression of differentially expressed classical neural stem cell (NSC) genes between adult SVZ subclusters

To gain more insights into the transcriptome of each cluster (n=11) we performed differential gene expression analysis. Heatmap of genes that were differentially expressed in each of the cluster revealed crucial markers (Fig 4.5; panel A). The radial glial populations (cluster 0, 1, 2, 6 and 10), fetal OPCs (cluster 8) and fetal astrocytes (cluster 3) from both the integrated datasets were corroborated to express classical markers representative of respective cell types (Fig4.5).

To focus on the SVZ NSC-like subclusters, we interrogated the genes differentially expressed in the subclusters, including C5, C4, C7 and C9. While C7 maintained expression of glial markers, including GFAP, S100B and APOE, C4 demonstrated expression of progenitor markers, including CDK4, SOX4 and HES6. Consistent with the transcriptomic similarity of C9 to fetal OPCs (C8), markers associated with oligodendrocyte differentiation lineage, such as PLP1, PTGDS and SPP1 were specific to C9. Interestingly, subcluster C5, the only adult NSC subcluster that contained fetal early radial glial cells, maintained a small transcriptome. Notably, C5 either exhibited very low or no expression of glial-like progenitor markers that were expressed in C7, including GFAP, SOX9 and EGFR but maintained higher expression of VIM, which was markedly lowly expressed in C7.

To look for cluster-specific expression of classical progenitor markers that we couldn't achieve in the SVZ NSC-like cluster globally (chapter 3), we assessed expression patterns of comprehensive neural progenitor markers and lineage-specific molecules amongst the subclusters (C5, C4, C7 and C9) only (Fig 4.6). Excitingly, this revealed classical NSC markers (PTPRZ1, CLU and OLIG1) uniformly expressed across all the subclusters and subcluster specific expression of markers associated with activated and lineage-specific progenitors (Fig 4.6; panel A and B).

4.3.3 Gene expression analysis reveals sub cluster-specific expression patterns of neurodevelopment-related genes

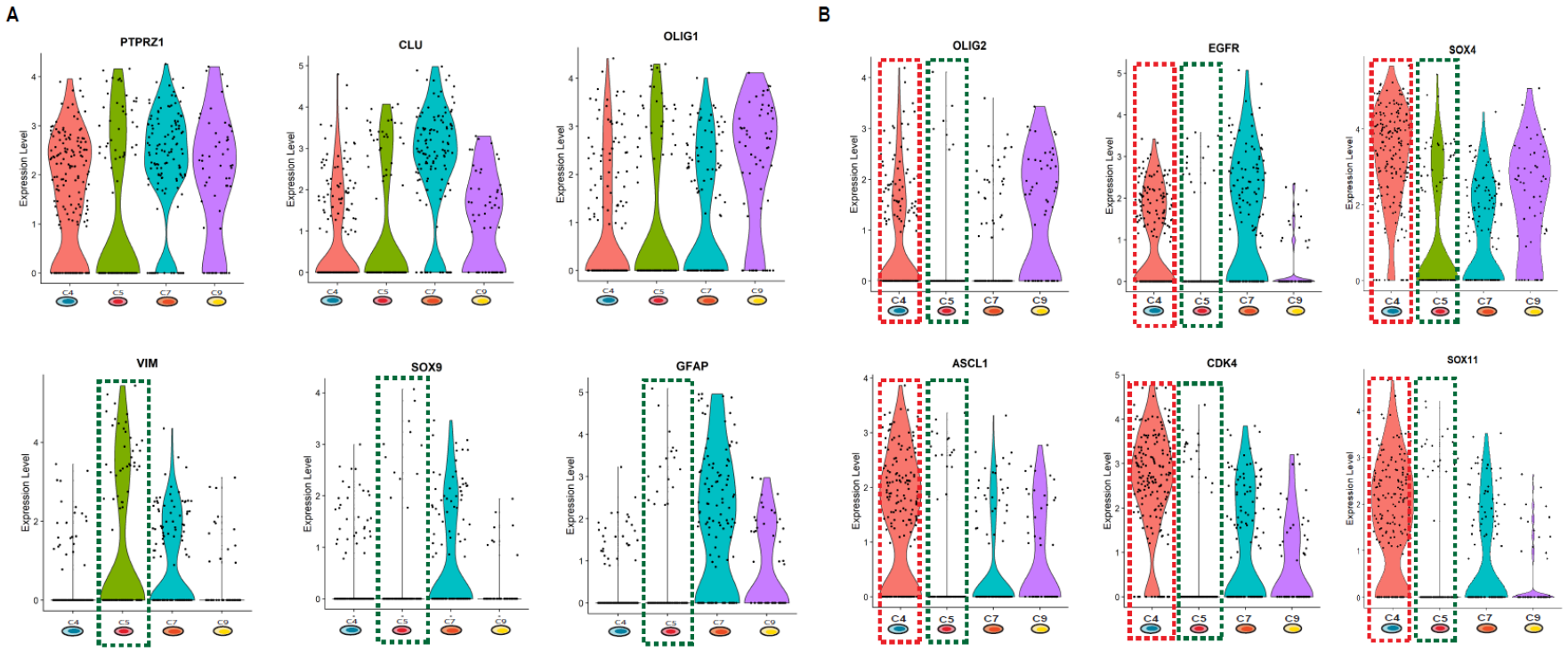


Figure 4.6 mRNA expression levels of key progenitor markers in the merged dataset
A. Top panel: Violin plots showing expression levels of classical radial glia/NSC markers, including receptor-like tyrosine-protein phosphatase zeta (PTPRZ1), clusterin (CLU) and oligodendrocyte transcription factor 1 (OLIG1). Ubiquitous expression of these markers was observed across all adult sub-clusters.
Bottom panel: Violin plots showing expression levels of classical RG markers, including, glial fibrillary acidic protein (GFAP), vimentin (VIM) and SRY-box transcription factor 9 (SOX9). Profound transcript levels of VIM were observed in C5 only.
B. Top panel: Violin plots showing expression levels of classical markers associated with activated progenitors, including oligodendrocyte transcription factor 2 (OLIG2), epidermal growth factor receptor (EGFR) and achaete-scute homolog 1 (ASCL1). These activated progenitor markers were predominantly expressed by the C4 (NPC) sub-cluster.
Bottom panel: Violin plots showing expression levels of classical cycling neuronal progenitor markers, including, cyclin dependent kinase 4 (CDK4), SRY-box transcription factor 4 (SOX4) and SRY-box transcription factor 11 (SOX11). In addition to expressing activated progenitor markers (panel A), adult C4 (NPC) also, exclusively, expressed pro-neuronal markers.

For example, C7 was marked by expression of activated and astrocytic lineage markers (SOX9, EGFR and GFAP) (astrocytic NSC – aNSC) (Fig 4.6; panel A and B) whereas C4 maintained profound levels of neuronal lineage related markers (ASCL1, CDK4, SOX4 and SOX11) (Fig 4.6; panel B)); both lineage-related set of markers were robustly specific to subcluster C7 and C4, respectively. On the contrary, C9 showed no significant expression of markers associated with astrocytic or neuronal identity but maintained decent expression of OLIG2, a key oligodendrocyte-lineage marker. Subcluster C5 was of particular interest and exhibited a unique profile: nearly absent for any of lineage-specific progenitor markers but maintained higher levels of VIM (Fig. 4.6; panel A).

Identification of gene expression patterns spanning major cellular lineages of the central nervous system was extremely exciting and encouraging. To further ensure this subcluster-specific expression and assess expression of other lineage-specific markers, we reasoned to expand our gene set by incorporating other lineage-specific canonical markers (Fig 4.7). Consistent with the previous expression pattern, glial gene set (CLU, CRYAB, ALDOC, NES, SOX9 and GFAP) was specific to C7.

However, C4, intriguingly, correlated with neuronal progenitor gene set (MASH1, SOX2, SOX4, SOX11, SYT1). Remarkably, C4 also maintained expression of the new-born neuron markers, such as ELAVL4, STMN2 and DCX (Fig 4.7; panel A). On the other hand, C9 enriched for classical OPC markers (SOX10, PTGDS and VCAN) (oligodendrocyte NSC – oNSC). Out of four subclusters, three subclusters distinctly exuberated lineage-related progenitor signatures. C5 did not demonstrate any lineage pledge, however, maintained higher expression levels of VIM compared to other subclusters (Fig 4.7) but otherwise was relatively relative to other subclusters as well as lineage-related progenitor markers.

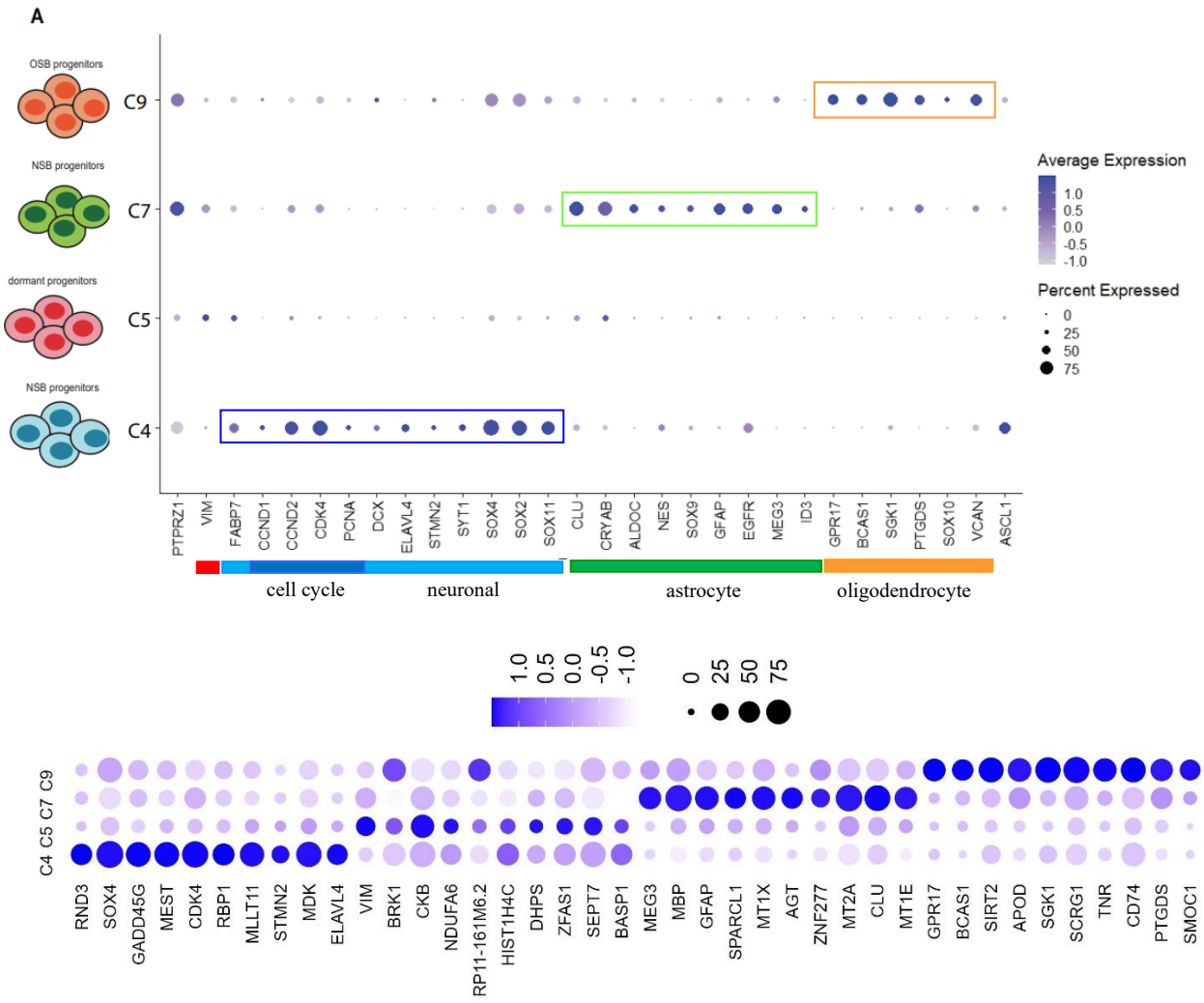


Figure 4.7 Lineage-specific expression patterns amongst adult SVZ subclusters
A. Dot plot representing average expression levels of key lineage-specific markers, including GFAP, SOX9, EGFR and PTPRZ1 for astrocytic signature-bearing NSCs (aNSC), VIM for early progenitors, PTPRZ1, OLIG1 and OLIG2 for oligodendrocyte signature-bearing NSCs (oNSCs) and CDK4, SOX4, ASCL1 and SOX11 for neuronal signature-bearing progenitors (nNSCs). Colour bar and dot size indicate average expression and percentage of cells expressing the marker, respectively.
B. Top 10 differentially expressed genes in each of the subclusters

In addition to assessing comprehensive progenitor markers in our subclusters, we employed markers of cell cycle, including CCND1, CCND2, PCNA and CDK4 to capture mitotically active states. Remarkably, cells in the subcluster C4 showed strong enrichment for cell cycle genes. This cycling nature of the C4 is in addition to the neuronal progenitor and new-born neuron markers demonstrated by C4 (Fig 4.7; panel A).

Notice that all three lineage-related signatures were significantly enriched in progenitor markers. In fact, gene expression profile of C7 highly correlated with fetal murine radial glial precursors (Yuzwa et al., 2017) with a strong glial/embryonic identity conserved across adult neurogenic niches (dentate gyrus and subventricular zone) (Borrett et al., 2022). Likewise, C4 demonstrated robust neuronal precursor profile (neuronal NSC – nNSC) with a strong indication developing new neurons.

In following these observations, we set out to establish extensive signatures based on intrinsic transcriptomic profile by computing differentially expressed genes locally between the four subclusters only, from here on referred to as local differential expression analysis (LDE). This LDE revealed a comprehensive profile of SVZ NSC subclusters uncovering both known and novel markers (Fig 4.8; panel A). Using these differentially expressed gene profiles, we performed gene set enrichment analysis (GSEA) (Fig 4.8; panel B).

4.3.4 Gene-set enrichment analysis (GSEA) revealed enrichment of unique fetal cell type signatures for each of the subclusters

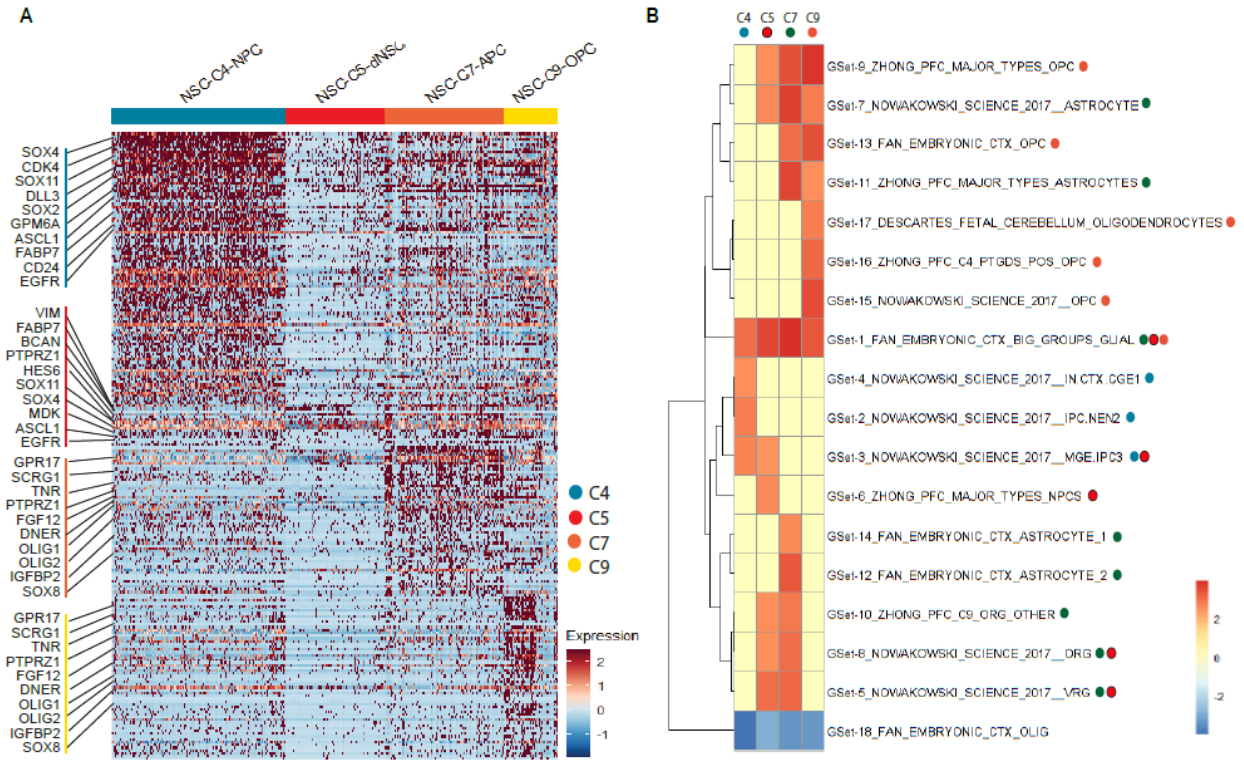


Figure 4.8 Differential expression and Gene-Set enrichment analysis of adult SVZ subclusters
A. Heatmap of gene expression for the four NSC sub-clusters.
B. Summary of Gene Set Enrichment Analysis (GSEA) using human brain cell types from single cell studies. Color bar indicates Normalized Enrichment Score (NES). Red and blue colors represent significant (FDR<0.0001) enrichment or down-regulation respectively, yellow indicates no significant result. Top dendrogram shows unsupervised hierarchical clustering of the data.

To get further insight into the adult SVZ NSC-like subclusters, we used curated gene sets of known human brain cell types from single-cell RNA-seq studies (Cao et al., 2020; Fan et al., 2018; Tomasz J Nowakowski et al., 2017; Zhong, Zhang, Fan, Wu, Yan, Dong, Zhang, Li, Sun, & Pan, 2018). The results of this Gene Set Enrichment Analysis (GSEA) are summarized in figure 4.8 (Fig 4.8; panel B). All 4 subclusters (C5, C4, C7 and C9) showed enrichment for the NSC-like cell group (GSet-1); C7-aNSC and C5-eNSC demonstrated the strongest correlation

owing to the core glial nature of fetal progenitors, and down-regulation for a differentiated cortical oligodendrocyte cell type (GSet-18).

Subcluster C4-nNSC showed enrichment for 3 neuronal progenitor programs (GSet-2,3,4). These gene sets corresponded to spatially diverse fetal intermediate progenitor cells, including medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and major prefrontal cortex (PFC) neuronal progenitor cells (NPCs).

While cluster C9-oNSC demonstrated clear enrichment for oligodendrocyte programs (n=5, Gset-15,16,17,13,9), including embryonic cortical OPCs, embryonic PTGDS+ OPCs and fetal cerebellum oligodendrocytes, cluster C7-aNSCL was up-regulated for 4 astrocytic cell types (Gset-12,14,11,7), including embryonic cortex astrocyte populations and fetal radial glia. Out of these 9 programs, 4 (Gset-11,13,7,9) showed enrichment in both C7-aNSC and C9-oNSC, although to a lesser extent whereas, C7-aNSC also enriched for 3 radial glia gene sets (Gset-5,8,10), like C5-eNSC. In addition, cluster C5 is enriched for another RG program (GSet-6; Neural Progenitor Cell). C5 not only shows similarity with C7-aNSC, but also with C4-nNSC (Gset-3).

Enrichment of diverse embryonic/fetal developmental programs across the adult SVZ NSC-like encouraged us to further explore the biological processes and cellular pathways driving this resemblance to fetal programs. We achieved this by performing pathway analysis using LDE analysis-derived top 500 genes for each of the subclusters (Zhou et al., 2019).

4.3.5 Neuronal signature bearing subcluster (C4-nNSC) revealed enrichment for neuronal developmental processes

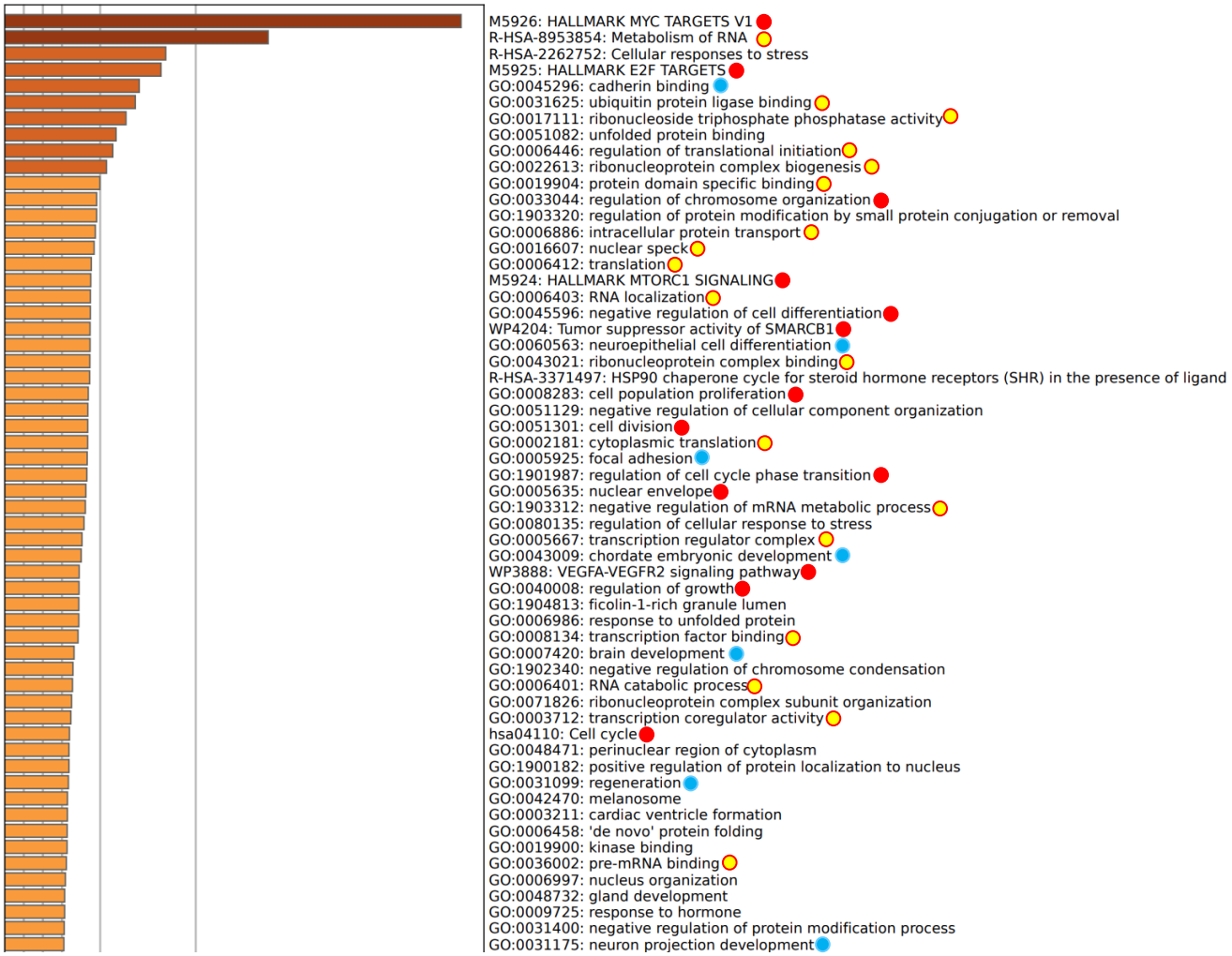


Figure 4.9 Pathway analysis of C4-nNSC
A. List of top statistically enriched terms (GO/KEGG terms, canonical pathways, hall mark gene sets, etc.,). Differentially expressed genes (top 500) in subcluster C4-nNSC were used to perform pathway analysis. Enriched terms corresponding to three major processes are marked with color dots as follows: RED: cell cycle/proliferation; YELLOW: transcription and translation; BLUE: neuron development and migration

We began by analyzing C4-nNSC subcluster. Pathway analysis for C4-nNSC subcluster results are shown in Fig 4.9 and 4.10. The top 500 differentially expressed genes yielded from LDE were utilized; significantly enriched biological pathways are shown in Fig 4.9. Interestingly, enriched pathways broadly belonged to four main biological categories:

development/proliferation, transcription and translation, neuron development and migration; enriched gene ontology terms (GO terms) belonging to each of these categories are colour labelled; red, yellow, and blue, respectively.

Top enriched signalling pathways included *M5926:* HALLMARK MYC TARGETS V1, *M5924:* HALLMARK MTORC1 SIGNALLING and *M5925:* HALLMARK E2F TARGETS, all of which are associated with cell cycle progression, DNA replication and proliferation (Liberzon et al., 2015). Consistently, strong enrichment for other proliferation, cell cycle and growth associated enriched terms included regulation of growth (GO:0040008), cell population proliferation (GO:0008283), regulation of cell cycle phase transition (GO:1901987), cell division (GO:00051301) and regulation of chromosome organization (GO:0033044).

This enrichment for growth and proliferation was also accompanied by intense transcription and translational activity in the C4-nNSC subcluster (Fig 4.9 and 4.10) showed by vigorous enrichment for regulation of translation initiation (GO: 0006446), translation (GO: 0006412), transcription coregulatory activity (GO: 0003712), to list a few; more related processes are highlighted with yellow dots (Figure 4.9).

To explore whether these proliferation associated signalling pathways are lineage specific, we explored these pathways further. Interestingly, the E2F pathway has been shown not only to regulate the cell cycle but also to be involved in adult neurogenesis and NSC activation exit from quiescence (O'Neil, 2022). Other studies have extended its role beyond proliferation and cell cycle control to regulating neuronal differentiation and migration (Ferguson et al., 2005; Takahashi et al., 2003). Similarly, the MYC pathway has been shown to stabilise larger neuroblasts and faster division rates by one of its targets (IGF2BF) (Samuels, Järvelin, Ish-Horowicz, & Davis, 2020), a highly conserved RNA-binding protein. Interestingly, IGF2BF3

was expressed in C4-nNSC only. These developmentally conserved signalling programs were further attested by the enrichment of brain development program (GO:0007420) and chordate embryonic development (GO: 0043009).

Enrichment of brain development programs, cell cycle regulation and neurogenic signalling pathways were further complemented and supported by the enrichment of additional exciting programs, including focal adhesion (GO:0005925), regeneration (GO:0031039) and neuron projection development (GO:0031175). Neuron projection development is a comprehensive term for processes ranging from neurite formation, development, and outgrowth (Ashburner et al., 2000; "The Gene Ontology resource: enriching a GOld mine," 2021). We reasoned that these biological processes could be associated with neuronal cytoskeletal dynamics, neurite formation, and migration via focal adhesion associated molecules. MANF (Tseng et al., 2017) and MLLT11 (Stanton-Turcotte et al., 2022) have been established to regulate neurite extension upon neuronal differentiation and migration coupled with neurite outgrowth during the maturation of cortical neurons. Strikingly, MANF expression was restricted to C4-nNSC subcluster only and MLLT11 was differentially expressed in the C4-nNSC subcluster and downregulated in the rest of the subclusters. The neuropathological hallmarks of Parkinson's disease (PD) include progressive degeneration of midbrain dopaminergic (mDA) neurons and their axons. STRAP (a serine threonine receptor-associated protein kinase) and NME1 (A nucleoside diphosphate kinase) is necessary and sufficient for the promotion of neurite growth (Anantha et al., 2020). Consistent with this, both STRAP and NME1 were differentially expressed in the C4-nNSC subcluster only.

To explore whether neurogenesis and neurite projection was coupled with neuronal migration, we assessed focal adhesion molecules in the C4-nNSC subcluster. Rac1 regulates

several aspects of neural development, including neuronal migration, axon formation and neural progenitor proliferation and survival (Kawauchi, Chihama, Nabeshima, & Hoshino, 2003; Kawauchi & Hoshino, 2008). During the migration, neurons acquire neuronal polarity and elongate their axons. Rac1 is reported to function in neural progenitors before neural differentiation and migration. Rac1 deficiency causes the reduction of neural progenitors due to enhanced cell cycle exit and decreased cell survival. Interestingly, Rac1 and Rac3 were differentially expressed in the C4-nNSC subcluster. In addition, microtubule proteins, including DCX and MAP1B, robustly expressed in C4-nNSC only, have been shown crucial for neuronal migration through regulating microtubule dynamics (Kawauchi et al., 2003).

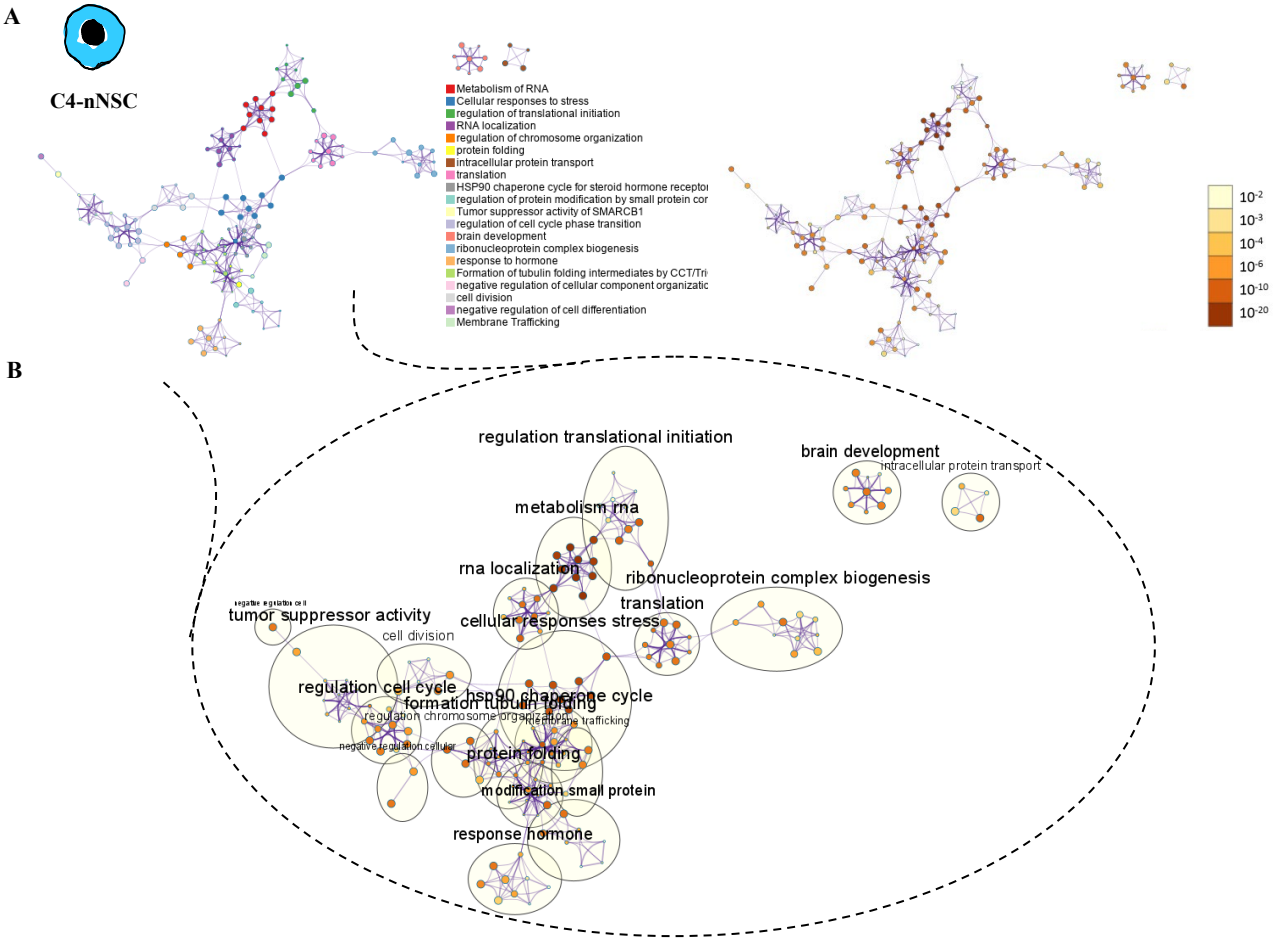


Figure 4.10 Biological processes enriched in C4-nNSC
A. Left panel: Selected of a subset of representative terms from the full cluster and converted them into a network layout. More specifically, each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network is visualized with Cytoscape with "force-directed" layout and with edge bundled for clarity. One term from each cluster is selected to have its term description shown as label. **Right panel:** Network layout as seen in left panel color coded for p-value.
B. Magnified and network layout as seen in panel A; with clusters circled and labelled for clarity. Graph was generated using Metascape

To uncover molecular entities underlying these pathways, we explored some top 50 genes. Classical neuronal markers that were specifically expressed in the C4-nNSC subcluster were further substantiated by LDE analysis, demonstrating expression of key neuronal transcriptional network members (SOX2, SOX4, SOX11 and MASH1) (Nakada, Hunsaker, Henke, & Johnson, 2004; Stevanovic et al., 2021). New-born neurons, both in human and mouse fetal brain, have been extensively characterised using single-cell technology. We assessed the expression of fetal neuroblast markers, for example DCX, FABP7, EOMES, TBR2, PAX6, TUBB, ELAVL2, ELAVL3 and ETV1. Interestingly, DCX, TUBB, ELAVL2, ELAVL3 and ETV1 expression was exclusively observed in C4-nNSC, however, intriguingly, expression of EOMES, PAX6, TBR2 and NEUROD6 was not observed (data not shown).

To ensure the maintenance of stem cell pools and proper development, crucial embryonic molecules are required to ensure proper development (Dichmann, Walentek, & Harland, 2015; Farioli-Vecchioli et al., 2012; Mesman, Van Hooft, & Smidt, 2017). Gene in the top 50 list, FABP7, MEST, HOXA9, TRA2B, BTG1 METLL1, EHBP1, TMSB15A, POUEF2, FAM60A, ODC1, PROX1, PAK3 and DLL3, SSR2 (contains binding sites for sox and pou proteins), all were enriched in C4-nNSC, revealing key molecular players evolutionarily conserved across the mammalian lifespan. However, these genes were accompanied by unique markers, including TUBB2B, UCHL1, TAGLN3, RTN3 and GAS1. GPM6A and NRXN (pre-synaptic neuronal

adhesion molecule) were revealed to be involved in axonal injury and functional recovery, axon regeneration and neuronal migration (Alhajlah, Thompson, & Ahmed, 2021; Lam et al., 2019; H. Liu et al., 2019; Marczenke et al., 2021; Mesman et al., 2017; Michibata et al., 2009). Interestingly, although evidence for neuronal migration, axonogenesis and neurite formation was solid, no indication of synaptic transmission or synapse formation related pathways/terms were enriched.

Next, we uncovered underlying pathways in C7-aNSC using the same method utilised to mine the C4-nNSC gene profile and the embedded pathways.

4.3.6 Astrocytic subcluster (C7-aNSC) was highly enriched for niche regulating factors, gliogenesis and wound healing response processes

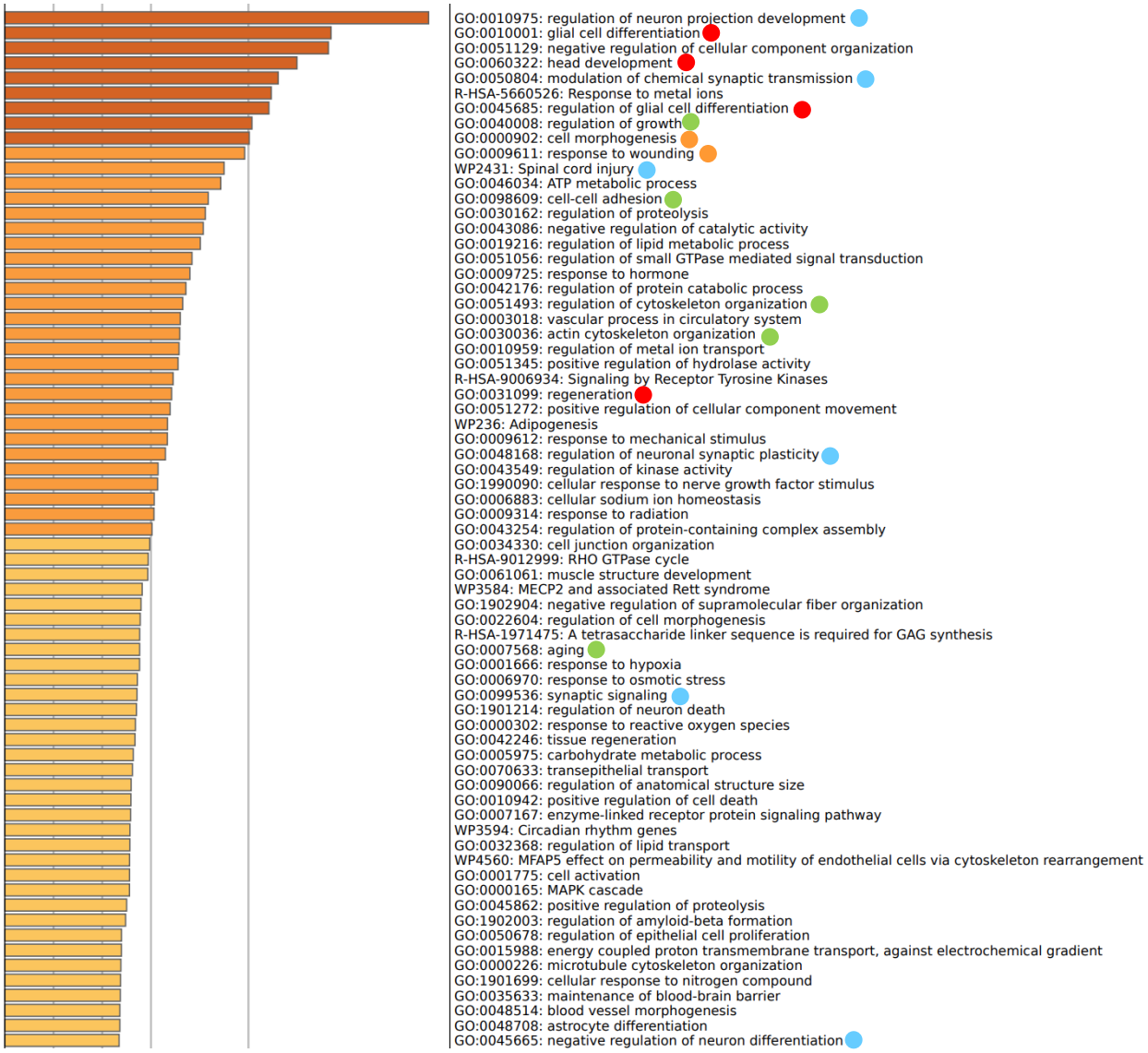


Figure 4.11 Pathway analysis of C7-aNSC
A. List of top statistically enriched terms (GO/KEGG terms, canonical pathways, hall mark gene sets, etc.,). Differentially expressed genes (top 500) in subcluster C7-aNSC were used to perform pathway analysis. Enriched terms corresponding to three major processes are marked with color dots as follows: RED: Gliogenesis; YELLOW: Matrisome and extracellular matrix; ORANGE: injury and wound healing; GREEN: tissue regeneration and remodeling and BLUE: synaptic transmission

Pathway analysis for C7-aNSC subcluster results are shown in Fig 4.11 and 4.12. Top 500 differentially expressed genes yielded from LDE were utilised; significantly enriched biological pathways are shown in Fig 4.10.

Top enriched pathways are shown in Fig 4.11 (only top 20 processes/pathways are shown here due to space constraints). Enriched pathways broadly belonged to four main biological categories: Gliogenesis (red), matrisome and extracellular matrix (yellow), injury and wound healing (orange) and tissue regeneration and remodelling (green) and synaptic transmission (blue). Enriched gene ontology terms (GO terms) belonging to each of these categories are colour labelled; red, yellow, orange, green and blue, respectively.

Development related terms enriched for C7-aNSC subcluster included head development (GO:0060322), WNT signalling pathway (GO:0016055), regulation of growth (GO:0040008), regulation of glial cell differentiation (GO:0045685) and reproductive structure development (GO:0048608). Interestingly, extracellular matrix related pathways/processes showed unique and profound enrichment as well. For example, NABA secreted factors (M5883), NABA matrisome associated (M5885) and a tetra-saccharide linker is required for GAG synthesis (R-HAS-1971475) pathways showed robust enrichment. Stemness has been shown to be regulated by specialized niches and distribution of extracellular matrix proteins and the fate of adult NSC is regulated by various signalling cues in the stem cell niche; for example, NSCs prefer to adhere to the adjacent basal lamina of blood vessels, where the extracellular matrix (ECM) such as laminin (LN), fibronectin and collagen are abundant. Genes regulating matrisome were revealed in C7-aNSC subcluster (AGT, CST3, CTSL1, SERPINE2, PTN, PLXNB, SEMA3B, CTSF, SDC3, CSPG5, NCAN, BCAN etc.) that included collagens, glycoproteins, and proteoglycans.

ECM proteins play a critical role in wound healing; cytokines such as PDGF, TGF and VEGF can activate signalling pathway to stimulate various processes. Consistently, genes underlying TGF-beta signalling pathway (ID1, ID3 and ID4), and growth factor stimulation pathway (FGF1, EGFR and PDGFA) were simultaneously enriched for C7-aNSC. Moreover N-

cadherin pathway (M266) was also enriched; N-cadherin has been shown to regulate adult SVZ NSC polarization leading to enhanced migration out of the SVZ into demyelinated lesions (Klingener et al., 2014). Intriguingly regulation of neuron death (GO:1901214) pathway was enriched as well.

It is also known that the spatial distribution of ECM controls migration and differentiation through ECM/receptor signalling (Lathia et al., 2007). Consistent with this, strong enrichment was observed for pathways, such as regeneration (GO:0031099), aging (GO:0007568), response to wounding (GO:0009611) and tissue regeneration (GO:0042246). Moreover, pathways such as, regulation of cytoskeletal organization (GO:0051493), microtubule cytoskeletal organization (GO:0000226), regulation of small GTPase mediated signal transduction (GO:0051056), signaling by receptor tyrosine kinase (RTK) (R-HAS-9006934), regulation of cell morphogenesis (GO:0022604) were also enriched (Fig 4.12).

Pathways driving C7-aNSC subcluster function and the molecular constituents orchestrating these pathways have revealed crucial biological functions underlying C7-aNSC function. Strong enrichment for pathways associated with gliogenesis, regeneration, extracellular matrix proteins, cytoskeletal organization, migration, and negative regulation of neuronal commitment.

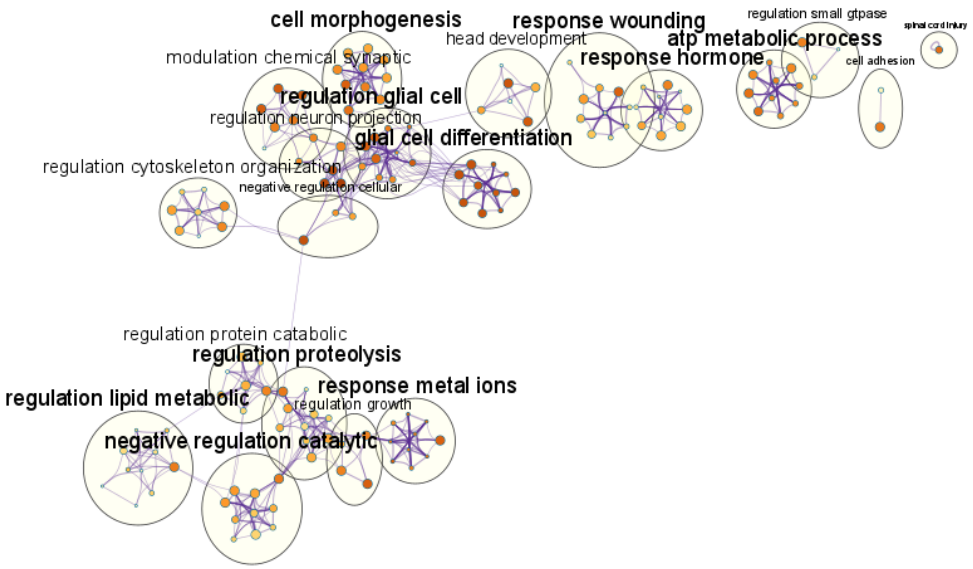


Figure 4.12 Biological processes enriched in C7-aNSC
A. Selected of a subset of representative terms from the full cluster and converted them into a network layout. More specifically, each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network is visualized with Cytoscape with "force-directed" layout and with edge bundled for clarity. One term from each cluster is selected to have its term description shown as label.

As discussed above, gliogenesis and astrocytic differentiation remained the only lineage related pathways enriched for C7-aNSC. NSCs are identified by their ability to give rise to multilineage descendants. Strikingly, pathway such as negative regulation of neuron differentiation (GO:0045665), was enriched. This, perhaps, suggests multilineage potential of the C7-aNSC subcluster demonstrated by its ability to upregulate and downregulate both gliogenic and neurogenic programs. More interestingly, instead of neuroblasts signatures, neuronal function supporting pathways (synaptic signaling (GO:0099536), regulation of neuronal synaptic plasticity (GO:0048168), regulation of neuron projection development (GO:0010975), regulation of chemical synaptic transmission ((GO:0050804)) were enriched (Fig4.12), hinting their potential role in supporting neuronal function as regenerative and repair substitutes.

Thus, C4-nNSC and C7-aNSC demonstrated exclusive signatures as two independent progenitor populations orchestrating different aspects of stem cell function. Next, we explore the

molecular entities of C9-oNSC subcluster and explore how this subcluster could potentially contribute to neural stem cell function.

4.3.7 Adult oligodendrocyte progenitor-like subcluster (C9-oNSC) demonstrated remyelinating and brain repair signature

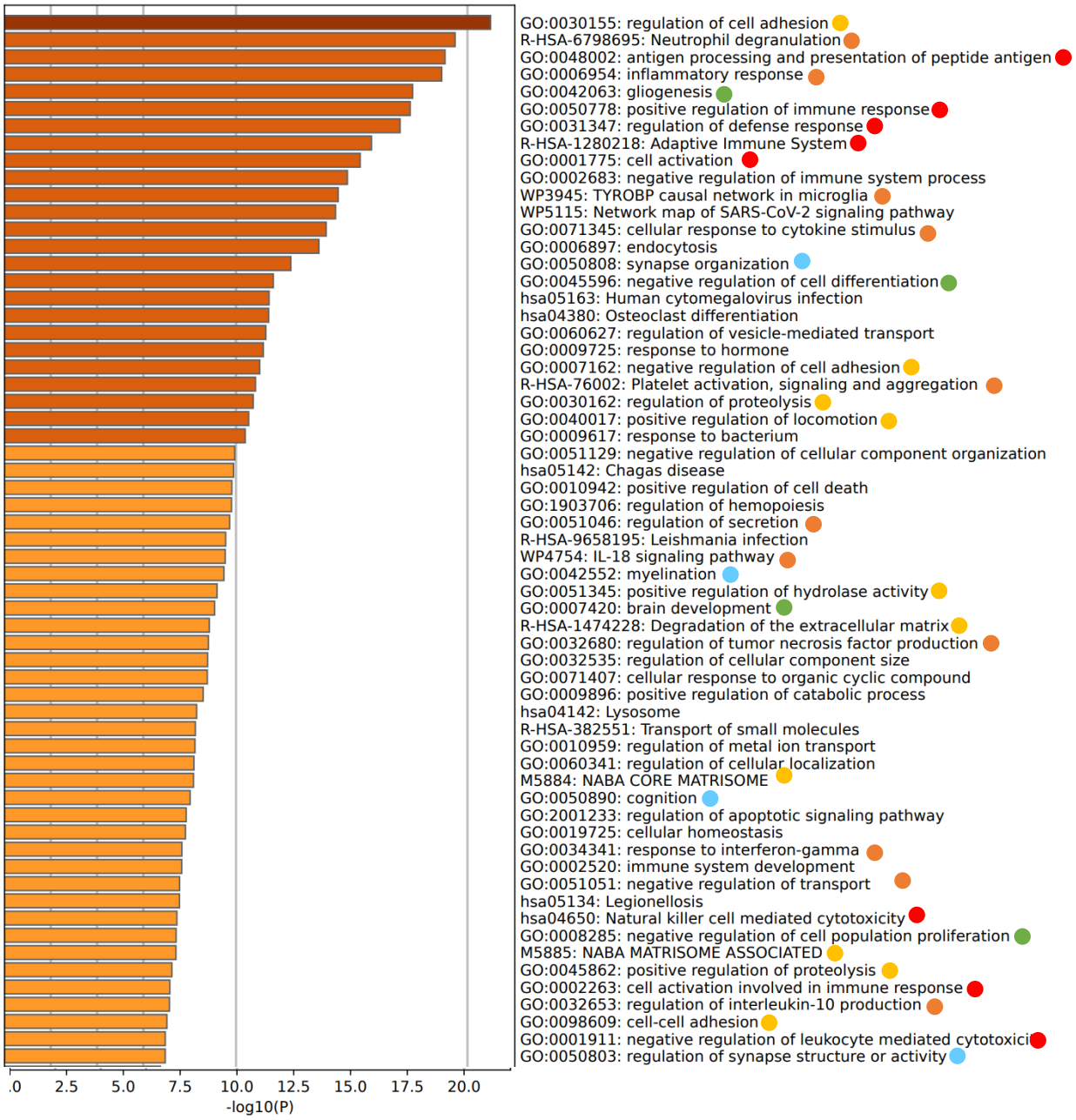


Figure 4.13 Pathway analysis of C9-oNSC
A. List of top statistically enriched terms (GO/KEGG terms, canonical pathways, hall mark gene sets, etc.,). Differentially expressed genes (top 500) in subcluster C9-oNSC were used to perform pathway analysis. Enriched terms corresponding to three major processes are marked with color dots as follows: RED: Immunogenic/Antigen presentation; YELLOW: inflammatory; GREEN: axon injury and remyelination

Pathway analysis for C4-nNSC subcluster results are shown in Fig 4.13 and 4.14. Top 500 differentially expressed genes yielded from LDE were utilised; significantly enriched biological processes/pathways are shown in Fig 4.13.

Top enriched processes are shown in Fig 4.13 (only 50-60 are shown here). Enriched processes/pathways broadly belonged to four main biological categories: Development and gliogenesis (green), matrisome and extracellular matrix (yellow), myelination and synaptic regulation (blue), immune modulation/antigen presentation (red) and inflammation (orange). Enriched gene ontology terms (GO terms) belonging to each of these categories are colour labelled; green, yellow, blue, red, and orange, respectively.

Consistent with C4 and C7 results, enrichment of developmental pathways (brain development (GO:0007420), tube morphogenesis (GO:0035239) and gliogenesis (GO:0042063) - in C7-aNSC only - was also seen in C9-oNSC. Further enrichment of developmental processes included oligodendrocyte development, positive regulation of myelination, myelin maintenance, axon ensheathment, ensheathment of neurons and positive regulation of myelination (GO:0032502). To extract molecular entities underlying these pathways, we looked at genes involved in these developmental processes and found MYRF, HEXB, SOX10, CXCR4, BCAS1, OLIG2, SIRT2, FA2H, CTSC, GAL3ST1, PLP1, SERINC5 etc.

Interestingly, similar trends were also seen for injury and wound healing, such as aging (GO:0007568), spinal cord injury (WP2431) and response to axon injury (GO:0048678) and response to wounding (G0:0009611), regeneration (GO:0031099), neuron projection

regeneration (GO:0031102) and axon regeneration (GO:0031103) (Fig 4.13; not all enriched pathways are shown here). Genes driving these pathways included, ACTB, AIF1, GPX1, TNR, TSPO, APOD, PTCH1, SRSF5, SOD2, TREM2, TYROBP, CD81, OMG etc. Age-dependent decline in remyelination potential of the central nervous system during ageing is associated with a declined differentiation capacity of oligodendrocyte progenitor cells (OPCs). Demyelination is the key pathological feature of the autoimmune inflammatory diseases of CNS such as multiple sclerosis (MS) and an early pathological hallmark of neurodegenerative diseases. As a result, demyelination may cause devastating axonal degeneration. Interestingly, top three differentially expressed genes in C9-oNSC: GPR17, BCAS1 and SIRT2 are well-known molecular players in remyelination. GPR17 receptors are sensors of local damage to the myelin sheath and play a role in the reconstruction and repair of demyelinating plaques caused by ongoing inflammatory processes. GPR17 receptors are present on nerve cells and precursor oligodendrocyte cells (Alavi, Shamsizadeh, Azhdari-Zarmehri, & Roohbakhsh, 2018; Dziedzic, Miller, Saluk-Bijak, & Bijak, 2020), whereas BCAS1 have been shown to identify an oligodendroglial subpopulation in the mouse and adult brain that are newly formed, myelinating oligodendrocytes, distinct from OPCs. BCAS1+ OPCs have also been shown to be restricted to the fetal and early postnatal human white matter have also been identified in cortical grey matter until in adults (Fard et al., 2017). Similarly, SIRT2 has been shown to promote remyelination during aging (X.-R. Ma et al., 2022). Another molecule RTN3 (differentially expressed in C9-oNSC) has been shown to enhance neurite outgrowth/axon regeneration and functional recovery after traumatic injury (Alhajlah et al., 2021).

In addition, core genes (COL9A1, SMOC1, THBS2, EDIL3, BCAN, TNR, FGL2, VWA1, LGI3, COL9A2, COL9A, SPOCK1, SRGN, VCAN, CHADL etc.) involved in ECM-

receptor interaction, collagens, integrins and proteoglycans were present in C9-oNSC transcriptome. In terms of matrisome and extracellular matrix proteins, C9-oNSC shared remarkable similarities with C7-aNSC. For example, both (NABA CORE MATRISOME; M5884) and NABA MATRISOME ASSOCIATED; M5885) pathways were enriched for both C7-aNSC and C9-oNSC (Fig 4.11 and Fig4.13). However, interestingly, pathways the underlying degradation of proteasome, extracellular matrix and collagen were uniquely seen in C9-oNSC (Fig 4.13). Moreover, neuron remodeling and regulation of tissue remodeling driven by C1QL1, C3, FARP2, C1QA, HAMP, PDK4, CSF1R, TF etc., were also seen in C9-oNSC only, further hinting at their function in tissue regeneration, remodeling, and morphogenesis. Genes regulating glial cell migration (TREM2, CCl3 and NTN1) were also seen.

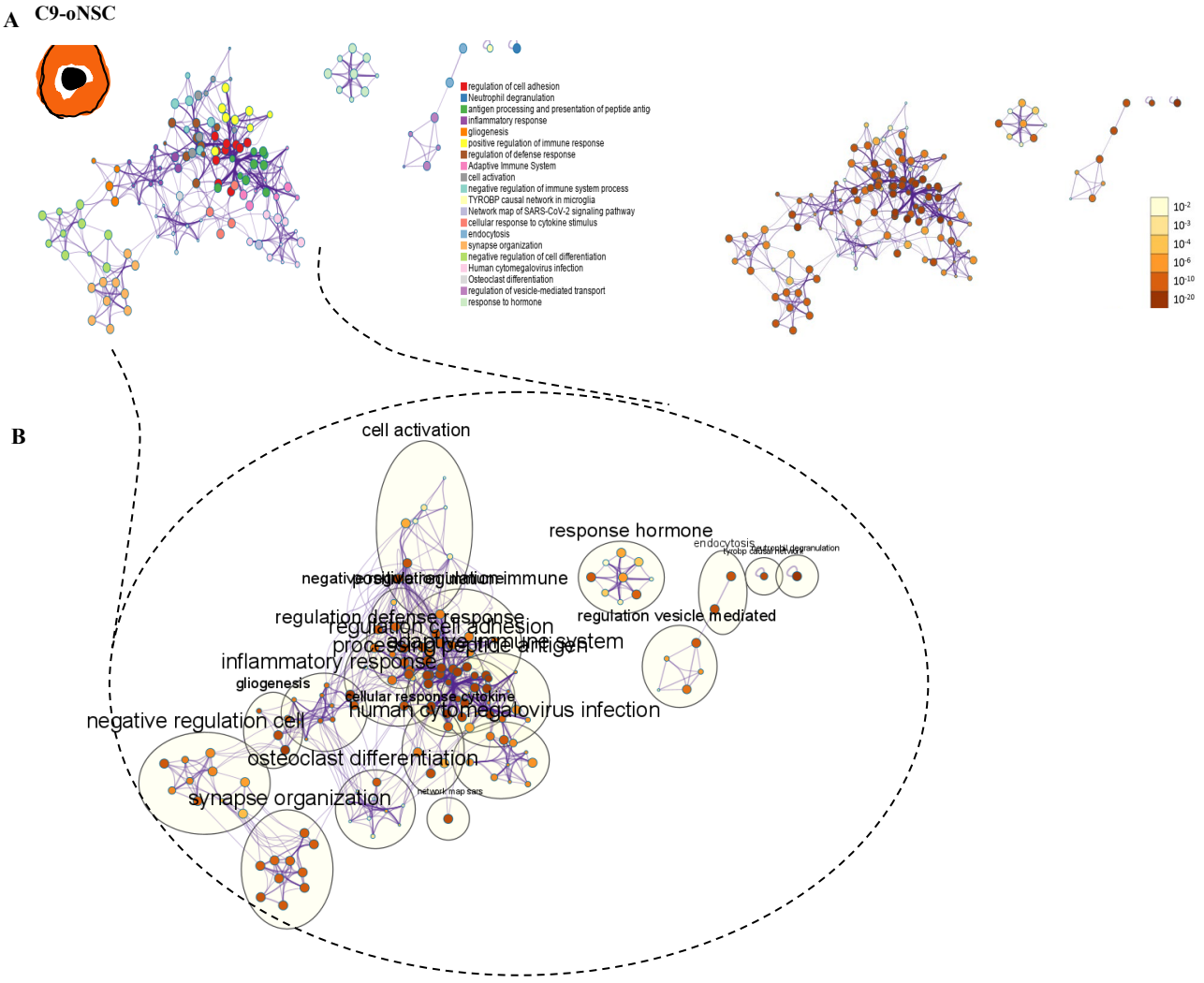


Figure 4.14 Biological processes enriched in C9-oNSC
A. Left panel: Selected of a subset of representative terms from the full cluster and converted them into a network layout. More specifically, each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network is visualized with Cytoscape with "force-directed" layout and with edge bundled for clarity. One term from each cluster is selected to have its term description shown as label. **Right panel:** Network layout as seen in left panel color coded for p-value.

Of key importance were pathways associated with synapse organization and synapse structure and activity (Fig 4.13 and 4.14). Key genes revealed, included APOE, FYN, VCP, NTN1, NLGN3, SEMA4D, PTPRO, PPT1, EPHB1, ABI3 etc. This is consistent with the diverse role of OPCs coming to light only recently that OPCs not only take part in myelination but also partake in synaptic organization, activity, and pruning (Akay, Effenberger, & Tsai, 2021; Auguste et al., 2022; Buchanan et al., 2021).

Further cell type enrichment analysis revealed strong correlation with embryonic microglia, macrophages and OPCs (Fig 4.15)

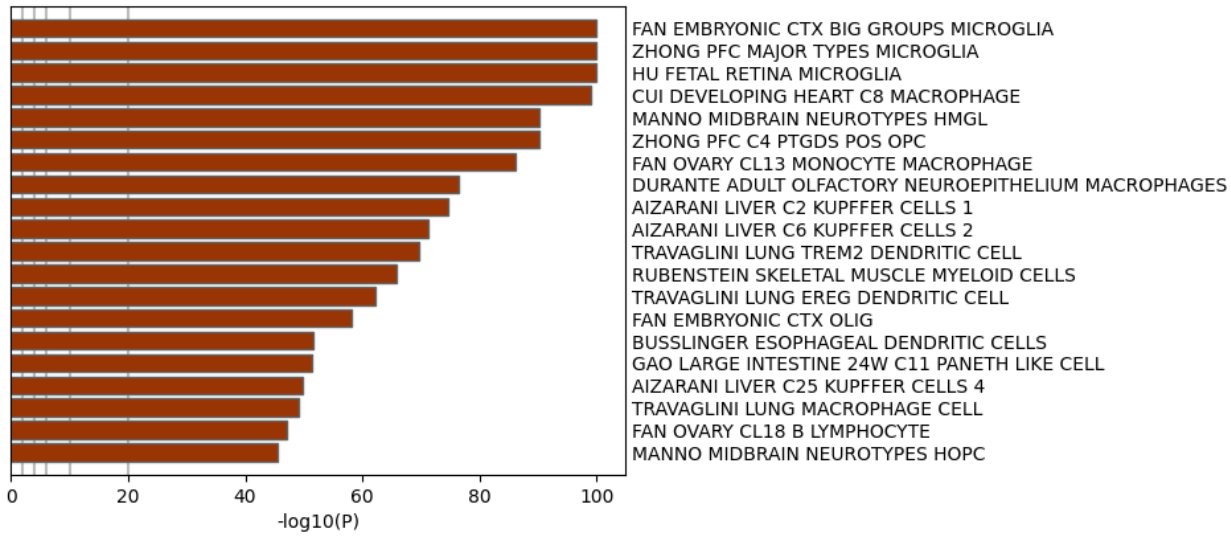


Figure 4.15 Summary of enrichment analysis for cell type signatures in C9-oNSCs

4.3.8 Oligodendrocyte progenitor subcluster (C9-oNSC) demonstrated immunogenic and inflammatory profile

Despite oligodendroglial nature, C9-oNSC subcluster harboured a unique transcriptomic profile. Apart from bearing strong oligodendroglial and developmental OPC signature, C9-oNSC maintained a strong immunogenic and inflammatory signature (Fig. 4.14, 4.15 and 4.16).

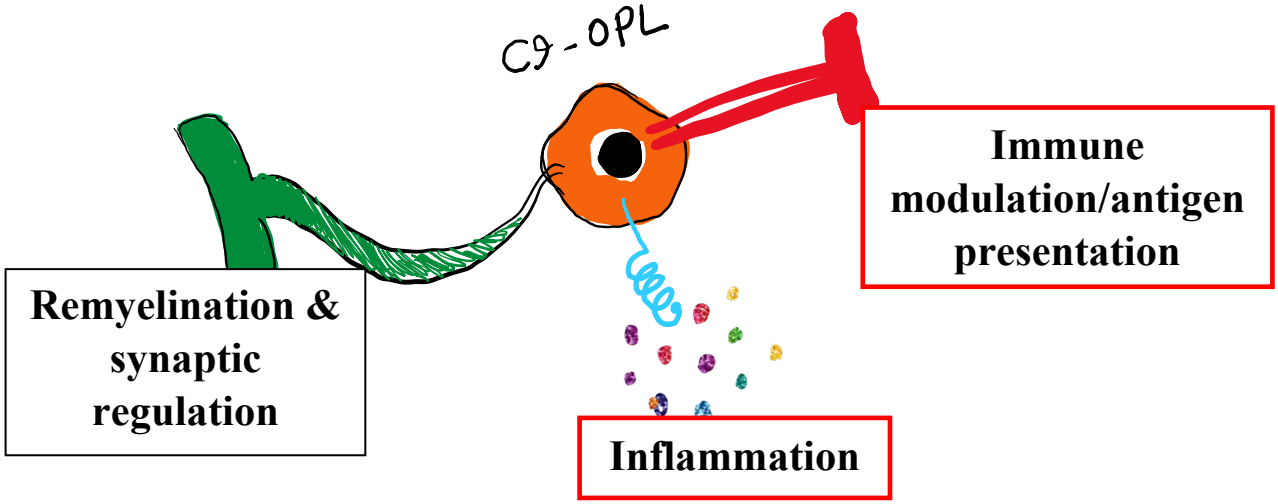


Figure 4.16 Schematic presentation of core C9-oNSC function as informed by pathway analysis

C9-oNSC showed robust enrichment for immune response activation, microglial crosstalk, and antigen presentation and inflammatory pathways (Fig 4.13). Transcriptome of C9-oNSC alongside pathway analysis revealed a myriad of molecules associated with antigen processing and presentation, including CD74, B2M, CTSS, HLA-A, HLA-B, HLA-C, HLA-DMA, HLA-DPA1, HLA-DRA, HLA-DRB1, HLA-DRB5, HLA-E, RAB33A, FECR1G etc. Oligodendroglial cells can exhibit immunogenic profiles in demyelinating diseases. OPCs play an important role in these demyelinating diseases by generating myelinating oligodendrocytes that may limit axonal degeneration, thereby promoting their differentiation and functional integration (Harrington, Bergles, & Calabresi, 2020). OPCs, like microglia, have demonstrated

their ability to constantly survey their environment and migrate to sites of CNS injury. Interestingly, OPCs also upregulate antigen presentation machinery in the demyelinating CNS and can regulate CD4 T cell proliferation and survival *in vitro*. Additionally, OPCs can cross-present antigens via MHC1 and activate CD8 T cells, further shaping the inflammatory milieu (Fernández-Castañeda et al., 2020). In addition to antigen presentation molecules, OPCs have also been shown to produce selective inflammatory cytokines and chemokines in areas of demyelination. Gene expression analysis of OPCs has demonstrated that increased expression of IL-1beta (a strong inducer of the innate immune response) has been detected within lesions and cerebrospinal fluid (CSF). Enrichment of pathways, including positive regulation of cytokine production (G0:0048518) and cellular response to cytokine stimulus (GO:0050896) was enriched in C9-oNSC demonstrated by genes, including F3, GSN, IL6ST, MT1X, MT2A, RORA, STXBP3, TLE4, CIB1, CDC42EP4, ERBN, ARID5B, IL17D, MBP, AGT, IL6ST, CD81 etc.

Many of the factors that influence OPC functions during neuroinflammation are downstream of the cytokines IFN-gamma, TNF-alpha, Il-1β. Moreover, interferon and TNF-alpha signalling are key inflammation and immune pathways indicative of wound healing process. While the inflammatory factors of these cytokines contribute to microglial-led degeneration, these same factors are not also necessary for effective myelin debris clearance but also for assisting with the migration of OPCs and supporting their maturation. Consistent with this, responses to interferon-gamma, IL-10 and TNF-signalling were all uniquely enriched in C9-oNSC (Brück et al., 1994; Cignarella et al., 2020; Domingues, Portugal, Socodato, & Relvas, 2016; Lampron et al., 2015; Nicholas, Stevens, Wing, & Compston, 2002). C9-oNSC also showed enrichment for pathways involved in macrophage and microglial cell activation (GO:0042116) and (GO:001774), respectively.

4.3.9 Unique NSC features were revealed in subcluster C5-eNSC (early NSC)

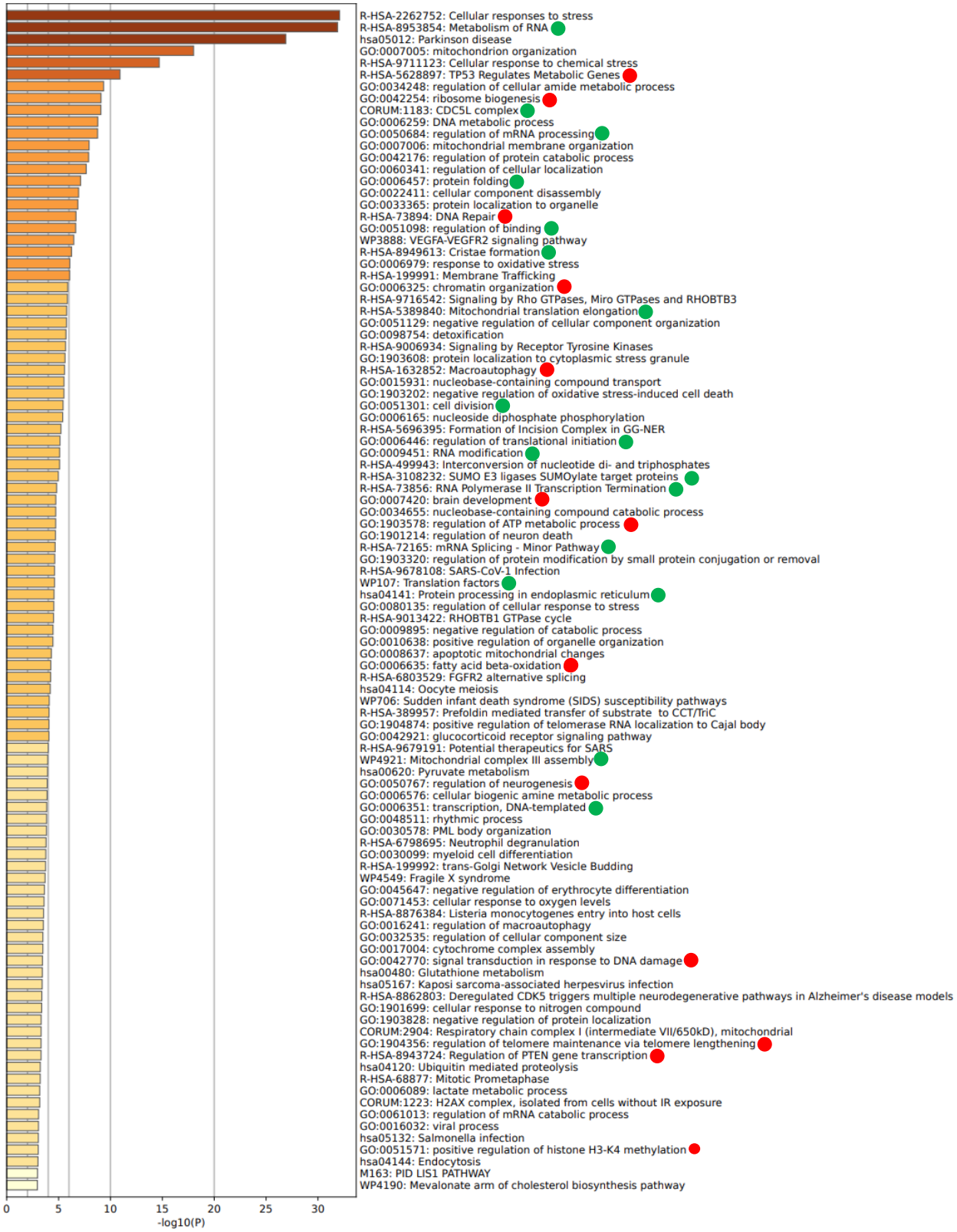


Figure 4.17 Pathway analysis of C5-eNSC (early NSC)
A. List of top statistically enriched terms (GO/KEGG terms, canonical pathways, hall mark gene sets, etc.,). Differentially expressed genes (top 500) in subcluster C5 were used to perform pathway and biological process analysis.

Pathway analysis for C5-eNSC subcluster results are shown in Fig 4.17 and 4.18. The top 500 differentially expressed genes yielded from LDE were utilised; significantly enriched biological pathways are shown in Fig 4.17.

The top 100 enriched pathways are shown in Fig 4.17. Unlike any of the subclusters that we explored previously, including C7-aNSC, C4-nNSC and C9-oNSC, pathway analysis revealed a distinct profile of subcluster C5. Firstly, no cellular processes associated with a any of the CNS lineages were expressed, rather core biological processes driving crucial cellular processes, including ribosome biogenesis, metabolic processes, DNA repair, mitochondrial processes, fatty acid oxidation and DNA binding regulation (Fig 4.17 and Fig 4.18).

On the contrary all the other three subcluster (nNSC, aNSC and oNSC) demonstrated specific function distinct from one another. However, shared fundamental core of developmental pathways, including brain development, aging, injury and repair, neurogenesis and, gliogenesis, oligodendroglial or neuronal lineage specificity C7, C9 and C4 respectively. While C5 pathway profile was devoid of any cell type specific function, several interesting biological processes were seen, including TP53 regulated metabolic genes (R-HAS-5628897), response to DNA damage (GO:0042770), fatty acid oxidation (GO:0006635), chromatin organization (GO:0006325), PTEN gene transcription (R-HAS-8943724), regulation of telomere maintenance (GO:1904356), ribosome biogenesis (GO:0042254), macroautophagy (and H3-K4 methylation (G0:0051571). However, two crucial pathways associated with neural stem cell function were

enriched (brain development (GO:0007420) and regulation of neurogenesis (GO:0050767)); highlighted by red dots.

Stem cells are required for lifelong homeostasis and regeneration of tissue. However, this function is believed to decline during aging. Quiescence has been defined as the fundamental property of stem cell to maintain genomic integrity and stem cell pool which is regulated by a complex interplay of different molecular mechanisms (Chakkalakal, Jones, Basson, & Brack, 2012; Cheung et al., 2012); for example, metabolic status, epigenome modulation, mitochondrial biogenesis, RNA processing, transcription activation, DNA replication and protein synthesis (Cheung & Rando, 2013). The transcriptome of C5 revealed enrichment of pathways regulating histone methylation (H3K4) and RNA processing (both rRNA and mRNA) and transcription and chromatin organization driven by CTNNB1, OGT, PHF20, NELFE, HSF1, HNRNPC, DHX9, SNRPG, PNN, TIA1, DDX1, GPX4, NAP1L1, DPF2, H4C3, HMGN3, BABAM1, ASF1A, CBX6 etc.

This chromatin remodeling and organization coupled with histone methylation has been is involved in chromatin accessibility in transcription and genetic regulation of stem cell potency and lineage (Bernstein et al., 2006). Consistent with this both transcriptional processing and mRNA splicing, and translational activity was also seen in C5 (Fig 4.17 – 4.19). Interestingly, C5 showed robust enrichment for transcriptional regulation by TP53; p53 is a master regulator of diverse cellular processes, especially those involved in the maintenance of genomic integrity and quiescence maintenance (Yan Liu et al., 2009). This is achieved by p53 regulating PI3kinase, Akt and MTOR pathways to mediate cell's adaptation to stress, which then negatively regulate Akt kinase and mTOR, leading to a decrease in cell growth (Puzio-Kuter, 2011). Genes underlying this pathway included COX5B, COX8A, COX2, RHEB, TVN, COX5A, PRDX5,

DDIT4, LAMTOR4 etc. Ribosome biogenesis is proportionally higher in stem cells than in differentiating cells; rRNA transcript is the highest in germline stem cells (Q. Zhang, Shalaby, & Buszczak, 2014). Interestingly, robust ribosome biogenesis was demonstrated in C5 alongside DNA repair machinery (Fig 4.17 and 4.18), C5 transcriptome was heavily dominated by ribosomal genes.

Nonsense-mediated decay (NMD), a post-translational mechanism that regulate gene expression, acts by degrading mRNA with premature termination codons or nonsense mutations (Mendell, Sharifi, Meyers, Martinez-Murillo, & Dietz, 2004). NMD is crucial for animal development in embryonic self-renewal and differentiation (Lou et al., 2014). Consistently, protein-protein interaction analysis revealed enrichment of NMD process in C5 (Fig 4.19).

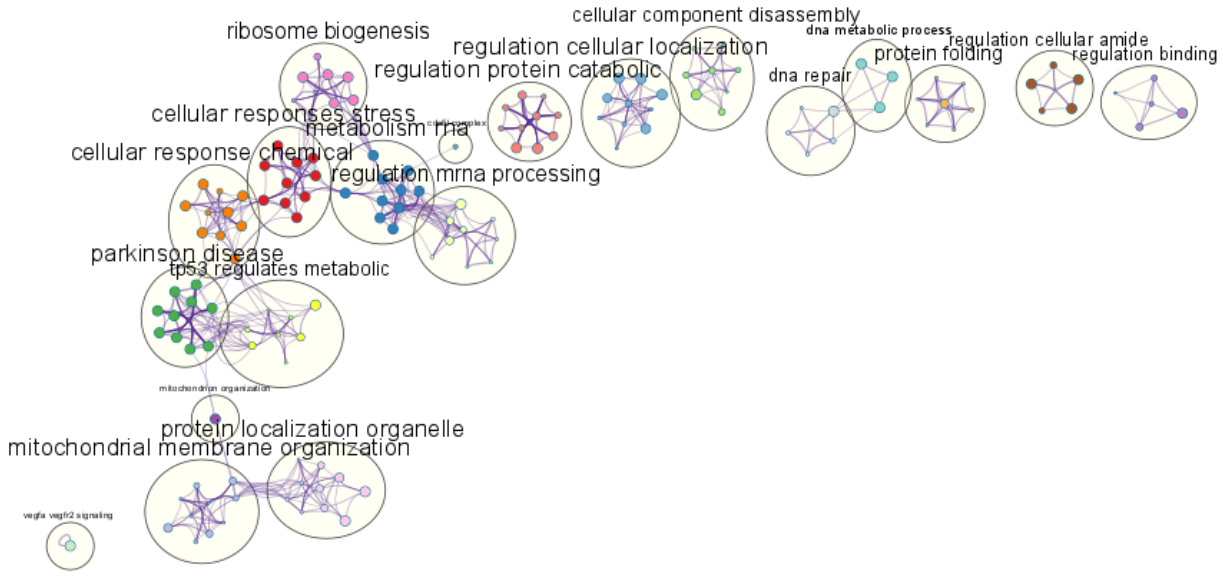


Figure 4.18 Biological processes enriched in C5-eNSC
A. Selected of a subset of representative terms from the full cluster and converted them into a network layout. More specifically, each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network is visualized with Cytoscape with "force-directed" layout and with edge bundled for clarity. One term from each cluster is selected to have its term description shown as label.

It is becoming increasingly clear that metabolism plays an instructive role in stem cell fate decision, for example, lipid metabolism and fatty acid oxidation is required in embryonic NSCs, and the breakdown of fatty acids has been shown to regulate the asymmetric division and maintenance of NSCs (Xie, Jones, Deeney, Hur, & Bankaitis, 2016). Interestingly, fatty acid oxidation pathway was enriched in C5 (Fig 4.19).

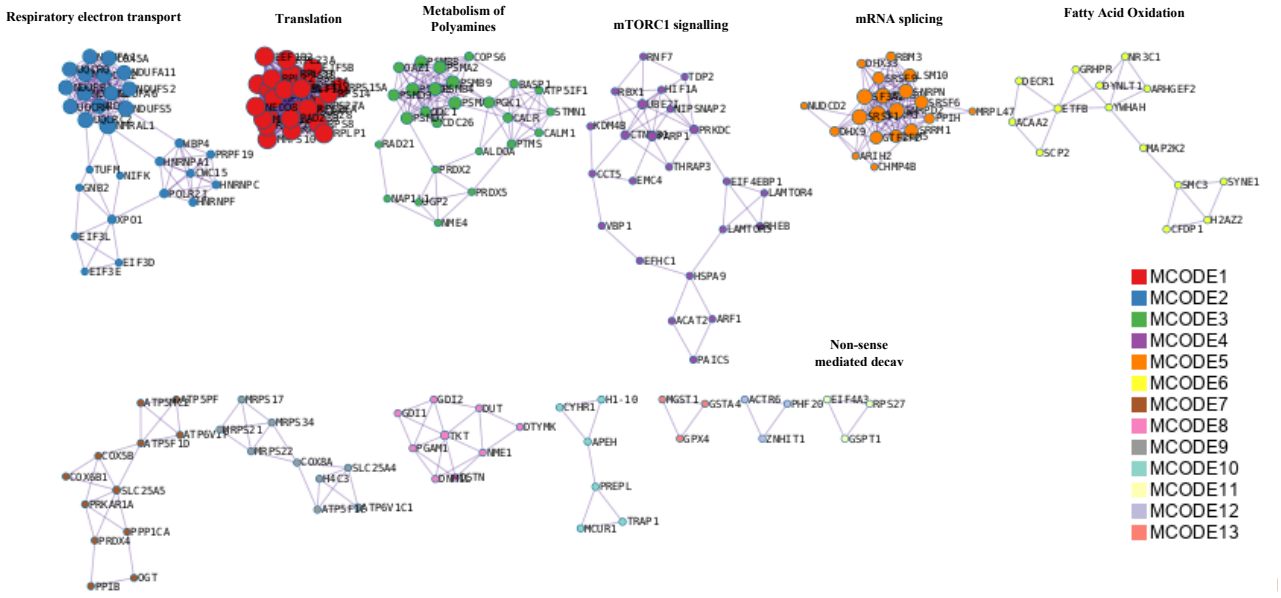


Figure 4.19 Protein-protein interaction and MCODE components identified for C5-eNSC.
For each given gene list, protein-protein interaction enrichment analysis has been carried out with the following databases: STRING, BioGrid, OmniPath, InWeb_IM.Only physical interactions in STRING (physical score > 0.132) and BioGrid are used. The resultant network contains the subset of proteins that form physical interactions with at least one other member in the list. If the network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm has been applied to identify densely connected network components. The MCODE networks identified for individual gene lists have been gathered.

The mammalian TOR (mTOR) pathway has emerged as a key regulator for cellular metabolism. Accumulating data have demonstrated that mTOR regulates several important cellular functions, including protein synthesis, autophagy, endocytosis, and nutrient uptake (Szwed, Kim, & Jacinto, 2021). Interestingly mTORC1 signalling was enriched in C5 (Fig 4.19).

4.3.10 Summary of biological processes and pathway analysis across the subclusters

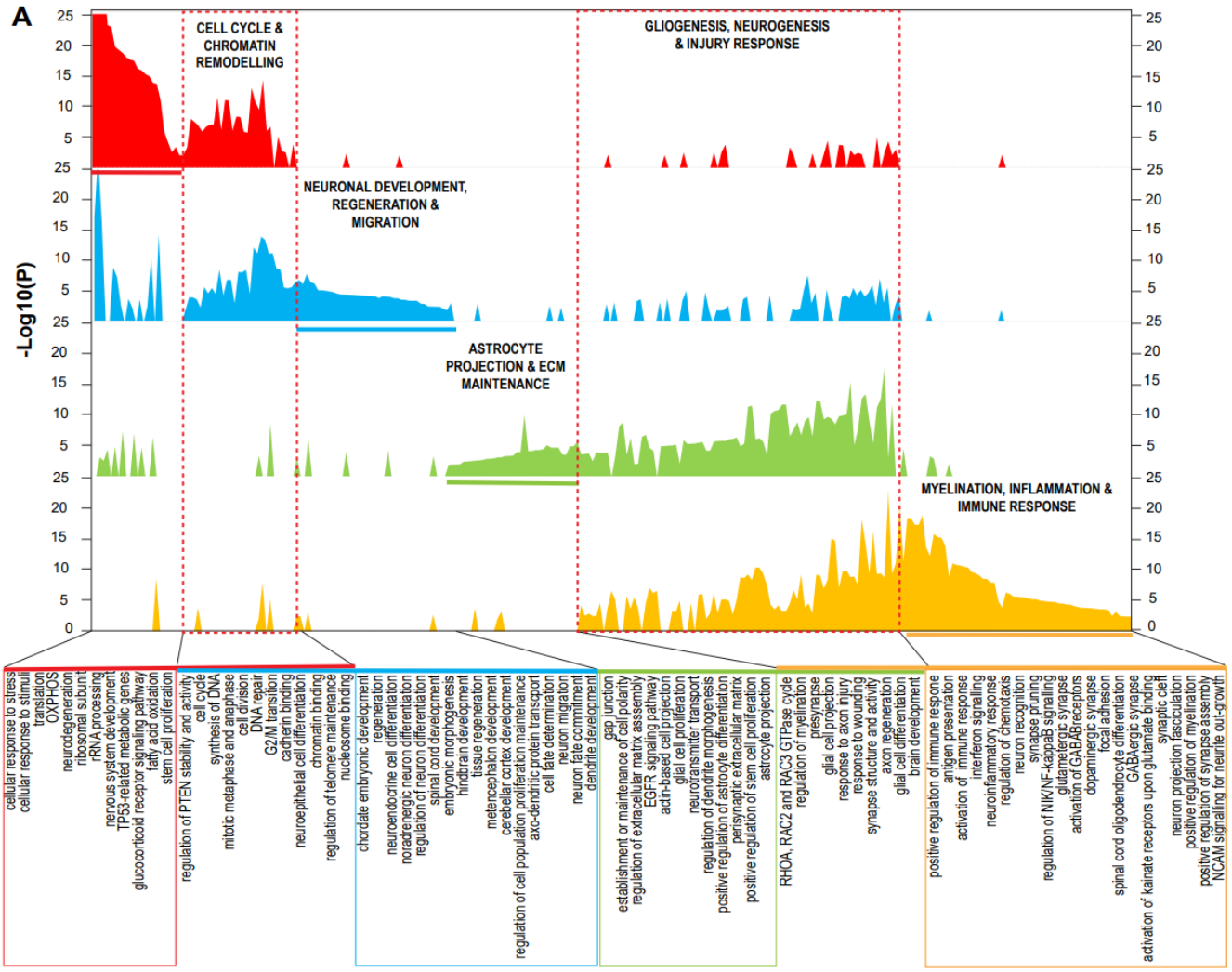


Figure 4.20 Enrichment of lineage-related developmental programs across the 4 subclusters (eNSCs, aNSCs, nNSCs and oNSCs)

In summary, to extricate the biological processes (BPs) and pathways driven by the genes identified in each of the subclusters, we performed pathway analysis by utilising top differentially expressed genes. Consistent with GSEA findings, all 4 subclusters demonstrated unique enrichment for lineage-specific BPs; nNSCs (neuronal), aNSCs (astrocytic) and oNSCs (oligodendrocytic) (Fig 4.20). nNSCs showed enrichment for key developmental processes,

including chordate embryonic development, embryonic morphogenesis, hindbrain development and cerebellar cortex development. Interestingly, neuron fate commitment, regulation of neuron differentiation and dendrite development were also exclusively enriched for nNSCs (Fig 4.20). On the contrary, astrocytic cell processes such as, EGFR signaling pathway, stem cell proliferation, growth factor binding, regulation of extracellular matrix, astrocyte projection and differentiation were distinctively enriched for aNSCs (Fig 4.20).

Intriguingly, unlike, foetal OPCs, adult oNSCs demonstrated a versatile BP profile demonstrating enrichment for not only core developmental glial processes, such as, brain development, myelination, gliogenesis, glial cell development, regulation of myelination, synapse assembly and organisation, but also uniquely showed enrichment for immune-inflammatory processes, such as glial cell activation, cytokine signaling, antigen processing and presentation, neuroinflammatory response, interferon signaling etc. Indeed, oligodendrocyte progenitors are at the center of inflammatory-immune processes regulating brain neuroinflammation and promoting CNS repair (Psenicka, Smith, Tinkey, & Williams, 2021).

However, BPs profile of C5 demonstrated no lineage specificity but showed enrichment for nervous system development (R-HAS-9675108), engaged in OXPHOS (HSA00190) and fatty acid oxidation, response to stress (R-HAS-2262752) and stimuli (R-HAS-8953897). It also demonstrated high rRNA processing (R-HSA-72312) and translation, neurodegeneration (hsa05022) and histone methylation. Strikingly, both eNSCs and nNSCs showed shared enrichment for stem cell proliferation and cell cycle/mitosis related processes (Fig 4.20). Hence, C5 demonstrated a unique molecular profile reflective of a quiescent progenitor population with

the capacity to undergo cell cycle. Owing to this molecular profile we refer to C5 as early NSC eNSC.

In addition to bearing unique biological profiles, a variety of BPs were shared amongst nNSCs, aNSCs and oNSCs. Interestingly, shared BPs revealed crucial neurodevelopmental processes (both neuronal and glial), such as, brain development (GO:0007420), regulation of nervous system development (GO:0051960), regulation of neurogenesis (GO:0050767), glial cell development (GO:0021782) and gliogenesis (GO: 0042063) (Fig 4.20). More interestingly, key processes associated with aging (GO:0007568) and brain repair and regeneration, such as, response to axon injury (GO:0048678), regeneration (GO:) and wound healing (GO:0042060) accompanied the developmental processes. These shared BPs along the neurodevelopment trajectory/injury response axis, were also accompanied by developmental migratory & motility and cell morphogenesis & guidance enriched including RHO GTPase cycle (cell morphogenesis (GO:0000902), neuron projection regeneration (GO:0031102) and guidance (GO:0097485), axon regeneration (GO:0031099) and development (GO: 0061564), suggesting a neuronal supportive role of glial cells.

Next, we set out to explore whether these lineage-specific progenitors exist in the normal adult human brain. To achieve this, we utilised adult human autopsy brain tissue and performed immunohistochemistry using subcluster-specific markers.

4.3.11 Mapping adult SVZ NSC-like subclusters revealed the presence of eNSC and aNSC, and scarcity of nNSC and oNSC in the adult human SVZ

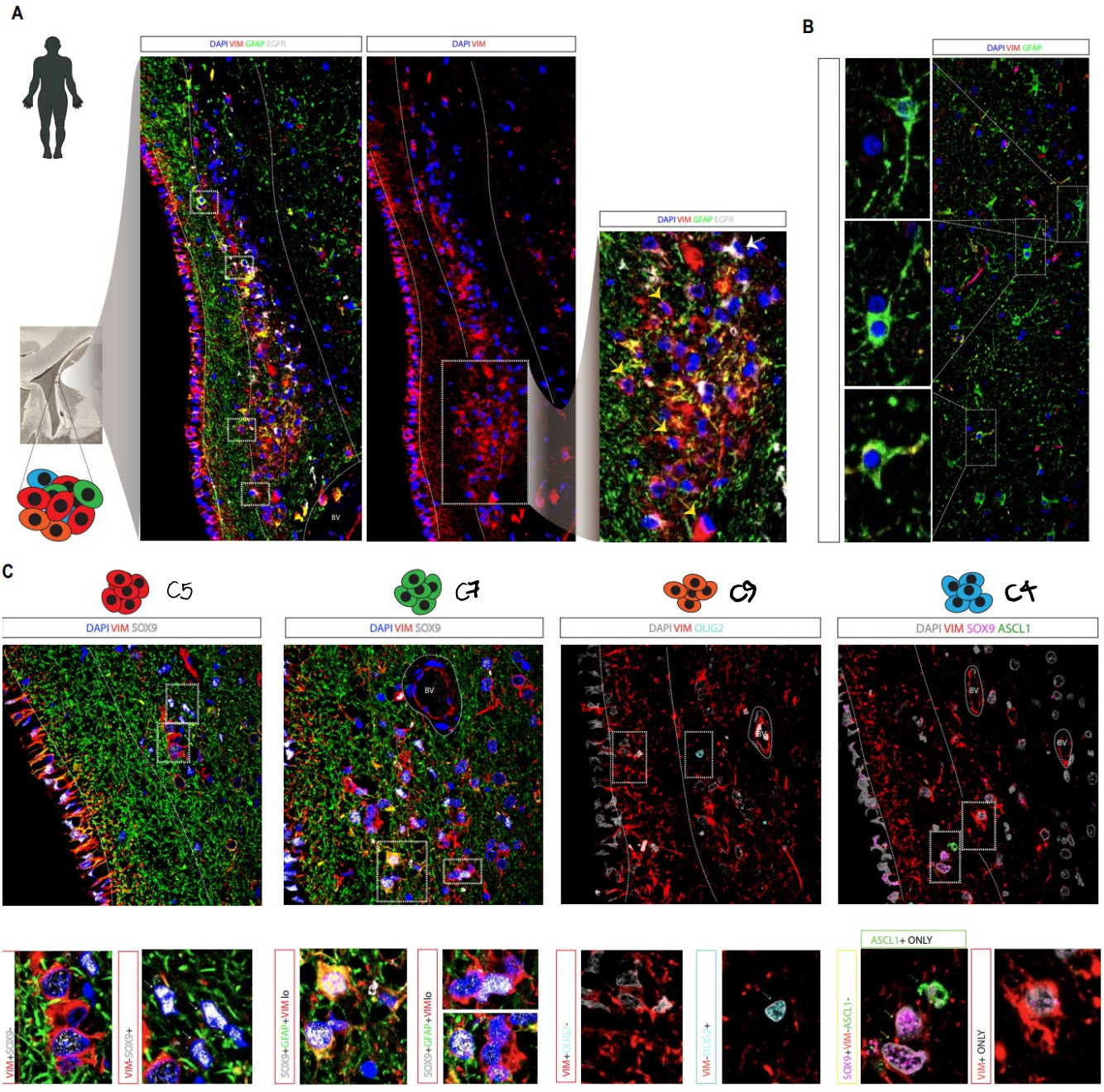


Figure 4.21 Delineating adult SVZ NSC-like subclusters *in situ*
A. Immunohistochemistry marking mutual exclusivity of VIM (red) and EGFR (white) positive cells.
B. Expression of GFAP positivity in the adjacent non-neurogenic zone. Cells demonstrating clear astrocytic morphology can seen positive for GFAP expression (green)
C. Identification of subclusters; from left to right: C5-eNSC, C7-aNSC, C9-oNSC and C4-nNSC. Cells positive for VIM (red) largely retained lower expression of SOX9 (white) - C5-eNSC, whereas SOX9hi (white) cells demonstrated lower to no expression of VIM (red) (C7-aNSC). C9-oNSC showed OLIG2 (cyan) positivity negative for VIM (red) and C4-nNSC positive for MASH1 (green), negative for VIM and SOX9 (magenta). Scale bar: 20X

To validate the adult NSC-like transcriptomic diversity *in situ,* we performed immunohistochemistry to delineate the subclusters in post-mortem adult human brain tissue. Same tissue utilised to validate SVZ cellular diversity *in situ* was used (n=3).

Both classical and SVZ transcriptomics-driven markers were selected to validate transcriptomic subcluster signatures of C5-eNSC, C4-nNSC, C7-aNSC and C9-oNSC. No single marker defined any subcluster as higher or lower expression patterns were seen across the subclusters. However, single marker specificity was seen in lineage-specific subclusters, such as C4 and C9. As informed by transcriptomics following panel was designed for each of the subcluster:

C5-eNSC: VIM+, GFAP-/lo, SOX9-/lo, EGFR-

C7-aNSC: VIM-/lo, GFAPhi, SOX9hi, EGFR+

C4-nNSC: VIM-, MASH1+, SOX9-

C9-oNSC: VIM-, OLIG2+, GPR17+ or OLIG2/GRP17+

All four subventricular zones were clearly established in the brain tissue sections confirming the anatomical SVZ identity. Both VIM and EGFR expression was first used to assess mutual exclusivity of both markers. GFAP, a well-known astrocytic marker, which is used to recognize and classify putative adult SVZ neural stem cells was also incorporated into our panel. Results are shown in Fig 4.21.

As expected, both VIM and EGFR positivity was seen to be mutually exclusive. While VIM expression dominated the astrocytic band of the subventricular zone (layer III). EGFR positive cells were observed to be nearly negative for VIM expression (Fig 4.21). GFAP

expression in the astrocytic band was seen, however, quite branchy, and fibrous. GFAP is a filament that is expressed in the astrocytic processes. In the adult SVZ, the neurogenic zone appeared to be covered in a fibrous network positive for GFAP in a fashion that processes were difficult to assign to a cell body (Fig 4.21; panel A). However, in the adjacent non-neurogenic parenchyma clear GFAP+ cells were seen demonstrating typical astrocytic morphology (Fig 4.21- panel B).

Interestingly, all four subclusters were observed in the astrocytic band of the adult SVZ (Fig 4.21; panel C). Mutual exclusivity of VIM and SOX9 was, again, clearly seen. This was consistent with our transcriptomics data where gradual decrease in VIM was seen in C7 compared to C5 and increase in SOX9. Both MASH1+ (C4-nNSC) and OLIG2+ (C9-oNSC) were also spotted in the astrocytic band of the SVZ confirming the identification of all four subclusters. MASH1+ cell was negative for VIM expression whereas OLIG2+ cell lacked expression of both VIM and SOX9 (Fig4.21; panel C).

Although all four subclusters were identified, the quantity of each of the subcluster varied dramatically. While C5 and C9 subclusters were noticeably present in the astrocytic band, C7 and C9 were rarely observed (Fig 4.21; panel C). As informed by transcriptomics, SOX9 and GFAP clearly defined C7-aNSC subcluster which lacked VIM expression. Consistently, while SOX9 and Vim exclusivity was clear (Fig4.22; panel A), GFAP+/VIM+ cells was rarely seen (Fig4.22; pane B).

The abundance of all four subclusters in the transcriptomics data but the lack of C9 and C4 in normal autopsy brain tissue was intriguing.

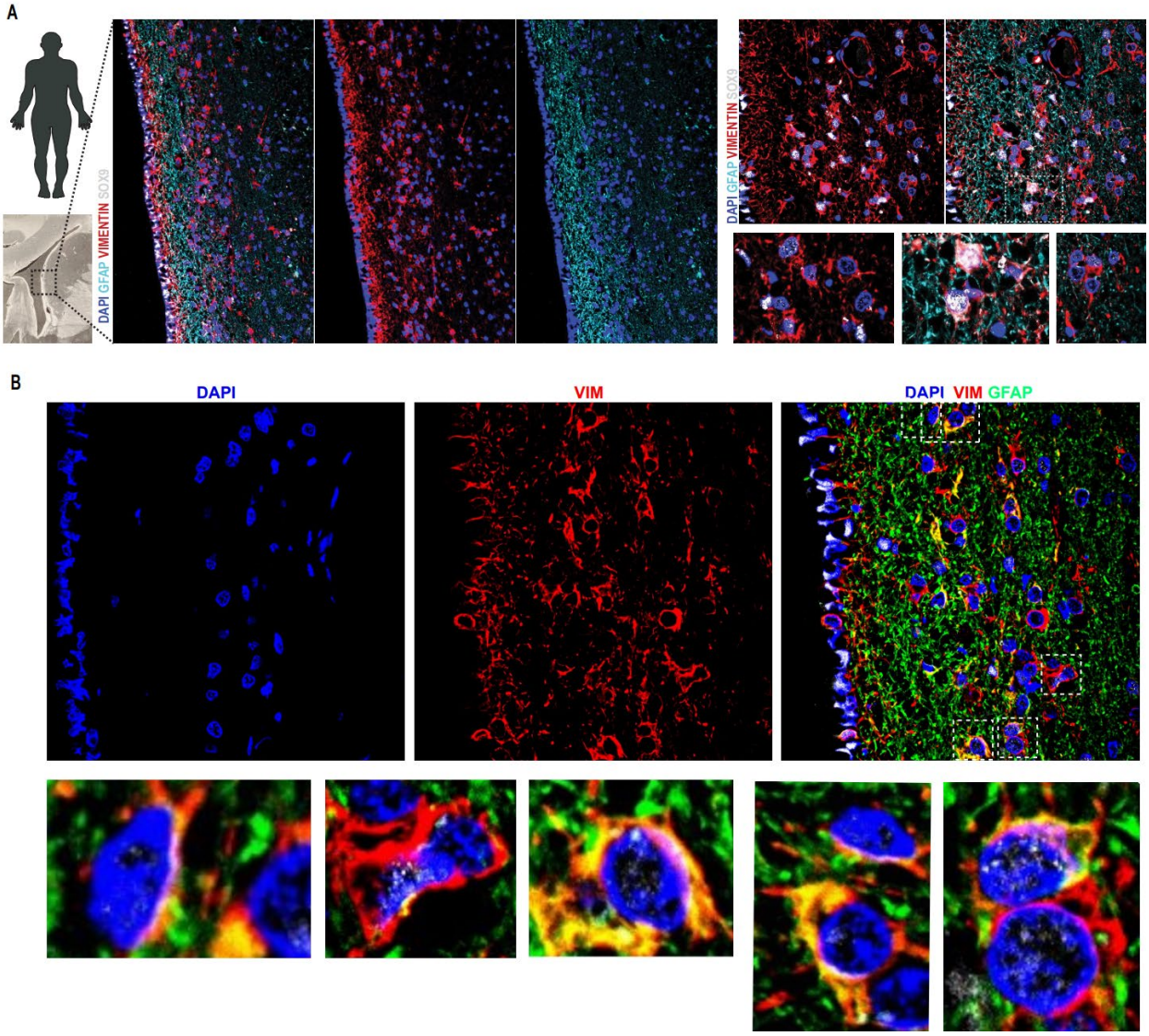


Figure 4.22 C5-eNSC and C7-aNSC are relatively abundant in the adult human SVZ
A. Immunohistochemistry marking GFAP (cyan) positivity and VIM (red); scale bar 63X.; and SOX9 (white). Scale bar 63X
B. Expression of GFAP (green) and VIM (red) in the astrocytic band. Scale bar 63X. Magnified images of highlighted cells showing mutual exclusivity of GFAP (green) and VIM (red).

4.3.12 C5-eNSC shared transcriptomic features with fetal early radial glia

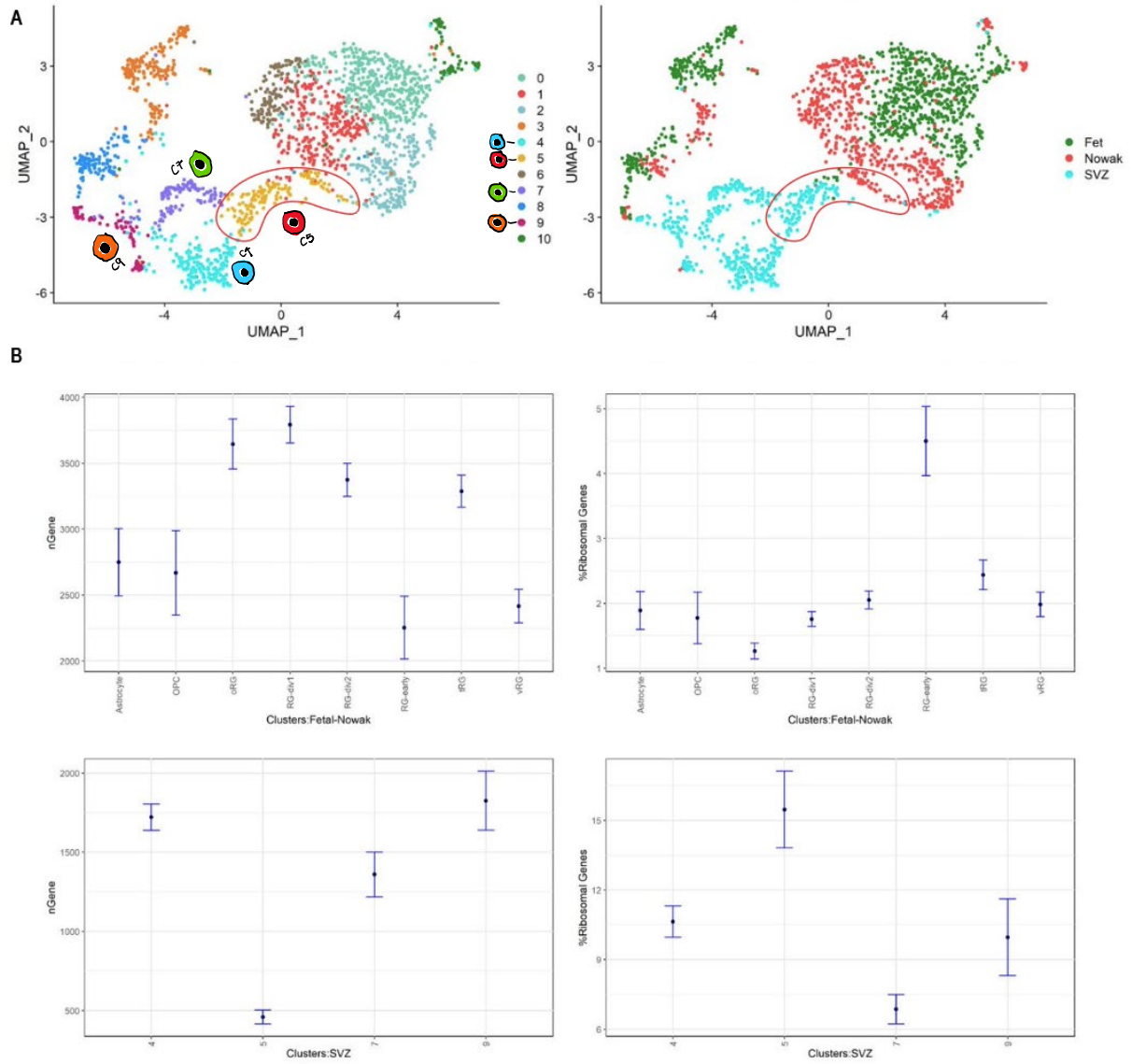


Figure 4.23 Transcriptome size and ribosomal protein content in C5-eNSC and fetal early RG (eRG)
A. Left panel: UMAP visualisation of conflated adult SVZ NSC-like cluster after applying clustering algorithm. **Right panel:** UMAP visualisation of conflated adult SVZ NSC-like cluster; cells are highlighted by datasets: adult SVZ NSC-like cluster (cyan) fetal datasets from Nowakowski et al. 2017 (red) and Couturier et al. 2020 (green)
B. Top panel: transcriptome size and ribosomal gene content in fetal eRG. **Bottom panel:** transcriptome size and ribosomal gene content in adult SVZ NSC-like subcluster C5.

C5-eNSC subcluster was composed of cells from both fetal and adult cells (Fig 4.23; panel A); cyan cells highlight adult SVZ cells and red highlights fetal cells (mainly from

Nowakowski et al. 2017). Intriguingly, fetal cells in the C5-eNSC subcluster were identified as early radial glial cells from the Nowakowski dataset. Moreover, only samples from embryonic gestational week (GW) 9 and below contributed to this cell type (Tomasz J. Nowakowski et al., 2017). We reasoned that exploring the transcriptomic similarity between the adult C5-eNSC and fetal C5-eNSC cells might aid in the profiling of this subcluster. Intriguingly, both fetal and adult cells in the C5-eNSC retained remarkably smaller transcriptomes compared to other clusters in the dataset (Fig 4.23; panel B). Moreover, high ribosomal gene content was seen in both fetal and adult C5-eNSC subcluster.

4.3.13 C5-eNSC preceded neurogenic C4-nNSC and gliogenic C9-oNSC subclusters on the lineage trajectory

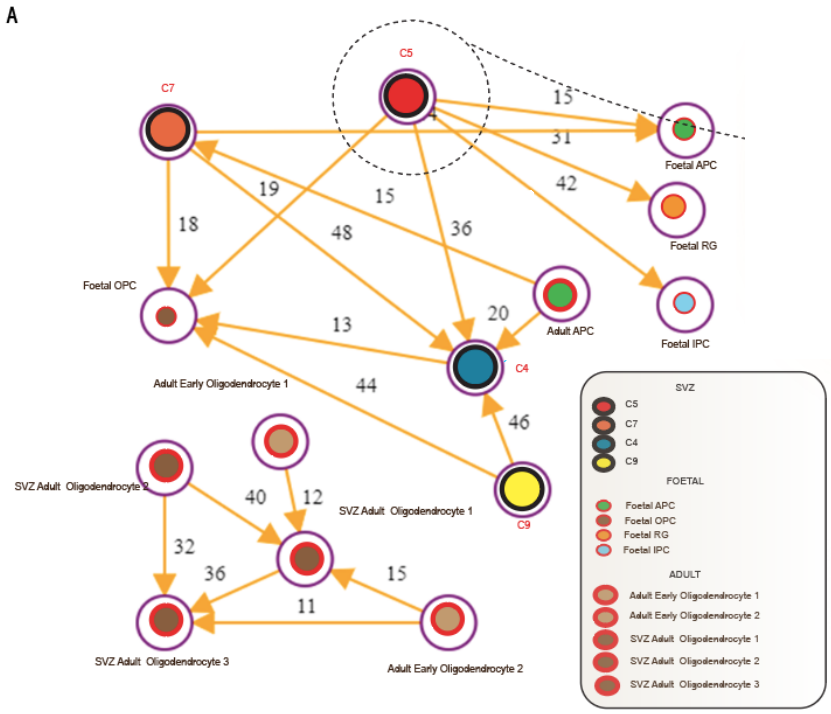


Figure 4.24 Velocity analysis revealed C7-aNSC and C5-eNSC as independent points of origin
A. Velocity analysis was performed on adult SVZ cells and fetal cells (Nowakowski et al. 2017 and Couturier et al. 2020). Clusters are colour coded: C5-eNSC (red), C9-oNSC (yellow), C7-aNSC (orange) and C4-nNSC (blue). Arrows indicate transition directionality of the predicted state, and the numbers indicate strength of transition directionality.
B. Schematic representation of velocity observations (as seen in panel A). C5 preceded all major radial glial populations and demonstrated directionality toward all fetal clusters.

Developmental hierarchies and lineage trajectories have been captured and reconstructed successfully using trajectory inference methods, particularly in dynamic systems, such as fetal brain and neoplastic structures (C. Couturier et al., 2020; La Manno, Soldatov, Zeisel, Braun, Hochgerner, Petukhov, Lidschreiber, Kastriti, Lönnerberg, & Furlan, 2018). To assess cellular lineage kinetics in the adult brain, we performed RNA velocity using scVelo algorithm on consolidated fetal and adult cells (Fig 4.24 and 4.24 (b)).

Lineage trajectory of foetal neural development is well-characterized, where trajectorial stream begins at the RG pool (vRG, oRG and tRG) and culminates in a mature cell type, such as mature neurons, particularly in neurogenesis. We assessed this by predicting future gene expression of clusters based on mathematical models informed by scVelo.

Remarkably, adult C5 preceded all major, well-annotated fetal progenitors and demonstrated transition directionality toward all 3 fetal developmental lineages: RG, APCs, OPCs, and IPCs (Fig 4.24 and 4.24 (b)). C7-aNSC followed a trajectory towards fetal OPCs and APCs only (Fig 4.24; panel A). Interestingly, no lineage flow was observed between C7-aNSC and C5-eNSC, suggesting an independent trajectory for both the C7-aNSC and C5-eNSC. However, C4-nNSC stream demonstrated multiple origins, particularly from fetal OPC and adult OPL populations, possibly hinting at progenitor plasticity with multilineage transition directionality.

The ability of adult C5-eNSC to give rise to fetal progenitors, the only progenitor sub-cluster with this versatile lineage potential, further prompted us to inquire deeper into the C5-

eNSC subcluster. To achieve this, we explored cell cycle status in both fetal and adult SVZ NSC-like cells.

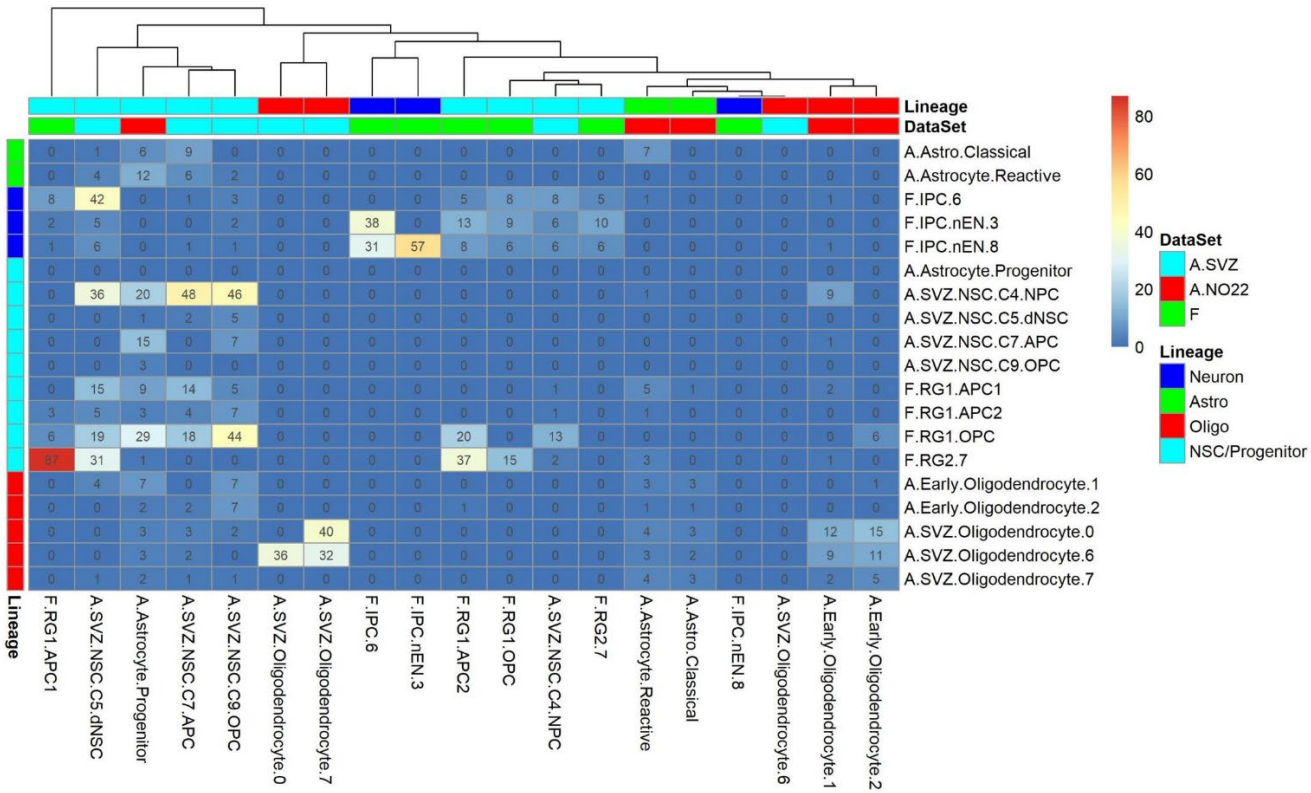


Figure 4.24 (b) Velocity Analysis
Supplementary velocity analysis heatmap for fetal, adult and SVZ cell types shown in Fig 4.24. Transition data reads from rows to columns, and the numberws are transition confidence.

4.3.14 Cycling cells identified in C5-eNSC and C4-nNSC

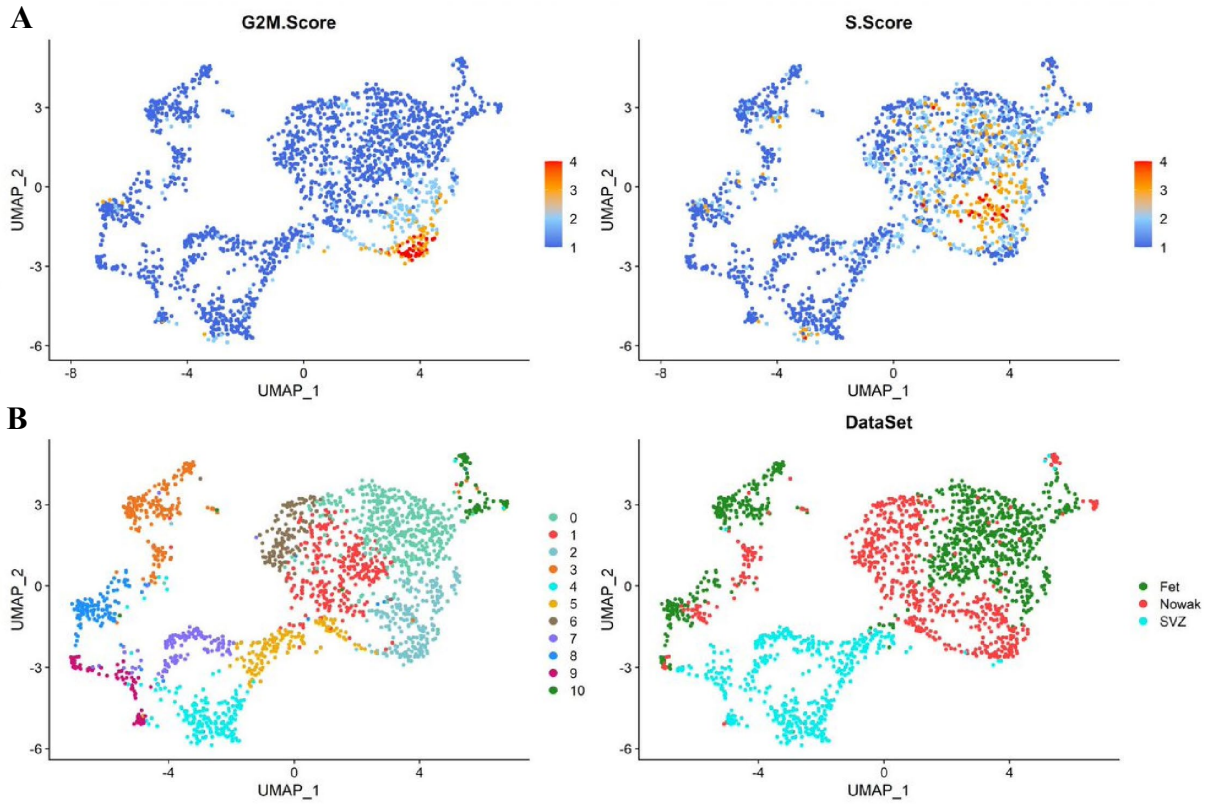


Figure 4.25 Cycling cells identified in C5-eNSC and C4-nNSC
A. Cell cycle phase scores based on expression of genes associated with G2M and S phase. Higher mitotic activity is observed in fetal cells; however, profound cell cycle activity is seen in subcluster C4-nNSC.
B. Left panel: UMAP visualisation of conflated adult SVZ NSC-like cluster after applying clustering algorithm. **Right panel:** UMAP visualisation of conflated adult SVZ NSC-like cluster; cells are highlighted by datasets: adult SVZ NSC-like cluster (cyan) fetal datasets from Nowakowski et al. 2017 (red) and Couturier et al. 2020 (green)

Next, to explore the cycling status of the adult SVZ NSC-like subclusters we performed cell cycle analysis. To achieve this, gene sets from two cell cycle phases (S phase and G2M phase) were used utilised to assess enrichment. As expected, strong enrichment of both cell cycles phases was seen in fetal subclusters (Fig 4.25; panel A). Robust enrichment of G2M phase was observed in RG-dividing 1 population (from Nowakowski), whereas S phase was enriched in RG-dividing 2 population (from Nowakowski and Couturier) (Fig 4.25; panel B). More importantly, profound G2M phase enrichment was seen in adult SVZ NSC-like subcluster C4-

nNSC. Moreover, subtle enrichment of S phase was also noticeable in C4-nNSC (Fig 4.25). Interestingly subtle cycling activity was also observed in C5-eNSC subcluster.

4.3.15 Human adult SVZ-derived cells demonstrated sphere-forming capacity and multi-lineage differentiation potential

Finally, we assessed stemness properties of SVZ-derived cells (Fig 4.26). Neural stem cells are assessed and identified based on their *in vitro*. For example, sphere forming NSCs capable of multilineage differentiation potential are recognised as bona fide neural stem cells (discussed thoroughly in chapter 1 and 3). To assess this behavior and property in adult SVZ-derived cells, we cultured adult SVZ tissue under restricted growth conditions.

Adult SVZ cells yielded spheres in culture (Fig 4.26; panel A). However, given the dearth of adult SVZ tissue, this was only achieved for 5 out of 10 SVZ samples. More interestingly, when grown in adherent culture conditions, these cells demonstrated radial morphology, a distinctive property of fetal radial glia, and showed expression of well known radial glial markers, including VIM, SOX9 and GFAP (Fig 4.26; panel A). Both forced differentiation and serum growth conditions resulted in differentiation of adult SVZ-derived cells resulted into progenies exclusively positive for each of the lineage-specific specialised markers, including MAP2 (neuronal marker), AQP4 (astrocytic marker) and APOD (oligodendrocyte marker) (Fig 4.26; panel A). Both adult SVZ and fetal brain-derived cells demonstrated similar behaviour in culture (Fig 4.26. panel B).

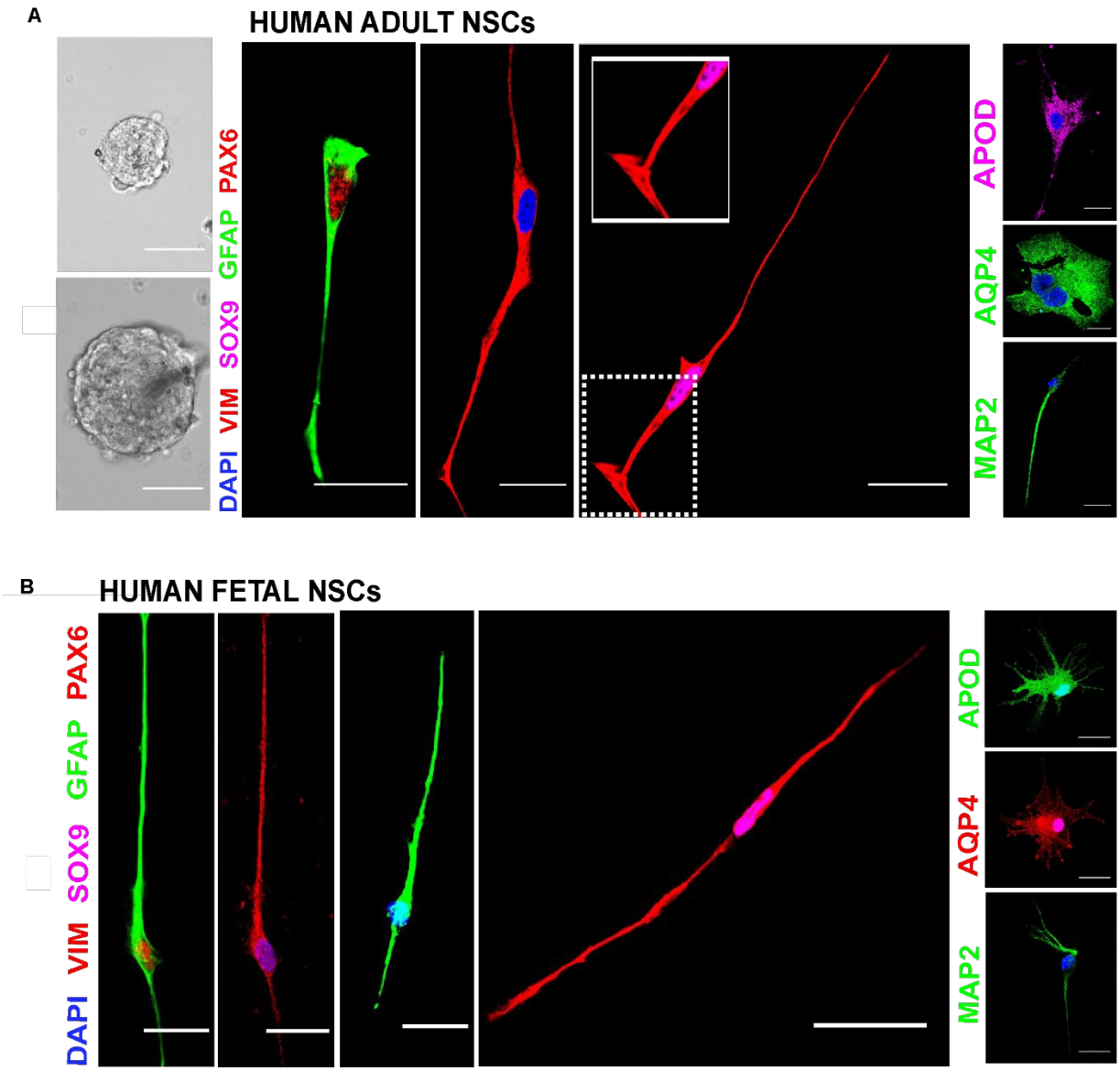


Figure 4.26 Sphere-forming capacity and multilineage differentiation potential of adult SVZ-derived cells *in vitro*
A. Left panel: Adult SVZ-derived cells yielded spheres *in vitro* **Right panel:** Adult SVZ-derived cells demonstrated radial morphology and expression of key progenitor markers, including VIM, SOX9, GFAP and PAX6. Multilineage differentiation. Both lineage specific differentiation driving cocktails and serum culture yielded three lineages: neuronal (MAP2; green), astrocytic (AQP4; green) and oligodendrocytic (APOD; magenta). Scale bar: 10um.
B. Fetal brain-derived cells demonstrated radial morphology and expression of key progenitor markers, including VIM, SOX9, GFAP and PAX6. Scale bar 10 um. Multilineage differentiation potential of fetal brain cells (GW16 – GW22). Both lineage specific differentiation driving cocktails and serum culture yielded three lineages: neuronal (MAP2; green), astrocytic (AQP4; red) and oligodendrocytic (APOD; green). Scale bar: 10um.

Summary:

In this chapter we have uncovered molecular heterogeneity of adult SVZ NSC-like cluster and revealed molecularly unique adult neural progenitors. We have achieved this by incorporating well-characterized developmental fetal cell types and exhaustive molecular analysis. This comprehensive approach to define neural stem cell heterogeneity resulted in four unique subclusters. To exhaustively characterize these subclusters we utilized both fetal developmental cell type signatures and endogenous molecular entities and processes (Fig 4.27).

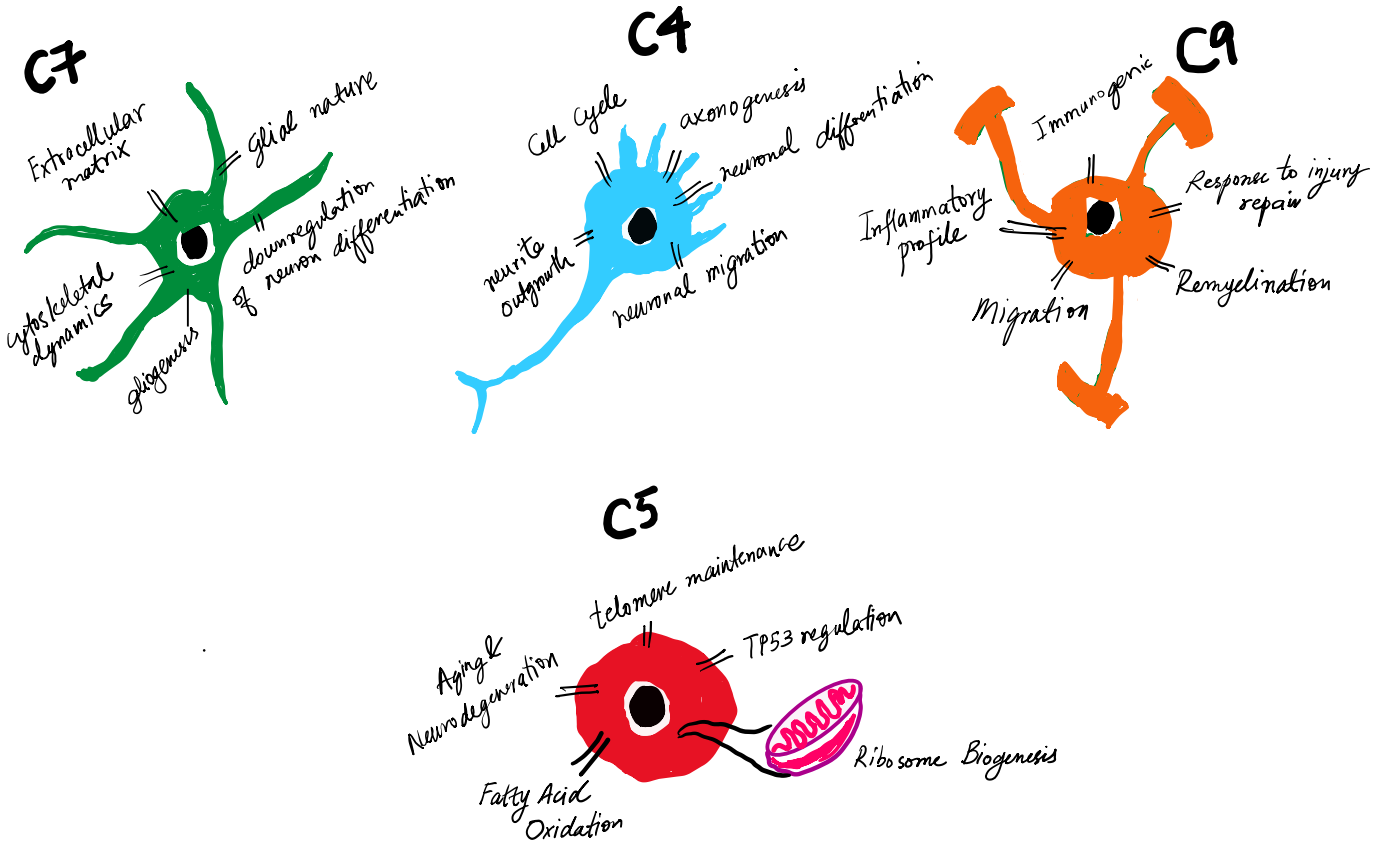


Figure 4.27 Schematic representation of the summary of adult SVZ NSC-like subclusters

Overall, we have achieved interesting revelations and insights into the diverse nature of adult SVZ-NSC diversity. Transcriptome and pathway analysis has revealed core programs shared by all subclusters and distinct molecular profile that distinguishes each subcluster which has further revealed lineage-related nature of C4, C7 and C9.

We pursued this in a stepwise manner by first cell type mapping to known and published cell types. Since the closest relatives that match the molecular profile and cellular behavior of stem cells are fetal/embryonic stem cells, we utilized established fetal development cell types and learned the transcriptomic similarities between adult and fetal neural stem cells.

We observed that while adult neural stem cells may share similarities with their fetal counterparts, they exhibit and maintain a unique profile. For example, the transcriptome of a new-born neuron in the fetal brain is reflective of migration along radial fibers toward cortical plate devoid of any synaptic function, whereas adult neuronal progenitor-like cells (C4-nNSC) demonstrate molecular constituents associated with response to wounding, repair, regeneration, axonogenesis and neurite outgrowth. This was further strengthened by processes supporting out-of-SVZ migration of these neuronal populations, such as E-cadherin and RAC-associated cytoskeletal dynamics. Recently, gliomas have been shown to use neuronal programs to hijack and colonize the brain along the neurodevelopmental/injury response axis (Venkataramani et al., 2022).

Likewise, the transcriptome of fetal OPCs reflect a relatively straightforward profile of OPCs that proliferate and differentiate into mature oligodendrocytes, however, adult C9-oNSC turned out to be a population that regulated a myriad of processes, including remyelination, inflammation and immune activation. Furthermore, activation of the migratory program and

response to brain injury and axon ensheathment and myelination further highlights the regenerative capacity and distinct function of oligodendroglial progenitors in the adult brain.

On the other hand, C7-aNSC demonstrated all the characteristics of traditionally defined neural stem cells, including complex matrisome and its contribution to niche ECM, its astroglia nature and regulation of cytoskeletal dynamics, and the activated status (demonstrated by EGFR expression), however, lacked migratory profile.

Activation of gliogenic and neurogenic programs in progenitors residing in the adult SVZ is an intriguing discovery as adult neurogenesis is believed to be likely impossible or rare. Consistent with this, *in situ* mapping of these subclusters showed the noticeable presence of C7-aNSC and C5-eNSC subclusters and rarely observed C4-nNSC or C9-oNSC in non-glioma SVZ. This strongly hints at the activation of lineage specific neuronal and oligodendroglia progenitors in the SVZ of glioma patients but lacked any differentiated cell types in the SVZ.

More interestingly, while almost all the subclusters corresponded to known progenitors, we uncovered a rare adult progenitor population that was made up of cells from both fetal and adult cells. We further saw this cell cluster was not captured in samples beyond gestational week (GW) 9 in fetal datasets, suggesting their role in early neurodevelopment and conserved nature. Both fetal and adult C5-eNSC cells demonstrated smaller transcriptomes and higher ribosomal gene expression. Excitingly, adult counterparts harbored processes associated with both quiescence and yet maintained active transcription and translation and shared cycling/ mitotically active status with C4-nNSC. Age-related quiescence or adult stem cell profile exhibited processes like autophagy, telomere maintenance and DNA repair mechanisms hinting at the aged profile and conserved nature of C5-eNSC.

To conclude, transcriptomic investigation of adult SVZ neural stem cell diversity has captured unique neural progenitors, which are noticeable in the SVZ of glioma patients, however, these progenitors were rarely observed in non-glioma SVZ suggesting a possible link between these progenitors and glioma.

In the next chapter, we will wind up and discuss our results from chapters 2, 3 and 4 in the context of neurogenesis and gliomagenesis and discuss the conclusions that could be drawn from our observations.

CHAPTER 5

DISCUSSION

The use of single-cell RNA-sequencing technology to gain unprecedented insights into cellular diversity and complexity has, indeed, given us a different perspective and transformed the way we view cellular and molecular biology. However, such depth not only uncovers the cellular intricacies but also discloses walloping amount of information, revealing our poor understanding. Such is the case in this study, where we attempted to answer some of the biggest and challenging questions both in the field of developmental neuroscience and neuro-oncology.

Although the chief objective of this study was to explore the possibility of cancer stem cells residing in the germinal zone (SVZ) in adult human SVZ specimens from glioblastoma patients, it demanded other questions to be answered first, for example, the status of adult human neural stem cells, which necessitated exploring the fetal brain, naturally, not without the challenges associated with acquisition and processing of adult human brain-derived samples. This developmental approach to understanding glioblastoma was desperately needed to answer two of the biggest outstanding questions in the field:

1. Are adult neural stems the origin of glioblastoma? If yes, then where these stem cells are and what do they look like?
2. If tumor is a hierarchically organized tissue, then where is the root of this hierarchy?

In this chapter we will review our findings and discuss our observations and interpretation. We will also see how these findings relate to the current understanding of disease and help us assess contemporary theories and speculations in the field.

Developmental v/s glioma trajectory

Parallels between CSCs/GSCs and normal stem cells have been drawn for a long time now, however, this similarity has been limited in terms of their function only (self-renewal and multi-lineage differentiation potential), and therefore, are referred to as "stem-like cells". This functional similarity is believed to be acquired by the earliest oncogenic cell(s) which gives rise to tumor thereafter. However, this long-held conventional view has been further refined by single-cell transcriptomic studies which have revealed that gliomas not only mimic the behavior of normal stem cells but also operate along the entire developmental framework. The term "cell-of-origin" has enormously dominated the realm of glioma research lately. This theory is mainly driven by the quest for quiescent cells that maintain and perpetuate tumors. Therefore, acquisition of adult SVZ tissue from adult glioma patients has brought to light the role of one the NSC harboring niches in the adult human brain.

Diverse nature of NSCs in the adult human brain

Performing cell-by-cell cellular census of the adult SVZ has revealed most of the cell types in the adult human SVZ and revealed unique adult NSCs-like progenitors, a cell type that has been long sought after. Our in-depth examination of the adult SVZ has enabled us to describe and define NSCs beyond a vague approximation which has been based on a single marker alone (GFAP). Based on this profile, our current definition of adult NSCs of the SVZ has been limited to GFAP-positive astrocytic cells in the SVZ and not much has been achieved from this understanding of the adult NSCs residing in the SVZ.

On the contrary, this study has brought to light the heterogenous nature of the adult SVZ progenitors; adult NSCs have long been thought to exist as a singular entity, an entity capable of

maintaining dormancy to facilitate self-renewal and give rise to multiple neural cell types. We successfully captured not only the potential dormant adult NSCs but also lineage-specific active progenitors, exhibiting multilineage differentiation tendencies in all three directions: astrocytic, oligodendrocyte and neuronal. In addition, developmental relationship between these populations demonstrated by lineage inference methods further supports their developmental potential, because, for a developmental hierarchy to exist, intermediate states along the continuum of cellular hierarchy are fundamental to the continuity of the cellular trajectory. Thus, this study has enabled us to define NSC diversity in the context of neurodevelopment at a level which has never been achieved before.

Neurodegeneration and brain repair

The presence of NSCs in diverse states is encouragingly indicative of developmental programs in the postmitotic human adult brain, but what is the functional significance of these progenitors? Unlike the human fetal brain, the adult human brain (a postmitotic organ) is naturally expected to be devoid of any active neurodevelopmental programs. The regenerative capacity of adult human brain has remained a challenge to explore, but sufficient regenerative capacity of adult mammalian brain has been demonstrated in mice. Use of scRNAseq technology in adult mouse SVZ has addressed questions related to increase in NSCs and NPCs numbers in response to various injuries; ischemic injuries have been shown to trigger transition of dormant NSCs into a state primed for activation, resulting in expansion of progenitor pools.

Interestingly, scarcity or absence of primed lineage-specific progenitors and abundance of eNSCs in normal human adult brain sections was remarkable. This discrepancy in the number of progenitor populations between glioma patient-derived SVZ and neuropathologically normal

SVZ sections is exciting and begs the question whether this is a glioma-related phenomenon only?

The prospect of progenitors generating stem cells seems unlikely at first glance. This is because our established perception of aNSCs is heavily dependent on morphological criteria, which dictates that adult NSCs should have radial glial cell-like (RGC-like) morphology.

Existence of latent developmental programs in glioma patients

If adult neural stem cells exist, they could only be caught in action. For adult neural stem cells to bounce back into action, there must be a need or demand for – repair or regeneration in the case of an adult system – which would serve as an activation trigger. Such demand would greatly vary from individual to individual based on various factors including brain tissue health, extent of wear and tear and overall homeostatic conditions. This data has captured adult neural stem cells in action in the neurogenic niche of the glioma patients and robustly demonstrated injury response, brain repair and neurodegeneration programs.

Perhaps, if not for cancer, we would not have been able to capture neural stem cells and recapitulate the lineage. This is because cancer itself is a dynamic process and perhaps a consequence of brain injury and repair.

It is only natural to assume that the source of the tissue being probed determines the nature of cellular entities and programs within that tissue. Although the SVZ tissue used to generate our SVZ dataset was derived from glioma patients, robust distinction between cancer and normal cells were made. When the CNV-carrying cells were removed from our dataset, significant decline in the number of cells in the lineage-specific pools, including C7-aNSC progenitors and C4-nNSC progenitors, including cycling cells, was evident. However, the pool

size of C5-eNSC remained unaffected. This incongruity in the pool size of progenitors and no significant change in dormant NSCs pool size between glioma and normal brain is astonishing. In fact, this only became evident when CNV-carrying cells were included back in the analysis, which gave our progenitor pool a significant boost; cell types/clusters made up of a small number of cells are generally considered inconsequential and ultimately go unrecognized and, therefore, are removed from the analysis.

The presence of VIM+ C5-eNSC and rarity of lineage-related progenitor pools both in glioma patient datasets (without cancer cells) and in neuropathologically normal SVZ sections strongly hints at the presence of dormant NSCs in the adult brain, but what purpose do these NSCs serve and how meaningful is their existence?

If the dataset from the glioma patients is any indication, it could be inferred that the SVZ of glioma patients is an active neurogenic site; it not only houses dormant NSCs but also is likely to engender lineage-specific glia and neuronal progenitors. Given that the lineage relationship between these progenitors and dormant NSCs has been reconstructed, existence of lineage-specific progenitors alludes to the dormant NSCs being their potential ancestors. This would suggest that dormant NSCs do retain neurogenic potential; but why is this phenomenon not prominent in a neuropathologically normal brain? As highlighted and discussed above, neurogenic potential of the adult human brain is virtually absent in the adult SVZ, and why should it not be? After all, it is a postmitotic organ, and dynamic phenomenon like neurogenesis and gliogenesis are profoundly observed only in active systems, such as fetal development. For this reason, it is much easier to witness and capture entire lineage hierarchies in dynamic, developing systems; cancer being one of them. If yes, then how do we expect to observe dynamic processes, such as neurogenesis, in a postmitotic, fully developed system like an adult

human brain? What use are dormant NSCs of in an adult human brain when the purpose of brain development has already been achieved during fetal development?

At this point it is inevitable to speculate the potential role of dormant NSCs in regeneration and repair, and inquire about the crude importance of dynamic phenomena, such as cancer, in studying latent processes like neurogenesis. In other words, it is, perhaps, difficult to validate the regenerative capacity of the adult human brain without capturing these programs in action when their function is not as prominent as it was during the peak of neurodevelopment? And that, if not for a dynamic system like cancer, the regenerative capacity of the adult brain could not have been observed.

Identification and characterization of a mysterious cell type (C5) embedded within the dynamism of C7, C4 and C5 is perhaps the most interesting of all. It has been a longstanding dream of neuroscientists to be able to identify adult NSCs. The profile of C5-DL has revealed the importance of conserved, fundamental biological processes that regulate cell fate and genomic integrity to preserve these long-living populations protected and conserve their pool. We have revealed crucial biological processes that quiescent stem cell engaged, such as fatty acid oxidation, lysosomal activity, ribosome biogenesis, transcription and post translational modification. To establish this functionally, isolation and maintenance of quiescent neural stem cells would remain a key to functionally validating this adult cell type, which, in the case of cancer therapy and regenerative medicine would be crucial. This would be further hindered by the rarity of tissue.

The origin of Glioblastoma has been speculated by various theories. Although all these theories agree upon cancer stem cells being the fundamental drivers of the disease, the nature of these assumed cell(s)-of-origin has been viewed differently. For instance, cancer stem cell(s) are

described as cells that are "stem-like" (not bona fide tissue-resident stem cells). These cells, prior to oncogenic transformation, could have been terminally differentiated cell(s) that after acquiring mutations, gained stem cell-like properties. According to another theory, this early transformed cell could have been a tissue-resident parenchymal progenitor(s) that had gone awry. Nevertheless, mounting evidence has shown that a tissue-specific stem/progenitor cell is more susceptible to oncogenic transformations.

Although previously reported, identification of CNV-carrying cells in the SVZ of glioma patients is an exciting finding. However, this identification of CNV-carrying cells is unique and reveals a crucial aspect of adult human SVZ and Glioblastoma. Firstly, previously reported mutation-carrying cells in the SVZ of glioma patients was a result of targeted sequencing of two hotspot mutations of telomerase reverse transcriptase (TERT) and 79 glioma-related genes along with chromosomal abnormalities (CNVs) in bulk SVZ tissue. They achieved this in triple-matched samples: the SVZ, the tumor and the blood in every patient. By comparing somatic mutations only between SVZ and the tumor, they observed that SVZ had a lower mutation-burden as compared to the tumor in the same patient. Secondly, although their study highlights the involvement of the neurogenic niche in gliomas, it does not reveal the stem cell type that could potentially be the origin.

Need for dynamic models to study dynamic processes

Neurogenesis and gliogenesis are highly dynamic processes that are well captured and characterized during development. Consequently, scientists heavily rely on cell type signatures built from single-cell sequencing dataset generated from fetal/embryonic transcriptomics dataset. However, there are many caveats to this approach, for example cellular behavior is dictated by the needs of the system and the demands of time; during corticogenesis, neuronal progenitors

express proteins that aid in migration out of the SVZ to populate the new forming cortical plate whereas, we have seen that adult neuronal-like progenitors displayed a transcriptome that reflected response to wound injury, cellular repair and out-of-SVZ migration with newly forming neurite outgrowths and active cell cycle status. Similarly, adult OPCs not only showed differentiation potential to become oligodendrocytes but also orchestrated injury repair, remyelination and synapse organization. Moreover, given age-related factors, adult OPCs regulated immune response and inflammation in adult brain.

Having said that, fetal and adult NSCs fundamental developmental programs that regulate processes like migration, stem cell maintenance and developmentally conserved lineage identity transcription programs. This fundamentally conserved similarity binds all subclusters together yet maintains a unique functional profile in adulthood.

Capturing NSCs in an idle adult system, inherently, appears to be a fool's errand. It is hardly surprising that such developmental programs would remain absent let alone manifestation of the entire developmental hierarchy. Therefore, declaring the absence of neurogenesis (birth of new neurons) and gliogenesis (birth of new glial cells) in a fully developed system based on casual immunostaining is a desperate attempt and a hasty conclusion.

Early NSCs in the adult human brain

The presence of eNSC that shared transcriptomic features with early radial glia in the fetal human brain is most intriguing. The metabolic status and the molecular profile of this subcluster would indicate an aged profile of these cells, hinting at the primordial/embryonic origin of these cells. Altered metabolic activity and cells under stress are generally associated with unhealthy cells, however, eNSCs displayed features of a cell that harbored an exhausted

molecular state and displayed mitotic capabilities shared with nNSCs. More interestingly, fetal eRG was only shown to be present during getational week 9 and under of fetal brain development.

As exciting as it may be, this population of adult NSCs represented a smaller fraction (less than 1%) of the total cells and could only be quantified by consolidating datasets from15 patients. But, in terms of their stem cell potential, do these cells hold functional significance and retain regenerative capacity? Lineage inference suggested that C5-eNSC preceded fetal early radial glia, which alludes to embryonic roots of the adult NSCs. Do these adult NSCs, after giving rise to fetal early radial, hibernate in a dormant state and continue to exist into adulthood to serve as the source of new progenitors in a postmitotic adult human brain?

The subventricular zone: The past (origin) and the present (maintenance) of tumor

The existence of both early NSC-like cells (eNSCs) and lineage-specific progenitors (nNSCs, aNSCs and oNSCs) is a strong reminder of developmental programs. Moreover, robust indication of involvement of injury response programs further props up the notion that adult human brain could be capable of repair and regeneration. Could the site of tumor be the site of injury that is being repaired with the help of replacement cells born in the SVZ but to be supplied to the site of injury? Is the enemy, the root of the disease in the SVZ? (Fig 4.28).

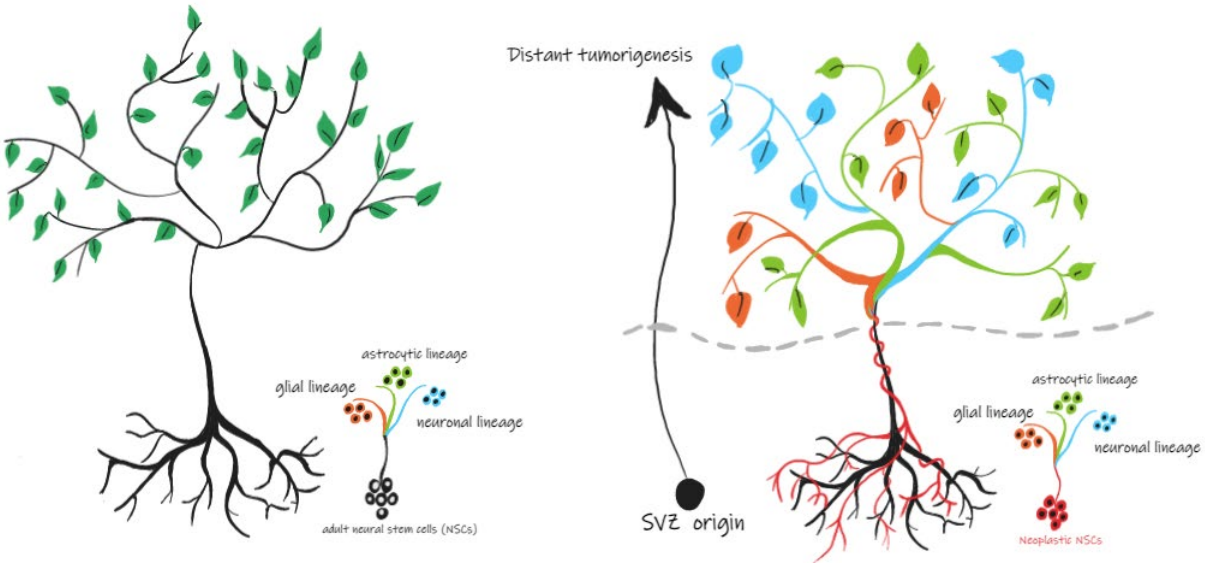


Figure 4.28 Neurodevelopmental roots of gliomagenesis in both the origin and maintenance of the tumor

Alternative explanation:

Although a transcriptomic snapshot of the adult human SVZ in glioma patients, this data has opened many questions and further challenges our understanding of the disease. Moreover, in the light of two contemporary, rather mutually exclusive, models, this data support the cancer stem cell model and underscores the importance of neurodevelopmental programs in the context of neural stem cells and their possible role in tumor maintenance.

This data also opens other avenues for alternative explanation. It should be noted that the inevitable proximity of the sampled SVZ to the tumor allows for the possibility of a reverse cell invasion i.e., invasion of cells from the tumor to the SVZ. Although, as informed by the neurodevelopmental programs, lineages emerge in the SVZ and the ensuing progeny migrate out of the SVZ to the cortical regions. Furthermore, such theories of white matter to SVZ invasion of glioma cells have been tested in mouse models where it has been demonstrated that glioma mutation-bearing progenitor cells in the SVZ migrate out of the SVZ and form tumors in the white matter. Moreover, using mutational burden as an indicator, cells in the SVZ have been shown to carry low mutational burden compared to the mutational burden of the cells in the tumor in glioma patients. Intriguingly, despite the possibility of tumor to SVZ invasion, gliomas are virtually never formed in this neurogenic zone. However, considering this observation, it could be possible that only the progenitors from the tumor gravitate towards the niche.

Nevertheless, the possibility of tumor to SVZ invasion of cells could not be ruled out and further interrogation into such invasive behavior are required to understand this possible phenomenon better.

Limitations of the study

Although encouraging, the data generated in this study and the observations can be confounded by methodological, conceptual and biological factors that need to be considered when interpreting these observations. Performing cellular census in the adult SVZ has revealed a wealth of information; the need for resolving the cell types/ states in the adult neurogenic niche is paramount to understanding both the process of oncogenesis and investigate the state of developmental cells and programs in the adult neurogenic zone. Although powerful, single-cell RNA-sequencing technology offers only a brief glimpse into the continuous realm of biology.

Conceptually, it is important to be aware of and understand the crucial difference between the existence of adult neural stem cells and their neurogenic potential. While the detection of new-born neurons plausibly suggests existence of a precursor i.e., neural stem cell/(s), the opposite doesn't necessarily hold true. This is particularly true when studying post-mitotic systems where rates and frequency of such developmental processes or birth of new cells is rare. The absence of new-born neurons does not rule out the possibility of existence of neural stem cells nor does it reflect the regenerative potential of the NSCs.

As much as we have captured unique cellular populations in the adult neurogenic zone, and profiled them based on the transcriptome and underlying biological processes, there are significant limitations to this study:

- Although transcriptomics revealed cellular entities in greater detail and depth by capturing subtle molecular differences, translating transcriptomics to proteomics to validate cell types *in situ* may not reflect the exact nature of cell states and thus

necessitates cautious interpretation of these data. To overcome this, technologies, such as *in situ* transcriptomics, could be used in the future.

- Given the small size of the stem cell pool, there is a strong need to increase the sample size, which owing to the rarity of the tissue and the cells could be a massive limitation. This could be overcome using previous knowledge of cell proportions in the tissue of interest. However, lack/absence of such studies makes it further difficult to work with an *a priori* estimation of stem cells expected in a dataset. For example, in hippocampal neurogenic studies, similar estimation strategies are employed to estimate the numbers of new-born neurons expected in a dataset. This is achieved by modelling estimation of the minimal number of sequenced cells required to observe a rare cell type at a certain frequency with a negative binomial or multinomial distribution.

  Another key variable related to the sample size that could impact detection of rare cell types is subject stratification. Detection of dynamic programs, such as neurogenesis, is influenced by various factors, including genetic background, existing pathologies, inflammatory status, medication, and lifestyle parameters. Evidently, given the rarity of such cell types, these results should be interpreted with caution.

  On the other hand, the opportunity to be able to work with freshly resected samples gives us a unique chance to capture cell types/ states which otherwise go undetected due to loss of cell/tissue quality associated with postmortem delay – a major obstacle in detecting neurogenesis in adult humans.

- Probing functional aspect of a cell type is fundamental to establishing the true identity and nature of a cell type. Given the limitations of the technology used, we have characterized the identified progenitor/stem cell populations based on their mRNA

content only. For example, lineage-specific molecular profile is reflective of markers that are associated with different lineages. In the case, whether a progenitor cell (npOPC and opNSC) associated with a certain lineage will give rise to a progeny of that lineage remains to be validated whether in culture or in *in vivo* settings.

However, *in vitro* manipulation of fragile cell types, including adult neural stem cells is particularly challenging as purification of various cell types/states requires using methods (such as, FACS sorting) that exert considerable strain on the cell(s) thus affecting the overall health and their survival.

- The similarities between the cancer stem cells and normal neural stem cells pose a huge challenge in making the distinction between the two in biological settings as no cancer-associated markers are known to exist. However, this has been overcome using computational approaches, such as copy-number calling (CNV) methods. Despite robust quality control checks and stringent CNV calling, the possibility that there could still be undetected CNV harboring cells cannot be eliminated with full confidence. This could result from either overestimation or underestimation of CNV. This is because CNV calling was performed on RNA sequencing data, not DNA, though it's a practice that is widely undertaken.
- To establish the identity of adult SVZ tissue-derived cells, we utilized neurosphere assay. Because the neurospheres were achieved using the SVZ-derived cells from the glioma patients, it could potentially be a mixed population of normal and CNV-carrying NSCs as, currently, there is no way to differentiate between a normal and CNV carrying cell with a 100% certainty (either phenotypically or immunohistochemically). However, brain tumor initiating cells have been shown to demonstrate prolific growth capacity *in vitro*.

On the contrary, SVZ-derived spheres showed latent growth patterns and slow growth dynamics.

- Functional regenerative capacity of adult neural stem cells cannot be conclusively determined as further maintenance and manipulation of adult stem cells would require further optimization which would greatly depend on our ability to mimic complex niche conditions *in vitro*. Moreover, neural stem cell identity of these populations has been established based on the transcriptomic profile and biological processes driven by the genes, and does not consider, the proteome, epigenome, and functional validation. More studies into the true functional nature of these populations are needed.
- We have gained valuable insights into the adult neurogenic, however, it should be kept in mind that although the SVZ samples were sampled from tumor-free (MRI-based), it has been harvested from glioma patients. Whether the tumor had influenced the states and microenvironment of the SVZ remains undetermined.

CHAPTER 6

CONCLUSION

This study has shed light on two of the outstanding questions in the field of neuroscience, and we attempted to kill two birds with one stone revealing not only the identity of adult neural stem cells but also their status in the SVZ of glioma patients. By building a cartograph of the adult neurogenic niche, we have extricated the neural progenitor populations and demonstrated the presence of lineage-specific progenitor states, nNSCs, aNSCs and oNSCs, in the SVZ of glioma patients – a region that is notoriously known to harbor the cell-of-origin.

We have gained, thus far, novel insights into the existence of neural progenitors, bearing glioma related CNVs in the SVZ of glioma patients and broken the progenitors down into their lineage-specific identities, taking the regional identity to the cellular level. Furthermore, we also show upregulation of programs associated with neuron development and oligodendrocyte development, primed for axonogenesis and synapse formation, respectively. We also reveal a unique progenitor population in the adult human SVZ that resembled fetal early radial glia (a rare population found during gestational week 9 and under) and preceded the lineage-specific progenitors on the trajectory scale.

More excitingly, we demonstrated the presence of neuronal and oligodendrocyte-like adult progenitors (nNSCs and oNSCs) in abundance in the SVZ of the glioma patients, in contrast to the rarity of these progenitors in adult human normal autopsy brain tissue. However, both eNSCs and aNSCs were plentiful in both glioma and normal autopsy tissue, suggesting a residential role of eNSCs and aNSCs as bona fide progenitors.

These findings lay a foundation to understanding some of the challenging questions centered around the cell of origin in gliomagenesis, such as, how do the most primordial cancer stem cells in the SVZ maintain and perpetuate the tumor mass? Are the neurodevelopmental programs in the guise of brain repair and injury response programs behind this? Is cancer a

consequence of neurodegeneration? To answer these imperative questions, this study has paved the way by unravelling crucial aspects of adult human neural progenitors of the SVZ and their potential to propagate neural lineages. Further enrichment and manipulation thereafter could reveal important biological insights into the functional role of these progenitors.

If the SVZ and the tumor turns out to be a single neurodevelopmental trajectory laid out across these two regions, mimicking neurodevelopment for brain repair, then the enemy and the root of the disease is in the SVZ. This would challenge the way we see the disease and would require novel and challenging therapeutic interventions to target the root of the disease in the SVZ. This, however, is going to be challenging given the challenges associated with acquisition of the SVZ tissue and manipulation thereafter as faithful recapitulation of SVZ progenitor function *in vitro* could prove to be difficult to achieve. Nevertheless, this study has opened doors to difficult yet exciting questions and challenges to be pursued.

METHODS

7.1 Ethics statement

Adult SVZ samples were harvested under a protocol approved by the Montreal Neurological Hospital's research ethics board. Consent was given by all patients.

7.2 Adult human intraoperative sample collection and dissection

Pre-operative magnetic resonance imaging (MRI) was performed for surgical planning. We obtained SVZ samples from 14 patients undergoing surgical resection of IDH wild-type glioblastoma, IDH-mutant glioma, or metastatic carcinoma. All samples were obtained from radiologically normal brain regions. Samples were processed as described above. Clustering was done using the Louvain algorithm, differential expression using the Wilcoxon test, and CNA analysis using the InferCNV (V.1.4.0).

7.3 Adult human SVZ sample processing and culture conditions

V-SVZ specimens were washed three times in sterile PBS containing penicillin and streptomycin. Specimens were then minced into pieces of less than 1mm in size, before being digested in a collagenase solution containing DNAase (Cal Biochem EMD Chemicals) and $MgCl_2$ for 1 hour at 37◦C. The digested specimens were washed with sterile PBS, and large debris were removed with a 70µm strainer. Residual RBCs were removed by density gradient using percoll (GE-Healthcare Bio-science AB). Samples were washed two more times in sterile PBS. An aliquot of cells was collected for single-cell mRNA-sequencing and the remaining cells were cultured in complete neurocult-proliferation media (Neurocult basal medium containing: Neurocult NS-A proliferation supplement at a concentration of 1/10 dilution, 20ng/ml recombinant EGF, 20ng/ml, recombinant bFGF, and 2µg/ml Heparin) from Stem Cells Technologies for all the downstream experiments.

7.4 Human autopsy brain tissue

Human autopsy brain specimens were obtained from de-identified excess diagnostic brain tissue that had been slated for incineration (n=3). Brains were cut in the coronal plane and immersed in 3% paraformaldehyde (PFA) or formalin for 1–2 weeks and then portions of their lateral ventricular walls were excised. These were further processed for immunolabeling, embedded in paraffin, and 5 µm thick sections were cut using a microtome (SLEE).

7.5 Human fetal sample collection and processing

De-identified human samples of fetal telencephalon (13–21 gestational weeks) were obtained from the University of Washington Birth Defects Research Laboratory (Seattle, Washington, USA), Center Hospitalier Universitaire Sainte-Justine (Montreal, Quebec, Canada), and the University of Calgary (Calgary, Alberta, Canada). The collection and use of the specimens following parental consent was approved by The Conjoint Health Research Ethics Board at the University of Calgary and studies were carried out with guidelines approved by McGill University and the Canadian Institutes for Health Research (CIHR). Briefly, fetal brain tissue was minced and treated with DNase (Roche, Nutley, NL) and trypsin (Invitrogen, Carlsbad, California, USA) before being passed through a nylon mesh. An aliquot of cells was collected for single-cell mRNA-sequencing and the remaining cells were cultured in complete neurocult-proliferation media (Neurocult basal medium containing: Neurocult NS-A proliferation supplement at a concentration of 1/10 dilution, 20ng/ml recombinant EGF, 20ng/ml, recombinant bFGF, and 2µg/ml Heparin) from Stem Cells Technologies for all the downstream experiments.

7.6 Adult and fetal single cell capture and library preparation for sequencing

For each sample, fetal or adult, an aliquot of cells was taken and stained for viability with calcein-AM and ethidium-homodimer1 (P/N L3224 Thermo Fisher Scientific). Stained cells were loaded onto hemocytometers (Incyto DHC-N01-5) and visualized through the bright field, GFP, RFP of a EVOS FL Auto microscope (ThermoFisher).

Following the Single Cell 3' Reagent Kits v2 User Guide (CG0052 10x Genomics) (Zheng et al., 2017), a single cell RNA library was generated using the GemCode Single-Cell Instrument (10x Genomics, Pleasanton, CA, USA) and Single Cell 3' Library & Gel Bead Kit v2 and Chip Kit (P/N 120236 P/N 120237 10x Genomics). Briefly, cell suspension was added to the reverse transcription (RT) reagents, then loaded onto the appropriate well on a 10X Chip. Afterward, gel beads and segregation oil were loaded to their designated wells. The 10X chip was then inserted into the GemCode instrument and nanoliter-scale droplets called GEMs (Gel bead-in-emulsions) were generated. The GEMS were then transferred from the 10X chip to PCR tubes and reverse transcribed in a T1000 Thermal cycler (Bio-Rad P/N 1861096) programmed at 53◦C for 45min, 85◦C for 5min then held at 4◦C. For bulk tumor, RT was performed within 6 hours of the extraction of the sample from the patient. After RT, GEMs were broken, and the single-strand cDNA was harvested with reagents containing DynaBeads (P/N 37002D Thermo Fisher Scientific). cDNA was then PCR amplified by a thermal cycler program of 98◦C for 3min, 10-to14 cycles of (98◦C for 15s, 67◦C for 20s, 72◦C for 1min), 72◦C for 1min then held at 4◦C. The full-length cDNA was quality controlled for sized distribution and yield (LabChip GX Perkin-Elmer). Subsequently, the full-length cDNA was fragmented, end repaired, A-tailed and index adaptor ligated, with SPRIselect Reagent Kit (P/N B23318 Beckman Coulter) cleanup in between steps. Post ligation product was PCR

amplified with thermal cycler programmed at 98◦C for 45s, 10to14 cycles of [98◦C for 20s, 54◦C for 30s, 72◦C for 20s], 72◦C for 1min and held at 4◦C. The sequencing ready library was purified with SPRIselect, quality controlled for sized distribution and yield (LabChip GX Perkin-Elmer) and quantified using qPCR (KAPA Biosystems Library Quantification Kit for Illumina platforms P/N KK4824). For sample QC and other parameters please see Table S2. Finally, the sequencing was done using Illumina HiSeq4000 or HiSeq2500 instrument (Illumina) using the following parameter: 26 bp Read1, 8 bp I7 Index, 0 bp I5 Index and 98 bp Read2. Cell barcodes and UMI (unique molecular identifiers) barcodes were de-multiplexed and single-end reads aligned to the reference genome, GRCh38, using the CellRanger pipeline (10X Genomics). The resulting cell-gene matrix contains UMI counts by gene and by cell barcode.

7.7 Clustering

For each cell, counts (Number of UMIs) per gene were normalized to the total counts of the cell and scaled by 10000. The data were natural-log transformed (log(counts+1)). All cells expressing more than 200 and less than 3500 genes, with lower than 8% expression for mitochondrial genes were kept, leading to 10385 cells in total. The 35 most significant principle components were selected, using jackstraw method and elbow visualization, to cluster the cells based on a shared nearest neighbour (SNN), applying Louvain algorithm (Stuart et al., 2019; Waltman & Van Eck, 2013). Markers, differentially expressed genes for each cluster compared to all other clusters, were identified using FindAllMarkers function with default Wilcoxon test (Stuart et al., 2019). Cell types were assigned to each cluster using known genes as shown in Fig. 4D; these were consistent with markers previously reported [14]. The results were visualized using t-distributed stochastic neighbour embedding (t-SNE).

7.8 RNA Velocity

We performed velocity analysis using partition-based graph abstraction (PAGA) (Wolf et al., 2019). PAGA-based velocity generates simpler graphs compared to single cell approaches. It can be especially beneficial here, as we have different samples with different ages and single-cell approaches can be more challenging. Single-end reads were aligned to the reference genome, GRCh38, using the CellRanger pipeline form 10X Genomics as previously described (Couturier et al., 2022). Spliced and un-spliced counts were calculated and saved in loom file format using Velocyto package [http://velocyto.org/]. The loom files from single samples were combined using scVelo package [https://scvelo.readthedocs.io/en/stable/about/] and were normalized with default parameters using. First and second-order moments were calculated for each cell across its 30 nearest neighbors in PCA. Velocity has been estimated and velocity graph constructed using default parameters. Transitions between clusters are usually range between 0 and 1, and a higher value means a better transition confidence. Just for the matter of visualization we multiply the values by 100.

7.9 Copy number variation calling:

InferCNV (V.1.4.0) was used to detect large-scale chromosomal CNAs at single-cell level using gene expression data. Gene expression values were smoothed on a moving window of 101 genes and CNAs were analyzed relative to the reference cells (Tickle, Tirosh, Georgescu, Brown, & Haas, 2019). The three largest clusters of myeloid cells (3 out of 8 clusters), and the largest cluster of oligodendrocytes (1 out of 3 clusters) were used as reference cells. The most variable chromosomes (greatest number of alterations) were chromosomes 7 and 10. The cells with any chromosomal alterations on chromosome 7 (gain) and chromosome 10 (loss) were defined as altered cells.

7.10 Fetal and adult adherent culture conditions:

Spheres were collected and gently dissociated with a pipette to achieve single-cell suspension in 1.5 ml of mTeSR 1 (Stem Cells Tech.) media. Single-cell suspension was seeded on coverslips coated with Matrigel (Stem Cells Tech.) with DMEM/F-12 (Gibco) in a 24 well-plate for at least 1 hour at 37^0C in a CO2 supplied incubator.

7.11 Fetal and adult neurosphere assay:

Intraoperative specimens were dissociated as mentioned above and incubated in non-adherent neurosphere conditions as described (Gritti et al. 2002). Each well in a 24 well-plate contained 1.5 ml of neurosphere medium (Gritti et al. 2002) and a cell concentration of 50,000 cells per well. Neurospheres were observed by week 3 post culture. Neurosphere growth was observed to be slower as compared to the fetal neurospheres (neurospheres were seen in 3 days post-culture). All the spheres used were between 150 to 200 μm in size. Conditioned neurosphere medium was used only in clonal neurosphere assays for which complete neurosphere medium was exposed to mature neurospheres for 24 h. The resultant medium was combined in a 50:50 mixture with fresh complete neurosphere medium to generate conditioned neurosphere medium.

7.12 Pathway and Process enrichment analysis

Pathway and Process enrichment analysis was performed using Metascape and Cytoscape (Zhou et al., 2019). For each given gene list (top 500 differentially expressed genes for each subcluster), pathway and process enrichment analysis was carried out with the following ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM and WikiPathways. All genes in the genome have been used as the

enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the cumulative hypergeometric distribution, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testings. Kappa scores are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.

7.13 Protein-Protein interaction enrichment analysis

For each given gene list, protein-protein interaction enrichment analysis has been carried out with the following databases: STRING[6], BioGrid[7], OmniPath[8], InWeb_IM[9].Only physical interactions in STRING (physical score > 0.132) and BioGrid are used. The resultant network contains the subset of proteins that form physical interactions with at least one other member in the list. If the network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm, has been applied to identify densely connected network components. The MCODE networks identified for individual gene lists have been gathered and are shown in Figure 3. Pathway and process enrichment analysis has been applied to each MCODE component independently, and the three best-scoring terms by p-value have been retained as the functional description of the corresponding components.

7.14 Fetal and adult multi-lineage differentiation assay

Spheres from fetal or adult V-SVZ cells were seeded on cover slips coated with Laminin in 24 well plates. Different recipes used to drive neuronal, astrocytic and oligodendrocyte differentiation are as follows:

Neuronal differentiation: Spheres were grown in neuro-media 1 (NCC, proliferation supplement (Stem Cell Tech.) and FGF (Invitrogen)) without EGF (invitrogen) for 10 days. Neuro-media 1 was replaced with neuro-media 2: (NS-A: Neurobasal 1:1), N2(0.5X), B-27(1X) and FGF (10ng/ml)) for 4 days. Finally, neuro-media 2 was replaced by Neurobasal, B27(1X) (Gibco) and BDNF (20ng/ml) (R&D Systems) – Half of this media was refreshed every 2-3 days.

Oligodendrocyte differentiation: Spheres were grown in oligo-media 1 (NCC, proliferation supplement (Stem Cell Tech.), EGF and FGF) for 24-48 hours. Oligo-media 1 was replaced with oligo-media 2: DMEM/F-12 (Gibco), N2 (1X) (Gibco), forskolin (10mM) (Tocris Bioscience), FGF-2 (10ng/ml) (Life technologies) and PDGF (10ng/ml) - Invitrogen for 14 days. Finally, oligo-media 2 was replaced by DMEM/F-12 (Gibco), N2 (1X) (Gibco), T3 (30ng/ml) (Sigma Aldrich), Ascorbic acid (20mM) (Sigma Aldrich) and PDGF (10ng/ml) for 1 week. PDGF was withdrawn on day 22. Oligodendrocytes were observed after a few weeks.

Astrocytic differentiation: Spheres were treated with 5% serum without EGF and FGF for 2-3 weeks.

7.15 Immunocytochemistry

Cells were grown on cover slips coated with laminin for 48 hours, then fixed in 3% PFA. Cells attached to the cover slips were then washed in phosphate-buffered saline (PBS), blocked with 0.5% bovine serum albumin (BSA), and permeabilized with 0.5% Triton X-100 added into the blocking buffer to detect intracellular antigens. Cells were incubated with primary antibodies overnight at 4°C in a humid chamber. Cells were washed with the blocking buffer and incubated with secondary antibodies for 30 minutes. Cover slips were mounted on glass slides using ProLong™ Diamond Antifade Mountant with DAPI

(Invitrogen) to counterstain cell nuclei. Fluorescent images were acquired using ZEISS LSM 700 laser scanning confocal microscope with a 63X objective.

7.16 Immunohistochemistry

Slides with brain sections were baked overnight at 60°C then de-paraffinized and rehydrated using a graded series of xylene and ethanol, respectively. For heat-mediated antigen retrieval, slides were incubated in citrate buffer at 125°C for 20 minutes in a decloaking chamber (Biocare Medical), followed by a cool-down period. Slides were rinsed in distilled water and PBS. The samples were then blocked using Protein Block (Spring Bioscience) for 30 minutes. The sections were incubated with primary antibodies diluted in 2% BSA in PBS overnight in a humid chamber at 4°C. The slides were washed using the IF buffer (containing 0.05% Tween-20 and 0.2% Triton X-100 in PBS) and incubated with secondary antibodies diluted in 2% BSA in PBS for 1 hour at room temperature. Following additional wash steps with the IF buffer, the slides were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen) to counterstain cell nuclei. Fluorescent images were acquired using ZEISS LSM 700 laser scanning confocal microscope with a 63X objective.

7.17 Multiplex immunolabeling of adult and fetal brain tissues and scanning

For multiplex whole slide immunofluorescence staining and scanning, fetal and adult human tissues were de-paraffinized and heat-induced antigen retrieval (HIER) was done using citric acid buffer for 3min at 125^0C and for 10min at 100^0C for subsequent retrievals. Slides were cooled and washed once with washing buffer (PBS with 0.5% Triton-X100 and 0.05% Tween-20) and blocked for 10 min (Perkin Elmer). Primary antibodies were diluted in blocking buffer in pre-titrated concentrations and tissues were stained in a humidifier chamber at 37^0C for 25min or

at 4^0C overnight. Slides were washed thrice with washing buffer on a shaker before a HRP-conjugated secondary antibody polymer (Perkin Elmer) was added and incubated for ten min. Finally, a TSA reagent (Opal reagents, Perkin Elmer) with the appropriate fluorescence spectral wavelength was added for a reaction time of ten minutes before being washed thrice with 0.05% Tween-PBS on a shaker and twice more with PBS only. A final rinse in water was carried out before DAPI(Sigma) was added at a concentration of 1:1000 before mounting.

After the slides were mounted, they were imaged using the LSM 700 confocal microscope using the 63x/20x objective. Imaging was performed using confocal microscope.

7.18 Antibodies

Following antibodies were used for immunolabelling: GFAP (ab4674 -Abcam), VIM (BL202 – EMD millipore), PAX6 (ab5790 - Abcam), SOX9 (ab185966 - Abcam), HES1 (NBP1-47791 - Novus Biologicals), FAM107A (ab185459 - Abcam), QC10 (PR060412B – Covance), GFAPδ (ab9598 – EMD Milllipore Corp.), GFAP (ab4674 – Abcam), APOD (ab191275 – Abcam), TUJ1 (G712A – Promega), AQP4 (ab9512 – Abcam), MAP2 (ab5392 – Abcam), CDK1 (92G2) 2978 – Cell Signalling), PCNA (ab29 – Abcam), MCM2 (ab4461 – Abcam), MKI67 (550609 – BD Biosciences). OLIG2 (ab9610 – EMD Millipore), MASH1 (ab74065 – abcam), TMEM119 (E3E4T – cell signaling), EGFR (ab32198 – Abcam), CLDN5 (LS-C352946 – LS Bio), ANXA1 (ab214486 – Abcam), MBP (ab40390 – Abcam), TAGLN2 (ab121146 – Abcam).

ABBREVIATIONS

Abbreviations

WHO	world health organization
TCGA	the cancer genome atlas research network
***t*SNE**	t-distributed stochastic neighbour embedding
NSC	neural stem cells
RG	radial glia
vRG	ventricular radial glia
tRG	truncated radial glia
oRG	outer radial glia
SVZ	subventricular zone
IP	intermediate progenitors
IN	interneurons
GO	gene ontology
CSCs	cancer stem cells
BTIC	brain tumor initiating cells
scRNAseq	single-cell mRNA-sequencing
CNV	copy number variation
IDH	isocitrate dehydrogenase
GBM	glioblastoma multiforme
CP	cortical plate
V-SVZ	ventricular-subventricular zone
AB	astrocytic band
CN	caudate nucleus
HG	hypocellular gap
EPL	ependymal layer
GW	gestational week
TMZ	temozolomide

GSC	glioblastoma stem cells
OPC	oligodendrocyte progenitor cells
ICD-O	international classification of diseases for oncology
C5	cluster 5
C4	cluster 4
C7	cluster 7
C9	cluster 9
eNSC	early neural stem cells
aNSC	astrocytic neural stem cells
nNSC	neuronal neural stem cells
oNSC	oligodendrocyte neural stem cells

References

Akay, L. A., Effenberger, A. H., & Tsai, L.-H. (2021). Cell of all trades: oligodendrocyte precursor cells in synaptic, vascular, and immune function. *Genes & Development, 35*(3-4), 180-198.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences, 100*(7), 3983-3988.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., & Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology, 10*(5), 619-624.

Alavi, M. S., Shamsizadeh, A., Azhdari-Zarmehri, H., & Roohbakhsh, A. (2018). Orphan G protein-coupled receptors: The role in CNS disorders. *Biomedicine & Pharmacotherapy, 98*, 222-232.

Alhajlah, S., Thompson, A. M., & Ahmed, Z. (2021). Overexpression of Reticulon 3 Enhances CNS Axon Regeneration and Functional Recovery after Traumatic Injury. *Cells, 10*(8), 2015.

Altman, J. (1962). Are new neurons formed in the brains of adult mammals? *science, 135*(3509), 1127-1128.

Altman, J., & Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *Journal of comparative neurology, 124*(3), 319-335.

Alvarez-Buylla, A., García-Verdugo, J. M., & Tramontin, A. D. (2001). A unified hypothesis on the lineage of neural stem cells. *Nature Reviews Neuroscience, 2*(4), 287-293.

Anantha, J., Goulding, S. R., Wyatt, S. L., Concannon, R. M., Collins, L. M., Sullivan, A. M., & O'Keeffe, G. W. (2020). STRAP and NME1 mediate the neurite growth-promoting effects of the neurotrophic factor GDF5. *Iscience, 23*(9), 101457.

Andor, N., Graham, T. A., Jansen, M., Xia, L. C., Aktipis, C. A., Petritsch, C., . . . Maley, C. C. (2016). Pan-cancer analysis of the extent and consequences of intratumor heterogeneity. *Nature medicine, 22*(1), 105-113.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., . . . Eppig, J. T. (2000). Gene ontology: tool for the unification of biology. *Nature genetics, 25*(1), 25-29.

Auguste, Y. S., Ferro, A., Kahng, J. A., Xavier, A. M., Dixon, J. R., Vrudhula, U., . . . Pedmale, U. V. (2022). Oligodendrocyte precursor cells engulf synapses during circuit remodeling in mice. *Nature Neuroscience*, 1-6.

Barbie, D. A., Tamayo, P., Boehm, J. S., Kim, S. Y., Moody, S. E., Dunn, I. F., . . . Scholl, C. (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature, 462*(7269), 108-112.

Beck, B., & Blanpain, C. (2013). Unravelling cancer stem cell potential. *Nature reviews cancer, 13*(10), 727-738. doi:10.1038/nrc3597

Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., . . . Plath, K. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *cell, 125*(2), 315-326.

Blomfield, I. M., Rocamonde, B., del Mar Masdeu, M., Mulugeta, E., Vaga, S., van den Berg, D. L., . . . Urbán, N. (2019). Id4 promotes the elimination of the pro-activation factor Ascl1 to maintain quiescence of adult hippocampal stem cells. *Elife, 8*, e48561.

Bocangel, D. B., Finkelstein, S., Schold, S. C., Bhakat, K. K., Mitra, S., & Kokkinakis, D. M. (2002). Multifaceted resistance of gliomas to temozolomide. *Clinical cancer research, 8*(8), 2725-2734.

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., . . . Mann, J. J. (2018). Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell, 22*(4), 589-599.e585. doi:10.1016/j.stem.2018.03.015

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine, 3*(7), 730-737.

Borrett, M. J., Innes, B. T., Jeong, D., Tahmasian, N., Storer, M. A., Bader, G. D., . . . Miller, F. D. (2020). Single-cell profiling shows murine forebrain neural stem cells reacquire a developmental state when activated for adult neurogenesis. *Cell reports, 32*(6), 108022.

Borrett, M. J., Innes, B. T., Tahmasian, N., Bader, G. D., Kaplan, D. R., & Miller, F. D. (2022). A Shared Transcriptional Identity for Forebrain and Dentate Gyrus Neural Stem Cells from Embryogenesis to Adulthood. *Eneuro, 9*(1).

Bralten, L. B., & French, P. J. (2011). Genetic alterations in glioma. *Cancers, 3*(1), 1129-1140.

Brown, T. J., Brennan, M. C., Li, M., Church, E. W., Brandmeir, N. J., Rakszawski, K. L., . . . Sawaya, R. (2016). Association of the extent of resection with survival in glioblastoma: a systematic review and meta-analysis. *JAMA Oncology, 2*(11), 1460-1469.

Brück, W., Schmied, M., Suchanek, G., Brück, Y., Breitschopf, H., Poser, S., . . . Lassmann, H. (1994). Oligodendrocytes in the early course of multiple sclerosis. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 35*(1), 65-73.

Buchanan, J., Elabbady, L., Collman, F., Jorstad, N. L., Bakken, T. E., Ott, C., . . . Brittan, D. (2021). Oligodendrocyte precursor cells prune axons in the mouse neocortex. *BioRxiv*.

Burd, G. D., & Nottebohm, F. (1985). Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. *Journal of comparative neurology, 240*(2), 143-152.

Cao, J., O'Day, D. R., Pliner, H. A., Kingsley, P. D., Deng, M., Daza, R. M., . . . Zhang, F. (2020). A human cell atlas of fetal gene expression. *science, 370*(6518), eaba7721.

Carter, S. L., Eklund, A. C., Kohane, I. S., Harris, L. N., & Szallasi, Z. (2006). A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nature genetics, 38*(9), 1043-1048.

Casali, B. T., & Reed-Geaghan, E. G. (2021). Microglial function and regulation during development, homeostasis and Alzheimer's disease. *Cells, 10*(4), 957.

Cebrian-Silla, A., Nascimento, M. A., Redmond, S. A., Mansky, B., Wu, D., Obernier, K., . . . Lim, D. A. (2021). Single-cell analysis of the ventricular-subventricular zone reveals signatures of dorsal and ventral adult neurogenesis. *Elife, 10*, e67436.

Chaichana, K., Zamora-Berridi, G., Camara-Quintana, J., & Quiñones-Hinojosa, A. (2006). Neurosphere assays: growth factors and hormone differences in tumor and nontumor studies. *Stem cells, 24*(12), 2851-2857.

Chaichana, K. L., Cabrera-Aldana, E. E., Jusue-Torres, I., Wijesekera, O., Olivi, A., Rahman, M., & Quinones-Hinojosa, A. (2014). When gross total resection of a glioblastoma is possible, how much resection should be achieved? *World neurosurgery, 82*(1-2), e257-e265.

Chaker, Z., Codega, P., & Doetsch, F. (2016). A mosaic world: puzzles revealed by adult neural stem cell heterogeneity. *Wiley Interdisciplinary Reviews: Developmental Biology, 5*(6), 640-658.

Chakkalakal, J. V., Jones, K. M., Basson, M. A., & Brack, A. S. (2012). The aged niche disrupts muscle stem cell quiescence. *Nature, 490*(7420), 355-360.

Challagundla, K. B., Wise, P. M., Neviani, P., Chava, H., Murtadha, M., Xu, T., . . . Vannini, I. (2015). Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy. *JNCI: Journal of the National Cancer Institute, 107*(7).

Chen, X., Wu, H., Chen, H., Wang, Q., Xie, X.-j., & Shen, J. (2019). Astragaloside VI promotes neural stem cell proliferation and enhances neurological function recovery in transient cerebral ischemic injury via activating EGFR/MAPK signaling cascades. *Molecular Neurobiology, 56*(4), 3053-3067.

Cheung, T. H., Quach, N. L., Charville, G. W., Liu, L., Park, L., Edalati, A., . . . Rando, T. A. (2012). Maintenance of muscle stem-cell quiescence by microRNA-489. *Nature, 482*(7386), 524-528.

Cheung, T. H., & Rando, T. A. (2013). Molecular regulation of stem cell quiescence. *Nature reviews Molecular cell biology, 14*(6), 329-340.

Chinot, O. L., Wick, W., Mason, W., Henriksson, R., Saran, F., Nishikawa, R., . . . Cernea, D. (2014). Bevacizumab plus radiotherapy–temozolomide for newly diagnosed glioblastoma. *New England journal of medicine, 370*(8), 709-722.

Chung, W., Eum, H. H., Lee, H.-O., Lee, K.-M., Lee, H.-B., Kim, K.-T., . . . Park, Y. H. (2017). Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nature communications, 8*(1), 1-12.

Cignarella, F., Filipello, F., Bollman, B., Cantoni, C., Locca, A., Mikesell, R., . . . Benitez, B. A. (2020). TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis. *Acta neuropathologica, 140*(4), 513-534.

Clermont, Y., & Leblond, C. (1953). Renewal of spermatogonia in the rat. *American Journal of Anatomy, 93*(3), 475-501.

Cochard, L. M., Levros Jr, L.-C., Joppé, S. E., Pratesi, F., Aumont, A., & Fernandes, K. J. (2021). Manipulation of EGFR-induced signaling for the recruitment of quiescent neural stem cells in the adult mouse forebrain. *Frontiers in Neuroscience, 15*, 621076.

Cohen, A. L., Holmen, S. L., & Colman, H. (2013). IDH1 and IDH2 mutations in gliomas. *Current neurology and neuroscience reports, 13*(5), 345-345. doi:10.1007/s11910-013-0345-4

Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., & Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer research, 65*(23), 10946-10951.

Conover, J. C., & Todd, K. L. (2017). Chapter 6 - Neuronal Stem Cell Niches of the Brain. In A. Vishwakarma & J. M. Karp (Eds.), *Biology and Engineering of Stem Cell Niches* (pp. 75-91). Boston: Academic Press.

Corcoran, C., Rani, S., O'Brien, K., O'Neill, A., Prencipe, M., Sheikh, R., . . . Crown, J. (2012). Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PloS one, 7*(12), e50999.

Couturier, C., Ayyadhury, S., Le, P., Nadaf, J., Monlong, J., & Riva, G. (2020). Single-cell RNA-seq reveals that glioblastoma recapitulates a normal neurodevelopmental hierarchy. Nat Commun. In: Nature Publishing Group.

Couturier, C. P., Ayyadhury, S., Le, P. U., Nadaf, J., Monlong, J., Riva, G., . . . Petrecca, K. (2020). Single-cell RNA-seq reveals that glioblastoma recapitulates a normal neurodevelopmental hierarchy. *Nature communications, 11*(1), 3406. doi:10.1038/s41467-020-17186-5

Couturier, C. P., Nadaf, J., Li, Z., Baig, S., Riva, G., Le, P., . . . Allache, R. (2022). Glioblastoma scRNAseq Shows Treatment-induced, Immune-dependent Rise In Mesenchymal Cancer Cells, and Structural Variants in Distal Neural Stem Cells. *Neuro-oncology*.

Crespo, I., Vital, A. L., Gonzalez-Tablas, M., del Carmen Patino, M., Otero, A., Lopes, M. C., . . . Tabernero, M. D. (2015). Molecular and genomic alterations in glioblastoma multiforme. *The American journal of pathology, 185*(7), 1820-1833.

Curley, M. D., Therrien, V. A., Cummings, C. L., Sergent, P. A., Koulouris, C. R., Friel, A. M., . . . Rueda, B. R. (2009). CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. *Stem cells, 27*(12), 2875-2883.

Dagogo-Jack, I., & Shaw, A. T. (2018). Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology, 15*(2), 81-94. doi:10.1038/nrclinonc.2017.166

Darmanis, S., Sloan, S. A., Zhang, Y., Enge, M., Caneda, C., Shuer, L. M., . . . Quake, S. R. (2015). A survey of human brain transcriptome diversity at the single cell level. *Proceedings of the National Academy of Sciences, 112*(23), 7285-7290.

Daynac, M., Chicheportiche, A., Pineda, J. R., Gauthier, L. R., Boussin, F. D., & Mouthon, M.-A. (2013). Quiescent neural stem cells exit dormancy upon alteration of GABAAR signaling following radiation damage. *Stem cell research, 11*(1), 516-528.

Dennis, C., Suh, L., Rodriguez, M., Kril, J., & Sutherland, G. (2016). Human adult neurogenesis across the ages: an immunohistochemical study. *Neuropathology and applied neurobiology, 42*(7), 621-638.

Dichmann, D. S., Walentek, P., & Harland, R. M. (2015). The alternative splicing regulator Tra2b is required for somitogenesis and regulates splicing of an inhibitory Wnt11b isoform. *Cell reports, 10*(4), 527-536.

Doan, N. B., Nguyen, H. S., Al-Gizawiy, M. M., Mueller, W. M., Sabbadini, R. A., Rand, S. D., . . . Mirza, S. P. (2017). Acid ceramidase confers radioresistance to glioblastoma cells. *Oncology Reports, 38*(4), 1932-1940.

Doan, N. B., Nguyen, H. S., Alhajala, H. S., Jaber, B., Al-Gizawiy, M. M., Ahn, E.-Y. E., . . . Schmainda, K. M. (2018). Identification of radiation responsive genes and transcriptome profiling via complete RNA sequencing in a stable radioresistant U87 glioblastoma model. *Oncotarget, 9*(34), 23532.

Doetsch, F., Caille, I., Lim, D. A., García-Verdugo, J. M., & Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *cell, 97*(6), 703-716.

Doetsch, F., Garcıa-Verdugo, J. M., & Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *Journal of Neuroscience, 17*(13), 5046-5061.

Doetsch, F., García-Verdugo, J. M., & Alvarez-Buylla, A. (1999). Regeneration of a germinal layer in the adult mammalian brain. *Proceedings of the National Academy of Sciences, 96*(20), 11619-11624. doi:10.1073/pnas.96.20.11619

Domingues, H. S., Portugal, C. C., Socodato, R., & Relvas, J. B. (2016). Oligodendrocyte, astrocyte, and microglia crosstalk in myelin development, damage, and repair. *Frontiers in cell and developmental biology, 4*, 71.

Donega, V., van der Geest, A. T., Sluijs, J. A., van Dijk, R. E., Wang, C. C., Basak, O., . . . Hol, E. M. (2022). Single-cell profiling of human subventricular zone progenitors identifies SFRP1 as a target to re-activate progenitors. *Nature communications, 13*(1), 1-12.

Dulken, B. W., Leeman, D. S., Boutet, S. C., Hebestreit, K., & Brunet, A. (2017). Single-cell transcriptomic analysis defines heterogeneity and transcriptional dynamics in the adult neural stem cell lineage. *Cell reports, 18*(3), 777-790.

Durante, M. A., Kurtenbach, S., Sargi, Z. B., Harbour, J. W., Choi, R., Kurtenbach, S., . . . Goldstein, B. J. (2020). Single-cell analysis of olfactory neurogenesis and differentiation in adult humans. *Nature Neuroscience, 23*(3), 323-326. doi:10.1038/s41593-020-0587-9

Dziedzic, A., Miller, E., Saluk-Bijak, J., & Bijak, M. (2020). The gpr17 receptor—A promising goal for therapy and a potential marker of the neurodegenerative process in multiple sclerosis. *International journal of molecular sciences, 21*(5), 1852.

Eramo, A., Lotti, F., Sette, G., Pilozzi, E., Biffoni, M., Di Virgilio, A., . . . De Maria, R. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death & Differentiation, 15*(3), 504-514.

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A.-M., Nordborg, C., Peterson, D. A., & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nature medicine, 4*(11), 1313-1317.

Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S. N., Hidalgo, O. F., Vanaclocha, V., . . . Herman, J. G. (2000). Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *New England journal of medicine, 343*(19), 1350-1354.

Fan, X., Dong, J., Zhong, S., Wei, Y., Wu, Q., Yan, L., . . . Zhao, Y. (2018). Spatial transcriptomic survey of human embryonic cerebral cortex by single-cell RNA-seq analysis. *Cell Research, 28*(7), 730-745.

Fard, M. K., Van Der Meer, F., Sánchez, P., Cantuti-Castelvetri, L., Mandad, S., Jäkel, S., . . . Starost, L. (2017). BCAS1 expression defines a population of early myelinating oligodendrocytes in multiple sclerosis lesions. *Science translational medicine, 9*(419), eaam7816.

Farioli-Vecchioli, S., Micheli, L., Saraulli, D., Ceccarelli, M., Cannas, S., Scardigli, R., . . . Ciotti, M. T. (2012). Btg1 is required to maintain the pool of stem and progenitor cells of the dentate gyrus and subventricular zone. *Frontiers in Neuroscience, 6*, 124.

Feng, E., Sui, C., Wang, T., & Sun, G. (2017). Temozolomide with or without radiotherapy in patients with newly diagnosed glioblastoma multiforme: A meta-analysis. *European Neurology, 77*(3-4), 201-210.

Ferguson, K. L., McClellan, K. A., Vanderluit, J. L., McIntosh, W. C., Schuurmans, C., Polleux, F., & Slack, R. S. (2005). A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. *The EMBO journal, 24*(24), 4381-4391.

Fernández-Castañeda, A., Chappell, M. S., Rosen, D. A., Seki, S. M., Beiter, R. M., Johanson, D. M., . . . Overall, C. C. (2020). The active contribution of OPCs to neuroinflammation is mediated by LRP1. *Acta neuropathologica, 139*(2), 365-382.

Ferris, S. P., Hofmann, J. W., Solomon, D. A., & Perry, A. (2017). Characterization of gliomas: from morphology to molecules. *Virchows Archiv, 471*(2), 257-269. doi:10.1007/s00428-017-2181-4

Filbin, M. G., Tirosh, I., Hovestadt, V., Shaw, M. L., Escalante, L. E., Mathewson, N. D., . . . Hebert, C. M. (2018). Developmental and oncogenic programs in H3K27M gliomas dissected by single-cell RNA-seq. *science, 360*(6386), 331-335.

Fuchs, E., & Chen, T. (2013). A matter of life and death: self-renewal in stem cells. *EMBO reports, 14*(1), 39-48.

Fuentealba, L. C., Rompani, S. B., Parraguez, J. I., Obernier, K., Romero, R., Cepko, C. L., & Alvarez-Buylla, A. (2015). Embryonic origin of postnatal neural stem cells. *cell, 161*(7), 1644-1655.

Gage, F. H., Coates, P. W., Palmer, T. D., Kuhn, H. G., Fisher, L. J., Suhonen, J. O., . . . Ray, J. (1995). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proceedings of the National Academy of Sciences, 92*(25), 11879-11883.

Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., . . . Vescovi, A. (2004). Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research, 64*(19), 7011-7021.

The Gene Ontology resource: enriching a GOld mine. (2021). *Nucleic acids research, 49*(D1), D325-D334.

Gertz, C. C., Lui, J. H., LaMonica, B. E., Wang, X., & Kriegstein, A. R. (2014). Diverse behaviors of outer radial glia in developing ferret and human cortex. *Journal of Neuroscience, 34*(7), 2559-2570.

Gil-Perotín, S., Duran-Moreno, M., Cebrián-Silla, A., Ramírez, M., García-Belda, P., & García-Verdugo, J. M. (2013). Adult Neural Stem Cells From the Subventricular Zone: A Review of the Neurosphere Assay. *The Anatomical Record, 296*(9), 1435-1452. doi:https://doi.org/10.1002/ar.22746

Gilbert, M. R., Dignam, J. J., Armstrong, T. S., Wefel, J. S., Blumenthal, D. T., Vogelbaum, M. A., . . . Won, M. (2014). A randomized trial of bevacizumab for newly diagnosed glioblastoma. *New England journal of medicine, 370*(8), 699-708.

Gonçalves, J. T., Schafer, S. T., & Gage, F. H. (2016). Adult neurogenesis in the hippocampus: from stem cells to behavior. *cell, 167*(4), 897-914.

Götz, M., & Barde, Y.-A. (2005). Radial glial cells: Defined and majorintermediates between embryonicstem cells and cns neurons. *Neuron, 46*(3), 369-372.

Götz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. *Nature reviews Molecular cell biology, 6*(10), 777-788.

Grelat, A., Benoit, L., Wagner, S., Moigneu, C., Lledo, P.-M., & Alonso, M. (2018). Adult-born neurons boost odor–reward association. *Proceedings of the National Academy of Sciences, 115*(10), 2514-2519.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell, 144*(5), 646-674.

Haridy, Y., Witzmann, F., Asbach, P., Schoch, R. R., Fröbisch, N., & Rothschild, B. M. (2019). Triassic Cancer—Osteosarcoma in a 240-Million-Year-Old Stem-Turtle. *JAMA Oncology, 5*(3), 425-426. doi:10.1001/jamaoncol.2018.6766

Harrington, E. P., Bergles, D. E., & Calabresi, P. A. (2020). Immune cell modulation of oligodendrocyte lineage cells. *Neuroscience letters, 715*, 134601.

Hartmann, C., Meyer, J., Balss, J., Capper, D., Mueller, W., Christians, A., . . . Wick, W. (2009). Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta neuropathologica, 118*(4), 469-474.

Hegi, M. E., Diserens, A.-C., Gorlia, T., Hamou, M.-F., De Tribolet, N., Weller, M., . . . Mariani, L. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *New England journal of medicine, 352*(10), 997-1003.

Hegi, M. E., Liu, L., Herman, J. G., Stupp, R., Wick, W., Weller, M., . . . Gilbert, M. R. (2008). Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *Journal of clinical oncology, 26*(25), 4189-4199.

Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., & Kornblum, H. I. (2003). Cancerous stem cells can arise from pediatric brain tumors. *Proceedings of the National Academy of Sciences, 100*(25), 15178-15183.

Hochberg, F. H., & Pruitt, A. (1980). Assumptions in the radiotherapy of glioblastoma. *Neurology, 30*(9), 907-907.

Hochgerner, H., Zeisel, A., Lönnerberg, P., & Linnarsson, S. (2018). Conserved properties of dentate gyrus neurogenesis across postnatal development revealed by single-cell RNA sequencing. *Nature Neuroscience, 21*(2), 290-299.

Hou, L. C., Veeravagu, A., Hsu, A. R., & Victor, C. (2006). Recurrent glioblastoma multiforme: a review of natural history and management options. *Neurosurgical focus, 20*(4), E3.

Houillier, C., Wang, X., Kaloshi, G., Mokhtari, K., Guillevin, R., Laffaire, J., . . . Delattre, J. Y. (2010). IDH1 or IDH2 mutations predict longer survival and response to temozolomide in low-grade gliomas. *Neurology, 75*(17), 1560-1566. doi:10.1212/WNL.0b013e3181f96282

Hu, Y., Wang, X., Hu, B., Mao, Y., Chen, Y., Yan, L., . . . Wang, W. (2019). Dissecting the transcriptome landscape of the human fetal neural retina and retinal pigment epithelium by single-cell RNA-seq analysis. *PLoS biology, 17*(7), e3000365.

Ignatova, T. N., Kukekov, V. G., Laywell, E. D., Suslov, O. N., Vrionis, F. D., & Steindler, D. A. (2002). Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia, 39*(3), 193-206.

Jamal-Hanjani, M., Wilson, G. A., McGranahan, N., Birkbak, N. J., Watkins, T. B., Veeriah, S., . . . Rosenthal, R. (2017). Tracking the evolution of non–small-cell lung cancer. *New England journal of medicine, 376*(22), 2109-2121.

Kawauchi, T., Chihama, K., Nabeshima, Y.-i., & Hoshino, M. (2003). The in vivo roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. *The EMBO journal, 22*(16), 4190-4201.

Kawauchi, T., & Hoshino, M. (2008). Molecular pathways regulating cytoskeletal organization and morphological changes in migrating neurons. *Developmental neuroscience, 30*(1-3), 36-46.

Keane, L., Cheray, M., Blomgren, K., & Joseph, B. (2021). Multifaceted microglia — key players in primary brain tumour heterogeneity. *Nature Reviews Neurology, 17*(4), 243-259. doi:10.1038/s41582-021-00463-2

Kelly, S. M., Raudales, R., Moissidis, M., Kim, G., & Huang, Z. J. (2019). Multipotent radial glia progenitors and fate-restricted intermediate progenitors sequentially generate diverse cortical interneuron types. *BioRxiv*, 735019.

Kempermann, G., Gage, F. H., Aigner, L., Song, H., Curtis, M. A., Thuret, S., . . . Frisén, J. (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell, 23*(1), 25-30. doi:https://doi.org/10.1016/j.stem.2018.04.004

Kierdorf, K., & Prinz, M. (2013). Factors regulating microglia activation. Front Cell Neurosci. 2013; 7: 44. In.

Kim, H. J., Park, J. W., & Lee, J. H. (2021). Genetic Architectures and Cell-of-Origin in Glioblastoma. *Frontiers in Oncology, 10*. doi:10.3389/fonc.2020.615400

Klingener, M., Chavali, M., Singh, J., McMillan, N., Coomes, A., Dempsey, P. J., . . . Aguirre, A. (2014). N-cadherin promotes recruitment and migration of neural progenitor cells from the SVZ neural stem cell niche into demyelinated lesions. *Journal of Neuroscience, 34*(29), 9590-9606.

Komori, T., Sasaki, H., & Yoshida, K. (2016). Revised WHO classification of tumours of the central nervous system: summary of the revision and perspective. *Neurological Surgery, 44*(8), 625-635.

Kriegstein, A., & Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annual review of neuroscience, 32*, 149-184.

La Manno, G., Gyllborg, D., Codeluppi, S., Nishimura, K., Salto, C., Zeisel, A., . . . Villaescusa, J. C. (2016). Molecular diversity of midbrain development in mouse, human, and stem cells. *cell, 167*(2), 566-580. e519.

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., . . . Furlan, A. (2018). RNA velocity of single cells. *Nature, 560*(7719), 494-498.

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., . . . Kharchenko, P. V. (2018). RNA velocity of single cells. *Nature, 560*(7719), 494-498. doi:10.1038/s41586-018-0414-6

Labussiere, M., Idbaih, A., Wang, X.-W., Marie, Y., Boisselier, B., Falet, C., . . . Criniere, E. (2010). All the 1p19q codeleted gliomas are mutated on IDH1 or IDH2. *Neurology, 74*(23), 1886-1890.

Lam, M., Moslem, M., Bryois, J., Pronk, R., Uhlin, E., Dehnisch, I., . . . Falk, A. (2019). Single cell analysis of autism patient with bi-allelic NRXN1-alpha deletion reveals skewed fate choice in neural progenitors and impaired neuronal functionality. *Experimental Cell Research, 383*. doi:10.1016/j.yexcr.2019.06.014

Lamprecht, S., Schmidt, E. M., Blaj, C., Hermeking, H., Jung, A., Kirchner, T., & Horst, D. (2017). Multicolor lineage tracing reveals clonal architecture and dynamics in colon cancer. *Nature communications, 8*(1), 1-9.

Lampron, A., Larochelle, A., Laflamme, N., Préfontaine, P., Plante, M.-M., Sánchez, M. G., . . . Rivest, S. (2015). Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *Journal of Experimental Medicine, 212*(4), 481-495.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., . . . Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature, 367*(6464), 645-648.

Lathia, J. D., Patton, B., Eckley, D. M., Magnus, T., Mughal, M. R., Sasaki, T., . . . ffrench-Constant, C. (2007). Patterns of laminins and integrins in the embryonic ventricular zone of the CNS. *Journal of comparative neurology, 505*(6), 630-643.

Leblond, C., & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Sciences, 55*(4), 548-573.

Leblond, C., & Stevens, C. (1948). The constant renewal of the intestinal epithelium in the albino rat. *The Anatomical Record, 100*(3), 357-377.

Lee, D. R., Rhodes, C., Mitra, A., Zhang, Y., Maric, D., Dale, R. K., & Petros, T. J. (2022). Transcriptional heterogeneity of ventricular zone cells in the ganglionic eminences of the mouse forebrain. *Elife, 11*, e71864.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N. M., . . . Zhang, W. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer cell, 9*(5), 391-403.

Lee, J. H., Lee, J. E., Kahng, J. Y., Kim, S. H., Park, J. S., Yoon, S. J., . . . Lee, J. H. (2018). Human glioblastoma arises from subventricular zone cells with low-level driver mutations. *Nature, 560*(7717), 243-247. doi:10.1038/s41586-018-0389-3

Li, C., Heidt, D. G., Dalerba, P., Burant, C. F., Zhang, L., Adsay, V., . . . Simeone, D. M. (2007). Identification of pancreatic cancer stem cells. *Cancer research, 67*(3), 1030-1037.

Li, Q., & Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nature Reviews Immunology, 18*(4), 225-242. doi:10.1038/nri.2017.125

Li, W. L., Chu, M. W., Wu, A., Suzuki, Y., Imayoshi, I., & Komiyama, T. (2018). Adult-born neurons facilitate olfactory bulb pattern separation during task engagement. *Elife, 7*, e33006.

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J. P., & Tamayo, P. (2015). The molecular signatures database hallmark gene set collection. *Cell systems, 1*(6), 417-425.

Lim, D. A., & Alvarez-Buylla, A. (2016). The adult ventricular–subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis. *Cold Spring Harbor perspectives in biology, 8*(5), a018820.

Linder, D., & Gartler, S. M. (1965). Glucose-6-phosphate dehydrogenase mosaicism: utilization as a cell marker in the study of leiomyomas. *science, 150*(3692), 67-69.

Liu, H., Povysheva, N., Rose, M. E., Mi, Z., Banton, J. S., Li, W., . . . Zhang, F. (2019). Role of UCHL1 in axonal injury and functional recovery after cerebral ischemia. *Proceedings of the National Academy of Sciences, 116*(10), 4643-4650.

Liu, Y., Curtis, M., Gibbons, H., Mee, E., Bergin, P., Teoh, H., . . . Faull, R. (2008). Doublecortin expression in the normal and epileptic adult human brain. *European Journal of Neuroscience, 28*(11), 2254-2265.

Liu, Y., Elf, S. E., Miyata, Y., Sashida, G., Liu, Y., Huang, G., . . . Menendez, S. (2009). p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell, 4*(1), 37-48.

Lledo, P.-M., & Saghatelyan, A. (2005). Integrating new neurons into the adult olfactory bulb: joining the network, life–death decisions, and the effects of sensory experience. *Trends in neurosciences, 28*(5), 248-254.

Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., & Martin-Villalba, A. (2015). Single-cell transcriptomics reveals a population of dormant neural stem cells that become activated upon brain injury. *Cell Stem Cell, 17*(3), 329-340.

Lou, C. H., Shao, A., Shum, E. Y., Espinoza, J. L., Huang, L., Karam, R., & Wilkinson, M. F. (2014). Posttranscriptional control of the stem cell and neurogenic programs by the nonsense-mediated RNA decay pathway. *Cell reports, 6*(4), 748-764.

Louis, D. N., Perry, A., Reifenberger, G., Von Deimling, A., Figarella-Branger, D., Cavenee, W. K., . . . Ellison, D. W. (2016). The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta neuropathologica, 131*(6), 803-820.

Lozano-Ureña, A., Montalbán-Loro, R., Ferguson-Smith, A. C., & Ferrón, S. R. (2017). Genomic imprinting and the regulation of postnatal neurogenesis. *Brain Plasticity, 3*(1), 89-98.

Luo, Y., Coskun, V., Liang, A., Yu, J., Cheng, L., Ge, W., . . . Cui, Y. (2015). Single-cell transcriptome analyses reveal signals to activate dormant neural stem cells. *cell, 161*(5), 1175-1186.

Ma, H., Rao, L., Wang, H., Mao, Z., Lei, R., Yang, Z., . . . Deng, Y. (2013). Transcriptome analysis of glioma cells for the dynamic response to γ-irradiation and dual regulation of apoptosis genes: a new insight into radiotherapy for glioblastomas. *Cell death & disease, 4*(10), e895-e895.

Ma, X.-R., Zhu, X., Xiao, Y., Gu, H.-M., Zheng, S.-S., Li, L., . . . Wu, Y. (2022). Restoring nuclear entry of Sirtuin 2 in oligodendrocyte progenitor cells promotes remyelination during ageing. *Nature communications, 13*(1), 1-19.

Malanchi, I., Peinado, H., Kassen, D., Hussenet, T., Metzger, D., Chambon, P., . . . Birchmeier, W. (2008). Cutaneous cancer stem cell maintenance is dependent on β-catenin signalling. *Nature, 452*(7187), 650-653.

Mao, L., Li, J., Chen, W.-x., Cai, Y.-q., Yu, D.-d., Zhong, S.-l., . . . Tang, J.-h. (2016). Exosomes decrease sensitivity of breast cancer cells to adriamycin by delivering microRNAs. *Tumor Biology, 37*(4), 5247-5256.

Marczenke, M., Sunaga-Franze, D. Y., Popp, O., Althaus, I. W., Sauer, S., Mertins, P., . . . Willnow, T. E. (2021). GAS1 is required for NOTCH-dependent facilitation of SHH signaling in the ventral forebrain neuroepithelium. *Development, 148*(21), dev200080.

McLendon, R., Friedman, A., Bigner, D., Van Meir, E. G., Brat, D. J., M. Mastrogianakis, G., . . . National Human Genome Research, I. (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature, 455*(7216), 1061-1068. doi:10.1038/nature07385

Mendell, J. T., Sharifi, N. A., Meyers, J. L., Martinez-Murillo, F., & Dietz, H. C. (2004). Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise. *Nature genetics, 36*(10), 1073-1078.

Merlo, L. M., Pepper, J. W., Reid, B. J., & Maley, C. C. (2006). Cancer as an evolutionary and ecological process. *Nature reviews cancer, 6*(12), 924-935.

Mesman, S., Van Hooft, J. A., & Smidt, M. P. (2017). Mest/Peg1 is essential for the development and maintenance of a SNc neuronal subset. *Frontiers in molecular neuroscience, 9*, 166.

Metcalfe, S. (2000). Biopsy versus resection for malignant glioma. *The Cochrane database of systematic reviews*(2), CD002034-CD002034.

Michibata, H., Okuno, T., Konishi, N., Kyono, K., Wakimoto, K., Aoki, K., . . . Taniguchi, T. (2009). Human GPM6A is associated with differentiation and neuronal migration of neurons derived from human embryonic stem cells. *Stem cells and development, 18*(4), 629-640.

Milstein, C., Frangione, B., & Pink, J. (1967). *Studies on the variability of immunoglobulin sequence.* Paper presented at the Cold Spring Harbor Symposia on Quantitative Biology.

Ming, G.-l., & Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron, 70*(4), 687-702.

Mizrak, D., Levitin, H. M., Delgado, A. C., Crotet, V., Yuan, J., Chaker, Z., . . . Doetsch, F. (2019). Single-cell analysis of regional differences in adult V-SVZ neural stem cell lineages. *Cell reports, 26*(2), 394-406. e395.

Molinaro, A. M., Taylor, J. W., Wiencke, J. K., & Wrensch, M. R. (2019). Genetic and molecular epidemiology of adult diffuse glioma. *Nature Reviews Neurology, 15*(7), 405-417. doi:10.1038/s41582-019-0220-2

Moreno-Jiménez, E. P., Flor-García, M., Terreros-Roncal, J., Rábano, A., Cafini, F., Pallas-Bazarra, N., . . . Llorens-Martín, M. (2019). Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nature medicine, 25*(4), 554-560. doi:10.1038/s41591-019-0375-9

Morizur, L., Chicheportiche, A., Gauthier, L. R., Daynac, M., Boussin, F. D., & Mouthon, M.-A. (2018). Distinct molecular signatures of quiescent and activated adult neural stem cells reveal specific interactions with their microenvironment. *Stem cell reports, 11*(2), 565-577.

Muzio, L., Viotti, A., & Martino, G. (2021). Microglia in neuroinflammation and neurodegeneration: from understanding to therapy. *Frontiers in Neuroscience, 15*.

Nakada, Y., Hunsaker, T. L., Henke, R. M., & Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification.

Negredo, P. N., Yeo, R. W., & Brunet, A. (2020). Aging and Rejuvenation of Neural Stem Cells and Their Niches. *Cell Stem Cell*.

Nelson, B. R., Hodge, R. D., Daza, R. A., Tripathi, P. P., Arnold, S. J., Millen, K. J., & Hevner, R. F. (2020). Intermediate progenitors support migration of neural stem cells into dentate gyrus outer neurogenic niches. *Elife, 9*, e53777.

Nguyen, H. S., Shabani, S., Awad, A. J., Kaushal, M., & Doan, N. (2018). Molecular Markers of Therapy-Resistant Glioblastoma and Potential Strategy to Combat Resistance. *International journal of molecular sciences, 19*(6), 1765. doi:10.3390/ijms19061765

Nicholas, R., Stevens, S., Wing, M., & Compston, D. (2002). Microglia-derived IGF-2 prevents TNFα induced death of mature oligodendrocytes in vitro. *Journal of neuroimmunology, 124*(1-2), 36-44.

Noble, M., Pröschel, C., & Mayer-Pröschel, M. (2004). Getting a GR (i) P on oligodendrocyte development. *Developmental biology, 265*(1), 33-52.

Nowakowski, T. J., Bhaduri, A., Pollen, A. A., Alvarado, B., Mostajo-Radji, M. A., Di Lullo, E., . . . Velmeshev, D. (2017). Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. *science, 358*(6368), 1318-1323.

Nowakowski, T. J., Bhaduri, A., Pollen, A. A., Alvarado, B., Mostajo-Radji, M. A., Di Lullo, E., . . . Kriegstein, A. R. (2017). Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. *science, 358*(6368), 1318-1323. doi:10.1126/science.aap8809

Nowell, P. (1976). The clonal evolution of tumor cell populations. *science, 194*(4260), 23-28. doi:10.1126/science.959840

O'Neil, D. (2022). *The Role of Activator E2fs In Adult Neural Stem Cell Quiescence and Activation.* Université d'Ottawa/University of Ottawa,

O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature, 445*(7123), 106-110.

Oliver, L., Lalier, L., Salaud, C., Heymann, D., Cartron, P. F., & Vallette, F. M. (2020). Drug resistance in glioblastoma: are persisters the key to therapy? *Cancer Drug Resistance, 3*(3), 287-301. doi:10.20517/cdr.2020.29

Ozdemir-Kaynak, E., Qutub, A. A., & Yesil-Celiktas, O. (2018). Advances in Glioblastoma Multiforme Treatment: New Models for Nanoparticle Therapy. *Frontiers in Physiology, 9*(170). doi:10.3389/fphys.2018.00170

Palmer, T. D., Schwartz, P. H., Taupin, P., Kaspar, B., Stein, S. A., & Gage, F. H. (2001). Progenitor cells from human brain after death. *Nature, 411*(6833), 42-43. doi:10.1038/35075141

Parsons, D. W., Jones, S., Zhang, X., Lin, J. C.-H., Leary, R. J., Angenendt, P., . . . Gallia, G. L. (2008). An integrated genomic analysis of human glioblastoma multiforme. *science, 321*(5897), 1807-1812.

Patel, A. P., Tirosh, I., Trombetta, J. J., Shalek, A. K., Gillespie, S. M., Wakimoto, H., . . . Martuza, R. L.

(2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *science, 344*(6190), 1396-1401.

Paton, J. A., & Nottebohm, F. N. (1984). Neurons generated in the adult brain are recruited into functional circuits. *science, 225*(4666), 1046-1048.

Peretto, P., Merighi, A., Fasolo, A., & Bonfanti, L. (1997). Glial tubes in the rostral migratory stream of the adult rat. *Brain research bulletin, 42*(1), 9-21.

Pérez-Rodríguez, D. R., Blanco-Luquin, I., & Mendioroz, M. (2021). The participation of microglia in neurogenesis: a review. *Brain sciences, 11*(5), 658.

Phillips, H. S., Kharbanda, S., Chen, R., Forrest, W. F., Soriano, R. H., Wu, T. D., . . . Soroceanu, L. (2006). Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer cell, 9*(3), 157-173.

Pichlmeier, U., Bink, A., Schackert, G., & Stummer, W. (2008). Resection and survival in glioblastoma multiforme: an RTOG recursive partitioning analysis of ALA study patients. *Neuro-oncology, 10*(6), 1025-1034.

Pollen, A. A., Nowakowski, T. J., Chen, J., Retallack, H., Sandoval-Espinosa, C., Nicholas, C. R., . . . Diaz, A. (2015). Molecular identity of human outer radial glia during cortical development. *cell, 163*(1), 55-67.

Pollen, Alex A., Nowakowski, Tomasz J., Chen, J., Retallack, H., Sandoval-Espinosa, C., Nicholas, Cory R., . . . Kriegstein, Arnold R. (2015). Molecular Identity of Human Outer Radial Glia during Cortical Development. *cell, 163*(1), 55-67. doi:10.1016/j.cell.2015.09.004

Prince, M., Sivanandan, R., Kaczorowski, A., Wolf, G., Kaplan, M., Dalerba, P., . . . Ailles, L. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences, 104*(3), 973-978.

Psenicka, M. W., Smith, B. C., Tinkey, R. A., & Williams, J. L. (2021). Connecting Neuroinflammation and Neurodegeneration in Multiple Sclerosis: Are Oligodendrocyte Precursor Cells a Nexus of Disease? *Frontiers in Cellular Neuroscience*, 221.

Puzio-Kuter, A. M. (2011). The role of p53 in metabolic regulation. *Genes & cancer, 2*(4), 385-391.

Quiñones-Hinojosa, A., & Chaichana, K. (2007). The human subventricular zone: A source of new cells and a potential source of brain tumors. *Experimental Neurology, 205*(2), 313-324. doi:https://doi.org/10.1016/j.expneurol.2007.03.016

Quiñones-Hinojosa, A., Sanai, N., Soriano-Navarro, M., Gonzalez-Perez, O., Mirzadeh, Z., Gil-Perotin, S., . . . Alvarez-Buylla, A. (2006). Cellular composition and cytoarchitecture of the adult human subventricular zone: A niche of neural stem cells. *Journal of comparative neurology, 494*(3), 415-434. doi:https://doi.org/10.1002/cne.20798

Ransohoff, R. M., & Perry, V. H. (2009). Microglial physiology: unique stimuli, specialized responses. *Annual review of immunology, 27*, 119-145.

Reynolds, B. A., & Rietze, R. L. (2005). Neural stem cells and neurospheres—re-evaluating the relationship. *Nature methods, 2*(5), 333-336.

Reynolds, B. A., & Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *science, 255*(5052), 1707-1710.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., & De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature, 445*(7123), 111-115.

Richards, L., Kilpatrick, T., & Bartlett, P. (1992). De novo generation of neuronal cells from the adult mouse brain. *Proceedings of the National Academy of Sciences, 89*(18), 8591-8595.

Richterová, R., & Kolarovszki, B. (2016). Genetic Alterations of Glioblastoma. *Neurooncology: Newer Developments*, 83.

Samuels, T. J., Järvelin, A. I., Ish-Horowicz, D., & Davis, I. (2020). Imp/IGF2BP levels modulate individual neural stem cell growth and division through myc mRNA stability. *Elife, 9*, e51529.

Sanai, N., Polley, M.-Y., McDermott, M. W., Parsa, A. T., & Berger, M. S. (2011). An extent of resection threshold for newly diagnosed glioblastomas. *Journal of neurosurgery, 115*(1), 3-8.

Sanai, N., Tramontin, A. D., Quinones-Hinojosa, A., Barbaro, N. M., Gupta, N., Kunwar, S., . . . Manuel-García Verdugo, J. (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature, 427*(6976), 740-744.

Sanai, N., Tramontin, A. D., Quiñones-Hinojosa, A., Barbaro, N. M., Gupta, N., Kunwar, S., . . . Alvarez-Buylla, A. (2004). Unique astrocyte ribbon in adult human brain contains neural

stem cells but lacks chain migration. *Nature, 427*(6976), 740-744. doi:10.1038/nature02301

Sandberg, A. A., & Hossfeld, D. K. (1970). Chromosomal abnormalities in human neoplasia. *Annual review of medicine, 21*(1), 379-408.

Sawaya, R. (1999). Extent of resection in malignant gliomas: a critical summary. *Journal of neuro-oncology, 42*(3), 303.

Shalek, A. K., Satija, R., Shuga, J., Trombetta, J. J., Gennert, D., Lu, D., . . . Regev, A. (2014). Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature, 510*(7505), 363-369. doi:10.1038/nature13437

Shapiro, W. R. (1982). Treatment of neuroectodermal brain tumors. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 12*(3), 231-237.

Shin, J., Berg, D. A., Zhu, Y., Shin, J. Y., Song, J., Bonaguidi, M. A., . . . Ming, G.-l. (2015). Single-cell RNA-seq with waterfall reveals molecular cascades underlying adult neurogenesis. *Cell Stem Cell, 17*(3), 360-372.

hu, M., Xue, X., Nie, H., Wu, X., Sun, M., Qiao, L., . . . Zhao, Y. (2022). Single-cell RNA sequencing reveals Nestin+ active neural stem cells outside the central canal after spinal cord injury. *Science China Life Sciences, 65*(2), 295-308.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., . . . Dirks, P. B. (2004). Identification of human brain tumour initiating cells. *Nature, 432*(7015), 396-401.

Song, J., Olsen, R. H., Sun, J., Ming, G.-l., & Song, H. (2016). Neuronal circuitry mechanisms regulating adult mammalian neurogenesis. *Cold Spring Harbor perspectives in biology, 8*(8), a018937.

Sorrells, S. F., Paredes, M. F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K. W., . . . Alvarez-Buylla, A. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature, 555*(7696), 377-381. doi:10.1038/nature25975

Stanton-Turcotte, D., Hsu, K., Moore, S. A., Yamada, M., Fawcett, J. P., & Iulianella, A. (2022). Mllt11 regulates migration and neurite outgrowth of cortical projection neurons during development. *Journal of Neuroscience, 42*(19), 3931-3948.

Stevanovic, M., Drakulic, D., Lazic, A., Ninkovic, D. S., Schwirtlich, M., & Mojsin, M. (2021). SOX transcription factors as important regulators of neuronal and glial differentiation during nervous system development and adult neurogenesis. *Frontiers in molecular neuroscience, 14*, 654031.

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck III, W. M., . . . Satija, R. (2019). Comprehensive integration of single-cell data. *cell, 177*(7), 1888-1902. e1821.

Stummer, W., & Kamp, M. A. (2009). The importance of surgical resection in malignant glioma. *Current opinion in neurology, 22*(6), 645-649.

Stummer, W., Reulen, H., Meinel, T., Pichlmeier, U., Schumacher, W., & Tonn, J. i dr.(2008) Extent of resection and survival in glioblastoma multiforme: Identification of and adjustment for bias. *Neurosurgery*, 564-576.

Stummer, W., Reulen, H., Meinel, T., Pichlmeier, U., Schumacher, W., Tonn, J., . . . Woiciechowsky, C. (2008). Pietsch T; ALA-Glioma Study Group. Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias.

Stupp, R., Mason, W. P., Van Den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., . . . Bogdahn, U. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New England journal of medicine, 352*(10), 987-996.

Stupp, R., van den Bent, M. J., & Hegi, M. E. (2005). Optimal role of temozolomide in the treatment of malignant gliomas. *Current neurology and neuroscience reports, 5*(3), 198-206.

Szwed, A., Kim, E., & Jacinto, E. (2021). Regulation and metabolic functions of mTORC1 and mTORC2. *Physiological reviews, 101*(3), 1371-1426.

Takahashi, C., Bronson, R. T., Socolovsky, M., Contreras, B., Lee, K. Y., Jacks, T., . . . Ewen, M. E. (2003). Rb and N-ras function together to control differentiation in the mouse. *Molecular and cellular biology, 23*(15), 5256-5268.

Taylor, M. D., Poppleton, H., Fuller, C., Su, X., Liu, Y., Jensen, P., . . . Board, J. (2005). Radial glia cells are candidate stem cells of ependymoma. *Cancer cell, 8*(4), 323-335.

Temple, S. (2001). The development of neural stem cells. *Nature, 414*(6859), 112-117.

Tepe, B., Hill, M. C., Pekarek, B. T., Hunt, P. J., Martin, T. J., Martin, J. F., & Arenkiel, B. R. (2018). Single-cell RNA-seq of mouse olfactory bulb reveals cellular heterogeneity and activity-dependent molecular census of adult-born neurons. *Cell reports, 25*(10), 2689-2703. e2683.

Thakurela, S., Tiwari, N., Schick, S., Garding, A., Ivanek, R., Berninger, B., & Tiwari, V. K. (2016). Mapping gene regulatory circuitry of Pax6 during neurogenesis. *Cell Discovery, 2*(1), 15045. doi:10.1038/celldisc.2015.45

Tickle, T., Tirosh, I., Georgescu, C., Brown, M., & Haas, B. (2019). inferCNV of the Trinity CTAT Project. *Klarman Cell Observatory, Broad Institute of MIT and Harvard*.

Tirosh, I., Izar, B., Prakadan, S. M., Wadsworth, M. H., Treacy, D., Trombetta, J. J., . . . Murphy, G. (2016). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *science, 352*(6282), 189-196.

Tirosh, I., Venteicher, A. S., Hebert, C., Escalante, L. E., Patel, A. P., Yizhak, K., . . . Filbin, M. G. (2016). Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma. *Nature, 539*(7628), 309-313.

Tobin, M. K., Musaraca, K., Disouky, A., Shetti, A., Bheri, A., Honer, W. G., . . . Arfanakis, K. (2019). Human hippocampal neurogenesis persists in aged adults and Alzheimer's disease patients. *Cell Stem Cell, 24*(6), 974-982. e973.

Tong, C. K., & Alvarez-Buylla, A. (2014). SnapShot: adult neurogenesis in the V-SVZ. *Neuron, 81*(1), 220.

Treutlein, B., Brownfield, D. G., Wu, A. R., Neff, N. F., Mantalas, G. L., Espinoza, F. H., . . . Quake, S. R. (2014). Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature, 509*(7500), 371-375. doi:10.1038/nature13173

Tseng, K.-Y., Danilova, T., Domanskyi, A., Saarma, M., Lindahl, M., & Airavaara, M. (2017). MANF is essential for neurite extension and neuronal migration in the developing cortex. *Eneuro, 4*(5).

Urbán, N., Blomfield, I. M., & Guillemot, F. (2019). Quiescence of Adult Mammalian Neural Stem Cells: A Highly Regulated Rest. *Neuron, 104*(5), 834-848. doi:https://doi.org/10.1016/j.neuron.2019.09.026

Urbán, N., van den Berg, D. L., Forget, A., Andersen, J., Demmers, J. A., Hunt, C., . . . Guillemot, F. (2016). Return to quiescence of mouse neural stem cells by degradation of a proactivation protein. *science, 353*(6296), 292-295.

Van Den Berge, S. A., Middeldorp, J., Zhang, C. E., Curtis, M. A., Leonard, B. W., Mastroeni, D., . . . Hol, E. M. (2010). Longterm quiescent cells in the aged human subventricular neurogenic system specifically express GFAP-δ. *Aging cell, 9*(3), 313-326.

van Strien, M. E., van den Berge, S. A., & Hol, E. M. (2011). Migrating neuroblasts in the adult human brain: a stream reduced to a trickle. *Cell Research, 21*(11), 1523-1525. doi:10.1038/cr.2011.101

Venkataramani, V., Yang, Y., Schubert, M. C., Reyhan, E., Tetzlaff, S. K., Wißmann, N., . . . Pramatarov, R. L. (2022). Glioblastoma hijacks neuronal mechanisms for brain invasion. *cell, 185*(16), 2899-2917. e2831.

Venteicher, A. S., Tirosh, I., Hebert, C., Yizhak, K., Neftel, C., Filbin, M. G., . . . Suvà, M. L. (2017). Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *science, 355*(6332), eaai8478. doi:10.1126/science.aai8478

Verhaak, R. G. W., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., . . . Cancer Genome Atlas Research, N. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell, 17*(1), 98-110. doi:10.1016/j.ccr.2009.12.020

Vermeulen, L., e Melo, F. d. S., Richel, D. J., & Medema, J. P. (2012). The developing cancer stem-cell model: clinical challenges and opportunities. *The lancet oncology, 13*(2), e83-e89.

Vermeulen, L., Todaro, M., de Sousa Mello, F., Sprick, M. R., Kemper, K., Alea, M. P., . . . Medema, J. P. (2008). Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proceedings of the National Academy of Sciences, 105*(36), 13427-13432.

Wallner, K. E., Galicich, J. H., Krol, G., Arbit, E., & Malkin, M. G. (1989). Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. *International Journal of Radiation Oncology* Biology* Physics, 16*(6), 1405-1409.

Waltman, L., & Van Eck, N. J. (2013). A smart local moving algorithm for large-scale modularity-based community detection. *The European physical journal B, 86*(11), 1-14.

Wang, C., Liu, F., Liu, Y.-Y., Zhao, C.-H., You, Y., Wang, L., . . . Zhang, Q. (2011). Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. *Cell Research, 21*(11), 1534-1550.

Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., . . . Verhaak, R. G. W. (2017). Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. *Cancer cell, 32*(1), 42-56.e46. doi:10.1016/j.ccell.2017.06.003

Watanabe, T., Nobusawa, S., Kleihues, P., & Ohgaki, H. (2009). IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *The American journal of pathology, 174*(4), 1149-1153.

Watanabe, T., Vital, A., Nobusawa, S., Kleihues, P., & Ohgaki, H. (2009). Selective acquisition of IDH1 R132C mutations in astrocytomas associated with Li-Fraumeni syndrome. *Acta neuropathologica, 117*(6), 653-656.

Watt, F. M., Hogan, & LM, B. (2000). Out of Eden: stem cells and their niches. *science, 287*(5457), 1427-1430.

Wolbers, J. G. (2014). Novel strategies in glioblastoma surgery aim at safe, supra-maximum resection in conjunction with local therapies. *Chinese journal of cancer, 33*(1), 8.

Wolf, F. A., Hamey, F. K., Plass, M., Solana, J., Dahlin, J. S., Göttgens, B., . . . Theis, F. J. (2019). PAGA: graph abstraction reconciles clustering with trajectory inference through a topology preserving map of single cells. *Genome biology, 20*(1), 1-9.

Wong, E. T., Hess, K. R., Gleason, M. J., Jaeckle, K. A., Kyritsis, A. P., Prados, M. D., . . . Yung, W. A. (1999). Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials. *Journal of clinical oncology, 17*(8), 2572-2572.

Woodburn, S. C., Bollinger, J. L., & Wohleb, E. S. (2021). The semantics of microglia activation: neuroinflammation, homeostasis, and stress. *Journal of Neuroinflammation, 18*(1), 258. doi:10.1186/s12974-021-02309-6

Xie, Z., Jones, A., Deeney, J. T., Hur, S. K., & Bankaitis, V. A. (2016). Inborn errors of long-chain fatty acid β-oxidation link neural stem cell self-renewal to autism. *Cell reports, 14*(5), 991-999.

Yan, H., Bigner, D. D., Velculescu, V., & Parsons, D. W. (2009). Mutant metabolic enzymes are at the origin of gliomas. *Cancer research, 69*(24), 9157-9159.

Yan, H., Parsons, D. W., Jin, G., McLendon, R., Rasheed, B. A., Yuan, W., . . . Riggins, G. J. (2009). IDH1 and IDH2 mutations in gliomas. *New England journal of medicine, 360*(8), 765-773.

Yosida, T. H. (1975). Chromosomal alterations and development of experimental tumors. In *Geschwülste· Tumors II* (pp. 677-753): Springer.

Yuzwa, S. A., Borrett, M. J., Innes, B. T., Voronova, A., Ketela, T., Kaplan, D. R., . . . Miller, F. D. (2017). Developmental emergence of adult neural stem cells as revealed by single-cell transcriptional profiling. *Cell reports, 21*(13), 3970-3986.

Zeisel, A., Muñoz-Manchado, A. B., Codeluppi, S., Lönnerberg, P., La Manno, G., Juréus, A., . . . Linnarsson, S. (2015). Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *science, 347*(6226), 1138. doi:10.1126/science.aaa1934

Zhang, Q., Shalaby, N. A., & Buszczak, M. (2014). Changes in rRNA transcription influence proliferation and cell fate within a stem cell lineage. *science, 343*(6168), 298-301.

Zhang, S., Balch, C., Chan, M. W., Lai, H.-C., Matei, D., Schilder, J. M., . . . Nephew, K. P. (2008). Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer research, 68*(11), 4311-4320.

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J., . . . Pan, N. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature, 555*(7697), 524-528.

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J., . . . Wang, X. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature, 555*(7697), 524-528. doi:10.1038/nature25980

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., . . . Chanda, S. K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature communications, 10*(1), 1-10.

Zywitza, V., Misios, A., Bunatyan, L., Willnow, T. E., & Rajewsky, N. (2018). Single-cell transcriptomics characterizes cell types in the subventricular zone and uncovers molecular defects impairing adult neurogenesis. *Cell reports, 25*(9), 2457-2469. e2458.

Wang, L., Babikir, H., Müller, S., Yagnik, G., Shamardani, K., Catalan, F., . . . Kriegstein, A. (2019). The Phenotypes of Proliferating Glioblastoma Cells Reside on a Single Axis of VariationA Draft Single-cell Atlas of Human Glioma. *Cancer discovery, 9*(12), 1708-1719.

Wang, L., Jung, J., Babikir, H., Shamardani, K., Jain, S., Feng, X., . . . Raleigh, D. (2022). A single-cell atlas of glioblastoma evolution under therapy reveals cell-intrinsic and cell-extrinsic therapeutic targets. *Nature Cancer, 3*(12), 1534-1552.

Wang, X., Zhou, R., Xiong, Y., Zhou, L., Yan, X., Wang, M., . . . Huang, Z. (2021). Sequential fate-switches in stem-like cells drive the tumorigenic trajectory from human neural stem cells to malignant glioma. *Cell Research, 31*(6), 684-702.