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Urbán Laboratory
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Exploring the regulation of human neural stem cells quiescence using a CRISPR/Cas9 approach

Bachelor thesis

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1.1.1 Definition of quiescence

Quiescence is defined as a non-proliferative state, where cells are in a reversible cell cycle arrest (Li and Bhatia, 2011). Quiescence occurs in several tissues, but the amount of quiescent adult stem cells seems to be more widespread in low turnover tissues, such as skeletal muscle or brain, compared to rapidly renewing tissues (Clevers and Watt, 2018). For example, in tissues such as the gut or the skin, adult stem cells divide constantly and no quiescent stem cells have been found in homeostatic conditions (Li and Clevers, 2010). In contrast, the majority of neural stem cells (NSCs) in the adult brain is quiescent. As they tend to rather differentiate than self-renew, a tight regulation of quiescence and activation of NSCs is crucial to maintain both the stem cell pool and the neurogenic capacity of the ageing brain (Urbán et al., 2019).

The ability to re-enter the cell cycle is a unique feature of quiescence compared to other non-proliferating cell states such as senescence or terminally differentiated states (Cheung and Rando, 2013). Sequential rounds of cell division can lead to DNA mutations (Walter et al., 2015), protein and mitochondria damage (Cheung and Rando, 2013). Therefore, over time, quiescence is crucial for cells to protect the stem cell pool from exhaustion and to prevent accumulation of impaired DNA, damaged proteins and mitochondria that could lead to malignant transformation or senescence. (Cheung and Rando, 2013). It is important to accentuate the fact that quiescence is an actively maintained condition which includes a molecular program that constantly suppresses terminal differentiation, prevents senescence, and secures the potential to re-enter the cell cycle again, if required (Cheung and Rando, 2013). Furthermore, the quiescent state correlates with a low metabolic rate, which includes low levels of RNA and protein synthesis (Cheung and Rando, 2013).

1.1.2 Quiescence of neural stem cells in the adult brain

With the help of RNA sequencing, the ‘transcriptional signature’ of quiescent cells is getting revealed. In spite of the observation that quiescent stem cells have low RNA levels, transcriptomic studies of isolated quiescent NSCs have shown that quiescence involves active transcription of hundreds of genes (Cheung and Rando, 2013). The transcriptome of quiescent NSCs show high levels of transcripts which are involved in cells signalling, cell-cell

communication, cell adhesion and the extracellular matrix (ECM). Active NSCs, on the other hand, show an enrichment of genes relevant for transcription, translation, and DNA repair (Basak et al., 2018; Dulken et al., 2017; Codega et al., 2014). This approach also showed that quiescent NSCs are more molecularly diverse which could be connected to the concept of cells being at different depths of quiescence (Harris et al., 2021; Basak et al., 2018; Dulken et al., 2017; Artegiani et al., 2017).

The lack of expression of cell-cycle progression markers, for example *proliferating cell nuclear antigen* (*PCNA*), *minichromosome maintenance complex component 2* (*MCM2*), or *Ki67*, is characteristic for quiescent neural stem cells (Codega et al., 2014; Lugert et al., 2010). It was also found that neural stem cell can be labelled by retention of Bromodeoxyuridine (BrdU) or 5-ethynyl-2′-deoxyuridine (EdU), which labels DNA during cell division. This pathway reveals NSCs that have previously proliferated and subsequently entered quiescence, since the EdU labels the DNA during cell division and remains traceable (Urbán et al., 2016; Codega et al., 2014). In addition to genes related to the cell cycle regulation, also the transcription of genes involved in metabolism seem to differ in active and quiescent NSCs. Whereas active NSCs are thought to use glycolysis as their main energy source, quiescent NSCs seem to switch to oxidative phosphorylation. However, these metabolic changes still remain controversial in the field. (Basak et al., 2018; Dulken et al., 2017; Llorens-Bobadilla et al., 2015; Shin et al., 2015). Genes such as *ApoE, Aldoc, Id4* or *Id3* are considered as characteristic genes of NSC quiescence and are expressed at highest levels in deeply quiescent cells (Blomfield et al., 2019; Basak et al., 2018; Artegiani et al., 2017; Dulken et al., 2017; Llorens-Bobadilla et al., 2015; Shin et al., 2015) (see chapter: specific markers of neural stem cells).

1.2 Adult neurogenesis and the neural stem cell fate

Stem cells have the potential to self-renew or to differentiate into several different cell types. NSCs are defined as multipotent, undifferentiated cells, that produce progeny cells that terminally give rise to neurons, oligodendrocytes, and astrocytes (Gage, 2000). NSCs are also referred to as radial-glia-like stem cells (RGL), because they resemble radial glia cells in development (Berg et al., 2018). Understanding the mechanisms and various factors regulating NSCs, raises hope of developing treatments against neurodegenerative diseases such as Alzheimer’s Disease, Huntington’s Disease or Parkinson’s Disease (Shal et al., 2018). The majority of NSCs in mammals differentiate in the late embryonic or early postnatal brain and therefore disappear from most regions of the central nervous system. However, in some

mammals NSCs persist in the so-called neural stem cell niches (Altman and Das, 1965) (see chapter: the neural stem cell niche).

Neurogenesis is defined as the production of new functional neurons from progenitor cells. During the multiple developmental stages of subgranular-zone-NSCs, they can differentiate into granule neurons and then migrate and integrate into the granule cell layer (von Bohlen und Halbach, 2007). These stages are defined by the expression of specific protein markers (von Bohlen und Halbach, 2007) (see fig.1). The first stage is represented by NSCs, or Type I RGL, which are characterized by their expression of *glial fibrillary acidic protein* (*GFAP*), *Nestin* and *sex determining region Y- box 2 protein* (*Sox2*) (Encinas et al., 2011). RGLs can then differentiate into intermediate progenitor cells (IPCs, Type II), which are highly proliferative and show expression of either *doublecortin* (*DCX*) or *polysialylated neural cell adhesion molecule* (*PSA-NCAM*). IPCs can then transit to neuronal lineage committed cells or neuroblasts (Type III), which can show the expression of both *DCX* and *PSA-NCAM*, in addition to *neuron-specific class III beta-tubulin* (*Tuj-1*), or *NeuroD*, which all indicate immature neurons. Type 3 cells however, loose the expression of *Sox2* and *GFAP* (Encinas et al., 2011) (see fig.1). The final stage of the differentiation cascade is reached when type 3 cells mature into DG granule neurons expressing *calretinin*, *Prospero Homeobox 1* (*PROX1*) and *neuron-specific nuclear protein* (*NeuN*, a post-mitotic neuronal marker). *PROX1* is already expressed at the NSC stage and gets upregulated as they differentiate into neurons. The newly formed neurons are then able to further integrate into the hippocampus and then express *calbindin*, which is a marker of synaptic integration. Integrated neurons can influence the functions of the hippocampus, which includes memory, learning and the performance of spatiomotor skills (von Bohlen und Halbach, 2007).

In the adult human brain, neurogenesis is controversially discussed among researchers in the field of neuroscience. While some studies report high neurogenesis after birth but declines with age to an undetectable level (Sorrels et al., 2018), others indicate that neurogenesis persist into old age in the dentate gyrus (DG) of the human hippocampus (Boldrini et al., 2018). It was proposed that human neurogenesis is located in the subgranular zone (SGZ) of the DG (von Bohlen und Halbach, 2007). The newly generated neurons from the subventricular zone (SVZ) of the brain integrate into the olfactory bulb (Kornack and Rakic, 1999) (see chapter: The neural stem cell niche). This was also shown in humans (Eriksson et al., 1998). Reasons that contribute to the debate about the existence of neurogenesis in humans is the limited accessibility and the lack of an *in vitro* system to study neurogenesis excessively.

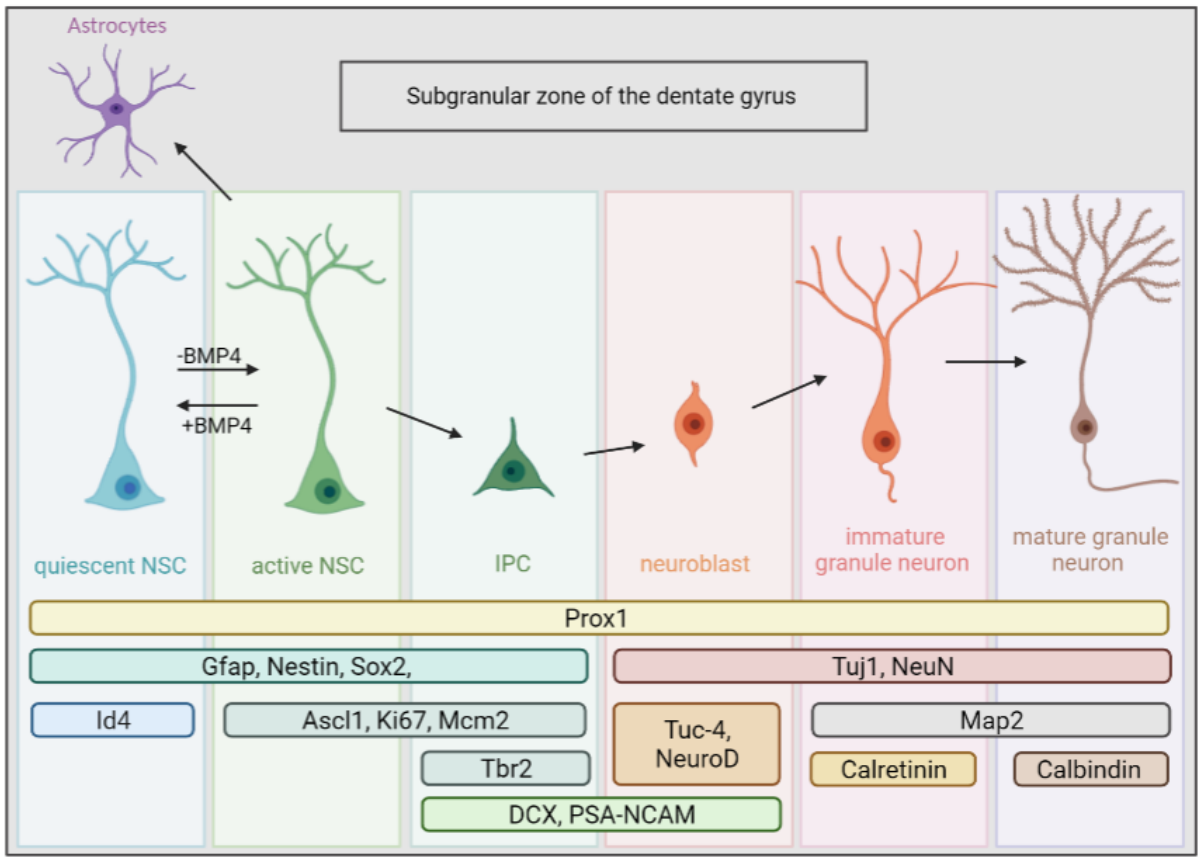


Figure 1: *Scheme of the neurogenic line of the SGZ and their markers. Created in BioRender.com*

1.3 The neural stem cell niche

Activation and quiescence of stem cells play an essential part in several organs, which are underlying tissue maintenance, regeneration, function, plasticity, aging, and disease (Cheung and Rando, 2013). A co-existence of quiescent and actively dividing stem cells can be found in the so-called adult stem cell niches (Li and Clevers, 2010). A stem cell niche is defined as a microenvironment of cells which organize in a three-dimensional structure, to provide maintenance and survival of undifferentiated stem cells. It also controls stem cell activity by extrinsic signals and provides a protective surrounding, promoting stemness (McKee and Chaudhry, 2017). In this stem cell niche, cell-intrinsic regulatory signals interact with extrinsic mechanisms and thereby regulate the stem cell fate. That comprises cell-to-cell communication and cell-matrix interactions (Ferraro et al., 2010). This specialized microenvironment can force the stem cell to either stay quiescent, proliferate, or differentiate (Yamashita and Fuller, 2005; Betschinger and Knoblich, 2004).

Several *in vitro* systems have been developed while facing challenges to culture and hereby mimic the *in vivo* niche, such as micro-patterned culture substrates, three-dimensional cultures, and the usage of different biomaterials to imitate the stiffness and three-dimensional structure of the stem cell niche (McKee and Chaudhry, 2017).

The two best described brain regions where adult mammalian NSCs generate new neurons and glial cells, is the SGZ of the dentate gyrus within the hippocampus and the ventricular-subventricular zone (V-SVZ), which is adjoining the lateral ventricles (Kempermann et al., 2015). The characteristics of the NSCs in both neural stem cell niches are long-term self-renewal, proliferative potential, and the feature to differentiate into the three major cell types of the central nervous system, which includes neurons, astrocytes, and oligodendrocytes (Mannino et al., 2021). NSCs of the SVZ niche are organized as a thin layer underneath the ependymal cells, which are in direct contact with the cerebral spinal fluid (CSF). The CSF functions as a transport system for trophic factors and neuroendocrine peptides, which play an important role in regulation of the NSC fate (Doetsch, 2003). In the hippocampal SGZ, NSCs can be found at the border between the hilus and the granule cell layer of the dentate gyrus (Walton, 2012). Through their apical processes, they can reach the granule layer, and their basal processes allow the interaction between SGZ-NSCs and the blood vessels below the DG (Walton, 2012). Different from the SVZ-NSCs, hippocampal NSCs are not connected with the CSF (Clarke and van der Kooy, 2011). In the SGZ niche, neuroblast and new neurons can be found close to the NSCs. In the V-SVZ however, they migrate away from the niche in direction of the olfactory bulb (Fuentealba et al., 2012).

Up to this day, our understanding of the neural stem cell niches in humans is still quite scarce and most studies on this topic are based on rodents. Certainly, many commonalities between humans and rodents exist, but there also have been unravelled several differences. For example, in both species, hippocampal NSCs differentiate into dentate granule neurons (Spalding et al., 2013). In the SVZ, neural progenitor cells are developing to become medium striatal spiny neurons in humans, whereas they are predestined to become olfactory interneurons in rodents. However, this has only been confirmed by one source so far and remains controversial (Ernst et al., 2014). In this thesis the focus lies on SGZ-NSCs unless specifically mentioned otherwise.

1.3.1 Specific markers of neural stem cells

Quiescent and active NSCs of both neuronal stem cell niches share several marker genes with IPCs but also astrocytes. The lack of a unique marker of quiescent NSCs makes their detection

difficult and the cells are normally recognized by a combination of astrocyte/NSC and IPC/NSC associated markers, as well as their morphology differences (Codega et al., 2014; Beckervordersandforth et al., 2010) (see fig.1). One important intrinsic regulator of NSC quiescence is *Achaete-Scute Family BHLH Transcription Factor 1* (*Ascl1*), a basic-helix-loop-helix (bHLH) transcription factor. It is present dividing progenitors in the adult hippocampus (Andersen et al., 2014). The mRNA levels of *Ascl1* were shown to be expressed independently of NSCs active or quiescent state but only reaches significant protein levels in active stem cells (Blomfield et al., 2019). *Ascl1* deletion fully blocks the activation of adult hippocampal stem cells and therefore inhibits the generation of new neurons and the depletion of the stem cell pool over time (Andersen et al., 2014). Inhibitor of differentiation/DNA binding Proteins (Id-Proteins) are inhibitors of bHLH transcription factors such as Ascl1 (Imayoshi and Kageyama, 2014). E-proteins are essential for Ascl1 to bind the DNA to act as transcription factor. Id-proteins have been shown to sequester E-proteins. Ascl1 can not bind DNA in its monomeric form, which then leads to its proteasomal degradation (Imayoshi and Kageyama, 2014). The Id family includes four genes, *Id 1-4*, that get expressed during development in multiple tissues and in the adult stem cell niches. *Id3* and *Id4* were shown to be highly expressed in quiescent hippocampal NSCs in rodents (Shin et al., 2015).

Bone morphogenic protein-4 (BMP4) was also shown to play a part in the maintenance of NSC quiescence in the hippocampus (Mira et al., 2010). BMPs are produced by granule neurons and NSCs themselves in the SGZ. They promote quiescence as well as long-term maintenance of NSCs, and at the same time suppress the proliferation of progenitor cells. By supplementing BMP4 to embryonic stem-cell-derived NSC cultures, a quiescent-like state can be achieved (Mira et al., 2010). BMP4 was shown to have an effect on the transcription of the four Id proteins (Blomfield et al., 2019). *Id2* and *Id3* were already expressed at high levels in proliferating cells. *Id1* and *Id2* were clearly upregulated by BMP4, whereas only *Id4* was shown to be absent in cells presenting high levels of Ascl1. This could point to a negative regulatory relationship between Ascl1 and Id4 (Blomfield et al., 2019). Direct transcriptional targets of Ascl1 are genes involved in the cell cycle progression were strongly downregulated in *Id4*-overexpressing NSCs (Blomfield et al., 2019). Overall, the degradation of Ascl1 seems to result in a downregulation of its targets, which leads to a cell cycle arrest of NSCs and loss of Id4 *in vivo* results in an increase in the activation of quiescent hippocampal adult NSCs (Blomfield et al., 2019). Establishing an *ASCL1*-reporter line in human NSCs would make it easier to trace and capture the mechanisms regulated by this important transcription factor. By linking the

ASCL1 protein with a fluorescent protein, visualization of the protein becomes more accurate and tractable.

NSCs of the SVZ and the SGZ additionally express *Paired box protein-6* (*Pax6*), *GFAP*, *Nestin*, *Vimentin* and *Sox2* (Urbán and Guillemot, 2014; Ferri et al., 2004). Fibroblast growth factors (FGFs) are known to have an effect on NSC proliferation and neurogenesis (Murphy et al., 1990). Besides FGF-2, also epidermal growth factors (EGF) influence the proliferation of NSCs. Active NSCs in the V-SVZ can be recognized by their expression of EGF-receptor. Pax6 has been detected in early neural progenitors and radial glia-like cells of the SGZ and SVZ of the adult brain (Maekawa et al., 2005). Sox2 is an essential factor for the proliferative ability of NSCs and seen as a stemness marker (Graham et al., 2003). GFAP on the other hand is seen as a quiescent marker but is also expressed in astrocytes, which indicates related properties of astrocytes and NSCs (Fukuda et al., 2003). *Nestin*, a type VI intermediate filament, and *Sox2* are also expressed in NSCs and IPCs. Both can be used as stemness markers for NSCs (Zhang and Jiao, 2015).

1.3.2 Regulation of quiescence

One important part of the local NSC niche and also regulator of the NSC fate is ECM, which is surrounding the stem cells. ECM components are spread throughout the brain parenchyma but can also be found encircling the blood vessels in special structures like the laminin-rich basal lamina. This then provides the NSCs with structure and gives them access to endothelial as well as blood-borne factors (Shen et al., 2008).

Direct interaction between signalling molecules expressed by activated NSCs, which are attached to their surface and transmembrane receptors, act as cell-bound signals that regulate NSC behaviour. One of the most important receptors found to regulate the quiescent state of NSCs is Notch (Kawaguchi et al., 2013). By eliminating the intracellular effector of Notch signalling, the recombination signal binding protein for immunoglobulin kapa region J, the regulatory effects on maintaining NSCs quiescence in both V-SVZ and SGZ have been demonstrated. The deletion led to an activation of quiescent NSCs and an increase in neurogenesis (Ehm et al. 2010; Imayoshi et al., 2010). An over-activation of Notch-deficient NSCs over a long period of time, led to the loss of the NSC pool and the arrest of neurogenesis (Ehm et al., 2010).

Noggin, an antagonist of BMP, is also expressed locally and promotes NSC proliferation (Mira et al., 2010). Milk fat globule-epidermal growth factor-8 (MFG-E8) is another factor that is thought to maintain quiescence in an autocrine or paracrine manner. It gets secreted through

integrin receptors by SGZ NSCs and astrocytes and in quiescent NSCs its expression is increased (Zhou et al., 2018). Neutrophin3, was also shown to promote quiescence and the long-term maintenance of V-SVZ NSCs. It gets secreted by the choroid plexus into the CSF and by endothelial cells of the brain vasculature (Delgado et al., 2014).

Neurotransmitters also play a regulatory part on the NSC fate. In both NSC niches, serotonin, dopamine, glutamate, gamma aminobutyric acid, acetylcholine, neuropeptide Y and noradrenaline have been shown to regulate NSC proliferation and neurogenesis (Berg et al., 2013).

Additionally, some transcription factors, cell cycle regulator and metabolites were also shown to influence the behaviour of NSCs. Ascl1 is expressed in the SGZ and the V-SVZ by active NSCs as well as early intermediate progenitor cells. It is essential for the activation of NSCs, as the deletion of *Ascl1* from adult NSCs has led to a stop of differentiation or proliferation of NSCs (Andersen et al., 2014). *Ascl1* is suppressed by *hairy and enhancer of split* (*HES*) family genes, which are effectors of the Notch signalling pathway that maintain NSC quiescence (Urban et al., 2016; Andersen et al., 2014; Imayoshi et al., 2010).

Aging also shows effects on the behaviour of NSCs. It is generally associated with a loss of homeostasis and decreased regenerative capacity, which can be connected to a decrease in stem cell function (Artegiani et al., 2017). Quiescent NSCs in the V-SVZ show age-related, cell-intrinsic changes such as a dysfunction of the lysosome. These changes are thought to lead to an increased accumulation of protein aggregates, resulting in neurodegenerative modifications and in some cases even disorders (Leeman et al., 2018).

1.4 Adult neural stem cells as a model to study quiescence

1.4.1 *In vitro* system

Due to the lack of availability and accessibility of human brain tissue and limitations of an *in vivo* model in living humans, studying neurogenesis and quiescence of NSCs in the adult human brain faces many challenges. *In vitro* models such as induced pluripotent stem cells or brain organoids, represent a possibility to study adult neurogenesis as well as quiescence of NSCs in humans up to a certain degree and can even give valuable insights in different neurogenic disorders (Wen et al., 2021). While there already exists a well-established functioning *in vitro* system for NSCs in mouse cells (Blomfield et al., 2018; Mira et al., 2010), there is up until today no published protocol for human NSCs. Therefore, one of the main goals

of the Urbán Laboratory, is to establish a working *in vitro* system for culturing human NSCs in a proliferative condition.

The human NSCs of the in vitro system were derived from human induced pluripotent stem cells based on the protocol published by Yu et al. in 2014. In this protocol they were able to show how to induce human pluripotent stem cells into an enriched population of hippocampal DG granule neurons (Yu et al., 2014). First, they used free-floating embryoid bodies (EBs), to provide the cells with a 3D structure, which was shown to stimulate the pregastrulational development and early gastrulation. After treating EBs with a cocktail of specific supplements that should mimic the signals of the developing brain, the EBs form neural rosettes that are dissociated and show a similar expression pattern as hippocampal NSCs. After 30 days the induced NSCs matured into neuroblast and postmitotic granule neurons, which was shown by an increase of the early neuronal markers *DCX* and T-box brain protein 1 (TBR1) levels, as well as *PROX1*, which is highly expressed in the dentate gyrus. After 60 days of differentiation, they were able generate neurons which even formed a neural network while kept in coculture with human hippocampal astrocytes. Those neurons showed the expression of *Neurogenic differentiation 1 Protein* (*NEUROD1*) and *DCX*, as well as *Microtubule-associated protein 2* (*MAP2*) and *PROX1*, indicating postmitotic hippocampal neurons. In the end, Yu and his team were able to induce human embryonic stem cells into PROX1+ hippocampal granule neurons. (Yu et al., 2014).

The Urbán Laboratory was able to adapt the protocol from Yu et al. and differentiated human induced pluripotent stem cells towards NSCs (unpublished protocol, see fig. 2). The human NSCs are cultured in two media conditions; one supplemented with FGF only as published in the protocol of Yu et al. in 2014 and media supplemented with EGF and FGF based on the well-established *in vitro* mouse model (Blomfield et al., 2019; Yu et al., 2014; Mira et al., 2010). EGF and FGF keep the NSCs in a proliferating active state.

1.4.2 Induction of quiescence

Developing a functional *in vitro* system for human NSCs which mimics the regulating factors of the stem cell niches known so far, provides a more transparent system to understand the mechanisms behind NSC's behaviour and states. In the established mouse *in vitro* model it was shown that addition of BMP4 is sufficient to induce the quiescence state. The BMP4-induced quiescent NSCs show a state of reversible cell cycle exit (Blomfield et al., 2019; Mira et al., 2010) (see fig.2), which show a similar transcriptional profile compared to that of quiescent mouse NSCs *in vivo* (Blomfield et al., 2019). The quiescent-like state of the BMP4

induced mouse NSCs was shown by immunofluorescent staining, western blot and RNAseq. The quiescent-like NSCs showed decreased levels of Ki67 and CyclinD1, both cell-cycle markers, and at the same time an increasing level of GFAP, which is a typical marker of RGLs (Blomfield et al., 2019). With the help of this mouse *in vitro* quiescence model the identification of intrinsic factors that promote NSC quiescence, such as Id4 for example, have been identified (Blomfield et al., 2019).

Adapting the mouse model to human NSCs could provide us with a system to identify the regulating factors behind the quiescence state as well as activation of human NSCs. The human *in vitro* model could give insights into the ability of acquiring quiescence and the regulation of potential active and quiescent NSC's states in human cells. The preliminary findings of the Urbán Laboratory regarding the novel *in vitro* system imply that human NSCs can be induced by BMP to enter a quiescent-like state. However, unlike mouse NSCs, human NSCs resume proliferation after a few days, even in the constant presence of BMP. Therefore, human NSCs may not be able to maintain dormancy for long periods of time in the novel *in vitro* system.

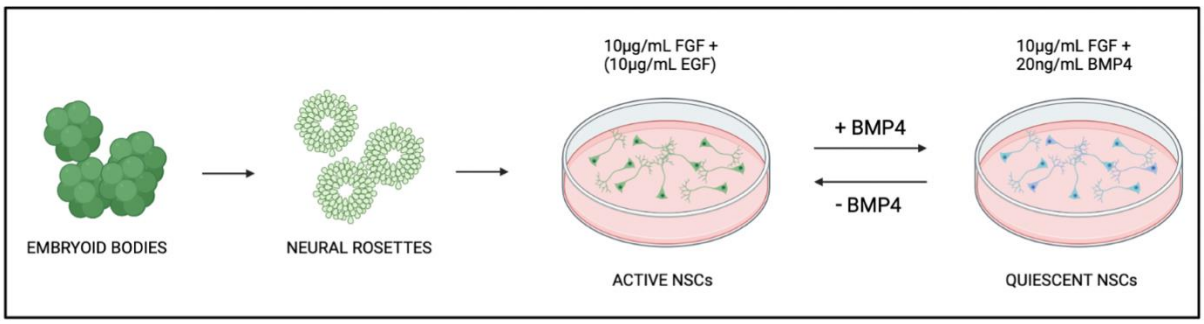


Figure 2: Scheme of the human in vitro system of quiescence induction of neural stem cells derived from human pluripotent stem cells. *Created in BioRender.com, adapted from Yu et al., 2014 and Blomfield et al., 2018.*

1.5 CRISPR/Cas9

Through recent breakthroughs of engineering transcription activator-like effector nucleases (TALENs) (Boch et al., 2009) and RNA-guided endonucleases of the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR/Cas9) system it is possible to induce site-specific double-strand breaks (DSB). Especially the CRISPR/Cas9 system allows easy, divers and efficient locus specific DSBs in various species and cell types (Jinek et al., 2012). The CRISPR system was derived from *streptococcus pyogenes* and uses

RNA-DNA Watson-Crick hybridization to identify the DNA target site to be cleaved (Porteus, 2016). The system consists of two components to initiate a DSB: a single guide RNA (sgRNA) and the Cas9 protein that cleaves the DNA strands leading to a DSB of the DNA (Kass and Jasin, 2010). To resolve the DSB, the affected cell uses one of two highly conserved repair mechanisms: non-homologous end joining (NHEJ) or homologous recombination (HR) (Kass and Jasin, 2010). NHEJ corrects defective breaks throughout the cell cycle by ligating DNA without end processing, which can result in small insertions or deletions. HR, on the other hand, leads to specific S or G2 phase DNA repair when an undamaged sister chromatid is available. By introducing an exogenous donor template, it is possible to initiate precise nucleotide changes to the genome and perform genome editing if the cell uses HR as repair mechanism (Dever et al., 2019). Therefore, this method provides us with the possibility to perform a knock in of fluorescent marked genes such as *Ascl1* to study its expression in active and quiescent NSCs. *Ascl1*-Venus and *Ascl1*-GFP knocked in the *Ascl1* locus, thereby replacing the *Ascl1* coding sequence, was already performed in mice (Imayoshi et a., 2013; Kim et al., 2007; Leung et al., 2007). Also, the generation of an *Ascl1Flpo* allele by inserting a P2A-Flpo-P2A-tTa DNA cassette immediately before the stop codon of the *Ascl1* gene in mice was successful by using a CRISPR/Cas9-based strategy (Li et al., 2021). So far, the generation of an *ASCL1*-fluorescent marked human neural stem cell reporter line was not published.

1.6 Fluorescence-activated cell sorting

Flow cytometric analysis and fluorescence-activated cell sorting (FACS) allow separation of cell populations based on fluorescent labelling, for example by surface antigens or intracellular reporters (Baumgarth and Roederer, 2000). A flow cytometer consists of three parts: first, the fluid system, which enables hydrodynamic focusing; second, the excitation source and optical emission systems from the wavelength filters to the detectors, which form the optical part; and finally, the electronic system which translates the signal to be analysed into a digital form using special computer software (Picot et al., 2012). The cells need to be in a suspension in order to pass dropwise across the laser beam and to get recognized individually by it. The excitation light source provides scattered and fluorescent lights by illuminating the cell, which can then be detected (Picot et al., 2012).

In summary, FACS offers an excellent possibility to sort cells and therefore, for example, to divide cells into positive and negative subpopulations after transfection.

1.7 Aims of this thesis

The regulation of quiescence and activation of NSCs is highly complex. Even though numerous regulatory effectors of NSC's behavior have been identified in mouse, the precise regulation of NSCs is still not fully understood. Due to the inaccessibility of the human brain and the lack of an *in vitro* model, even less is known about human NSC and their ability to acquire quiescence. To elucidate the regulator effects on quiescence of human NSCs, my aims for this thesis are:

1. To characterize the neural stem cells of the human *in vitro* system.
2. To minimize the differences between the mouse and the human *in vitro* quiescence induction system to understand the different behavior of mouse and human cells when acquiring quiescence.
3. To set up a CRISPR/Cas9 system for human NSCs, with the ambition of creating a fluorescently labelled *ASCL1*-reporter line.

2. Material and Methods

2.1 Handling and maintenance of human neural stem cells

2.1.1 Cell lines

The cell lines used were NSC derived from human induced pluripotent stem cells, cultured in FGF and FGF/EGF conditions. Hereafter called NSC FGF and NSC EGF/FGF. NSC FGF (Hippo NSCs Urbán Lab, IMBA, Austria) and NSC EGF/FGF (Hippo NSCs Urbán Lab, IMBA, Austria) were thawed and upscaled until the desired number of cells had been reached and the cells could be used for further experiments.

2.1.2 Cell culture

All cell culture work was performed in laminar flow hoods under sterile working conditions. The cells were incubated in a humidified atmosphere at 37 °C, 5 % CO_2. Maintenance media for human NSCs was Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 without glutamine (DMEM/F-12 without glutamine, 11540566, Fisher Scientific Austria, Vienna, Austria), supplemented with L-Glutamine (200 mM, 25030081, Life Tech Austria, Vienna, Austria), B-27 (B-27 Supplement (50X), serum free, 17504001, Thermo Fisher Scientific, Vienna, Austria), N2 (N2 supplement (100x), 17502001, Life Tech Austria, Vienna, Austria) and growth factors. The cells were maintained in two different media with growth factors, supplemented with FGF (fibroblast growth factor-2, human FGF basic (146AA), 10µg/ml, AF-100-18C, Eubio, Vienna Austria) only and supplemented with EGF (epidermal growth factor, animal-free human EGF, 10µg/ml , AF-100-15, eubio, Vienna, Austria) and FGF. Media was changed 24 hours after seeding the cells and from then on, every second day.

Before seeding, cell culture plates (CellStar 6-well, 12-well and 24-well cell culture multiwell plates, Greiner Bio-One, Schwerte, Germany, and Cell culture flasks, CellStar filter top cell culture flask T25 and T75, Greiner Bio-One, Kremsmünster, Austria) were first coated with Poly-L-Ornithine solution (POL, stock concentration: 100 µg/ml, final concentration: 15 µg/ml, P4957-50ML, Sigma-Aldrich, St. Louis, Missouri, USA), dissolved in Dulbecco's phosphate buffered saline (DPBS (1x, 14190-094, Thermo Fisher Scientific, Vienna, Austria)), then incubated overnight at 37 °C and 5 % CO_2. On the next day the POL solution was removed, and the plate washed three times with PBS (inhouse, media kitchen, IMBA, Vienna, Austria). Two hours before seeding the cells, the POL-coated plate was coated with laminin (stock concentration: 1000 µg/ml, final concentration: 10 µg/ml, Sigma-Aldrich, L2020-1MG) and

incubated again at 37 °C, 5 % CO_2. The coating was then removed, and fresh media was added to the plate to culture the cells.

2.1.3 Thawing of cells

The samples were taken out of liquid nitrogen and thawed at 37 °C in the water bath. The cells were then put into 4.5 ml of the respective media, either maintenance NSC media with FGF or NSC media with EGF/FGF. The cell suspension was then centrifuged at 300 x g for three minutes. The supernatant was aspirated and discarded, and the cell pellet was resuspended in 1 ml of the respective media. After this step, the cells were seeded into a cell culture plate (Greiner Bio-One) and cultivated in the incubator at 37 °C in 5 % CO_2.

2.1.4 Mycoplasma testing

The only way to detect mycoplasma in a cell culture is to specifically test for it. The PCR test for mycoplasma detection was performed by the inhouse facility ‘Molecular Biology Service’ of IMBA. Therefore, after thawing the cell lines, 500 µl of the NSC media was collected before changing the media and submitted to the facility.

2.1.5 Passaging of cells

To keep the cells proliferating and viable, it is essential to split them once they reach confluency, which is on average every five days. Therefore, the medium was aspirated from the plate and Accutase (StemPro Accutase Cell Dissociation Reagent, A1110501, Life Tech Austria, Vienna, Austria) was added to the wells and incubated for three minutes at 37 °C, 5 %. After checking in the microscope if all cells detached, the cell-Accutase suspension was diluted with either FGF only or EGF-FGF media depending on the cell line. The suspension was then collected and centrifuged for three minutes at 300 x g. The supernatants were discarded, and the cell pellets resuspended in fresh medium. According to the splitting ratio the cell suspension was then transferred to the prepared POL and laminin coated plates. In order to make sure the cells were distributed evenly in the plate, it was rocked in a cross-like movement. If the cells were getting confluent, they were split in 1:2 to 1:3 ratio.

2.2 Quiescence induction in mouse neural stem cells

2.2.1 Quiescence induction

For this experiment neural stem cells derived from the dentate gyrus of the adult mouse brain (strain C57BL/6J) were used. The mouse cells were kept in two media conditions: mouse aNSC complete media and human-like media. Composition of complete media is DMEM-F12, GlutaMAX™ (Thermofisher Scientific, 10565-018) supplemented with Penicillin-Streptomycin (1X, Sigma-Aldrich, P4333), Laminin (2µg/ml, Sigma-Aldrich, L2020), FGF (20 ng/ml, Peprotech, Hamburg, Germany, 450-33-1MG), EGF (20 ng/ml, Peprotech, 315-09-1MG), N2 supplement (1x with 430nM of insulin, inhouse, IMBA media kitchen) and Heparin (5µg/ml, Sigma-Aldrich, H3393). Human-like media is complete media supplemented with B27 (50X, Thermofisher Scientific) and had 10ng/ml FGF and EGF. One day after seeding cells on coverslips (20.000 cells/coverslip), quiescence was induction by adding BMP4 (20 ng/ml, Bio-Techne Ltd, Abingdon, United Kingdom, 5020-BP-010) for both complete and human-like media (Mira et al., 2010). Besides the BMP4-condition, one condition supplemented with FGF but without EGF and one condition with vehicle (0.2 % BSA+4mM HCl), as controls, were also kept in culture. The media was from then on changed every 24 hours. After zero days (D0), three days (D3), five days (D5) and ten days (D10) (see fig. 3), four coverslips for each condition, were fixed as described before (see: Fixing of the Coverslips), before performing ICC.

2.2.2 5-ethynyl-2′-deoxyuridine labelling of proliferating cells

Before fixing the coverslips, some of them were incubated with 10ng/ml of EdU (Carl Roth, Karlsruhe, Germany, 7845) and incubated for 30 minutes at 37 °C to assess proliferation rates. EdU can be used to detect proliferating cells that have undergone DNA synthesis, as it is incorporated into DNA of dividing cells (Chehrehasa et al., 2009).

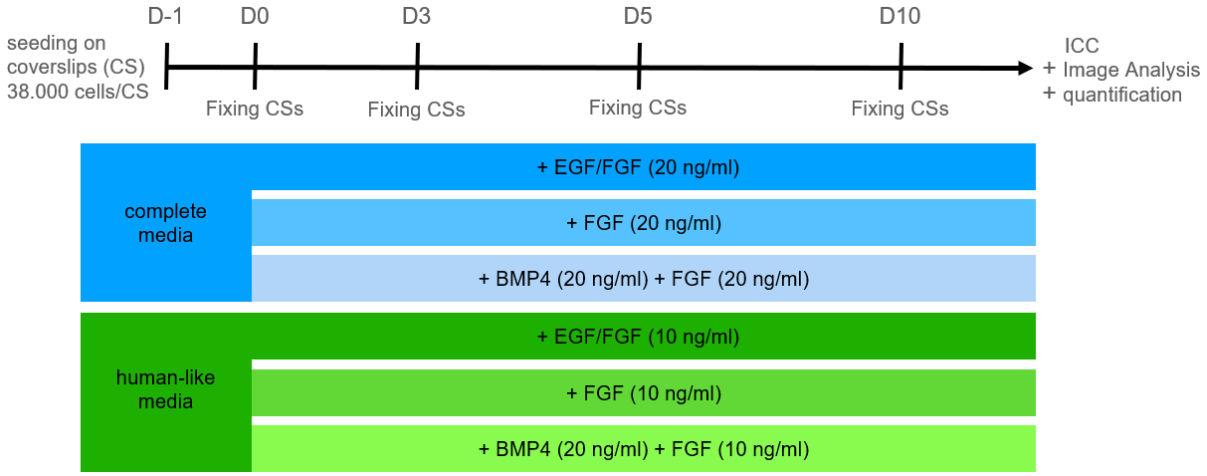


***Figure 3**: Experimental Set Up of Quiescence Induction Experiment*

2.3 Immunofluorescence cyto chemistry

For immunofluorescence cyto chemistry (ICC), 57,000 cells were seeded on a coverslip (round 13mm, VWR International, Pennsylvania, USA) and kept in culture under the respective conditions of the experiment.

Before the staining can begin, the cells were fixed on the coverslips. For that, media was aspirated and 500 µl of room temperature 4 % paraformaldehyde (PFA, P6148, Sigma-Aldrich) was added to each coverslip. Cells were incubated for ten minutes with PFA and then washed once with PBS (inhouse, IMBA media kitchen). The coverslips were stored in PBS + 0.02 % acide (Acetic acide 96 %, Merck, New York, USA) at 4 °C before using it further.

The chosen coverslips were transferred into a 24-well plate containing 500 µl of blocking solution (PBS-Triton (PBS-T, PBS + 0.1 % Triton (Triton X100, 28817.295, VWR™, Radnor, Pennsylvania, USA)) + 10 % Donkey Serum (Normal Donkey Serum, JAC017000121, Szabo-Scandic Handelsgmbh, Vienna, Austria)) and were incubated for 30 minutes at room temperature at 60 rpm on a shaker. In the meantime, a humid chamber was prepared, which acts as shield for the coverslips from light and contains a wet filter paper where a piece of parafilm is placed. Before incubating the coverslips overnight at 4 °C, the primary antibodies were diluted in blocking solution and 15 µl of it were placed on the parafilm and the chosen coverslip was placed on the drop, cells facing the drop.

On the next day, the coverslips were transferred to the 24-well plate and washed four times for five minutes at room temperature on the shaker at 60 rpm with PBS-T. Meanwhile, the secondary antibody solution was prepared the same way as the primary antibody solution.

After this the coverslips were again placed on the secondary antibody solution in the humid chamber and incubated for one hour at room temperature in the dark. From this step on everything was kept in the dark. The next step was to transfer the coverslips back to the 24-well plate again and wash it with PBS-T for five minutes on the shaker at 60 rpm. Then the coverslips were washed three more times with PBS again for five minutes on the shaker at 60 rpm. In the meantime, diamidino-2-phenylindole solution (DAPI, 1:10.000, Thermo Fisher Scientific) diluted in PBS 1/2 (PBS:MilliQ (1:1) (Milli-Q® IQ Water Purification System, Millipore, Burlington, Massachusetts, USA)) was prepared. PBS was removed and 500 µl of the DAPI solution were added per coverslip and incubated in the dark for five minutes on the shaker at 60 rpm. After that the coverslips were washed once again with PBS1/2 (PBS:MilliQ (1:1)) before mounting them on a glass slide with a drop of Glass Mountant (ProLong Glass Antifade Mountant, Invitrogen, Massachusetts, USA). This then should dry for at least 24 to 48 hours before starting with the imaging.

Table 1: *Used Primary Antibodies*

Target molecule	Species	Dilution	Company	Catalogue number #
Ascl1	mouse	1:100	BD Bioscience, New Jersey, USA	556604
Calbindin	mouse	1:100	swant®, Burgdorf, Swizerland	300
Calretinin	mouse	1:100	Abcam, Cambridge, United Kingdom	ab82198
Calretinin	rabbit	1:100	Abcam	ab92341
GFAP	rat	1:500	Invitrogen	130300
ID4	rabbit	1:500	Biocheck Hygienetechnisches Labor GmbH, Trierweiler, Germany	BCH-9/82-12
Ki-67	rat	1:200	Thermo Fisher Scientific	14-5698-82
MAP2	mouse	1:100	Sigma-Aldrich	M9942
Nanog	goat	1:200	R&DSystems, Minnesota, USA	AF1997
PROX1	rabbit	1:500	Millipore, Burlington, Massachusetts, USA	ab5475
Sox2	rat	1:500	Invitrogen	14-9811-82
TBR2	rabbit	1:200	Abcam	ab23345
TUJ1	rabbit	1:1000	BioLegend®, San Diego, California, USA	802001

***Table 2**: Used Secondary Antibodies*

Target molecule	Species	Conjugated to	Dilution	Company	Catalogue number
Mouse IgG	donkey	647	1:500	Jackson Laboratories, Bar Harbor, Maine, USA	715-606-151
Rabbit IgG	donkey	Cy3	1:500	Jackson Laboratories	711-166-152
rat IgG	donkey	488	1:1000	Invitrogen	405418

2.3.1 Imaging

To acquire multicolor fluorescent images, the cover slips were imaged with the Axio Imager.Z2 (upright) microscope (Carl Zeiss AG, Jena, Germany), which is equipped with Apoptome2 (Carl Zeiss AG, Jena, Germany), a structure illumination imaging technology. The images were taken as Z-stacks, five different focal lengths images with a distance of 2 µm and 0.4 distance between each of the levels, were taken. Z-stacking is a digital image processing technique that allows an image to be captured with greater depth of field by combining multiple images taken at different focal lengths.

2.3.2 Image processing

The image processing was done with the software Fiji (ImageJ, version: 2.1.0/1.53c). First, the Z-stacked apotome images were combined into one projection image with the algorithm ‘maximum intensity Z-projection’ run on Fiji and saved as TIFF. The next step was to set maximum and minimum intensity settings for each channel, by taking the secondary control (no primary, only secondary antibody) as a reference. This was done by using the Fiji tool ‘Brightness/Contrast. By using a Macro, one image of every channel and in addition a composite of all of the channels were saved as JPEG for further analysis.

2.3.3 Counting and quantification

To quantify the number of cells expressing the protein of interest, microscope images were counted manually with the ‘Cell Counter’ of Fiji and the data entered into a table of an Excel-file (Excel, Microsoft) and with the help of a Macro working for Fiji, which measured the intensities of the signal of the cytoplasm and the nucleus of the stained cells. The data was

then further analyzed statistically processed with RStudio (version: 1.4.1717) and Prism (version: 9.1.2 (225))

2.3.4 DNA extraction for single nucleotide polymorphism genotyping

For DNA extraction the Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin, USA) was used. The cell suspension was centrifuged at 15.000 x g for ten seconds. Supernatant was aspirated and the cell pellet was washed with PBS followed by vortexing. Then 600 µl of Nuclei Lysis Solution from the kit was added and the pellet was dissolved by pipetting up and down.

For the lysis and protein precipitation 3 µl of RNase Solution was added to the cells and incubated for 15-30 minutes at 37 °C. Then the suspension was cooled to room temperature before adding 200 µl of Protein Precipitation Solution. This mixture was vortexed and kept on ice for five minutes. After this step it was then centrifuged at 15.000 x g for four minutes.

For the DNA precipitation and rehydration, the supernatant was transferred to a fresh tube which contained 600 µl of room temperature isopropanol and then mixed carefully by inversion. Afterwards it was centrifuged again at 15.000 x g for one minute. The supernatant was aspirated and 600 µl of room temperature 70 % ethanol was added to the pellet and dissolved by pipetting up and down. The sample was centrifuged again at 15.000 x g for one minute. Then the ethanol was aspirated, and the pellet was air-dried for five to ten minutes at 55 °C on a thermo block. The DNA was then rehydrated in 100 µl of DNA Rehydration Solution for one hour at 65 °C on the thermo block.

2.3.5 DNA concentration

The DNA concentration was measured with the DeNovix DS 11 Spectrophotometer (DeNovix Inc., Wilmington, United States). For single nucleotide polymorphisms (SNP) genotyping the samples require a concentration of at least 100 ng/ml DNA. The SNP genotyping is performed inhouse by the stem cell core facility (SCCF, IMBA, Vienna, Austria).

2.4 Transfection of neural stem cells

2.4.1 Nucleofection

Before the nucleofection, new 6-well plates (Greiner Bio-One) were coated first with POL (stock concentration 100 µg/mL; final concentration: 15 µg/mL; Sigma-Aldrich) and incubated at 37 °C for around two to three hours before washing them with PBS (inhouse, media kitchen)

for three times. The plates were then coated with Sigma-Aldrich Laminin (stock concentration: 1 mg/ml; final concentration: 10 µg/ml). Two hours before the electroporation, the media of the chosen cells was changed and supplemented with Rock inhibitor (RI, 10 mM, 1:1000, Y-27632 dihydrochloride, R&D systems, 1254/10). For the electroporation, the Amaxa-4D Nucleofector (Lonza Group AG, Basel, Switzerland) and the nucleofection kit (P3 Primary Cell 4D-Nucleofector® X Kit S (32 RCT) Cat. No V4XP-3032) was used. The program used for the nucleofection was CA-137.

Right before nucleofecting the cells, laminin was aspirated from the 6-well plates, 2 ml of the media supplemented with RI were added per well and the plate was put back into the incubator to pre-warm. The chosen cells were then detached with Accutase (Life Tech Austria), incubated at 37 °C for around three minutes and then collected with the media supplemented with RI. 10 µl of the cell suspension was collected for counting. While the collected cells were then centrifuged at 300 x g for three minutes, trypan blue was added to the cell aliquot and the cells were counted with the Neubauer chamber (Karl Hecht, Sondheim von der Rhön, Germany). A cell number between 700.000 and one million cells is required per nucleofection reaction. After centrifuging, the required number of cells was then dispensed in a new tube and spinned again at 120 x g for five minutes. The cell pellet was resuspended in the nucleofection master mix containing 82 µl of Solution I, 18 µl of Supplement I from the kit and the respective DNA-Plasmid, which should have a concentration of at least 1 µg/µl. Because the nucleofection mix is toxic to the cells, the following steps had to be done as fast as possible. The cell/nucleofection suspension was transferred to the nucleofection cuvettes of the kit and electroporation was performed via inserting the cuvette into the Amaxa-4D nucleofector with the program CA-137. Upon electroporation, 500 µl of preheated media supplemented with RI were added immediately to the cuvette and the cells were left to rest for five minutes. The transfected cells were transferred to the already prepared plate, gently shook and placed back in the incubator at 37 °C and 5 % CO_2. On the next day, the media, still supplemented with RI, was changed.

Two different plasmids were used for the transfection: one transfection was done with 1 µl GFPmax plasmid (2µg/µl) provided by the kit and the second reaction was supplemented with 0.68 µl px458 pSpCas9(BB)-2A-GFP plasmid (2.9µg/µl, px458, plasmid preparation inhouse), which also has green fluorescent protein (GFP) inserted. A third transfection reaction was also transfected but without any plasmid, to see the reaction of the cells to the transfection itself, without the influence of additional DNA.

2.4.2 Checking transfection efficiency

To determine the efficiency of the transfection of the cells, the plate was checked with the Celigo Image Cytometer (Nexcelom Bioscience, Massachusetts, United States) every day for up to seven days post transfection, and pictures were taken for further analysis.

3. Results

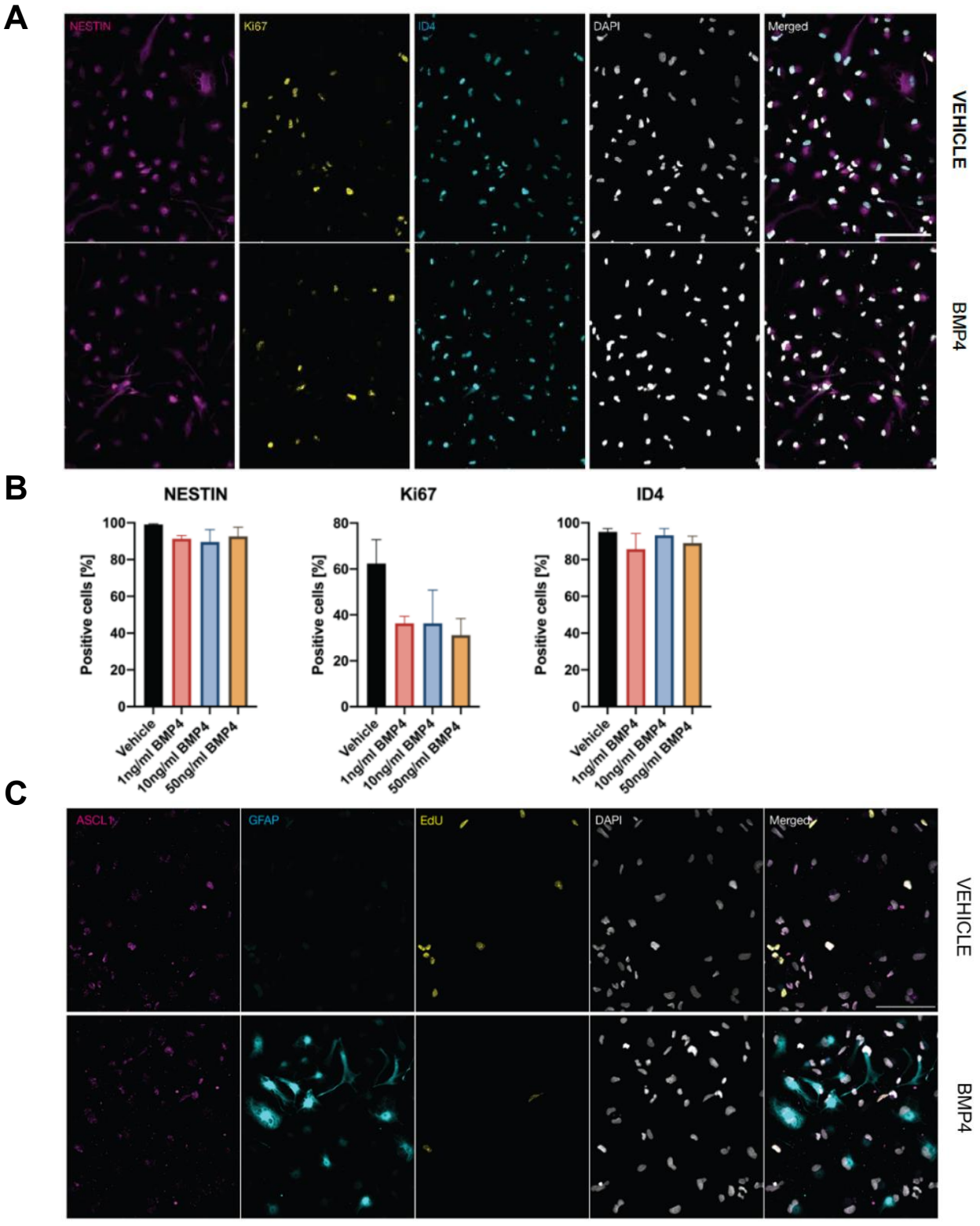
3.1 Characterization of *in vitro* generated human neural stem cells

Neural stem cells express stemness markers (SOX2 and NESTIN) and they are proliferating (KI67, EdU, proliferation markers) and express *ASCL1*. We characterized if in vitro derived human neural stem cells from pluripotent stem cells are expressing these specific markers. Stainings against these markers were performed previously. To get familiar with the image analysis pipeline, I analyzed these stainings using the macro (as described in Material & Methods) and counted proteins expressed in the cytoplasm (GFAP, NESTIN) as the macro does not properly work for that.

The results are presented in fig.4. In fig.4 A immunofluorescent images of NESTIN, Ki67 and ID4 are shown and in B for ASCL1, GFAP and EdU. The top row shows active NSCs, whereas the bottom row represents a BMP4 induced quiescent-like state of the NSCs. Quiescence was induced with different concentrations of BMP4 (1 ng/mL; 10 ng/ml; 50 ng/ml) for 72 hours. Quantification of the stainings showing the percentage of the positive cells of the respective staining are presented in fig.4 B and D. NESTIN and ID4 levels in active and quiescent-like are very similar. Ki67 expression decreases in the BMP4 condition compared to the active condition (vehicle). ASCL1 levels show a drop of expression in all the BMP4 conditions, but the BMP4 induction with 50 ng/mL, where cells show similar levels to the active state. EdU levels decrease in the BMP4 condition in comparison to the vehicle condition. Only the BMP4 quiescent like cells show expression of GFAP. The GFAP levels increase with the BMP4 concentration.

These results show that the active NSCs derived from human pluripotent stem cells, are proliferating (Ki67, EdU). When the cells are exposed to BMP4 for 72 hours, they should enter a quiescent-like state, as it was shown that BMP4 induces quiescence in mouse NSCs (Blomfield et al., 2019). In the quiescent-like state the human NSCs show a drop of proliferation markers (Ki67 and EdU) and at the same time an increasing level of GFAP, which is a typical marker of RGL and seen as a quiescent marker (Blomfield et al, 2019; Fukuda et al., 2003). These results indicate that quiescence induction by BMP4 of human NSCs, decreases their proliferation potential, while at the same time keeping their stemness (NESTIN) and increasing of their GFAP expression. This leads to the assumption that a quiescent-like state of human NSCs could be achieved by BMP4-induction. ID4 levels are very high in the active condition,

which is a different finding to what is known in mouse. Further testing is needed to also understand differences to the mouse *in vitro* system.



D

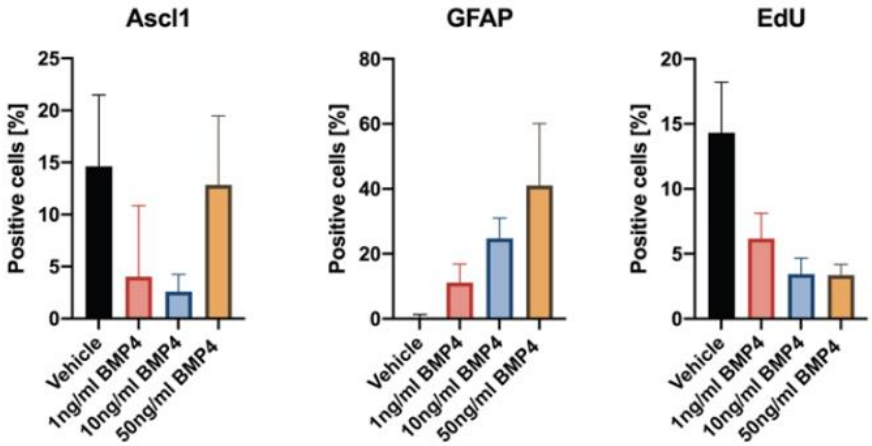


Figure 4: Establishing a human neural stem cell in vitro system to study their ability to enter quiescence. ***(A)*** *Immunofluorescent images of human neural stem cells derived from pluripotent stem cells stained against NESTIN (magenta), Ki67 (yellow) and ID4 (cyan). Top row represents active neural stem cells, bottom row represents BMP4 treated condition, which are supposed to enter a quiescent-like state.* ***(B, D)*** *Quantification of immunofluorescent staining showing the percentage of positive cells of the respective staining.* ***(C)*** *Immunofluorescent images of human neural stem cells derived from pluripotent stem cells stained against ASCL1 (magenta), GFAP (cyan) and EdU (yellow). Top row represents active neural stem cells, bottom row represents BMP4 treated condition, which are supposed to enter a quiescent-like state. Scale bars are 100µm.*

3.2 Antibody testing – neurodifferentiation

Neural stem cells give rise to neurons. To assess the differentiation potential of *in vitro* generated human neural stem cells, they were differentiated towards hippocampal neurons. To functionally validate the differentiation, we performed stainings against markers expressed in hippocampal neurons, like Calretinin and Calbindin. We used a combination of neuron markers specific for the hippocampal stem cell niche (Calretinin and Calbindin) and general neuronal markers (TUJ1, MAP2), to show that the human NSC differentiation towards neurons worked. Calretinin and Calbindin are both calcium binding proteins and are expressed in hippocampal granule neurons (Rogers et al., 1990). *Calretinin* can be found in mature granule neurons (hippocampal interneurons), whereas *Calbindin* is expressed rather in immature neurons (interneurons and Purkinje cells) (von Bohlen und Halbach, 2007). Microtubule-associated protein 2 (MAP2, pan neuronal marker which marks all neurons) is a microtubule-stabilizing protein found in postmitotic neurons (Soltani et al., 2005) and also beta-tubulin III (TUJ1) marks neurons (Encinas et al., 2011). PROX1 is a protein involved mechanically in the

differentiation versus proliferation decision of NSCs and promotes cell cycle exit and differentiation (Stergiopoulos et al., 2015) and it is expressed in the hippocampus.

As shown in fig.5 (A and B) the Calretinin antibody, the PROX1 antibody, as well as Calbindin antibody and GFAP antibody did not show any or only a very small percentage of positive cells in this experiment. The majority of cells were positive for Calretinin, MAP2 and TUJ1 were positive. The number of cells expressing *SOX2* and *KI67* were lower but visible. These results could point to a success in differentiating the NSCs of the human *in vitro* model into neurons.

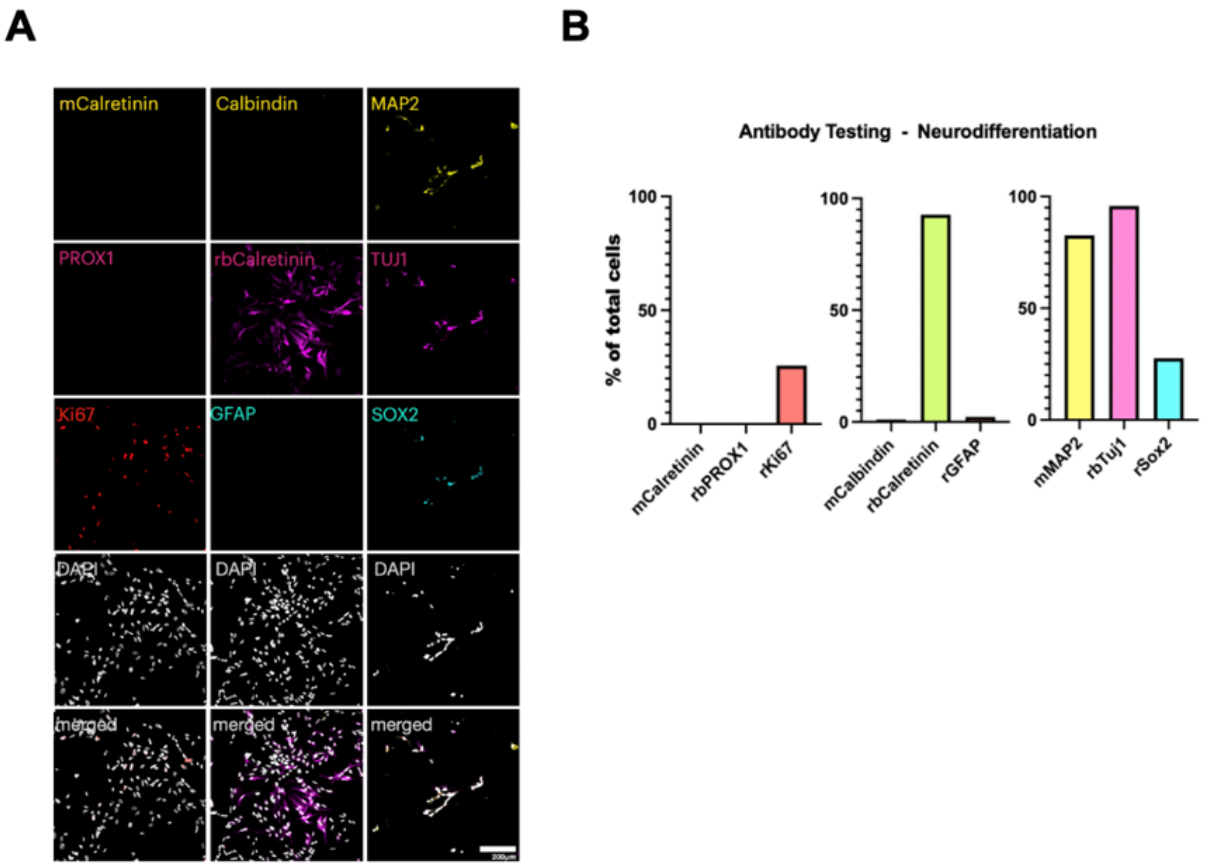


Figure 5: Differentiation of human NSCs of the in vitro model into neurons ***(A)*** *Immunofluorescent images of differentiated neurons from derived human NSCs for Calretinin, Calbindin, MAP2, PROX1, TUJ1, KI67, GFAP and SOX2. Scale bars represents 200µm.* ***(B)*** *Quantification of positive cells in percent. The graphs show the percentage of positive cells for the tested antibodies against Calretinin, Calbindin, MAP2, PROX1, TUJ1, KI67, GFAP and SOX2.*

3.3 Quiescence Induction of Mouse Neural Stem Cells

With the help of the novel human in vitro system of human NSCs, regulatory factors behind the quiescent state of these cells might be unravelled. As mentioned before, preliminary findings of the Urbán Laboratory in regard of this in vitro model, show that human NSCs can be induced by BMP4 to enter a quiescent-like state. However, unlike mouse NSCs, human NSCs resume proliferation after a few days, even in the constant presence of BMP. Therefore, human NSCs may not be able to maintain dormancy for long periods of time in the novel *in vitro* system (unpublished).

The human quiescent induction system is based on the quiescent induction system of mouse NSC. To determine any factors that could influence the different behavior of human NSCs regarding their entry into quiescence compared to the mouse induction system, we looked at the media composition. One major difference between these two systems is the B27 supplement in the human NSC media. To investigate if B27 contributes to this different behavior of human NSC regarding entering quiescence, we added B27 to mouse NSC, induced quiescence and compared the two media conditions, complete media and human-like media, which was supplemented with B27. Also, the concentration of EGF and FGF in the humanlike media was reduced to 10 µg/µl. The experimental set up is shown in fig. 6 (A). Besides the BMP4-quiescent inducing condition (BMP4), we kept cells in an EGF/FGF + vehicle condition (vehicle), which acts as a positive control. The third condition was FGF only (FGF), as it was shown before that removing EGF from the in vitro system induced several transcriptional changes in NSCs that had been specifically associated with BMP4 in the past (Blomfield et al., 2018; Mira et al., 2010). FGF on its own should be enough to keep the stemness of NSCs and also to keep them in a proliferating state (Blomfield et al., 2018). Cells were seeded on coverslips (D-1) and one day later quiescence was induced. An experimental timeline is shown in fig. 6 (A). On D0, D3, D5 and D5 a set of coverslips was fixed for further ICC analysis.

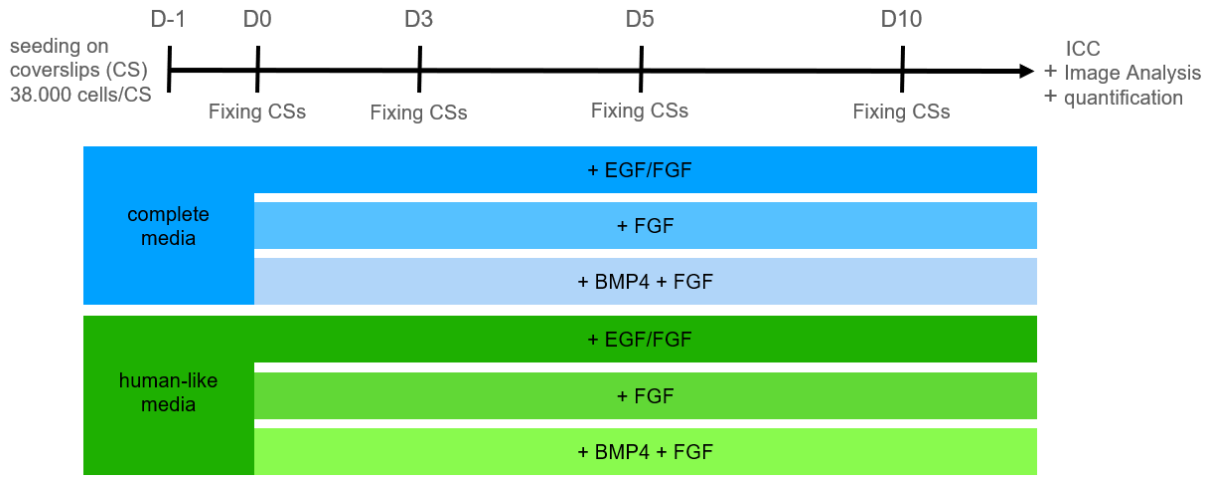
As shown in fig.6 (B) the cell densities of the different conditions and media are quite different from each other. The more time past, less cells survived in the FGF and BMP4 condition, whereas in the vehicle conditions the cells started to overgrow. In total the cells kept in human-like media seemed to grow denser and less cell death occurred compared to the complete media.

Cells were stained against ASCL1, ID4 and SOX2, the results are presented in fig.6 (C). As the cells were growing too dense in the vehicle condition, the acquired data from this condition of both media was excluded from the analysis after D3, as the fluorescent signal could not be

quantified. Additionally, FGF condition was also partly excluded from this analysis, as too much cell death occurred after D3 in both media. Also, ID4 staining was completely excluded from the image analysis, as it was not quantifiable and showed background signal that could point to unspecific binding of the secondary antibody. *ASCL1* levels were shown to decrease with the induction of quiescence with BMP4, and that ASCL1 protein only reaches significant levels in active NSCs (Blomfield et al., 2019). At D3, the vehicle condition and FGF condition of both media showed higher expression of ASCL1 than the BMP4 condition. In the complete media, there were no cell left in the BMP4 condition at D10.

SOX2-levels in both media are quite stable. D10 complete media BMP4 condition shows no signal because there were no cells left in this condition at that timepoint, as mentioned before. As the values of the three repetitions of each condition are very different from each other, and the complete and human-like media are very divers, it is not possible to draw reliable conclusions. Also changing the seeding density of vehicle condition should be considered to prevent overgrowth. Therefore, this experiment needs to be repeated.

A



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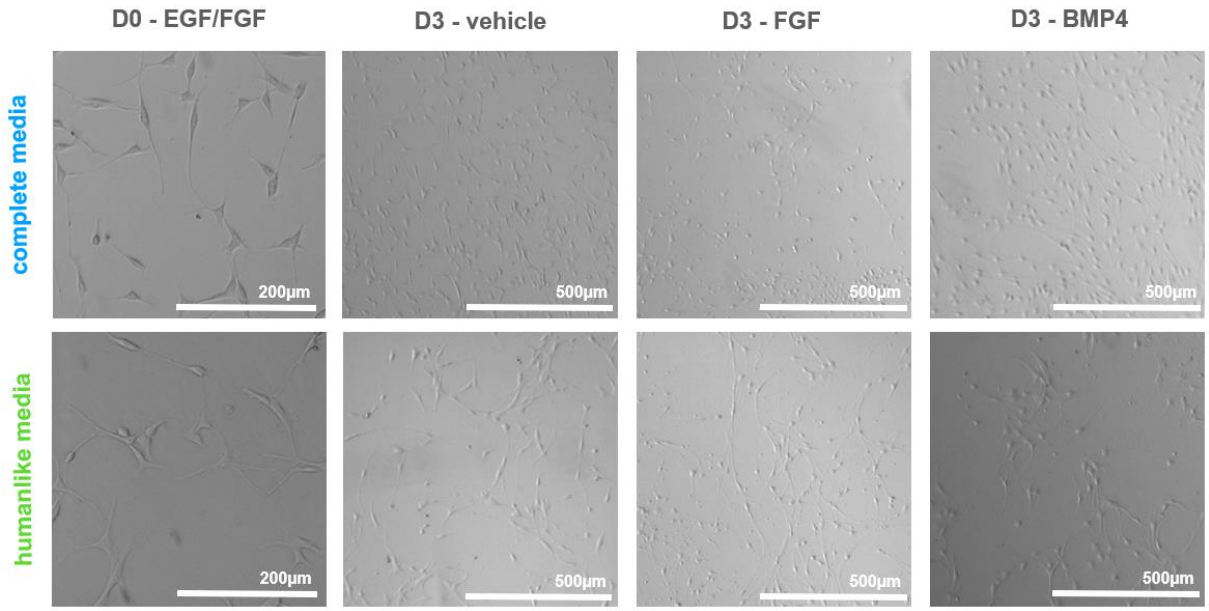
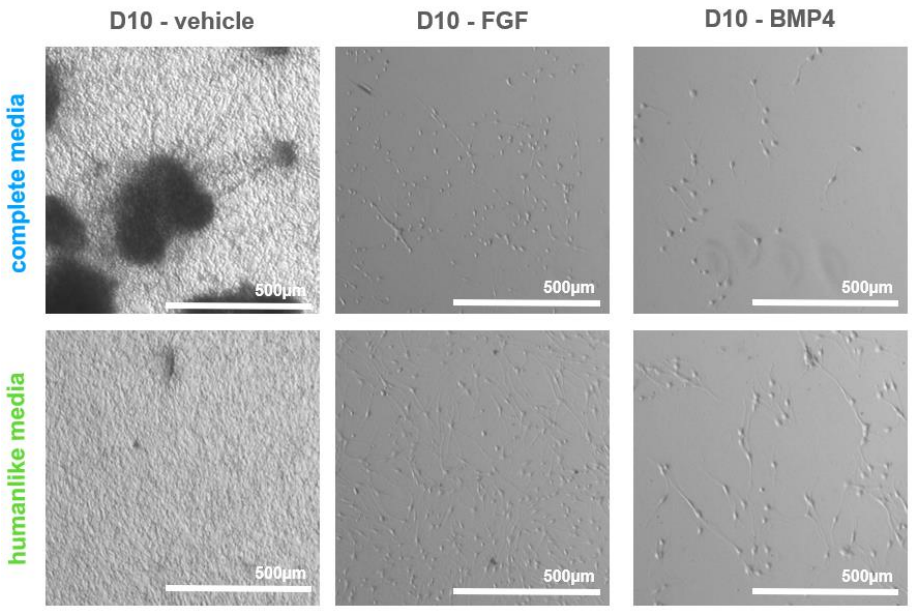
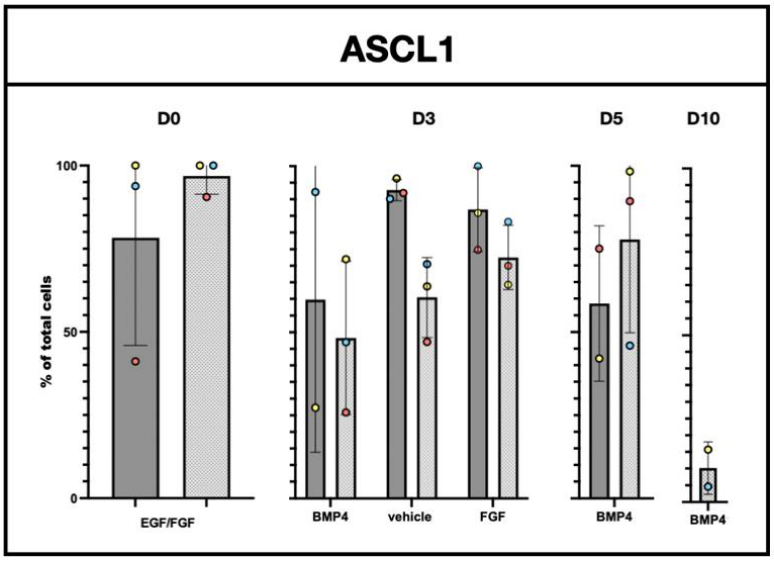

D0 - EGF/FGF
D3 - vehicle
D3 - FGF
D3 - BMP4
complete media
humanlike media
200µm
500µm
500µm
500µm
200µm
500µm
500µm
500µm

C


D10 - vehicle
D10 - FGF
D10 - BMP4
complete media
humanlike media
500µm
500µm
500µm
500µm
500µm
500µm

D



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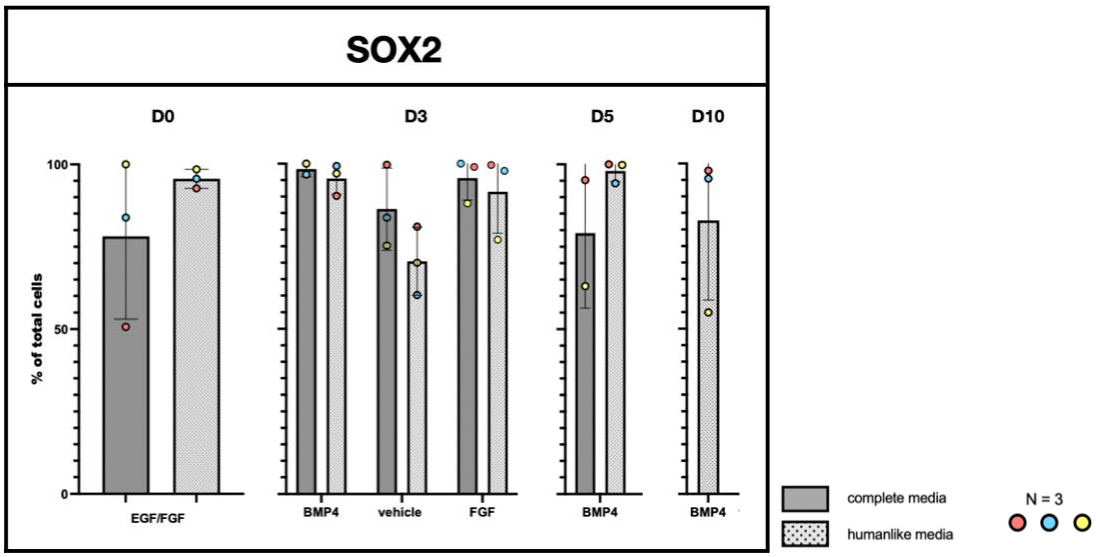

Figure 6: Comparison of complete media and humanlike media in quiescence induction of mouse NSCs (A) *Experimental set up of the B27-quiescence induction experiment.* ***(B, C)*** *Representative light microscope images of both media conditions of D0, D3 and D10. The images show cell density and morphology differences of the conditions (vehicle, FGF and BMP4).* ***(D, E)*** *Quantification of ASCL1 and SOX2 positive cells. Dark grey bars show complete media, light grey bars show humanlike media. All graphs indicate the positive cells for ASCL1 and SOX2 per timepoint (D0, D3, D5, D10) and per tested condition (vehicle, FGF and BMP4, as well as EGF/FGF, which acts as the control). After D3 vehicle and FGF condition were excluded from the analysis.*

3.4 Transfection

With the aim to establish a reporter line, fluorescently marking Ascl1, it is important to find a functioning delivery method to introduce the CRISPR/Cas9 system to genetically modify the human NSCs. We decided to test electroporating our cells by nucleofection (as described in Material Methods) as our delivery method.

The first transfection trial was set up in three different reaction conditions: one transfection was done with the GFPmax plasmid provided by the kit as a positive control, because it usually shows high efficiency results. To the second reaction, px458 plasmid (px458 pSpCas9(BB)-2A-GFP) was added, which also has the green fluorescent protein (GFP) inserted and is meant to be the backbone of the plasmid used for further trials with the human NSCs and the development of a reporter line in the future. The third transfection reaction was without any plasmid, to see the effects of the transfection itself on the cells, without the influence of additional DNA.

After performing the nucleofection of the human NSCs, the nucleofection efficiency was checked via fluorescent microscopy one day post transfection (dpT), three dpT, four dpT, five dpT and seven dpT. The highest GFP signal of the positive control (GFPmax) can be expected at three dpT, as the plasmid starts to get diluted through cell division and the GFP degrades from then on. This time course is shown in Fig. 7. The transfection with the GFPmax plasmid, as a positive control, worked very well for the human NSCs, whereas the px458 showed no success at all. The highest expression of the GFPmax was reached at day three post transfection. The control group (no plasmid) also worked, as it does not show any GFP signal.

The cell survival rate was good for all the conditions. As the transfection itself worked, shown by the positive control of the GFPmax plasmid and the cell survival rate of all the conditions, the nucleofection is a suitable delivery method for human NSCs. However, the experiment needs to be repeated and the px458 plasmid needs to be tested again, as the experiment was only performed once during this thesis due to lack of time.

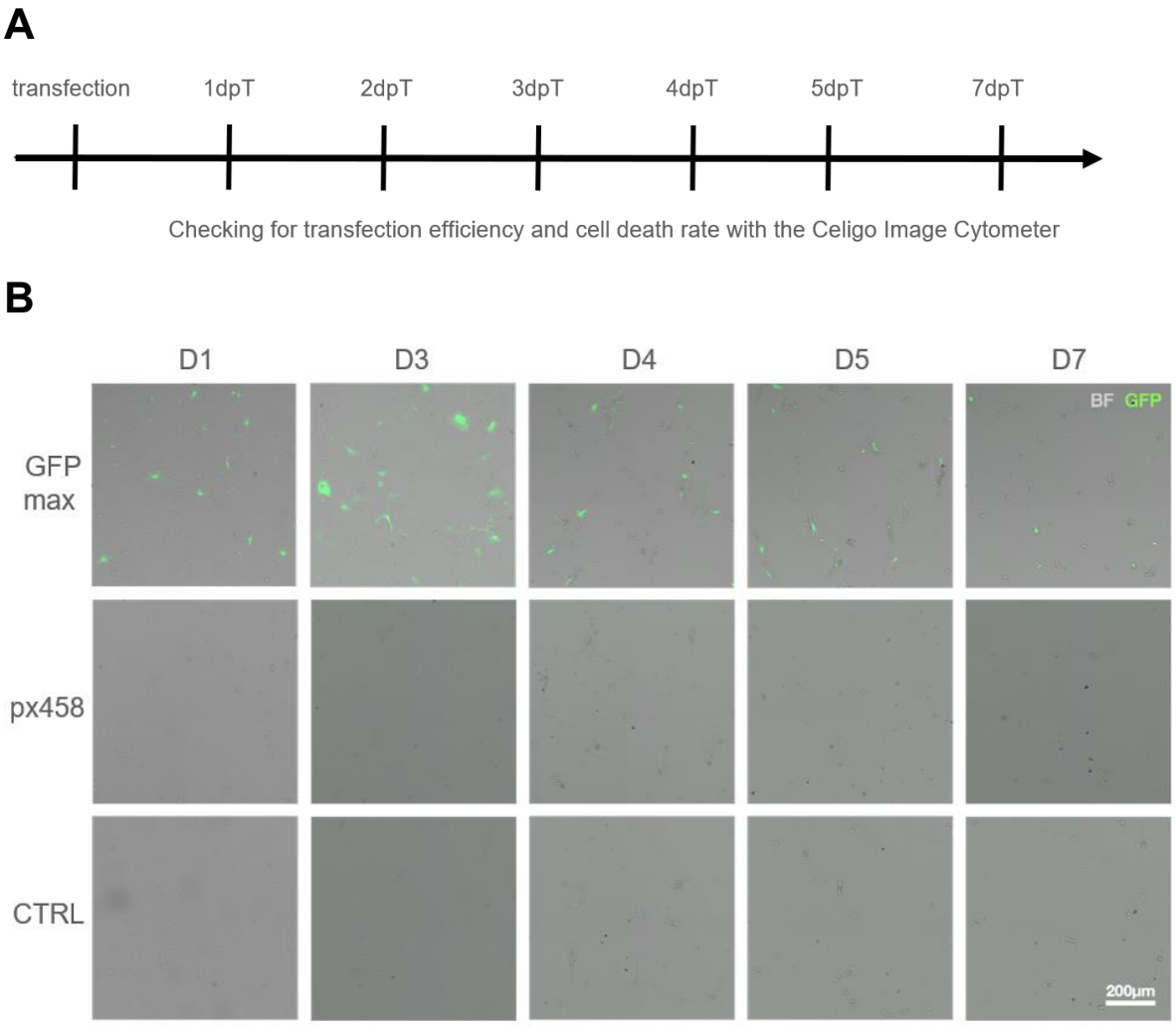

Figure 7: Assessment of nucleofection of human NSCs using GFP and fluorescent microscopy. *(**A**) experimental set up; (**B**) representative fluorescent images of the tested plasmids (GFPmax, px458 and control (CTRL)), one-, three-, four-, five- and seven-days post transfection. Images were merged; green indicates GFP, whereas grey shows the brightfield image (BF).*

4. Discussion

The work presented in this thesis includes the characterization of human neural stem cells in a novel *in vitro* system. It also addresses the comparability of the mouse BMP4-induced quiescence *in vitro* model and its application on human NSC to induce quiescence. Furthermore, this thesis discusses first attempts to transfect human neural stem cells and possible ways to subsequently sort these transfected cells, as first steps to establish an Ascl1-knock-in reporter line.

The neuronal niche represents an extremely complex environment and regulates activity and quiescence in NSCs through numerous factors. Many of these regulatory effects are still unknown or poorly understood. Therefore, it is even more important to have an *in vitro* system to better reproduce and trace certain regulators of quiescence and activity of NSCs. By adapting the well-established *in vitro* mouse system to human NSCs, we hope to understand the mechanisms behind the behavior of human NSCs better and their potential ability to acquire quiescence *in vitro*. To prove that cells derived from human pluripotent stem cells are really hippocampal NSCs, immunofluorescent stainings were performed to show that they express characteristic markers of these cells. NSCs express stemness markers, such as SOX2 and Nestin. In addition, they are also proliferating and therefore express proliferation markers such as Ki67. By quantifying those markers in the human NSCs, we showed that we are able to derive human NSCs in the Urbán Laboratory. When exposed to BMP4, the cells started to increase expression of quiescence associated markers such as GFAP, and a decrease of proliferation markers (Ki65, EdU) was noticeable. Therefore, we can assume that this *in vitro* model acts as a great tool to study human NSCs and will further contribute to understand their ability to acquire quiescence. However, further testing is needed to understand, for example, expression differences of ID4 between human and mouse models, as in mouse the ID4 levels are low in active and increase in BMP4-exposed NSCs. In human cells we don’t see a difference of ID4 expression in active and quiescent-like NSCs.

Furthermore, the Urbán Laboratory was interested in differentiating those derived human NSCs further into neurons, to show that they are again really NSCs and can give rise to neurons. After following the differentiation protocol, the cells were characterized by immunofluorescent staining, where we tested different antibodies. Especially stainings against markers expressed in hippocampal neurons (PROX1, Calretinin and Calbindin), were of huge

interest. MAP2 and TUJ1 are also found in neurons and were therefore also stained against in our experiment. All these antibodies have been tested before on slices from the mouse brain, which act as positive control for the tested antibodies. While PROX1, Calbindin, MAP2, TUJ1 and Calretinin were clearly working (data not shown), the mouse Calretinin antibody gave a very weak signal with high background. Therefore, this antibody could also not work anymore with the cell stainings. This assumption is also strengthened by the fact that Calretinin antibody, raised in mouse, worked.
As presented in the results, the cells showed high levels of the neuronal markers MAP2, Tuj1 and Calretinin (rabbit). On the other hand, the cells showed low expression levels of *Ki67* and *Sox2*, which could point to their loss of stemness (*Sox2*) and their lack of proliferation (*Ki67*), as neurons are in a postmitotic state. The antibodies Calretinin (mouse) and Calbindin (mouse) showed no signal. *Calbindin* is expressed by mature neurons. The *in vitro* derived neurons in this experiment were six weeks old. Therefore, these neurons could be not mature enough to express *Calbindin* and only express it at later stages. To test this, we would need to maintain neurons longer and stain again against Calbindin.

As previously shown by the Urbán laboratory, human NSCs are either not fully entering quiescence or returning from quiescence (unpublished). As the human quiescent induction system is based on the quiescent induction system of mouse NSCs, it is important to understand which factors of the human system might contribute to this different behavior of human NSC regarding the entering of quiescent state. As B27-supplement in the human media is a major difference to the mouse media. We performed a quiescence induction time course experiment with mouse NSCs and compared the two media (complete media and humanlike media with B27). To distinguish any influencing effects of B27 on the cells compared to the complete media without B27 and to see, if the quiescence induction was working, we fixed the cells after zero, three, five and ten days and performed immunofluorescent stainings. We repeated the experiment three times in total. As presented in the results, the outcome was quite unsatisfactory. The main problem we faced during this experiment, was the massive cell death that occurred, mainly in the FGF condition, but also in the BMP4 condition. While the vehicle condition (EGF + FGF) was overgrowing already from day three, FGF and BMP4 often showed very little to no cells. The cell death rate was higher in complete media than in the humanlike media. This could point to a compensatory effect of B27 supplement for the FGF. The batch of FGF used for this experiment, also caused problems in different experiments performed in the Urbán Laboratory at that time. Hence, repeating the experiment with a new

batch of FGF is necessary to exclude possible influences caused by it but also other factors of the media. Another aspect to think about is why the stainings differ sometimes quite a lot between the three repetitions of this experiment. The values of *SOX2* and *ASCL1* expressing cells seem to be closer together of all three repetitions in the humanlike media, compared to the complete media. This makes it difficult to compare the three repetitions, but also the two media conditions are then less transferable to each other. Additionally, the ID4 staining was excluded from the analysis, as the the images showed unspecific background signaling. This could point to unspecific binding of the secondary antibody.

The last major aim of this thesis was to establish an *ASCL1*-knock-in reporter line in human NSCs. By fluorescently marking the *Ascl1*-gene, we hope to be able to better tract and understand the regulatory function of this transcription factor on human NSCs. First a functioning delivery method needs to be found, to then be able to introduce the CRISPR/Cas9 system and the gene of interest (*Ascl1* with a fluorescent marker) into the human NSCs. We performed nucleofection on our cells and tested three different conditions. One part of the cells was transfected with GFPmax plasmid, as a positive control. The other part was transfected with px458 plasmid, which we want to use as the backbone of the plasmid for the knock-in experiment. The last condition was transfected without any plasmid, to see the reaction of the cells to the nucleofection without the influence of external DNA. In conclusion, the cell death rate after transfection was acceptable, which indicates that this delivery method is well tolerated by our cells. While the GFPmax showed very high transfection efficiency, even after one week post transfection, our plasmid of interest, px458, did not work at all. Numerous factors could play a role in this. Although the experiment was performed only once for this thesis due to time constraints, it is necessary to repeat it in the future. The event leading to the unsatisfactory results may have happened while pipetting the nucleofection reaction mix. Also, the promotor inserted in the px458 plasmid might not have worked for our cells. The next steps for this experiment are therefore, to repeat it again and see if the px458 works then. If not, a different plasmid needs to be used to further establish the reporter line. Other delivery methods, besides nucleofection, such as lentiviral transfection or lipofection, could also be options to establish this reporter line and might show higher efficiency and lower cell death rate then nucleofection. In addition, a functioning sorting method, to distinguish positively transfected cells afterwards needs to be tested. FAC sorting seems to be the first choice, but also puromycin selection for example could be considered and needs to be tested. Once the

delivery method and sorting method are fully established, a designed guide RNA will be used to establish an Ascl1-tagged reporter line.

In summary, with this thesis I contributed to the characterization of a novel human NSC model and to establish a quiescence induction protocol in human NSCs. To understand the influence of different media supplements, I performed quiescence induction experiments and tested the effect of B27 on mouse NSC and their ability to induce quiescence. Furthermore, I started setting up nucleofection and FACS for human NSCs, methods that are required to perform CRISPR/Cas9. This will in the future contribute to the generation of a fluorescently labelled reporter line.

5. Abstract

5.1 Abstract (English)

Quiescence, a dormant cell cycle rest of adult stem cells, maintains the stem cell pool and tissue homeostasis throughout life. Adult neural stem cells reside beside others in the subgranular zone in the hippocampus of the adult brain. The majority of these cells are quiescent, and their activation is tightly regulated by a variety of signals, that are yet not fully understood. Active neural stem cells rather differentiate than self-renew. This highlights the importance of a tight quiescence regulation of NSCs, as an imbalance in their activation, is linked to a loss of the stem cell pool and in the long term loss of the generation of new neurons.

We characterized hippocampal neural stem cells derived from human pluripotent stem cells. Additionally, these neural stem cells were differentiated towards neurons. We could show that these neurons are indeed expressing neuron-specific markers. Therefore, we can derive human neural stem cells *in-vitro*, which will contribute to our understanding of their ability to acquire quiescence.

Furthermore, we induced quiescence *in-vitro* to understand the effect of B27 on mouse neural stem cells and their potential to enter quiescence. We did not observe any difference in the quiescence induction. However, due to variability of the replicates, it is currently not possible to draw a valid conclusion and the experiment needs to be repeated.

The last aim of this thesis was setting up nucleofection and FACS of human neural stem cells to generate a reporter line. First nucleofection trials showed a good survival rate of the cells. Further trials are needed, which could not be done due to time restrictions.

In summary, this *in-vitro* model is a tool to study human neural stem cell quiescence. Developing a reporter line will allow a more tractable system to study the mechanism of human neural stem cells entering quiescence.

5.2 Abstract (German)

“Quiescence“ ist eine Ruhephase im Zellzyklus adulter Stammzellen. Diese Phase ist essenziell für den lebenslangen Erhalt des Stammzellpools und der Gewebehomöostase. Im erwachsenen Gehirn befinden sich adulte neurale Stammzellen unter anderem in der subgranulären Zone des Hippocampus. Die Mehrheit dieser adulten neuralen Stammzellen ruht und ihre Aktivierung wird durch eine Vielzahl von Signalen gesteuert, die noch nicht vollständig bekannt sind. Zudem führt die Aktivierung zum Verlust des Stammzellenpools mit der Zeit, da neuralen Stammzellen eher differenzieren als sich selbst zu erneuern. Dies unterstreicht die Bedeutung von “Quiescence“ für die langfristige Erzeugung neuer Neuronen.

Wir haben neurale Stammzellen des Hippocampus charakterisiert, die aus menschlichen pluripotenten Stammzellen gewonnen wurden. Des weiteren wurden diese neuralen Stammzellen zu Neuronen differenziert und wir konnten die Expression von neuronen-spezifischen Markern zeigen. Dieses humane neurale Stammzell-*in-vitro*-Modell ermöglicht es uns, die Fähigkeit zur Erlangung von “Quiescence“ zu untersuchen.

Darüber hinaus wurden Experimente zur Induktion von “Quiescence“ *in-vitro* durchgeführt, um den Einfluss von B27 in neuralen Stammzellen der Maus und deren Eintritt in die Ruhephase zu verstehen. Dies verdeutlicht die Unterschiede beim “Quiescence“-Eintritt *in vitro* zwischen dem menschlichen und dem Maussystem. Die Ergebnisse zeigen, dass das Experiment wiederholt werden muss, da es nicht möglich war, logische Schlussfolgerungen aus den Daten zu ziehen.

Der letzte Teil dieser Arbeit befasste sich mit Nukleofektion und FAC Sorting von menschlichen neuralen Stammzellen mit dem Ziel, eine Reporterlinie zu generieren. Erste Nukleofektionsversuche zeigten eine gute Überlebensrate der Zellen. Es sind weitere Versuche erforderlich, die aus Zeitgründen nicht durchgeführt werden konnten.

Zusammenfassend lässt sich sagen, dass das humane *In-vitro*-Modell eine gute Methode zur Untersuchung neuraler Stammzellen darstellt. Ein gut etabliertes und charakterisiertes *In-vitro*-Modell wird die Entwicklung einer Reporterlinie ermöglichen, die wiederum ein leichter handhabbares System zur Untersuchung der “Quiescence“ Regulierung menschlicher neuraler Stammzellen bietet.

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7. List of Abbreviations

Ascl1	Achaete-Scute Family BHLH Transcription Factor 1
bHLH	basic-helix-loop-helix
BMP4	Bone morphogenic protein-4
BrdU	Bromodeoxyuridine
Cas9	CRISPR-associated Protein 9
CSF	cerebral spinal fluid
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCX	doublecortin
DPBS	Dulbecco's phosphate buffered saline
DSB	double-strand breaks
DG	dentate gyrus
EB	embryoid bodies
ECM	extracellular matrix
EdU	5-ethynyl-2′-deoxyuridine
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HES	hairy and enhancer of split
HR	homologous recombination
ICC	immunofluorescence cyto chemistry
Id	Inhibitor of differentiation/DNA binding Protein
IPC	Intermediate progenitor cell
MAP2	Microtubule-associated protein 2
MCM2	minichromosome maintenance complex component 2
MFG-E8	Milk fat globule-epidermal growth factor-8
NHEJ	non-homologous end joining
NEUROD1	Neurogenic differentiation 1 Protein
NSC	neural stem cell
PAX6	paired box preotein-6
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
POL	Poly-L-Ornithine
Prox1	prospero homebox 1
PSA-NCAM	polysialylated neural cell adhesion molecule
RGL	radial-glia-like stem cell
Sox2	sex determining region Y- box 2
sgRNA	single guide RNA
SGZ	subgranular zone
SVZ/ V-SVZ	subventricular zone/ ventricular subventricular zone
TALENs	transcription activator-like effector nucleases
TBR2	T-box brain protein 2
Tuj1	neuron-specific class III beta-tubulin

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