Identification and evaluation of cell type-specific markers for human adult hippocampal neurogenesis (AHN)

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1. Abstract

In human brain, most neurons are generated during fetal development, however adult neurogenesis, the lifelong self-renewal of neural stem cells (NSCs) and their maturation into neurons, remains a long-debated topic (*Spalding et al., 2013; Kempermann, 2015; Boldrini et al., 2018; Sorrells et al., 2018*). In rodents, two neurogenic niches are so far robustly identified, the subventricular zone (SVZ), along the walls of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyri in the hippocampus (*Gage et al., 2000; Gillotin et al., 2021*). However, the subgranular zone of the dentate gyrus is the only putatively neurogenic region in the adult human brain. This process is referred to as adult hippocampal neurogenesis (AHN). So far, to address the controversial topic of adult neurogenesis in the human hippocampus, several techniques have been used, such as neuronal birth-dating, marker-based immunohistochemistry and more recently single-nucleus RNA-sequencing (scRNA seq). Even if similar techniques have been employed, different results and conclusions have been reached, pointing towards the challenges and the limitations of the approaches and markers used to investigate this controversial topic in neuroscience. Therefore, it is absolutely necessary to find new and more reliable and specific markers to investigate the presence or the absence of neurogenesis in the adult human brain. The present study aims to address this necessity by focusing on the closest established and characterized neurogenic event to the adult neurogenesis in human, namely the human fetal neurogenesis, which occurs during embryonic development. With this rational, a meta-analysis was performed taking into consideration studies where human fetal brain samples were subjected to scRNA sequencing. The hypothesis behind this study is that neurogenic cell type-specific markers might be conserved between fetal and adult neurogenesis in human (*Urbán and Guillemot, 2014*). Hence, a list of candidate markers for NSCs, neural progenitor cells (NPCs) and immature neurons (ImNs) was obtained via *in silico* cross-referencing of previously published single-cell transcriptomic datasets. Subsequently, these candidate markers were further evaluated by analysing their transcriptional profile along the differentiation of two distinct human stem cell lines, induced-pluripotent stem cells (iPSCs) and RenCells, selecting those with an expected expression profile for each specific cell type of interest. In addition, the expression of the selected candidate markers, one for each cell type, was validated via immunofluorescence in the same two human stem cell lines. The final candidate markers were also tested in mouse brain section at two different aging stages, in healthy and Alzheimer's mouse model brain. Based on our preliminary results, the selected gene sets may offer a useful resource for identifying neurogenic markers conserved in fetal and adult human brain. Yet,

further evaluation and validation follow-up studies are necessary, in order to systematically delineate commonalities and differences between embryonic and adult neurogenesis on a quest for more specific neurogenic markers.

2. Layman's summary

In human brain, most neurons are generated during fetal development, however adult neurogenesis, the proliferation of neural stem cells (NSCs) and their maturation into neurons during the entire lifespan remains a long-debated topic (*Spalding et al., 2013; Kempermann, 2015; Boldrini et al., 2018; Sorrells et al., 2018*). In rodents, neurogenesis is so far identified in two distinct regions, the subventricular zone (SVZ), along the walls of the two lateral inner brain cavities, known as ventricles, and the subgranular zone (SGZ) of the dentate gyri, in a brain structure called hippocampus (*Gage et al., 2000; Gillotin et al., 2021*). However, the SGZ of the DG is the only presumed region in the adult human brain where neurogenesis occurs. This process is referred as adult hippocampal neurogenesis (AHN). So far, to address the controversial topic of adult neurogenesis in the human hippocampus, several different techniques have been used. However, different results and conclusions have been reached, pointing out the challenges and the limitations of the approaches and markers used to investigate this controversial topic in neuroscience. Therefore, it is absolutely necessary to find new and more reliable and specific markers to investigate the presence or the absence of neurogenesis in the adult human brain. The present study aims to address this necessity by focusing on the closest known neurogenesis event to adult neurogenesis in human, namely the fetal neurogenesis, which occurs during fetal development. With this rational, an analysis was performed taking into consideration studies where human fetal brain samples were sequenced at a single cell level. The hypothesis behind this study is that markers specific to cell type involved in neurogenesis might be conserved between fetal and adult neurogenesis in human (*Urbán and Guillemot, 2014*). Hence, a list of candidate markers for NSCs, neural progenitor cells (NPCs) and immature neurons (ImNs) was obtained via cross-referencing of previously published datasets. Subsequently, these candidate markers were further evaluated by analysing their gene expression profile along the differentiation of two distinct human stem cell lines, induced-pluripotent stem cells (iPSCs) and RenCells, from neural progenitor to neurons, selecting those with a gene expression profile in line with the expected one for each specific cell type of interest. In addition, the protein expression of the selected candidate markers, one for each cell type, was validated in the same two human stem cell lines. The protein expression of the final candidate markers was also tested in mouse brain section at two different aging stages, in healthy and Alzheimer's mouse model brain. Based on our preliminary results, the selected gene may offer a useful resource for identifying markers conserved in fetal and adult human neurogenesis. Yet, further evaluation and validation follow-up studies are necessary, in order to clearly delineate

commonalities and differences between embryonic and adult neurogenesis on a quest for more specific neurogenic markers.

3. Introduction

Neurogenesis refers to the formation of new neurons from neural stem cells (NSCs) and neural progenitor cells (NPCs) and occurs throughout the life span in specific regions of the mammalian brain (*Gage et al., 2019*). Neurogenesis occurs at higher rates in early years and declines with aging. Although abundant during development, the presence of adult neurogenesis in the human brain is still debated (*Kempermann et al., 2019*).

3.1. Adult neurogenesis

Adult neurogenesis has been shown to occur in mammals in two major active neurogenic niches: the subventricular zone (SVZ), along the walls of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyri in the hippocampus (*Gage, 2000; Gillotin et al., 2021*).

In rodents, NSCs resident in the SVZ generate neuroblasts that migrate to the olfactory bulbs, where they differentiate into interneurons and astrocytes. Whether the same process happens in the adult human brain remains a very dynamically studied topic (*Sanai et al., 2004; Wang et al., 2011; Gillotin et al., 2021*). However, it has been proposed that new born neurons generated in the human SVZ migrate to the striatum where they integrate as interneurons (*Bergmann et al., 2015; Ernst et al., 2014; Donega et al., 2022*).

In the SGZ of the dentate gyrus (DG) in the hippocampus, NSCs that generate excitatory granule neurons and astrocytes (*Cameron et al., 1993*) have been observed in many species, such as rodents and non-human primates.

The SGZ is particularly of great interest since it is the most studied and so far the only putative neurogenic niche in adult human brain. Although promising evidence for adult neurogenesis in the human SGZ, a consensus still need to be reached (*Eriksson et al., 1998; Gould et al., 1998; Kornack and Rakic, 1999; Leuner et al., 2007, Boldrini et al., 2018; Gillotin et al., 2021*). This process is namely referred as adult hippocampal neurogenesis (AHN).

The process of AHN involves different cell types and molecular players. Active NSCs, also called radial glia-like (RGL) or Type I, proliferate and differentiate sequentially into three different neural progenitor cells (NPCs): type IIa, type IIb (intermediate progenitor cells, IPCs) and type III (neuroblasts), with increasing neuronal commitment, generating immature neurons that will eventually reach the stage of mature excitatory granule neurons (*Dimou and Gotz, 2014; Kriegstein and Alvarez-Buylla, 2009; Urban and Guillemot, 2014; Gillotin et al., 2021*). Along the differentiation

process, each neuronal cell type is characterised by the transient expression of specific markers, morphology and topology (*Urban and Guillemot, 2014; Toda et al., 2019; Gillotin et al., 2021*).

The final stage of AHN is the integration of the newly formed neurons into the hippocampal try-synaptic circuit, which links the DG to the rest of the hippocampus and to the entorhinal cortex. This final stage is characterized by neurites elongation and synapsis formation (*Christian et al., 2014; Toni and Schinder, 2015; Gillotin et al., 2021*). Evidence in mice showed that only a small part of the newly born neurons eventually will survive and integrate into the functional hippocampal circuits (*Kempermann et al., 2003*).

The integration of new neurons is thought to be responsible for the plasticity of the hippocampal circuit. Interestingly, recent evidence indicates that adult neurogenesis modulates DG inputs and induces global remapping in the dentate gyrus in rodents. (*Luna et al., 2019*). New born neurons in the DG contribute to cognition functions, such as memory, learning, pattern separation and mood regulation (*Akers et al., 2014; Clelland et al., 2009; Deng et al., 2009; Hill et al., 2015; Epp et al., 2016; Franca et al., 2017; Gillotin et al., 2021*).

Moreover, neurogenesis, and therefore these processes, can be modulated by external stimuli, such as environment, stress, mood and drugs (*Snyder et al., 2009; Miller et al., 2015; Anacker et al., 2011*)

3.2. AHN in relation to physical exercise, aging and neurodegenerative diseases

Studies in rodents have robustly proved how neurogenesis and physical exercise have a beneficial effect on cognition, memory and Alzheimer's disease (AD) *(Kempermann et al., 2018; Dery et al., 2013; Lucassen et al., 2020*).

In addition, studies in animal models have established the link between impaired AHN and cognitive deficits and thereby impaired learning and memory functions (*Toda et al., 2019; Dupret et al., 2008; Tronel et al., 2012; Gillotin et al., 2021*).

However, in humans, the link between AHN to cognitive function and brain disease is largely elusive. Nevertheless, evidence supports a correlation between neurogenesis changes, AD condition and cognitive status. In particular, it has been shown that higher numbers of neuroblasts are associated with better cognitive status and a drop of neuroblasts number occurs in individual with cognitive impairments (*Tobin et al., 2019*).

Growing knowledge about this process showed that also in human, AHN decrease is associated with cognitive decline along ageing (*Bergmann et al., 2015*). Indeed, AHN changes are shown to be linked

with the pathogenesis of several neuropsychiatric diseases such as autism, schizophrenia, Alzheimer's disease and depression (*Moreno-Jimenez et al., 2019; Tobin et al., 2019; Berger et al., 2020*). In particular, immunohistological studies on human post-mortem hippocampus samples showed how neurogenetic cellular populations drop sharply along aging and in neurodegenerative conditions, such as AD or mild cognitive impairment (MCI) (*Moreno-Jiménez et al., 2019; Tobin et al., 2019*).

Therefore, the integration of new born neurons could be relevant for the prevention of cognitive decline due to the physiological aging and also for the treatment of cerebral trauma, stroke, major neuropsychiatric disorders, depression and dementia, opening the venue of new therapeutic approaches

3.3. Adult neurogenesis across species

Neuronal cell birth in the adult was first observed between the 1960s and the 1980s, based on tritiated (3H) thymidine incorporation into dividing DNA in the brain of adult rodents and birds (*Altman et al., 1965; Goldman et al. 1983*). However, the detection of new born cells in the hippocampus of non-human primates was unsuccessful, and hence it was assumed that adult neurogenesis was likely absent also in humans (*Rakic et al., 1985*).

By using different techniques, such as non-radioactive thymidine analogues and antibodies directed against specific cell cycle and cell type markers, neurogenesis was robustly confirmed in rodent hippocampus (*Lois et al., 1994; Cameron et al., 1993*) and detected also in the macaque brain (*Nowakowski et al., 1981; Rakic et al., 1981*).

However, it has been shown high variability in the number of newly born neurons among different species, as well as in the rate of neurogenesis (*Sorrells et al., 2021; Snyder, 2019; Gillotin et al., 2021*). The developmental time window of new born neurons differs between species and most likely also decreases at a different speed during the lifespan (*Snyder, 2019*).

For instance, in rodents, it has been shown that the neurogenesis rate decreases sharply during the first months after birth and it remains constant at lower rate throughout life (*Snyder, 2019; Ben Abdallah et al., 2010; Kuhn et al., 1996; Gillotin et al., 2021*).

In contrast, in non-humans primates the neurogenesis rate decreases drastically after birth and it slowly continues to decrease down to sexual maturation, after which it remains constantly low (*Dennis et al., 2016; Snyder, 2019; Charvet and Finlay, 2018; Gillotin et al., 2021*).

3.4. Ongoing human adult neurogenesis debate: historical evidence overview

Adult neurogenesis has been so far challenging to study in humans. Whether new neurons are only generated during neural development or also occurs throughout adulthood is of great interest and still remains controversial. Big steps forward in igniting this debate were represented by the introduction of new and more sophisticated approaches and techniques.

3.4.1. BrdU retrospective birth-dating

The first evidence in favour of the thesis that human neurogenesis continues also during adulthood comes from a study by Eriksson et al. (1998) of 5 patients with cancer. In this study, the patients received intravenous infusions of very low doses of the thymidine analogue, bromodeoxyuridine (BrdU), in order to determine tumor staging and assess the proliferative activity of the tumor cells, however without receiving any other treatment affecting cell generation. Afterwards, BrdU-positive cells were counted post-mortem along the hippocampus, between 16 and 781 days post-BrdU injection (*Eriksson et al., 1998*). The concept behind this technique is that BrdU is integrated into the DNA of proliferating cells and can be assessed using immunohistochemistry. Finding a BrdU-positive neuron implies that that neuron was generated from a precursor cell that divided at the same time at which BrdU was administered, since BrdU has a short biological half-life (*Bauer and Patterson et al., 2005; Kempermaan et al., 2018*).

From this analysis, BrdU-positive cells were found in the SGZ, the granule cell layer, and hilus of the DG (*Eriksson et al., 1998*). The conclusion was that adult neurogenesis is present in the human hippocampus in the same region and levels as observed in studies in rats (*Eriksson et al., 1998*).

However, this study presents some limitations since BrdU labelling was considered as a positive proof of the presence of new born cells, without considering the limitations of the technique used in the study. Firstly, the number of sample and brain sections per sample analysed were very few. Secondly, all the patients in the study were terminally ill and no healthy patient was included in the study as control. Moreover, the study did not take into account the intrinsic limitations of BrdU labelling. Although it stains dividing cells, it is not a specific marker of cell division, but rather a proxy of DNA synthesis, independently form the mitosis, such as DNA repair or apoptotic processes (*Duque et al., 2021*). Postmitotic dying neurons can re-enter the cell cycle, getting labelled with BrdU. Therefore, BrdU positive cells were counted, but these cells might have not been new born neurons (*Sorrells et al., 2021*). Several of them co-stained also with neuron-specific markers, but it is still not

certain if these are new born neurons, or due to cell division-independent BrdU integration or because of technical problems of BrdU detection (*Breunig et al., 2007; Sorrells et al., 2021*).

Moreover, BrdU staining needs deep tissue processing that can give positive signals in human tissue, even in the absence of BrdU (*Sorrells et al., 2018; Sorrells et al., 2021*). Finally, this experimental approach cannot be repeated to validate the results for evident ethical limitations in the administration of a toxic compound in humans.

3.4.2. 14C birth-dating

The second piece of evidence in support of AHN by Spalding et al. (2013) used carbon 14 (14C) birth-dating as a method of investigation of neurogenesis in adult humans. This approach exploited the nuclear-bomb-tests-derived rise in atmospheric 14C and its integration into DNA, allowing cellular age measurements. This method was applied to neuronal DNA collected from 55 individuals. This technique consisted in the isolation of NeuN+ neuronal nuclei, which afterward were analysed in bulk with mass spectrometry and the average birth dates were determined (*Spalding et al., 2013*).

This study reported that several new born neurons are generated daily in the adult human hippocampus, with a small decrease along aging (*Spalding et al., 2013*). Anyway, the results achieved from this technique are different from histological studies, which showed a drastic decrease in markers expression of newly born neurons during early postnatal development (*Knoth et al., 2010; Dennis et al., 2016; Sorrells et al., 2018; Sorrells et al., 2021*).

However, many technical limitations must be taken into account when considering these results. NeuN can also be expressed by subpopulations of oligodendrocytes and microglia (*Parakalan et al., 2012; Zhang et al., 2014*). Furthermore, positive results do not certainly indicate neurogenesis, since integration of 14C into the DNA, as well as for BrdU, does not happen exclusively during cell proliferation but also during DNA repair, DNA methylation or in postmitotic neurons, which re-enter the cell cycle, processes that have been shown to occur often in the hippocampus (*Munzel et al., 2010; Guo et al. 2011; Duque et al, 2021*).

Another problem is that 14C birth-dating technique is complex and requires particular equipment and for these reason and the fact that such studies are difficult to be reproduced, the 14C birth-dating method has not been repeated yet (*Lucassen et al., 2020; Sorrells et al., 2018*).

3.4.3. *In vitro* cell-based models

A third indirect evidence of AHN in human is represented by studies *in vitro* in which NSCs and neuroblasts isolated from human post-mortem hippocampi were differentiated into neurons, confirming their neurogenic potential. (*Azari et al., 2016; Hermann et al., 2006*).

Moreover, another study assessed the neurogenic potential of human NSCs coming from DG samples surgically resected from epileptic patients (*Coras et al., 2010*). It was shown how NSCs isolated from adult human hippocampus were proliferating. However, they presented variable proliferative capacity *in vitro*. Surprisingly, highly proliferative stem cell came from patients who had preserved memory performances prior to epilepsy surgery, while stem cell derived from patients with impaired memory performances showed decreased proliferative potential

3.4.4. Cell type-specific markers

Another widely explored approach to investigate adult neurogenesis is represented by immunohistochemistry of specific cell markers of progenitor cells or immature neurons.

This technique relies on the principle that when a new born neuron differentiates from a progenitor cell, it undergoes distinct maturational changes, which can be represented by protein markers, with the assumption that some of these maturing cells will eventually differentiate into mature neurons (*Frisen et al., 2019*). It is clear that this histological approach on human is performed on post-mortem or surgical resected brain tissue, with its own limitations, in particular represented by post-mortem delay and fixation (*Kempermann et al., 2018; Duque et al., 2021*).

However, while Eriksson et al. (1998) and Spalding et al. (2013) focused on the final result of neurogenesis in order to infer the existence of newly formed neurons, using a birth-dating technique, in which the DNA of dividing precursor cells was labelled, either by BrdU or 14C, the histological marker studies mainly by Sorrells et al. (2018) and Boldrini et al. (2018) based their findings regarding neurogenesis on earlier points of adult neurogenesis, the progenitor cells, their proliferation potential and early immature neurons (*Kempermann et al., 2018*).

Although Sorrells et al. (2018) and Boldrini et al. (2018) used the same approach, the first one reached the conclusion of the absence of evidence of AHN, based on the lack of detection of the immature neuronal markers DCX and PSA-NCAM in the adult human hippocampus. In contrast, Boldrini et al. (2018), using very similar immunohistochemical methods, obtained positive results as evidence of the existence of AHN throughout the human life span (*Kempermann et al., 2018*).

These opposing findings triggered a considerable debate in the scientific community. Some studies report a drastic decline after birth and a little or no significant presence during adulthood (*Knoth et al., 2010; Dennis et al., 2016; Sorrells et al., 2018; Paredes et al., 2018*). Others, on the other side, support sustained neurogenesis throughout life (*Boldrini et al.,2018; Moreno-Jimenez et al., 2019; Tobin et al., 2019*).

In particular, Sorrells et al. (2018) found little evidence of progenitors or immature neurons in the human hippocampal DG, arguing that neurogenesis drastically decreases to irrelevant levels during childhood, and for this reason the human hippocampus differs from that in other species, in which adult neurogenesis is maintained during the adulthood (*Cipriani et al., 2018; Paredes et al., 2018; Sorrells et al.,2018; Kempermann et al., 2018*). Hence, this finding proposes that newly born neurons in the adult human hippocampus are therefore missing or very rare (*Sorrells et al., 2018; Paredes et al., 2018*).

According to this school of thought with respect to scepticism about AHN, while pre-natal neurogenesis is robust and elevated, post-natal neurogenesis decreases drastically after birth with the consequent lower integration of new neurons throughout life (*Sorrells et al., 2018; Snyder, 2019; Gillotin et al., 2021*).

On the other side, Boldrini et al. (2018) sustains the opposite conclusion and supported that neurogenesis is present in human during adulthood, proposing that new neurons are generated throughout life, in line with the evidence found by Moreno-Jimenez et al. (2019) and Tobin et al. (2019).

According to this pro AHN view, several studies have proposed the existence of immature neurons in the adult human hippocampus: although the number of these cells decreases during the aging process, it persists at least until the ninth decade of life, supporting previous evidence (*Spalding et al., 2013; Boldrini et al., 2018; Frisen et al., 2019*).

Recently, Kempermann et al. (2018) presented an overview where they highlighted the significance of the influence of human brain tissue processing and the characteristics of the samples on the results, such as post mortem delay (PDM), agonal stage, tissue fixation, methodological controls, stereology and the limitations of the techniques and markers used so far. This represented an attempt to give a possible explanation for the discrepancies between the before mentioned histological studies.

Therefore, the main challenge to investigate neurogenesis in the human brain is the lack of performing tools to directly and specifically birth-date, track or mark neural progenitor cells. The markers used to study the generation and integration of new neurons into the adult human brain are not specific and/or reliable by themselves since they present evident limitations (***Figure 1***).

3.5. AHN marker limitations: necessity for new and more specific

So far, to address the controversial views on adult neurogenesis in the human hippocampus, several markers have been used. Even if the same markers were used, different results and conclusions have been reached, pointing out their relative challenges and limitations.

DCX

The AHN studies have abundantly made use of doublecortin (DCX) as a marker for new born immature neurons to infer the presence of neurogenesis during adulthood (*Brown et al., 2003*).

DCX is a cytoskeletal binding protein. In particular it is a microtubule-associated protein, involved in cytoskeletal organization via stabilization of actin and microtubules filaments (*Moores et al., 2006*) and it is fundamental for neuronal migration (*Gleeson et al., 1999; Bai et al., 2003*). Therefore, it has an important role during neurodevelopment.

However, there are some limitations in the use of DCX as a specific marker for adult neurogenesis (*Balthazartand, 2014; Vellema et al., 2014; R. X. Liu et al., 2020; Sorrells et al., 2021*).

It has been shown that DCX can be constantly expressed throughout life in some cells. DCX+ cells could be a subgroup of immature cells, which are not newly generated but instead they underwent a prolonged maturation in adulthood (*La Rosa et al., 2020; Duque et al., 2021*). This suggests that neurons might have a very slow or delayed maturation rate, probably associated with neural plasticity (*La Rosa et al., 2020; Sorrells et al., 2021*).

In addition, in rodent, non-human primate and human non-newly born DCX+ cells can be also found in other brain regions, besides the common established neurogenic niches. For instance, in the adult rodent piriform cortex or the human amygdala (*Zhang et al., 2009; Gómez-Climent etal., 2011; Klempin et al., 2011; Martí-Mengual et al., 2013;Piumatti et al., 2018; Rotheneichner et al., 2018; Sorrells et al.,2019; Sorrells et al., 2021*).

Moreover, several pieces of evidence show how in the adult mammalian brain of several different species, including humans, mature neurons can also re-express DCX, although they are not newly born (*Zhang et al., 2009; Gómez-Climent et al., 2011; Klempin et al., 2011; Martí-Mengual et al.,*

2013; Piumatti et al., 2018; Rotheneichner et al.,2018; Sorrells et al., 2019; Sorrells et al., 2021). This evidence supports the hypothesis that mature neurons in the GCL might undergo "de-maturation", therefore starting to re-express immature markers, such as DCX (*Ohira et al., 2019; Sorrells et al., 2021*). Even if DCX+ cells might be newly integrated neurons into adult neural circuits, it is not given that they are surely newly born neurons (*Rotheneichner et al., 2018; Benedetti et al., 2020; Sorrells et al., 2021*).

Furthermore, DCX has been shown to not be expressed exclusively in immature adult neurons (*Duque et al., 2021*). Some DCX+ cells do not co-stain with neuronal markers (Sorrells et al., 2021). DCX expression has been observed in microglia (*Liu et al.,2018; Sorrells et al., 2018; Unger et al., 2018*), astrocytes (*Verwer et al., 2007*) and in oligodendrocyte precursors (Boulanger and Messier, 2017). Therefore, DCX+ cells negative for neuronal markers could be glial cells, which are smaller and morphologically ramified, which can be improperly interpreted as immature neurons (*Sorrells et al., 2021*).

Recently, the evidence of non-neuronal expression of DCX has been also consolidated via single-cell RNA sequencing, where DCX expression was seen to be present in several cell types of the analysed adult human prefrontal cortex and hippocampus samples (*Franjic et al., 2021; Sorrells et al., 2021*).

Therefore, although DCX is often widely used as a marker for "immature" neurons, there is no clear link among cell proliferation, DCX+ cells number and neurogenesis rate. Hence, DCX expression by itself is quite certainly not enough to fully infer adult neurogenesis.

PSA-NCAM

The neural cell adhesion molecule (PSA-NCAM) is another cell marker that has been commonly used to identify immature newly born neurons.

However, the expression of PSAN-CAM is also linked to neuronal plasticity and remodelling processes, therefore its expression is not exclusively linked to neurogenesis (*Theodosis et al., 1999; Sorrells et al., 2021*). Its expression has been detected in the hippocampus but also in many other regions of the adult human brain (*Sorrells et al., 2018; Mathern et al., 2002; Varea et al., 2011; Sorrells et al., 2021*). PSA-NCAM+ cells in the human adult DG, also co-labelled with DCX and NeuN, were broadly distributed in the hippocampus, and showed large, round nuclei and complex morphology, suggesting a mature neuron identity (*Paredes et al., 2018*). In particular, PSA-NCAM has been shown to be highly expressed in several mature inhibitory interneurons (*Duque et al., 2021*).

Therefore, PSA-NCAM expression, as well as DCX, is not undoubtedly correspondent to newly born neurons in the adult. In humans, immature newly born neurons may require several months in order to mature and in the meanwhile they might still express for a long time such immature markers (*Sorrells et al., 2021*). Hence, a prolonged maturation period of several months implies an higher number of detectable DCX+ PSA-NCAM+ cells, even if they are not newly born neurons anymore (*Sorrells et al., 2021*).

CR and CB

A subgroup of the DCX+ cells co-label with another immature neuron markers, calretinin (CR), and not with the mature neuron marker, calbindin (CB) (*Sorrells et al., 2021*). in the adult mouse hippocampus, it has been shown that these markers are switched on one after the other during newly born neuron maturation (*Brandt et al., 2003*).

However, the subsequential expression of CR and CB markers has not yet been proved to be identical in the human brain. Indeed, it is not clear whether these different labelled groups of cells are part of the same origin in the adult human brain (*Sorrells et al., 2021*). Moreover, several CR+ and CB+ cells are detected outside of the GCL, for instance, in the inner molecular layer (*Sorrells et al., 2021*).

In addition, CR and CB are found to be expressed also in adult mature interneurons in the hippocampus, besides in subpopulations of GABAergic interneurons in the cerebral cortex, both in mice and humans (*Gulyás et al.,1996; Tóth et al., 2010; Sorrells et al., 2021; Duque et al., 2021*).

KI67

Neural progenitor cells are proliferative cells therefore, markers of proliferation, such as Ki-67 and MCM2 are useful for this purpose.

However, co-labelling with other specific cell type markers are necessary to assess what cell type is dividing, hence these proliferation markers are limited (*Sorrells et al., 2021*). Ki-67 expression can be also detected in precursors of oligodendrocytes, microglia, and parenchymal astrocytes, since they keep to divide during adulthood, as well as in postmitotic neurons, during DNA repair (*Paredes et al., 2018*).

In the study by Sorrells et al. (2018), Ki-67+ cells are distributed across the DG in the adult human hippocampus, without an increased cellularity in the neurogenic human SGZ.

PCNA

PCNA expression has been used as another proliferation marker for NPCs (*Curtis et al., 2007*).

However, in humans it can be also expressed in non-dividing cells, such as adult postmitotic multi-ciliated ependymal cells (*Sanai et al., 2007*) and it is also expressed upon DNA repair and programmed cell death (*Bologna-Molina et al., 2013; Sorrells et al., 2021*).

SOX2 and Nestin

SOX2 or Nestin expression has been commonly used to label neural progenitor cells in rodents.

However, in humans, these two proteins are often expressed in dividing glial cells, in some mature astrocytes and in subpopulation of mature neurons and intermediate progenitors, respectively (*Komitova and Eriksson, 2004; Paredes et al., 2018; Duque et al., 2021*) .

Table 1. Markers frequently used to study adult neurogenesis and their limitations

Marker	Common use	Limitations to marker interpretation	References
Ki-67	Cell proliferation	Includes dividing cells of non-neuronal lineage; also expressed in postmitotic neurons for DNA repair	Namba et al., 2005; Schwartz et al., 2007; Munakata et al., 2013; Boldrini et al., 2018
MCM2	Cell proliferation	Can be expressed on cell cycle reentry occasionally prior to cell death; also expressed in postmitotic neurons for DNA repair	Schwartz et al., 2007; Bonda et al., 2009
PCNA	Cell proliferation	May also be expressed during DNA repair and programmed cell death or in nondividing cells	Lin et al., 2001; Sanai et al., 2007; Bologna-Molina et al., 2013
Nestin	Neural stem/progenitor cells	Also expressed in mature glia and a subpopulation of mature neurons and intermediate progenitors	Doetsch et al., 1997; Farzanehfar et al., 2017; Krishnasamy et al., 2017
Vimentin	Neural stem/progenitor cells	Can be expressed in mature astrocytes	Schnitzer et al., 1981; Souza et al., 2017
SOX2	Neural progenitors	Also expressed in dividing glia and mature astrocytes	Komitova and Eriksson, 2004; Cruz et al., 2014
BLBP	Neural progenitors	Also expressed in astrocytes and many adult brain regions	Gerstner et al., 2008
TUJ1	Immature neurons	Expression may be retained in mature neurons, expressed in mature basket cells in SGZ	Ambrogini et al., 2004; Seri et al., 2004
DCX	Immature neurons	Expressed at low levels in oligodendrocytes and microglia, can be reexpressed in mature neurons, also can indicate prolonged maturation instead new neuron generation	Rotheneichner et al., 2018; Sorrells et al., 2018, 2019; Ohira et al., 2019
PSA-NCAM	Immature neurons	Also expressed in a subpopulation of mature neurons, particularly inhibitory	Varea et al., 2007
CR	Immature neurons	Also a marker of a subtype of mature inhibitory interneurons, possible reexpression in mature neurons	Tóth et al., 2010; Rocco et al., 2016; Ohira et al., 2019
CB	Mature neurons	Also a marker of a subtype of mature inhibitory interneurons	Tóth et al., 2010; Rocco et al., 2016
NeuN	Mature neurons	Does not recognize all neuronal cell types, can be expressed in subpopulations of oligodendrocytes and microglia	Cannon and Greenamyre, 2009; Parakalan et al., 2012; Zhang et al., 2014

Figure 1 – Neurogenic cell-type markers established in rodents studies and their limitation to study adult human neurogenesis - Sorrells et al. (2021)

Hence, because of these technical and methodological limitations, it is clear how the results of studies on adult neurogenesis on human brain tissue can be controversial, contradictory and with high variability, even when the samples and conditions examined are relatable with each other.

It is therefore absolutely urgent the necessity of finding new and more reliable and specific markers to state once and for all the presence or the absence of adult hippocampal neurogenesis in the human brain. The present study aims to propose new and more reliable and unbiased tools to investigate the existence of AHN in human brain.

3.6. single cell RNA sequencing: a powerful tool for cell type identification

Single cell RNA sequencing (scRNA seq), a more recent and promising approach relying on transcriptome analysis at single cell level, became of great interest in the last years as tool to infer the presence of neurogenesis in the human dentate gyrus.

This tool, by providing the transcriptional profile at single cell level allows for an unbiased characterization of the cell types and cell states present in the region of interest. scRNA sequencing in embryonic and adult mouse and human brain demonstrated to be very powerful to identify and characterize the players involved in the neurogenesis process, such as neuronal progenitors and immature neurons (*Hochgerner et al., 2018; Duque et al., 2021; Donega et al., 2022*).

Therefore, this technique has the potential to uncover the entire neurogenic process in an unbiased way, rather than relying on a few debatable cellular markers. Hence, transcriptome analysis could be the key tool to finally clarify the adult neurogenesis dispute in human brain (*Kempermann et al., 2018; Kuhn et al., 2018; Lee et al., 2018; Paredes et al., 2018, Duque et al., 2021*).

That is the reason why single-cell transcriptomic analysis may be fundamental in order to discover additional and robust markers that characterize the different cell types, which consequentially rise from the differentiation of the NSCs in the human DG (*Paredes et al., 2018*).

However, this technique presents some limitations as well. For instance, technical variability and sampling bias can have an influence on scRNA seq results (*Kelley et al., 2018*). Furthermore, transcriptional level is not always coupled with translational level (*Greenbaum et al., 2003; Maier et al., 2009*). Indeed, since neurogenesis is expected to be a rare but still present and ongoing phenomenon in adult human brain, it is therefore necessary to sequence a high number of cells with a lot of sequencing depth. This is fundamental in order to increase the chances to detect neurogenic cellular populations with their characteristic transcriptional profile that set them apart. Moreover, the availability of high quality adult human brain samples is another limitation for this technique. It is therefore important the establishment of biobanks where well preserved post-mortem brain tissue are stored and readily usable.

Given the difficulties to investigate AHN in human brain, a promising approach could be represented by focusing on relevant and similar processes to adult hippocampal neurogenesis that occurs in the human brain, in order to identify neurogenic cellular population and their cell-type specific markers.

In the human brain, the closest known neurogenic event to adult neurogenesis is represented by the fetal neurogenesis, which occurs during embryonic development. So far, it is a process well established and characterized in literature for its fundamental role in the brain development.

As shown in ***Figure 2***, the immunostaining for immature neuron markers DCX and PSA-NCAM and proliferative marker KI67 show an enrichment of positive cells in the human SGZ, in correspondence of perinatal age and a consequent decrease along aging until adulthood. These results could be explained, either by the decrease of neurogenic populations and therefore neurogenesis in the human brain along aging, or by the change of neurogenic cell type specific markers expression from fetal to adult neurogenesis.

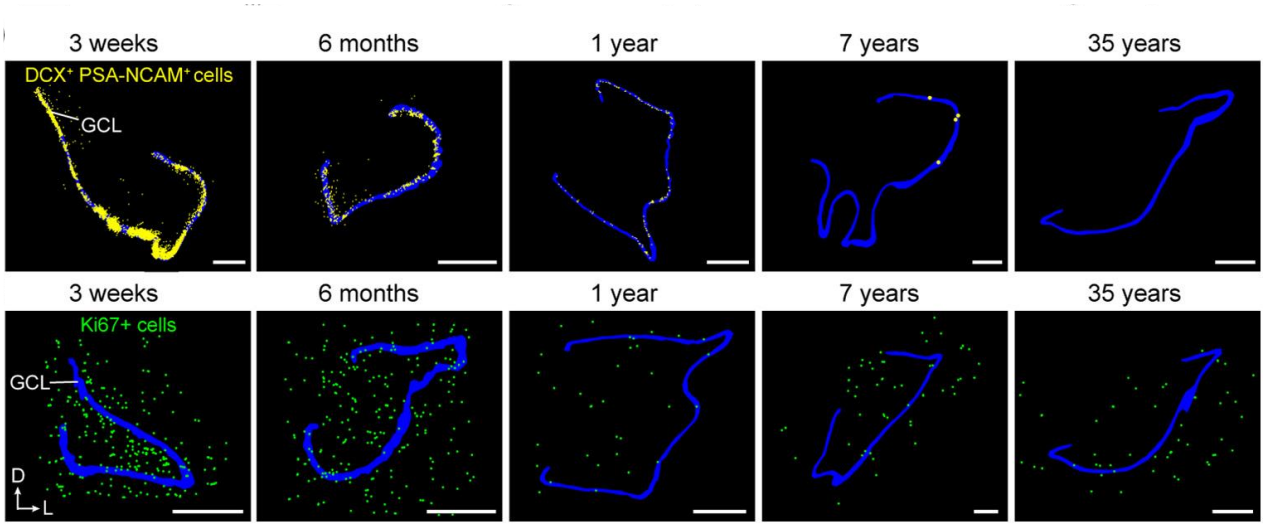


Figure 2 – Immature neurons markers (DCX, PSA-NCAM) and proliferative marker (KI67) expression in the human DG throughout life form 3 weeks old to 35 years old individual - Sorrells et al. (2021)

For this reason, the hypothesis formulated to address the research question of the present study was that neurogenic cell type-specific markers might be conserved between fetal and adult neurogenesis. In support of this hypothesis, it has been shown in literature that neurogenesis in mouse fetal and adult brain presents same regulators, some of them with conserved function, such as TBR2, NEUROG2 and PROX1, besides conserved pathways involved in neurogenesis, such as WNT and NOTCH pathways (*Noelia Urbán and François Guillemot, 2014*).

Therefore, this approach presumably leads to higher chances in the identification of relevant and specific markers of cell types involved in the adult hippocampal neurogenesis by means of the single cell RNA sequencing analysis on human fetal brain.

4. Materials and methods

4.1. Meta-analysis

A systematic literature search was performed to identify relevant transcriptomic studies related to human fetal neurogenesis.

The systematic literature search was conducted in PubMed (https://pubmed.ncbi.nlm.nih.gov), using as key words 'single cell/ nucleus RNA sequencing', 'fetal', 'brain', and 'human', with no limitation on the year of publication, leading to a total of 19 hits (***Figure 3***).

A first step of identification and screening was carried out, manually checking the relevance and pertinence of the papers (***Figure 3***). For inclusion, the studies had to fulfil the following criteria: single-cell transcriptomic dataset availability, presence of transcriptomic profile of the cell types of interest, and pertinence of the analysis conducted (i.e. exclusion of studies reporting differentially expressed genes in control *vs* disease condition). After this initial screening, 10 papers were selected for data extraction.

The extracted gene sets were organized into cell-type-specific groups, and they underwent an initial step of processing. Further 2 studies were removed because ambiguous cell-type imputation (***Figure 3***).

For each study, gene expression data of the following cell types were collected: neural stem cells (NSCs), neural progenitor cells (NPCs), and immature neurons (ImNs), resulting in a total of 9 , 12 , and 7 datasets for NSCs, NPCs, and ImNs, respectively (***Table 1***).

Paper	NSCs	NPCs	Neuroblasts	ImNs	Dataset number per article
Darmanis_2015	NA	- Replicating neuronal progenitors	NA	- Quiescent newly born neurons	2
Liu_2016	- Radial glia	- Dividing radial glia - Intermediate progenitors	NA	- Maturing excitatory neurons	4
Nowakowski_2017	- vRG - RG-div2 - tRG - RG-div1	- IPC-div2 - IPC-div1	NA	- nEN-early1 - nEN-early2 - nEN-late	10

	- oRG				
Zhong_2018	NA	- NPCs	NA	NA	1
Polioudakis_2019	- vRG - oRG	- IP - PgS - PgG2M	NA	- ExM - ExM-U	7
Couturies_2020	NA	- ENP	NA	NA	1
Eze_2021	- RG	- IPC	NA	NA	2
Yu_2021	NA	- NPCs	NA	NA	1
Dataset number per cell type	9	12	0	7	

Table 1 – Datasets sorting in cell-type-specific groups (Neural Stem Cell – NSCs, Neural Progenitor Cell - NPCs, Immature Neuron - ImNs)

Furthermore, a first data selection was carried out depending on the number of genes provided per cell type. More specifically, only the top 500 expressed genes, according to fold change ranking higher than 0, were selected from gene lists with more than 500 genes, whereas the entire gene list was selected from gene sets providing fewer than 500 genes.

Studies containing multiple datasets under the same cell-type category underwent a further processing step: reduced lists of genes were obtained by comparing the datasets within a category and selecting the list of overlapping genes, for each study, for each cell-type. This resulted in max 1 list of genes per category (NSCs, NPCs, ImNs) per study.

4.2. Candidate gene selection

To identify candidate markers for each cell type of interest, lists of candidate genes were obtained by comparing across different studies the list of genes within a category and selecting the list of overlapping genes. For each category, the genes present in at least 75% of the studies were extracted, resulting in 31 candidate markers for NSCs, 19 for NPCs and 18 for ImNs (***Table 2***).

NSCs	***NPCs***	***ImNs***
HES1	MKI67	SATB2
VIM	CENPF	NEUROD6
BCAN	SMC4	
DDAH1	EOMES	
MOXD1	TMPO	
CLU		

ATP1A2
TAGLN2
ANXA2
GFAP
ZFP36L1
SOX9
PAX6
ID4
DBI
FAM107A
FOS
GATM
CDO1
LIPG
SLC1A3
GLI3
GPX3
CREB5
TMEM47
TMEM132B
DOK5
SFRP1
CYR61
IQGAP2
LRRC3B

Table 2 – Candidate gene markers present in at least 75% of papers for each cell-type-specific group ordered by decreasing average fold change (NSCs, NPCs, ImNs)

The expression of each candidate gene was then evaluated by using the following visualization tools: LIBD Stem Cell Browser (http://stemcell.libd.org), which allows the visualization of human iPSCs transcriptomics during corticogenesis, dentate gyrus from Linnarsson Lab

(http://linnarssonlab.org/dentate/), which allows visualization of single-cell RNA-seq data from mouse DG (Hochgerner et al., 2018), and SCope (http://scope.aertslab.org/), which was used to visualize our single-cell RNA-seq unpublished data from adult human DG.

The expression of candidate genes in the LIBD Stem Cell Browser was expected to show a decreasing expression for NSCs candidate markers, a first increasing and then decreasing trend of expression for NPCs markers and increasing expression for ImNs, along the iPSCs differentiation into mature neurons. This evaluation was then compared with the candidate markers expression in single cell RNA sequencing analysis of mouse dentate gyrus (http://linnarssonlab.org/dentate), expecting specific expression of the candidate markers in the corresponding clusters of the UMAP. From this evaluation 6 plausible candidate markers for NSCS, 4 for NPCs and 1 for ImNs were selected (***Table 3***).

NSCs	***NPCs***	***ImNs***
HES1	MKI67	NEUROD6
VIM	CENPF	
MOXD1	SMC4	
PAX6	EOMES	
ID4		
DBI		

Table 3 – Final candidate gene markers selection per cell-type-specific group (NSCs, NPCs, ImNs) ordered by decreasing average fold change

4.3. Human stem cell lines

ReNcell™ VM Human Neural Progenitor Cells (Millipore; RRID: CVCL_E921) were cultured in ReNcell NSCs Maintenance Medium (Millipore) supplemented with 20 ng/mL epidermal growth factor (EGF) (PeproTech) and 20 ng/mL fibroblast growth factor 2 (FGF-2) (PeproTech) in laminin-coated flasks (10-20 mg/ml; Sigma-Aldrich; Greiner Bio-one) at 37 °C under 95% O2 and 5% CO2. When culturing ReNcells for staining, cells were plated onto poly-L-ornithine (PLO; 0,1 mg/mL; Sigma-Aldrich)/laminin-coated coverslips in a 12 well plate. Undifferentiated ReNcells were split using 1x Accutase (Sigma-Aldrich) every 3-4 days (>80% confluent). Removal of FGF-2 and EGF from the culture medium was deployed to induce differentiation. ReNcells at four different time points of differentiation (day 0, day 7, day 14, day 30) were obtained for quantitative PCR and immunofluorescence experiments.

Human iPSCs (iPSC EPITHELIAL-1, Sigma-Aldrich) were cultured in mTeSR™1 (Stemcell) in Matrigel-coated plates (Corning® Matrigel® hESC-Qualified). The differentiation into NPCs was induced by culturing the cells in Neural Maintenance Medium (Fisher), supplemented with 10 uM SB431542 (Sigma-Aldrich) and 1 uM LDN (2MG StemMACS LDN-193189, MILTENYI) at 37 °C under 95% O2 and 5% CO2. The cells were split using 0.5 mM EDTA (Sigma-Aldrich) at 70% confluency. The differentiation of NPCs into neurons was induced by culturing the cells in Neural Maintenance Medium (Fisher), supplemented with 20 ng/mL BDNF (Stemcell), 20 ng/mL GDNF (Stemcell), 200 uM cAMP (Stemcell) and 200 uM ascorbic acid (Sigma-Aldrich) in poly-L-ornithine (PLO; 0,01%; Sigma-Aldrich)/laminin-coated wells 1 mg/ml; Sigma-Aldrich) at 37 °C under 95% O2 and 5% CO2. iPSCs, iPSCs-derived NPCs (day 30) and iPSCs-derived neurons (day 70) were fixed at the respective differentiation time points for immunofluorescence experiments.

4.4. RNA extraction, reverse transcription and quantitative PCR of RenCells

The candidate markers for NSCs, NPCs and ImNs were validated via qPCR in RenCells at four different time points of differentiation (D0, D7, D14, D30). The RenCells were obtained as previously described.

RNA extraction from cultured RenCells was performed using the miRVana Paris Kit (AM1556, Invitrogen) according to the manufacturer's instructions. Briefly, the medium was aspirated and the cells were subsequently rinsed with 1x PBS and collected in TRIzol (Invitrogen). The cells were homogenized in the TRIzol solution and incubated in chloroform for 5 min at RT. After centrifugation of 1 min at 12.000 g, 1.25 volumes of 100% ethanol were added to the aqueous phase. The samples were loaded and processed on miRVana spin columns (miRVana Paris Kit) and processed according to the manufacturer's instructions. Finally, the RNA was eluted in Elution Solution and its concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

Reverse transcription of 200 ng mRNA was performed using Superscript II Reverse Transcriptase (Invitro-gen) with 500 µg/mL Oligo(dT) 12-18 (Invitrogen) as suggested by the manufacturer in a T100™ Thermal Cycler (BioRad).

Next, semi-quantitative real-time PCR (qPCR) was carried out using a total of 2.5 ng cDNA as template. Each reaction was performed using 1X SYBR™ Green PCR Master Mix (Applied Biosystems) and 25 pmol Fw and Rev primer (See ***Table 4*** for sequences) in MicroAmp™ Optical 96-Well or 384-Well Reaction Plates (Applied Biosystems) in the 7300 Real-Time PCR system (96-well plates; Applied

Biosystems) or the Quantstudio 5 Real-time PCR system (384-well plates; Applied Biosystems) The lightcycler program used can be found in ***Table 5***. Average CT (Cycle Threshold) values of technical triplicates per gene were normalized to the mean expression of two housekeeping genes (hGAPDH and h18S). Fold changes were subsequently calculated using the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*). Three technical replicates were performed for each biological replicate of each condition.

Marker		***Sequence***
hHES1	Fw	AGTGAAGCACCTCCGGAAC
	Rv	TCACCTCGTTCATGCACTC
hVIM	Fw	TCCAAACTTTTCCTCCCTGA
	Rv	AGGTCATCGTGATGCTGAGA
hMOXD1	Fw	GGGATGCCTGAGTTCCAGTC
	Rv	CCACTTGGCTTTTCGGCTTC
hPAX6	Fw	AACGATAACATACCAAGCGTGT
	Rv	GGTCTGCCCGTTCAACATC
hDBI	Fw	ATGTTGGACTTCACGGGCAA
	Rv	GCTTTCATGGCATCTTCCTTGG
hID4	Fw	TCCCGCCCAACAAGAAAGTC
	Rv	CCAGGATGTAGTCGATAACGTG
hMKI67	Fw	GAGGTGTGCAGAAAATCCAAA
	Rv	CTGTCCCTATGACTTCTGGTTGT
hCENPF	Fw	GCAGAGGTTAAAGCCTTGCAG
	Rv	ATGTCGCGGTGATTCATGGT
hSMC4	Fw	ACTGCAAGCATCCAGCGTTT
	Rv	TTACCAGCTTTCTCAGCCACA
hEOMES	Fw	TCAAATTCCACCGCCACCAA
	Rv	GCAGTGGGATTGAGTCCGTT
hNEUROD6	Fw	AGCCCTCAGTTTGAAGGTCC

hGAPDH	Rv	CAACCTGAACATGGCACCCT
	Fw	TCAAGAAGGTGGTGAAGCAGG
	Rv	ACCAGGAAATGAGCTTGACAAA
h18S	Fw	TTCGAGGCCCTGTAATTGGA
	Rv	GCAGCAACTTAATATACGCTAT

Table 4 – Human primers for qPCR to determine the respective neurogenic cell type-specific candidate markers and housekeeper genes (hGAPDH, h18S) expression along RenCells differentiation.

	Cycles	***Target (°C)***	***Acquisition mode***	***Hold (hh:mm:ss)***	***Ramp rat (°C/s)***
Act	1	95	none	00:10:00	3
Ampl	40	95	none	00:00:10	3
		60	single	00:00:30	2,5
Melt	1	95	none	00:00:10	3
		60	none	00:01:00	2,5
		95	Continuous	-	0,11

Table 5 – Lightcycler qPCR program: Activation, Amplification and Melting steps.

4.5. Immunofluorescence in human cell lines

The human cell lines fixed on coverslips (RenCells D0, D14, D30 differentiation time points and iPSCs, iPSCs-derived NPCs and iPSCs-derived neurons) were initially permeabilized in 1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 15 min at RT and then blocked in 1% (v/v) Triton X-100 (Sigma-Aldrich), 10% (v/v) normal goat serum (Jackson ImmunoResearch) in PBS for 2 h at RT. Primary antibody mix (***Table 6***) incubation was performed in 0.3% (v/v) Triton X-100, 3% (v/v) normal goat serum in PBS overnight at 4°C in a humified chamber. This was followed by washes with 1% (v/v) Triton X-100 and afterward incubated in secondary antibody mix (***Table 7***) in 0.3% (v/v) Triton X-100, 3% (v/v) normal goat serum in PBS for 2 h at RT. Finally, samples were incubated in DAPI (Sigma-Aldrich) and mounted with Mowiol (Merck Millipore). Sections were dried overnight at RT and imaged within one week at a DMI6000 CS Confocal microscope (Leica).

Primary Antibodies	***Dilution***	***Catalogue number***	***Host species***
Anti-Eomes	1:250	(ab23345, Abcam)	Rabbit
Anti-GFAP	1:400	(MAB360, Sigma)	Mouse

Anti-GFP	1:500	(ab13970, Abcam)	Chicken
Anti-Id4	1:100	(BS-6669R, Thermofisher)	Rabbit
Anti-Tra181	1:200	(MA1-024, Invitrogen)	Mouse

Table 6 – Primary antibodies used in the immunofluorescence. Tra181 is a control marker for NSCs

Secondary Antibodies	***Dilution***	***Catalogue number***	***Host species***
Anti-Chicken (488)	1:500	(A11039, Invitrogen)	Goat
Anti-Mouse (568)	1:500	(A11004, Invitrogen)	Goat
Anti-Mouse (647)	1:500	(115-606-062, Jackson ImmunoResearch)	Goat
Anti-Rabbit (488)	1:500	(111-487-003, DyLight)	Goat
Anti-Rabbit (594)	1:500	(711-585-152, Jackson ImmunoResearch)	Donkey

Table 7 – Secondary antibodies used in the immunofluorescence

4.6. Animal models

Mice were bred according to standard laboratory approaches and housed under standard 12-hour light-dark conditions.

For neural stem cell and neuronal progenitor cell visualization *in vivo* and cell isolation from the adult dentate gyrus, the characterized Nestin:GFP mice (MGI:5523870) were used (Mignone et al., 2004). For Nestin:GFP+-niche cell visualization in the dentate gyrus of AD model, Nestin:GFP mice were crossed with AppNL-G-F mice (NesxNLGF; RRID:IMSR_RBRC06344). All animal experiments were approved by the ethical and animal care committees of the Netherlands institute for Neuroscience, Koninklijke Nederlandse Akademie van Wetenschappen and Centrale Commissie Dierproeven (AVD8010020201090).

The AD mouse models used in the study was AppNL-G-F strain. AppNL-G-F mice is a more recently established App knock-in model that expresses the APP KM670/671NL (Swedish), APP I716F (Iberian), APP E693G (Arctic) mutations. AppNL-G-F animals develop plaques around the age of 3 months and behavioural deficits from 6 months onward (*Saito et al., 2014*).

DGs of Nes:GFP and NesxNLGF mice 3 and 9 months old were dissected and snap-frozen in liquid nitrogen for RNA extraction.

2.5 and 9.5 months old Nes:GFP and NesxNLGF mice were perfused with 1x PBS followed by 4% paraformaldehyde (PFA; Thermo Scientific). The brains were dissected and post-fixed ON in 4% PFA after which they were stored in 1X phosphate-buffered saline (PBS) for vibratome sectioning (Leica).

4.7. RNA extraction, reverse transcription and quantitative PCR of mouse DG

The candidate markers for NSCs, NPCs and ImNs were afterwards validated via qPCR in mouse DG samples of 4 different conditions (3 mo Nes:GFP, 9 mo Nes:GFP, 3 mo NesxNLGF, 9 mo NesxNLGF). The dentate gyrus from mouse brain was micro-dissected as previously described.

RNA extraction from whole dentate gyri was performed using the miRVana Paris Kit (AM1556, Invitrogen) according to the manufacturer's instructions. Briefly, frozen tissue was homogenized in 350 ml disruption buffer (miRVana Paris Kit) supplemented with 1:10 diluted cOmplete™ Protease Inhibitor Cocktail (Roche) and 1:100 diluted Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich). Subsequently, the samples were denatured and incubated at RT with acid phenol:chloroform. Following centrifugation at 12.000 g for 20 min and collection of the aqueous phase, 1.25 volumes 100% ethanol were added. After mixing, the samples were loaded onto the columns supplemented in the mirVana PARIS kit and processed further as indicated by the manufacturer. Finally, the RNA was eluted in Elution Solution and its concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

Reverse transcription of 200 ng mRNA was performed using Superscript II Reverse Transcriptase (Invitro-gen) with 500 µg/mL Oligo(dT) 12-18 (Invitrogen) as suggested by the manufacturer in a T100™ Thermal Cycler (BioRad).

Next, semi-quantitative real-time PCR (qPCR) was carried out using a total of 2.5 ng cDNA as template. Each reaction was performed using 1X SYBR™ Green PCR Master Mix (Applied Biosystems) and 25 pmol Fw and Rev primer (See ***Table 8*** for sequences) in MicroAmp™ Optical 96-Well or 384-Well Reaction Plates (Applied Biosystems) in the 7300 Real-Time PCR system (96-well plates; Applied Biosystems) or the Quantstudio 5 Real-time PCR system (384-well plates; Applied Biosystems) The lightcycler program used can be found in ***Table 5***. Average CT (Cycle Threshold) values of technical triplicates per gene were normalized to the mean expression of two housekeeping genes (mActb and mGapdh). Fold changes were subsequently calculated using the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*). Three technical replicates were performed for each of the four biological replicates of each condition.

Marker		***Sequence***
mVim	Fw	CTGCCTCTGCCAACCTTTTC
	Rv	GTCCATCTCTGGTCTCAACCG
mId4	Fw	CAGTGCGATATGAACGACTGC
	Rv	GACTTTCTTGTTGGGCGGGAT
mEomes	Fw	CGGCAAAGCGGACAATAACA
	Rv	TGGGAGCCAGTGTTAGGAGA
mNeurod6	Fw	TTGCGAAAAGTGGTCCCCTG
	Rv	GGACGAACGTGAGCAGATCC
mGapdh	Fw	TTGATGGCAACAATCTCCAC
	Rv	CGTCCCGTAGACAAAATGGT
mActb	Fw	AGCCATGTACGTAGCCATCC
	Rv	CTCTCAGCTGTGGTGGTGAA

Table 8 – Mouse primers for qPCR to determine the respective neurogenic cell type-specific candidate markers (mVim, mId4, mEomes, mNeurod6) and housekeeper genes (mGapdh, mActb) expression in 3 mo Nes:GFP, 9 mo Nes:GFP, 3 mo NesxNLGF, 9 mo NesxNLGF mouse DG samples.

4.8. Immunofluorescence in mouse brain sections

Coronal brain sections (40 mm thick vibratome-prepared sections: 2.5 mo Nes:GFP, 9.5 mo Nes:GFP, 2.5 mo NesxNLGF, 9.5 mo NesxNLGF) were initially permeabilized in 1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 15 min at RT and then blocked in 1% (v/v) Triton X-100 (Sigma-Aldrich), 10% (v/v) normal goat serum (Jackson ImmunoResearch) in PBS for 2 h at RT. Primary antibody mix (***Table 6***) incubation was performed in 0.3% (v/v) Triton X-100, 3% (v/v) normal goat serum in PBS overnight at 4°C in a humified chamber. This was followed by washes with 1% (v/v) Triton X-100 and afterward incubated in secondary antibody mix (***Table 7***) in 0.3% (v/v) Triton X-100, 3% (v/v) normal goat serum in PBS for 2 h at RT. Finally, samples were incubated in DAPI (Sigma-Aldrich) and mounted with Mowiol (Merck Millipore). Sections were dried overnight at RT and imaged within one week at a DMI6000 CS Confocal microscope (Leica).

5. Results

5.1. Meta-analysis of human neurogenic cell type-specific candidate markers

The hypothesis followed in the meta-analysis was that in developing human fetal brain there is a higher presence of proliferating and differentiating progenitor cells to sustain the brain development and embryonic neurogenesis and that the cell type-specific markers are conserved throughout life until the adulthood, in what is called adult hippocampal neurogenesis. This is the rationale which supports the meta-analysis performed in this study, in order to discover new and specific cell-type-specific cell markers for NSCs, NPCs and ImNs. The resulting flowchart illustrating the meta-analysis performed in the present study is shown in ***Figure 3***.

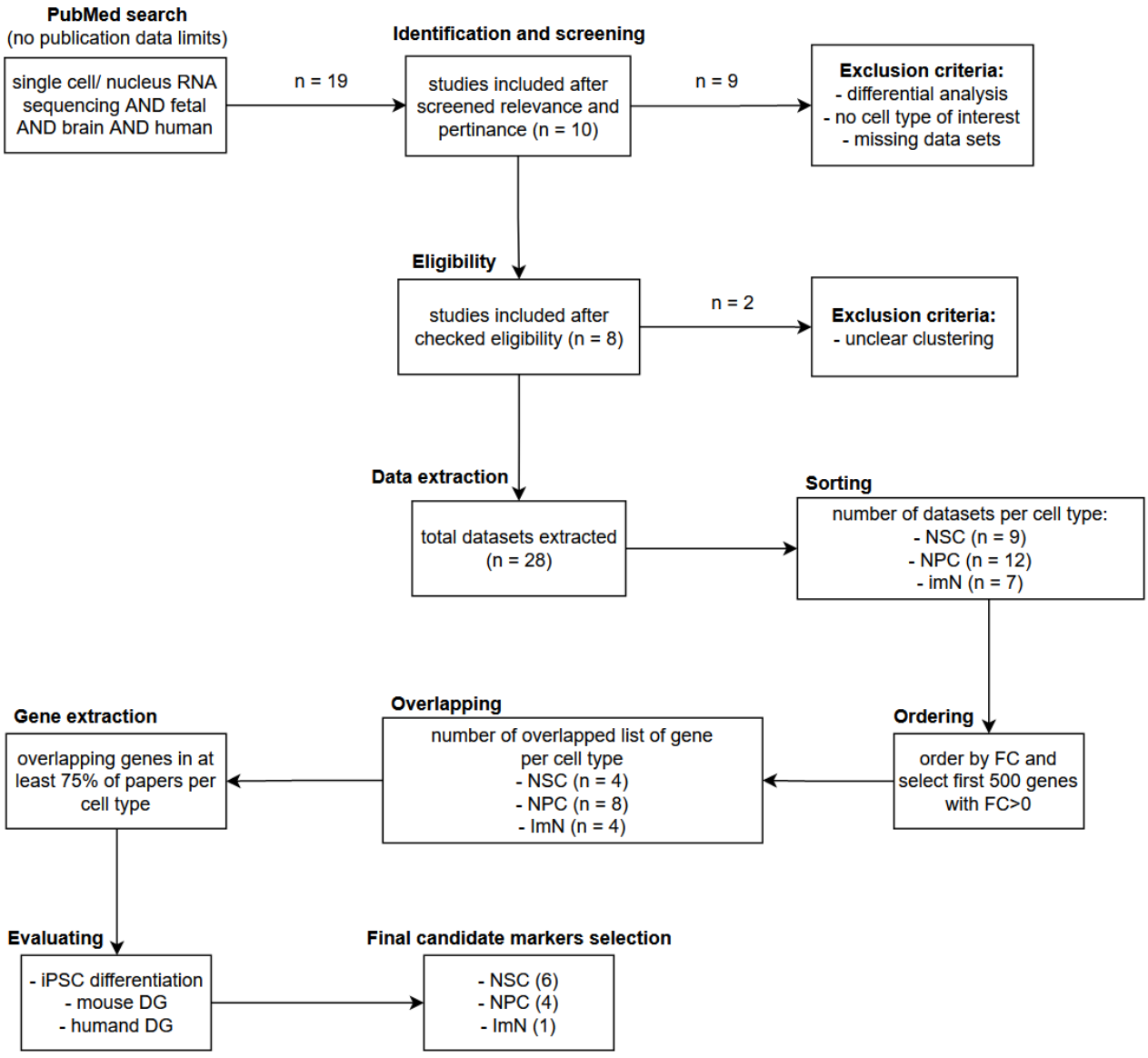


Figure 3 – Flow chart of the meta-analysis and candidate genes selection

From this analysis, 8 relevant articles were took in consideration, from which 28 datasets were extracted and sorted in the 4 cell type clusters as shown in *Errore. L'origine riferimento non è stata trovata.**9***.

Papers	***RGC/ NSCs***	***NPCs/ IPC***	***Neuroblasts***	***Immature N***	***Total dataset #***
Darmanis_2015	NA	1	NA	1	2
Liu_2016	1	2	NA	1	4
Nowakowski_2017	5	2	NA	3	10
Zhong_2018	NA	1	NA	NA	1
Polioudakis_2019	2	3	NA	2	7
Couturies_2020	NA	1	NA	NA	1
Eze_2021	1	1	NA	NA	2
Yu_2021	NA	1	NA	NA	1
Total datasets #	9	12	0	7	

Table 9 - Papers and datasets resulting from the meta-analysis

This analysis resulted in 11 candidate markers, 6 for NSCs cell type, 4 for NPCs and 1 for ImNs cell type, as shown in ***Table*** .

NSCs	***NPCs***	***ImNs***
HES1	MKI67	NEUROD6
VIM	CENPF	
MOXD1	SMC4	
PAX6	EOMES	
ID4		
DBI		

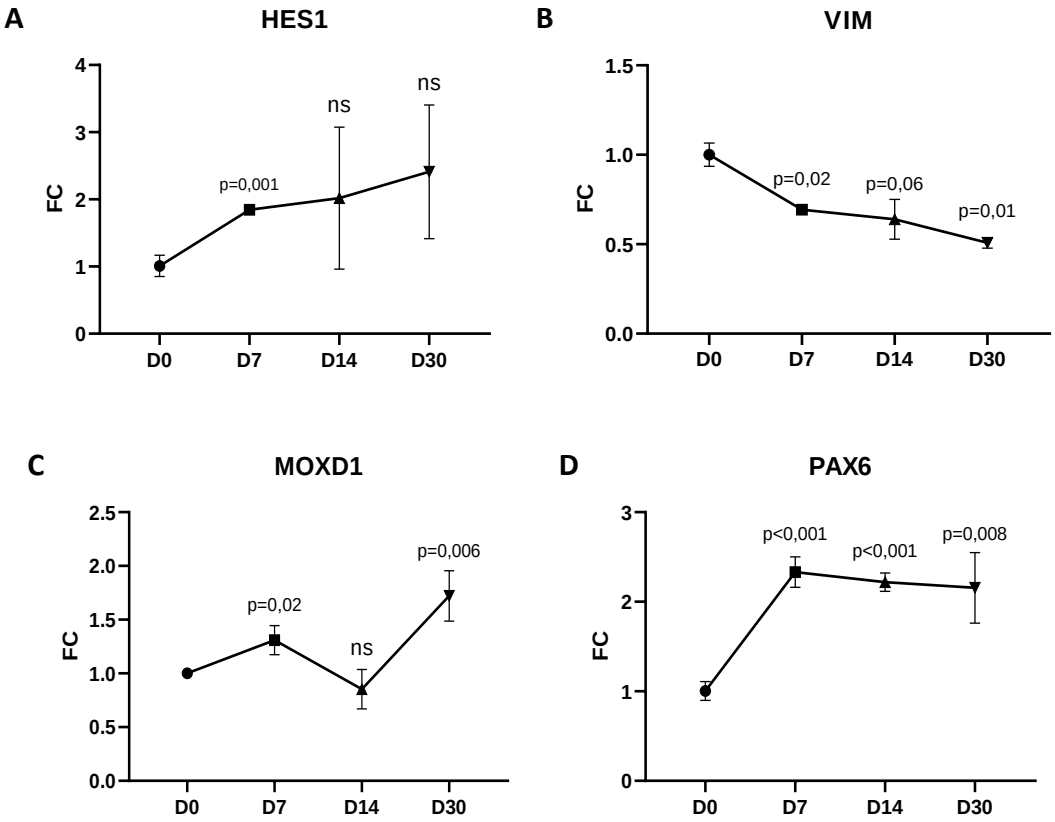
Table 10 – Final candidate markers per cell-type-specific groups (NSCs, NPCs, ImNs)

5.2. Neurogenic cell type-specific candidate markers expression along RenCells differentiation

To investigate the specificity of the candidate markers for NSCs, NPCs and ImNs selected from the previous meta-analysis for the respective neural differentiation stage, the transcription trend of each candidate markers was determined via qPCR along immortalized human neural stem cell line differentiation (RenCells) at four different time points (D0, D7, D14, D30), ideally representative of the different neuronal differentiation stages.

5.2.1. NSCs candidate markers

The transcription of the NSCs candidate markers (HES1, VIM, MOXD1, PAX6, ID4, DBI) along RenCells differentiation at four different time points (D0, D7, D14, D30) is expected to decrease as the RenCells differentiate from a NSCs-like phenotype to mature neurons. From these results, VIM follows very nicely this expected transcription trend (***Figure 6B***). Also ID4, whose transcription decreases later in the differentiation process from D14 seems to be a promising NSCs candidate marker (***Figure 4E***). Therefore, these two genes could be promising candidate markers for NSCs to further validate. Whereas the other NSCs candidate markers have a completely different transcription trend, which increases along the RenCells differentiation (***Figure 4A, 4C, 4D, 4F***).



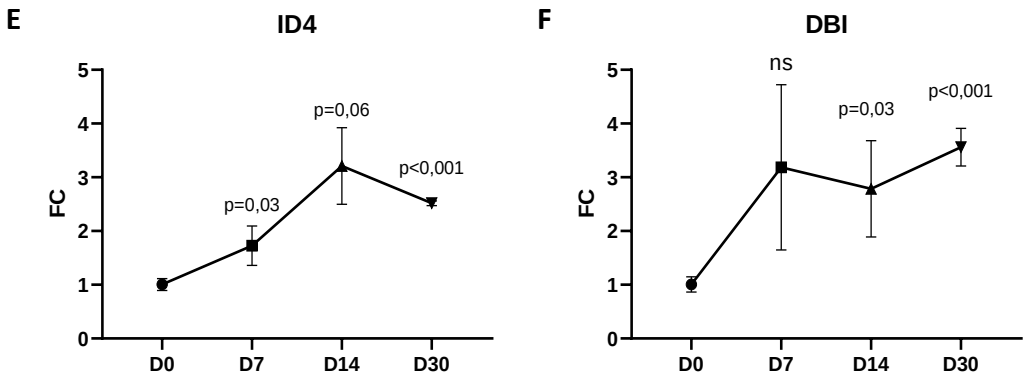


Figure 4 – NSCs candidate markers HES1, VIM, MOXD1, PAX6, ID4, DBI transcriptional profile along RenCell differentiation at 4 different time points (D0, D7, D14, D30). 3 biological replicates for each time point, except for VIM expression with 2 biological replicates per time point.

However, after a further evaluation of VIM and ID4 expression in differentiating human iPSCs (http://stemcell.libd.org), mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/), VIM was excluded from further validation due to its poor specificity for NSCs population and because of its documented limitation as a specific NSCs marker (*Souza et al., 2017*) (***Supplementary Table 1***). Therefore, only ID4 was further carried on in the validation process of being a good NSCs candidate marker.

5.2.2. NPCs candidate markers

The transcription of the NPCs candidate markers (MKI67, CENPF, EOMES, SMC4) along RenCells differentiation at four different time points (D0, D7, D14, D30) is expected to increase and decrease as the RenCells differentiate from a NSCs-like phenotype to mature neurons, passing for a NPCs differentiation stage. From these results, EOMES follows very nicely this expected transcription trend (***Figure 5C***). Also the candidate MKI67 and CENPF, whose transcription sharply decreases immediately after the induction of differentiation from D0, seem to be promising NPCs candidate markers (***Figure 5A, 5B***). Therefore, these three genes could represent promising candidate markers for NPCs to further validate. Whereas, the other NPCs candidate marker SMC4 has an opposite transcription trend along the RenCells differentiation (***Figure 5C***).

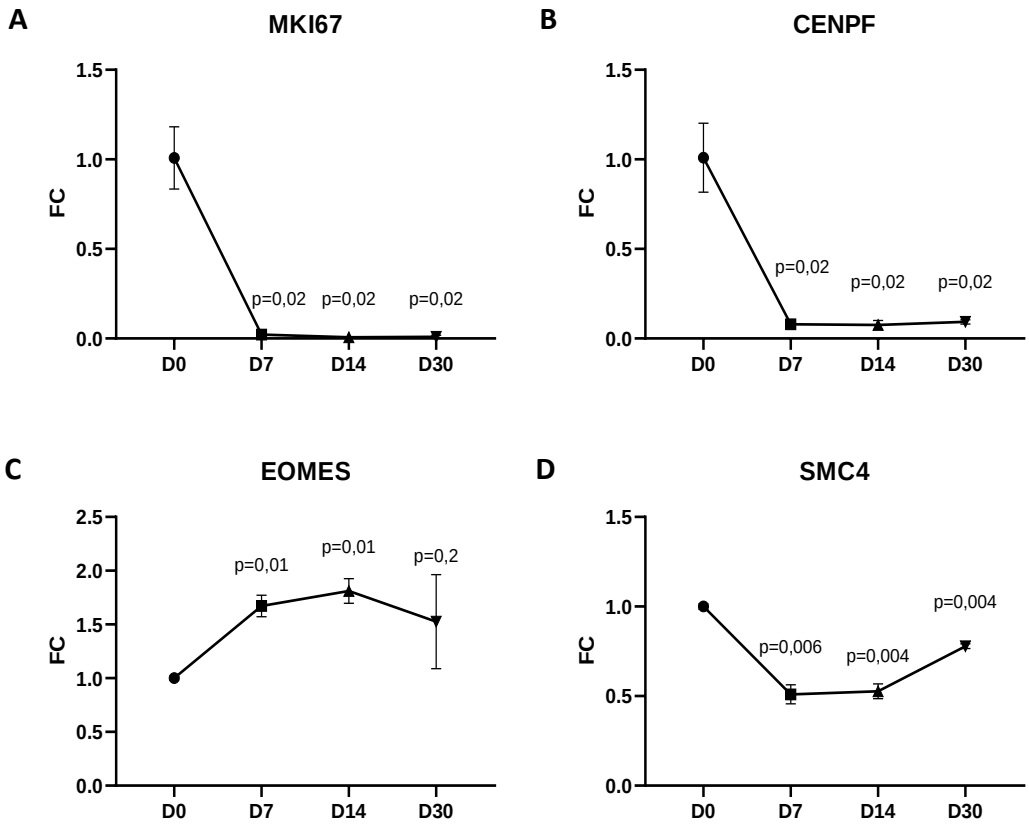


Figure 5 – NPCs candidate markers MKI67, CENPF, EOMES, SMC4 transcriptional profile along RenCell differentiation at 4 different time points (D0, D7, D14, D30). 2 biological replicates for each time point.

However, after a further evaluation of MKI67, CENPF and EOMES expression in differentiating human iPSCs (http://stemcell.libd.org), mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/), MKI67 and CENPF were excluded from further validation due to their poor specificity for NPCs population, since they are protein generally involved in the mitotic process and because of the documented limitation of MKI67 as a specific NPCs marker (*Munakata et al., 2013*) (***Supplementary Table 2***). Therefore, only EOMES was further carried on in the validation process of being a good NPCs candidate marker.

5.2.3. ImNs candidate markers

The transcription of the ImNs candidate marker (NEUROD6) along RenCells differentiation at four different time points (D0, D7, D14, D30) is expected to increase as the RenCells differentiate from a NSCs-like phenotype to mature neurons. Indeed, from these results, NEUROD6 follows very nicely this expected transcription trend (***Figure 6***). Hence, this gene could be a promising candidate marker for ImNs to further validate. Also a further evaluation of NEUDO6 expression in differentiating human iPSCs (http://stemcell.libd.org), mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/) confirmed the

adequacy of this gene to be further carried on in the validation process of being a good ImNs candidate marker (***Supplementary Table 3***).

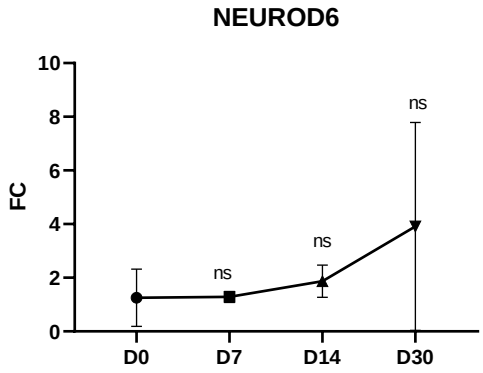


Figure 6 – ImNs candidate marker NEUROD6 transcriptional profile along RenCells differentiation at 4 different time points (D0, D7, D14, D30). 2 biological replicates for each time point.

5.3. NSCs candidate marker ID4: immunofluorescence along RenCells differentiation

To evaluate the specificity of the candidate marker ID4 for human NSCs, the immunofluorescence against ID4 was performed on RenCells at three different time points of differentiation: D0, D14, D30. ID4 is expected to localize in the nucleus since it is an inhibitor of the bHLH transcription factor ASCL1 (*Imayoshi and Kageyama, 2014; Ling et al., 2014*). Indeed, TRA181 was used as a reference marker to label NSCs, since it is an established pluripotency marker (*Trusler et al., 2018*). Therefore, if the candidate marker ID4 co-labelled the same cell type as for TRA181, this would be indicative of the specificity of ID4 for NSCs. Indeed, the expected expression trend for ID4 to be a good NSCs marker was a peak of its expression at the time point D0 and then a decrease along the RenCells differentiation, as for the reference marker TRA181 expression. This is because the RenCells, as they differentiate, change their phenotype from pluripotent stem cells, at the differentiation time point D0, into neurons, at the differentiation time point D30, passing through a NPCs stage at the differentiation time point D14.

The expected decreasing expression trend of ID4 was confirmed by the results of the immunofluorescence, where ID4 signal decreases along the RenCells differentiation from D0 to D30, with a peak at D0 (***Figure 7***). In contrast, the reference marker TRA181 was observed to increase its expression along RenCells differentiation reaching the peak at D30, not accordingly to what would have been expected (***Figure 7***). A possible explanation is represented by the genetic manipulation performed on the human mesencephalic neuronal progenitor cell line (RenCells), in order to immortalize it via the transduction of v-myc by mean of a retrovirus. In support of this hypothesis,

in literature it has been shown a positive correlation between MYC and TRA181 expression (*Liao et al., 2018*)

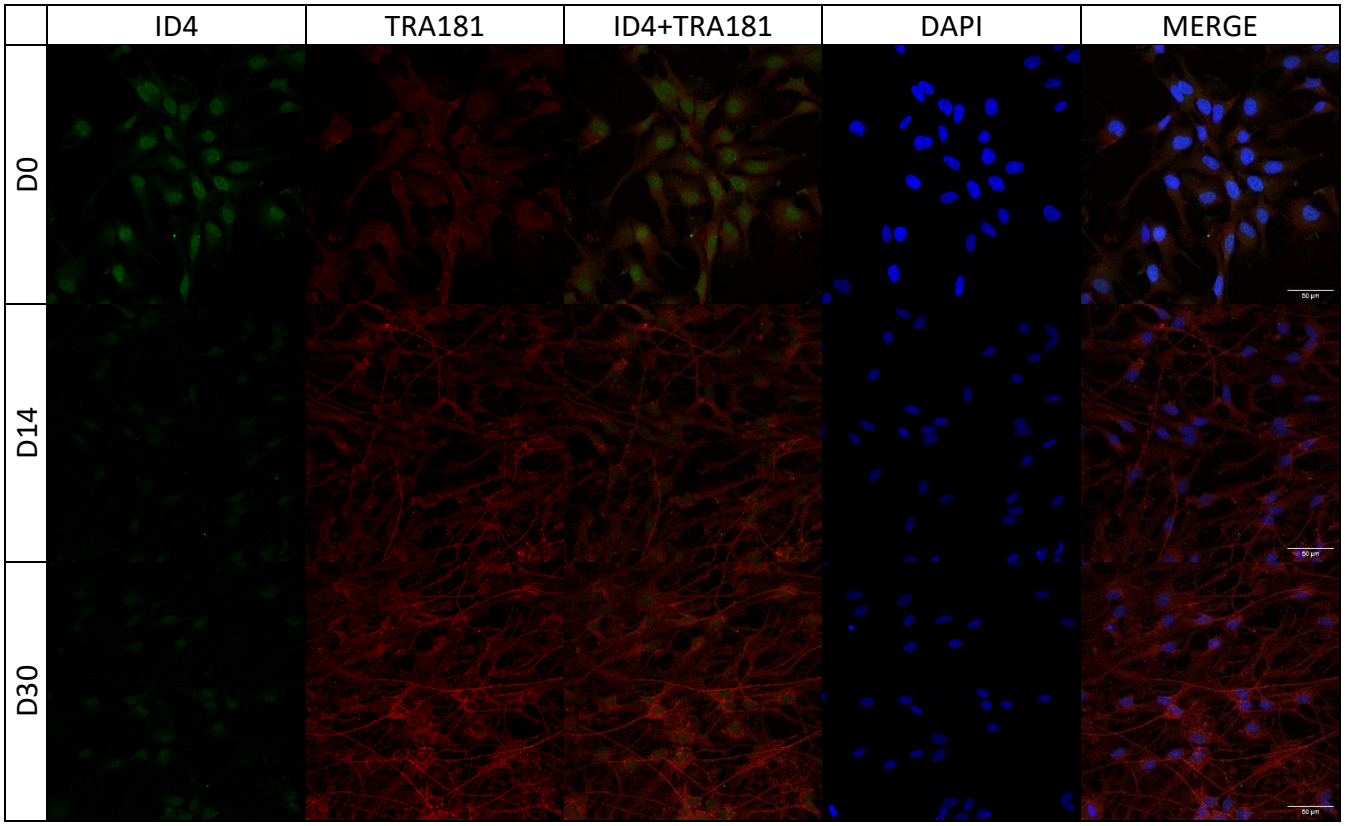


Figure 7 – Immunofluorescence of ID4 (green) and TRA181 (red) in RenCells at three different time points (D0, D14, D30).

Moreover, from these results it is evident that the expression trend of ID4 is not coupled with its transcriptional trend along RenCells differentiation (***Figure 4E***). This can be explained by the fact that not always transcription and translation are coupled and simultaneous. Different molecular mechanisms are responsible for such uncoupling, such as RNA degradation and silencing, resulting in a lower or delayed protein synthesis (*Johnson et al., 2022*).

5.4. NPCs candidate marker EOMES: immunofluorescence along RenCell differentiation

To evaluate the specificity of the candidate marker EOMES for human NPCs, an immunofluorescence against EOMES was performed on RenCells at three different time points of differentiation: D0, D14, D30. EOMES is expected to mainly localize in the nucleus since it is a transcription factor, which control gene expression (*www.proteinatlas.org*). Indeed, TUBB was used as a reference marker to indicate the mature neural phenotype along RenCells differentiation, since it stains the neural cytoskeleton, with its peak of expression at D30.

The expected expression trend for EOMES to be a good NPCs marker was a peak of its expression at the time point D14 and then a decrease along the RenCells differentiation, differently from the expression of the reference mature neurons marker TUBB.

The results of the immunofluorescence of EOMES was partially in line with the expected expression trend, as it increases along the RenCells differentiation (***Figure 13***). Accordingly, the mature neural marker TUBB was also observed to increase its expression along RenCells differentiation (***Figure 13***).

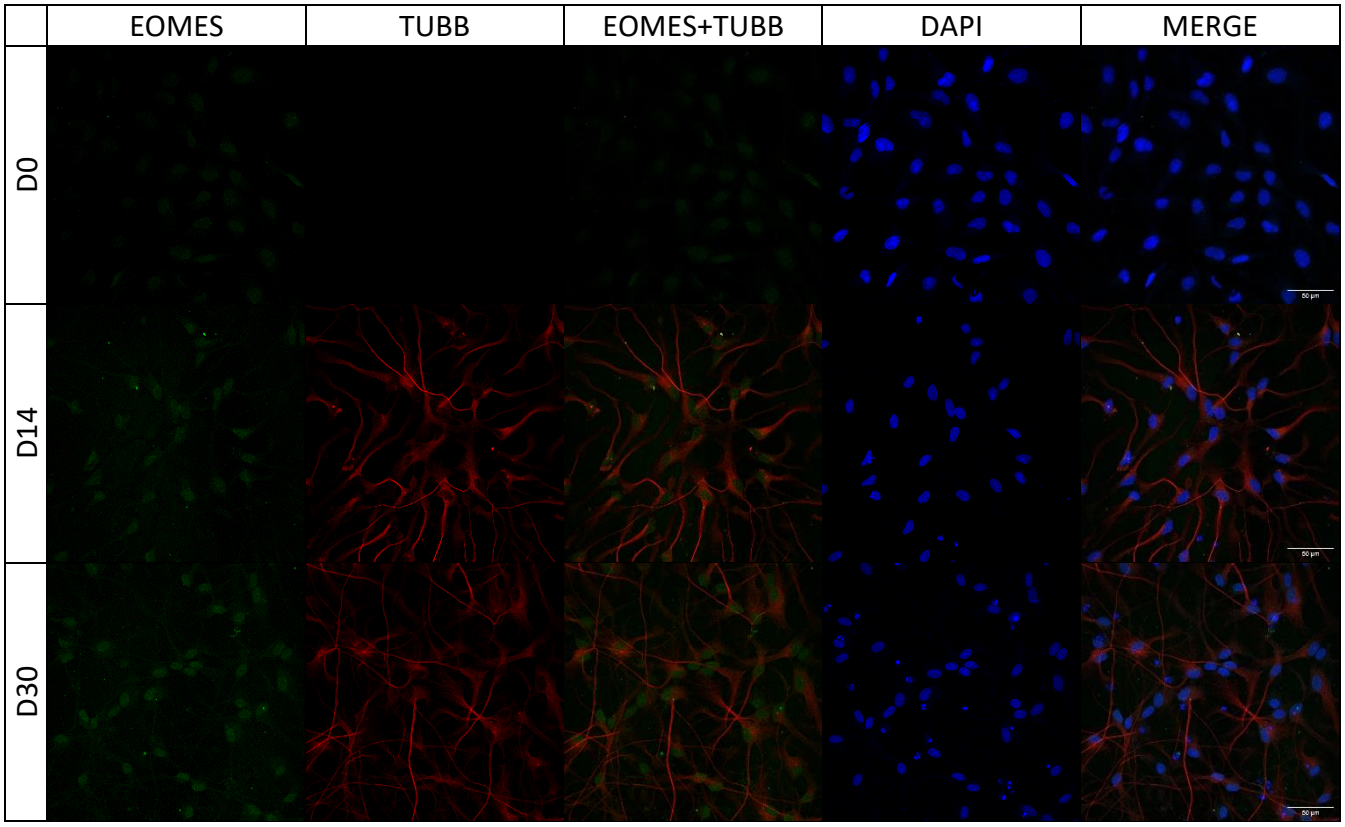


Figure 8 – Immunofluorescence of EOMES (green) and TUBB (red) in RenCells at three different time points (D0, D14, D30).

Moreover, from these results it is also evident that the expression trend of EOMES is not coupled with its transcriptional trend along RenCells differentiation (***Figure 5C***). Also in this case it can be explained by the fact that not always transcription and translation are coupled and simultaneous (*Johnson et al., 2022*).

5.5. NSCs candidate marker ID4: immunofluorescence along iPSCs differentiation

To evaluate the specificity of the candidate marker ID4 for NSCs also in another human cell line, an immunofluorescence against ID4 was performed on iPSCs-derived cell line at three different time points of differentiation: iPSCs, NPCs and neurons. ID4 also in this case is expected to localize in the nucleus and TRA181 was again used as a reference marker for pluripotency. Moreover, the expected

expression trend for ID4 to be a good NSCs marker was also in this case a peak of its expression at the iPSCs stage and then a decrease along the iPSCs differentiation into NPCs and neurons, as for the reference marker TRA181 expression.

The expected expression trend of ID4 was confirmed also in this case by the results of the immunofluorescence, where it decreases along the iPSCs differentiation (***Figure 8***). Accordingly, the reference marker TRA181 was observed to decrease its expression along iPSCs differentiation, as expected (***Figure 8***).

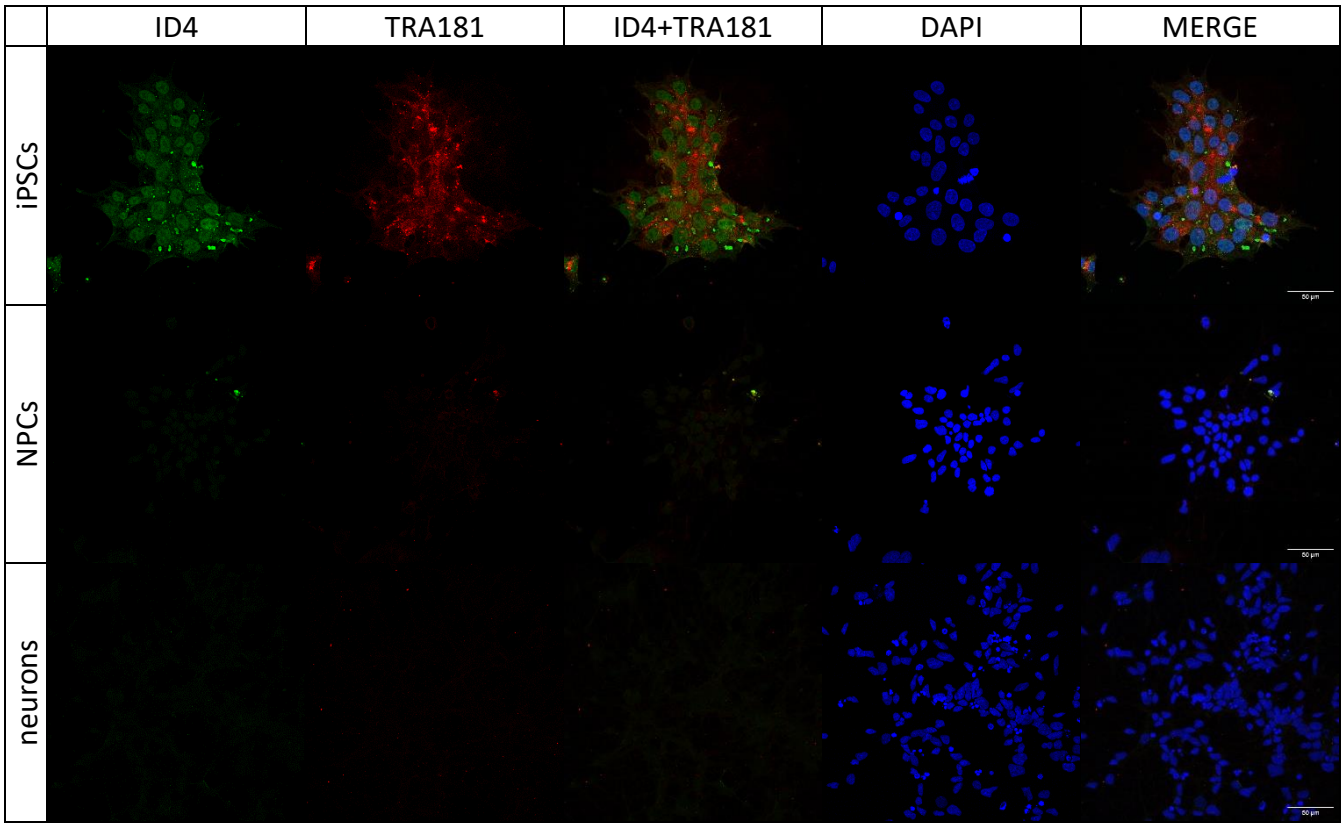


Figure 9 – Immunofluorescence of ID4 (green) and TRA181 (red) along iPSCs reprogramming at three different time points (iPSCs, NPCs, neurons).

Moreover, from these results it is also evident that the expression trend of ID4 is not coupled with its transcriptional trend along iPSCs differentiation (***Supplementary Table 1***). Also in this case it can be explained by the fact that not always transcription and translation are coupled and simultaneous (*Johnson et al., 2022*).

5.6. Neurogenic cell type-specific candidate markers expression in young, old, WT and AD mice

Since the neurogenesis and therefore neurogenic populations has been shown to decrease along aging and in AD condition (*Moreno-Jiménez et al., 2019; Tobin et al., 2019*), it was interesting to

investigate the transcription trend of the candidate markers for NSCs, NPCs and ImNs selected from the previous meta-analysis along aging and comparing the healthy condition with the AD one. Therefore, a qPCR was performed on DG samples collected from young (3 mo) and old (9 mo) WT and AD mouse model, in order to investigate whether the transcription of the neurogenic cell type-specific candidate markers was accordingly decreased by aging and AD conditions.

5.6.1. NSCs candidate marker Id4

The transcription of the NSCs candidate markers Id4 in mouse DG along aging was expected to decrease in order to be a good NSCs marker. However, from these results, both the transcriptional levels of the candidate markers for NSCs seem not to be affected by the aging process, both in the WT and AD model (***Figure 9***).

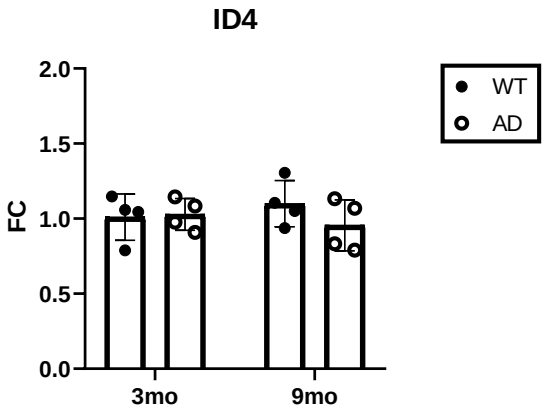


Figure 10 – NSCs candidate marker Id4 expression profile along aging (3mo and 9mo) in WT and AD mouse DG.

5.6.2. NPCs candidate marker Eomes

The transcription of the NPCs candidate marker Eomes in mouse DG along aging was expected to decrease in order to be a good NPCs marker. From these results, the transcriptional level of the candidate marker for NPCs decreases along the aging process, both in the WT and AD model, with statistical significance for the old AD condition (***Figure 10***).

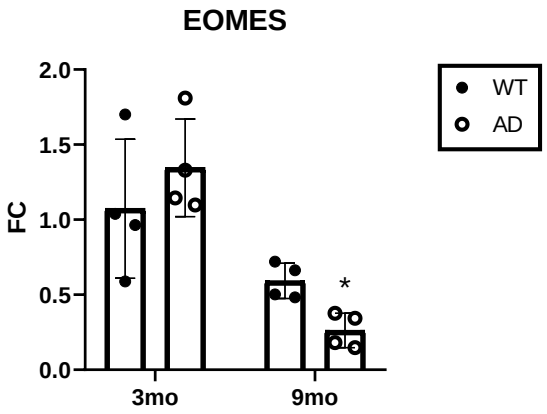


Figure 11 - NPCs candidate marker Eomes expression profile along aging in (3mo and 9mo) WT and AD mouse DG.

5.6.3. ImNs candidate marker Neurod6

The transcription of the ImNs candidate marker Neurod6 in mouse DG along aging also in this case was expected to decrease in order to be a good ImNs marker. However, from these results, the transcriptional level of the candidate marker for ImNs seems to be constant along the aging process, both in the WT and AD model (***Figure 11***).

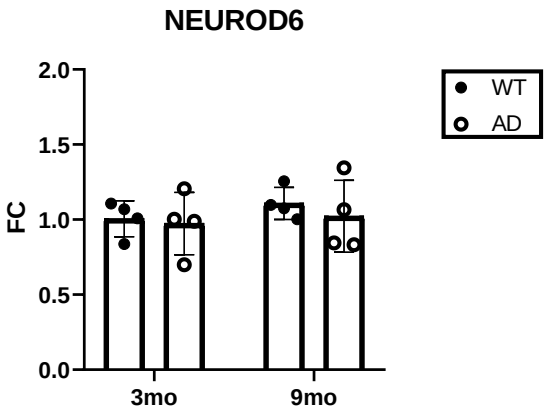


Figure 12 - ImNs candidate marker Neurod6 expression profile along aging (3mo and 9mo) WT and AD mouse DG.

5.7. NSCs candidate marker Id4: immunofluorescence in young, old, WT and AD mouse model

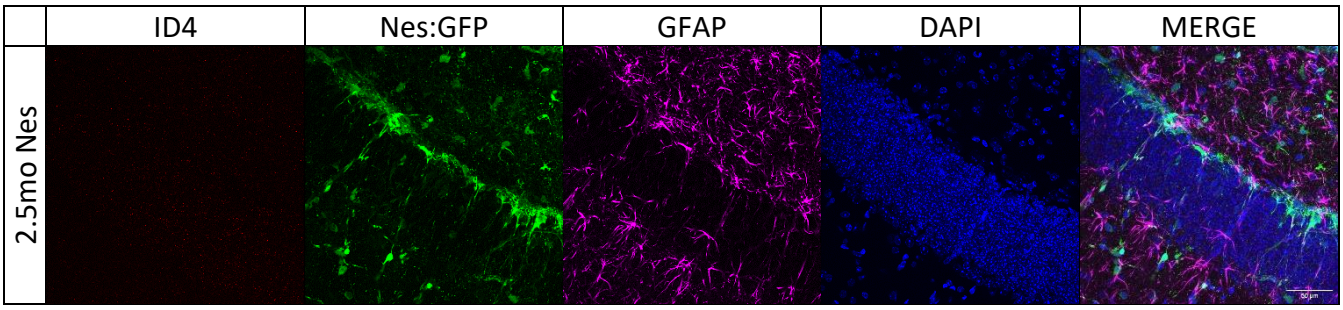
To evaluate the specificity of the candidate marker Id4 for NSCs located in the DG *in vivo*, an immunofluorescence against Id4 was performed on 2.5 mo Nestin:GFP mouse brain section. The conjugation between Nestin and GFP was useful to identify easily the NSCs in the mouse brain sections, using Nestin as a specific NSCs marker, to verify if this latter and the NSCs candidate marker

Id4 co-localized. However, Id4 is expected to be expressed both in NSCs and astrocytes in the mouse DG (***Supplementary Table 1***).

From this experiment, any Id4+ cells were detected in the 2.5 mo Nestin:GFP mouse brain section (***Figure 12A***).

Moreover, to investigate the Id4 expression in mouse DG along the aging process, an immunofluorescence against Id4 was also performed on 9.5 mo Nestin:GFP mouse brain sections, in order to evaluate if there is a link between the Id4 expression and the aging process. The expected expression trend of Id4 to be a good NSCs marker was higher in young mouse and decreased in older one, as for Nestin expression. Also in this case, any Id4+ cells were detected in the 9.5 mo Nestin:GFP mouse brain section (***Figure 12B***).

In addition, to investigate the Id4 expression in the DG of an AD mouse model and along the aging process, an immunofluorescence against Id4 was performed on the brain sections of the mouse Nestin:GFPxNLGF AD model at two different time points: 2.5 mo and 9.5 mo, in order to evaluate if there is a link between the Id4 expression and the aging process in the AD condition. The expected trend of expression for Id4 to be a good NSCs marker was also in this case higher in young mouse and decreased in older one, as for Nestin expression, but overall lower in the AD condition compared to the WT one, since it has been shown, besides a decrease of progenitor cells number throughout life in mouse brain, also a stronger decrease in AD condition, which is responsible for the neurogenesis impairment (*Kuhn et al., 1996; Drapeau et al., 2003; Ben Abdallah et al., 2010; Knoth et al., 2010; Boldrini et al., 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019; Denoth-Lippuner and Jessberger, 2021*). Also in this case, any Id4+ cells were detected both in the 2.5 mo and 9.5 mo Nestin:GFPxNLGF mouse brain sections (***Figure 12C, 12D***).



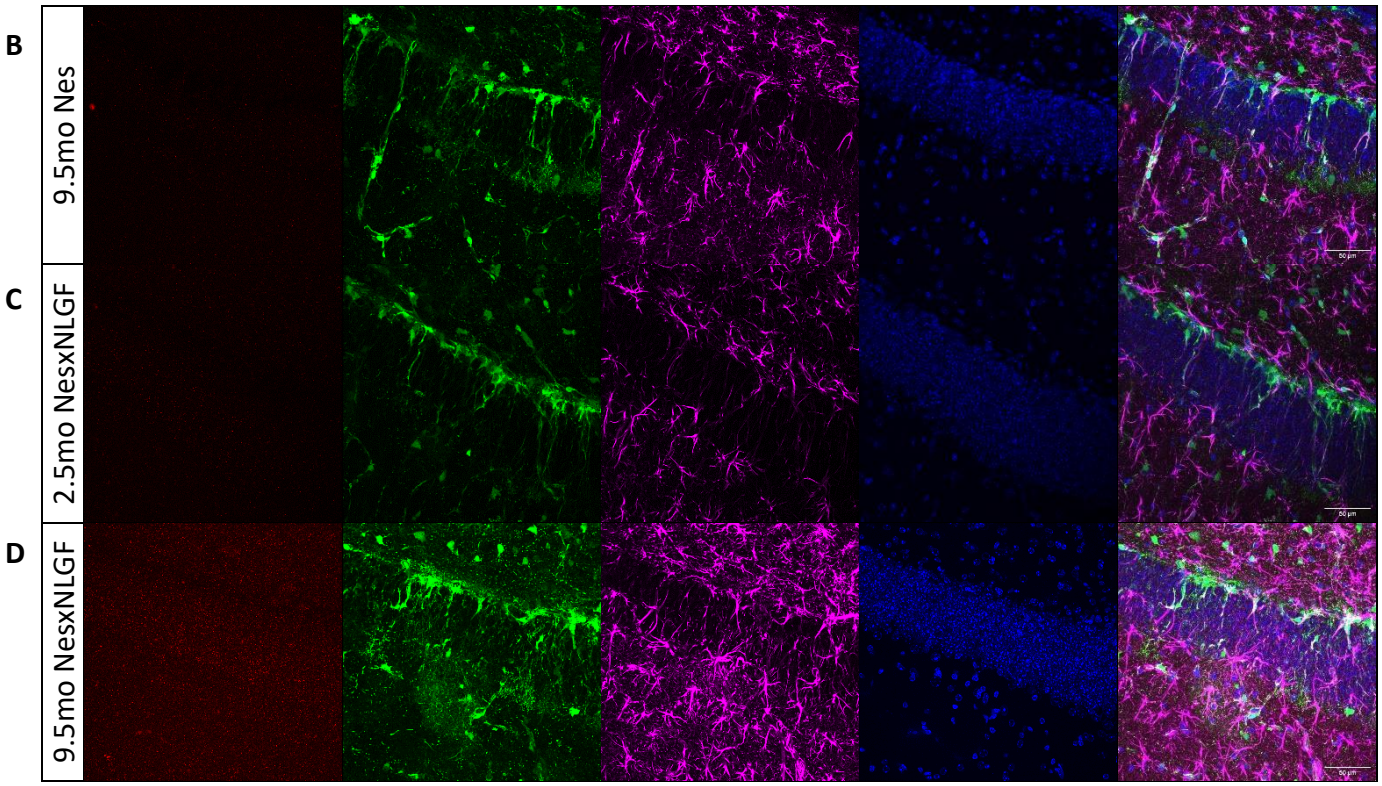


Figure 13 – Immunofluorescence of Id4 (red), Nes:GFP (green) and GFAP (magenta) in mouse brain section in four different conditions (2.5 mo Nes:GFP, 9.5 mo Nes:GFP, 2.5 mo NesxLGF, 9.5 mo NesxNLGF).

6. Discussion and conclusions

Adult hippocampal neurogenesis in human is still nowadays a very debated topic with several pieces of evidence both in support and against it. The contradicting results are mainly due to the lack of reliable and reproducible techniques and approaches to study this process in the adult human brain. In particular, the absence of specific and reliable markers to identify specific cell type involved in the AHN is among biggest limitations to address this controversial research question in neuroscience. Therefore, the goal of the present study was to identify novel neurogenic cell type-specific markers to investigate human adult hippocampal neurogenesis.

To address this objective, a meta-analysis was performed taking into consideration studies that performed scRNA sequencing in human fetal brain, selecting the list of genes characteristic of the cell type clusters involved in the hippocampal neurogenesis, such as NSCs, NPCs and ImNs. The underlying hypothesis was that in the developing fetal brain there are more progenitor cells than in the adult brain, increasing the chances to identify such neurogenic cellular population in the human brain. According to our hypothesis, it was assumed that the marker specific for those cell types are conserved throughout life.

The biggest challenging aspect encountered during the meta-analysis performed in the present study has been the absence of unique and standardized nomenclature to refer to the different cell types involved in neurogenesis. This complicated the data collection and the sorting of the datasets into the corresponding groups. Categorization was largely based on Penning et al. (2022), who provided a unifying summary of terminology (***Figure 14***), proposing a standardized nomenclature, gathering together the different terms for each cell type. Still, this challenge most probably had a big impact on the final results of the present study.

TABLE 1 | Terminology used to describe aNSCs and their progeny.

Terminology Our review	Others	Cellular state	Reference(s)
Adult neural stem cells (aNSCs)	Radial glia-like cells Quiescent neural stem cells Type-1 cells	Quiescent/Activated/Proliferative	Kempermann et al. (2004) and Encinas et al. (2011)
Neural progenitor cells (NPCs)	Intermediate progenitors Transiently amplifying neural progenitors Type 2a cells (glial phenotype) Type 2b cells (neuronal phenotype)	Proliferative	Kempermann et al. (2004), Encinas et al. (2011), and Berg et al. (2018)
Oligodendrocyte precursor cells	NG2-positive cells	Proliferative	Dawson et al. (2000) and Belachew et al. (2003)
Neuroblasts	Type 3 cells	Largely postmitotic	Kempermann et al. (2004), Encinas et al. (2011), and Berg et al. (2018)

Figure 14 – "Adult Neural Stem Cell Regulation by Small Non-coding RNAs: Physiological Significance and Pathological Implications" – Penning et al. (2022)

Moreover, the data collected for the meta-analysis are not perfectly comparable across the papers taken into consideration, since the fold change of each gene expression is affected by several variables and conditions, which are different across studies. The variability across datasets was normalized and relatively corrected as previously described in this thesis, however, this heterogeneity of datasets may have had a great influence on the outcomes of the present study.

From the meta-analysis results and following a first filtering step based on candidate transcriptional level along iPSCs differentiation, in mouse DG and human DG, a set of promising candidates for each cell type was selected. Afterwards, their transcription was evaluated also along RenCell differentiation, selecting those that showed transcriptional profile in line with the respective cell type of interest. One candidate marker per cell type was eventually identified: ID4 for NSCs, EOMES for NPCs and NEUROD6 for ImNs.

NSCs candidate marker: ID4

ID4 is an inhibitor of the transcription factor ASCL1, which promotes the activation and proliferation of adult NSCs (Urban and Guillemot, 2014). Therefore, because of its function, it could represent a valid candidate marker for NSCs. ID4 is already known for its role in the neurogenesis, in particular for keeping the adult hippocampal NSCs in a quiescent state and therefore repressing the neurogenesis (*Blomfield et al., 2019*). In particular, the Id (Inhibitor of differentiation/DNA binding) proteins are inhibitors of bHLH transcription factors, such as ASCL1 (*Imayoshi and Kageyama, 2014; Ling et al., 2014*). Id proteins contain a conserved HLH domain with which they dimerize with some bHLH proteins. In detail, they lack the DNA binding domain and therefore prevent bHLHs from binding DNA and other bHLH factors (*Benezra et al., 1990*). However, increased ID4 expression lead

to an increase in astrocytes population in the DG instead of neurons (*Zhang et al., 2019*). This is in line with the premature aging process, where activated NSCs in the hippocampus of aged mice give rise to astrocytes rather than neurons (*Encinas et al., 2011*).

In this study, it was shown that ID4 transcription increased along RenCells differentiation, reaching the peak at D14, after which it decreased. This could be explained by the fact that ID4 is a inhibitory factor necessary to keep NSCs quiescent in the adult hippocampus (*Zhang et al., 2019*). However, ID4 in the developing hippocampus is necessary for the proliferation of NSCs, in particular for the transition of the mitotic G1-S phase in early cortical progenitors (*Yun et al., 2004*). Therefore, since RenCells is originated from developing fetal brain (Millipore; RRID: CVCL_E921) and ID4 role is to inhibit the differentiation and promote proliferation in the early stages of this cell line, this culd explain its increasing transcription trend followed by a further decrease along RenCells differentiation. Indeed, ID4 represents an example of neurogenetic regulator conserved throughout life, which changes its function between embryonic and adult brain.

Accordingly, ID4 transcription increased along iPSCs differentiation, reaching the peak at differentiated neuronal stage. This trend can be explained for the same reason as mentioned before, in particular by its role in promoting proliferation..

Furthermore, from the validation of the candidate marker for NSCs ID4 in the human cell line RenCells via immunofluorescence, it has been shown how ID4 expression decreased along RenCells differentiation. In particular, the expression peak was reached at D0, corroborating its presumable specificity for NSCs and its role in promoting proliferation in this fetal-derived cell line. However, from this study, it is evident how ID4 expression is not coupled with its transcription. Different molecular mechanisms could be responsible for such uncoupling, such as RNA degradation and silencing, resulting in a lower or delayed protein synthesis (*Johnson et al., 2022*).

Also from the validation of ID4 in human iPSCs, it has been shown how its expression decreased along iPSCs differentiation. In particular, the expression peak was reached also in this case at D0, indicating a presumable specificity for NSCs. This expression trend for ID4 was in line with the established reference marker for progenitor cells, TRA181, which also decreased along iPSCs differentiation. However, as well as for the RenCells, its expression was not couple with its transcription, probably for the same molecular mechanisms explained before.

Moreover, the transcription trend of ID4 along aging WT and AD mouse DG was evaluated via qPCR, expecting a decrease along ageing and in AD condition (*Tobin t al., 2019*). However, any significant

differences of ID4 expression were shown along aging or in AD condition. Most probably these results are due to the small sample number or the absence of any effect by age and AD pathology on the expression of this candidate marker for NSCs.

However, given the previously described uncoupling between transcription and translation for ID4, its expression was validated in mouse brain sections via immunofluorescence, in order to investigate its cell type specificity and its expression trend along aging and in the AD pathology. Four different conditions were taken into consideration, 2.5mo WT, 9.5mo WT mice and 2.5mo AD and 9.5mo AD mice. However, there were no ID4 positive detected cells in the mouse brain sections. This is most probably not due to the lack of ID4 positive cells in the mouse brain, since in literature they have been found using a different protocol and antibody (*Cole et al., 2022*), but most probably because the immunofluorescence for ID4 did not work. Probably because of some technical problems, such as the absence of antigen retrieval, in order to properly reveal the antigen reactive with the antibody, or maybe because the antibody itself is not reactive on mouse samples besides human, since it is just predicted to but never validated before. However, the staining for the astrocytic marker, GFAP, and the established reference marker for progenitor cells, Nestin, worked perfectly in mouse brain sections.

NPCs candidate marker: EOMES

EOMES is already an established marker for NPCs in adult brain. In particular, it is a transcription factor involved in the proliferation and differentiation of intermediate progenitor cells in adult hippocampal neurogenesis (*Hodge et al., 2008*) and during cortical development (*Hevner et al., 2019*). Therefore, since this candidate marker resulted from the meta-analysis performed in the present study on fetal human brain sequencing, this finding supports the previously described hypothesis that some neurogenic cell type-specific markers might be conserved throughout life (*Urban and Guillemot, 2014*).

In this study, it was shown that EOMES transcription increased along iPSCs differentiation, reaching the peak in correspondence of the NPCs stage and afterwards decreasing to the original level, as the iPSCs differentiate into neurons. This transcriptional trend was also observed along RenCells differentiation, where the peak was reached at D14, before decreasing to the original level at D30. This transcriptional trend of EOMES in both the cell lines taken into account is in line with its role as transcription factor involved in the NPCs proliferation and determination.

From the validation of the candidate marker for NPCs EOMES in the human cell line RenCells via immunofluorescence, it has been shown how EOMES expression increased along RenCells differentiation. In particular, it reached the peak at D30, where the RenCells differentiate into neurons, supporting its presumable specificity for NPCs. However, from this study, also in this case its translational profile was not coupled with its transcription, probably for the same molecular mechanisms explained before.

For sure, it would be meaningful to validated the expression of the candidate marker for NPCs EOMES also along iPSCs differentiation to confirm the previous findings also in another human cell line.

Moreover, the transcription trend of EOMES along aging WT and AD mouse DG was evaluated via qPCR, expecting a decrease along ageing and in AD condition (*Tobin t al., 2019*). A decrease of EOMES transcription was observed along aging, in particular in a statistically significant way in the AD condition. This is in line with the expected decrease of NPCs population and neurogenesis along aging and in AD condition (*Tobin et al., 2019*).

In addition, given the previously described uncoupling between transcription and translation for EOMES, it would be meaningful to validated EOMES expression along aging WT and AD mouse brain sections via immunofluorescence, in order to investigate its cell type specificity and whether also its expression trend is decreased along aging and in the AD pathology, as seen for its expression.

ImNs candidate marker: NEUROD6

NEUROD6 is a neurogenic bHLH transcription factor responsible for neuronal differentiation and long-term neuronal survival, via the network of molecular chaperones, during fetal brain development (*Uittenbogaard et al., 2010*). Therefore, it could represent a valid candidate marker for ImNs.

In the present study, it was only investigated the transcription trend of NEUROD6 along aging WT and AD mouse DG via qPCR, expecting a decrease along ageing and in AD condition (*Tobin et al., 2019*). However, any significant differences of NEUROD6 expression were shown along aging or in AD condition. Most probably these results are due to the small sample number or the absence of any effect by age and AD pathology on the expression of this candidate marker for ImNs.

In conclusion, both ID4 and EOMES, so far in the validation process on human cell lines, seem to be promising good candidate markers for the respective cell types, NSCs and NPCs.

In addition, it would be worthy to further test and validate also the candidate marker for ImNs NEUROD6 that resulted from the present meta-analysis on the different models taken into consideration so far.

Evidently, more follow-up validation is necessary to robustly establish these markers as specific tools to investigate neurogenic population and AHN in the human brain.

7. Limitations and follow-up studies

The present study presents some limitations. First of all, as mentioned before the lack of a standardized nomenclature to refer specifically to each cell type involved in the AHN has been problematic in the sorting of the datasets performed in the meta-analysis (*Penning et al., 2022*). This is the reason why, we cannot currently exclude the partially inaccurate sorting of the datasets collected from the articles taken into consideration during the meta-analysis in the right cell type group.

In addition, the datasets among papers are not perfectly comparable to each other. Each one was the result of scRNA sequencing of different samples and conditions. Therefore, even after the filtering and thresholding were applied to datasets to minimize the variability among them, this crucial aspect must be taken into account.

Moreover, only few datasets where available in order to perform a systematic meta-analysis. This is due to the recent appearance of the scRNA sequencing as a powerful tool to identify different cell types in a specific tissue (*Svensson et al., 2017*). In particular, such studies in human brain, and more specifically in fetal samples, looking at rare population of progenitor cells are very few and recent (*Wang et al., 2017*). Therefore, a greater collection of datasets relevant to the present study would contribute more significantly to the final results for better, more specific and more reliable neurogenic cell type-specific candidate markers.

Another limitation is the inherent susceptibility to technical variability and sampling bias of the scRNA sequencing approach (*Kelley et al., 2018*). In addition, since scRNA sequencing analyse the transcriptional profile of cellular populations, it does not provide a realistic representation of the protein expression characteristic of each cell type (*Greenbaum et al., 2003; Maier et al., 2009*). This may interfere with conclusions on gene expression, since uncoupling between transcription and translation has been reported in different cell types (*Greenbaum et al., 2003; Maier et al., 2009*). It is therefore fundamental to keep in mind these discrepancies when investigating protein markers basing the selection on transcriptional analysis.

Furthermore, the RenCell line is a mixed cell culture since along the differentiation, neurons, astrocytes and oligodendrocytes are generated (*Song et al., 2019*). This aspect must be taken into consideration when this cell line is used as a model to test possible candidate markers, both looking at the transcription and the translation, especially if the candidate markers could be expressed in

all of these cellular populations. Indeed, the RenCells line is originated from the mesencephalic human neuronal progenitor cell, which when differentiated gives rise to cortical neurons (*Donato et al., 2007*). It is therefore crucial to consider the different nature of these neurons when in the present study neurogenic cell type-specific markers for hippocampal neurons are investigated, which could have a different transcriptional profile than the cortical ones (*Zeisel et al., 2015*).

An additional limitation to consider is the possibility that embryonic neurogenesis might be different than adult neurogenesis (*Götz et al., 2016*). Although the initial hypothesis was based on the similarity between these two neurogenic events in human brain, it is not possible to exclude a discrepancy and therefore a difference in their marker profile. Therefore, it is necessary to keep in mind that the *in vitro* experiments in the present study actually are modelling embryonic and not adult neurogenesis, since the cell lines used are multipotent neural stem cell lines. This aspect could have an impact on the results of the evaluation and validation process of new cell type-specific candidate markers for neurogenic cell populations, leading to the selection of markers specific for embryonic neurogenesis. It is therefore fundamental to validate and test the present candidate markers also on adult human brain samples in order to have a better readout of their specificity for adult neurogenic cell populations.

In addition, the reactivity of the antibody against Id4 used in the present study in mouse samples is doubtful, since it was only predicted by the company to be reactive against mouse, besides human species. In the present study this particular antibody worked in human cell lines, such as RenCells and iPSCs, but it did not in mouse brain sections, supporting the idea that it might be not reactive in mouse samples. However, in order to test this hypothesis, it would be necessary to test this antibody in a mouse positive control tissue for Id4, such as the thyroid gland (*Rigolet et al., 1998*).

Last, it would be of great interest to perform immunofluorescence against Id4 in fetal and adult human DG sections in order to eventually identify putative Id4+ NSCs also in the human brain. The integration of the findings of the present study into such future approaches would offer support to the presence of NSCs in the adult human dentate gyrus, a possible proxy of adult hippocampal neurogenesis.

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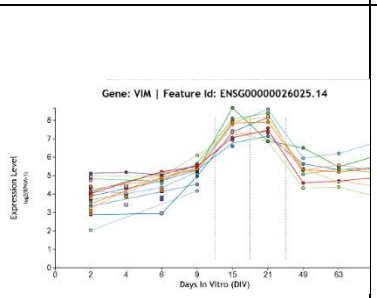
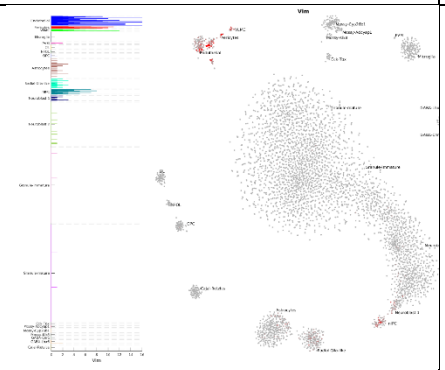
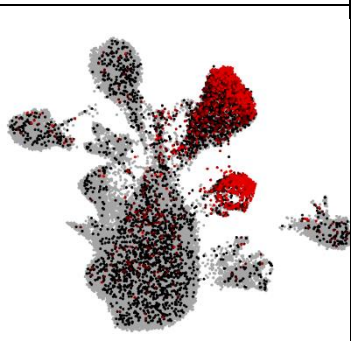
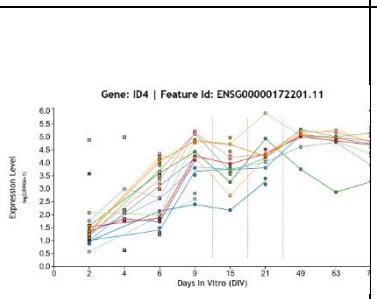
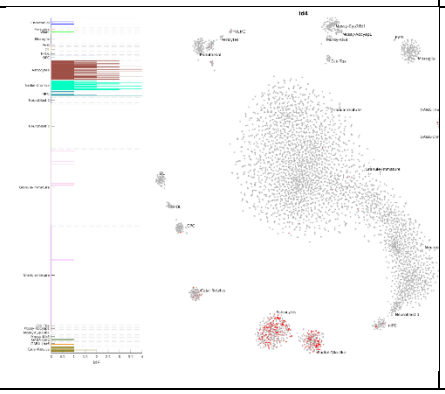
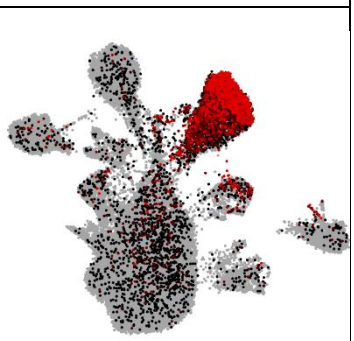
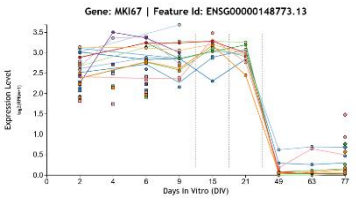
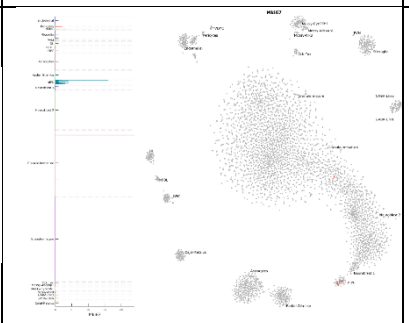
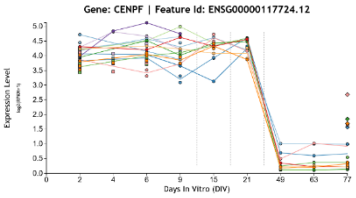
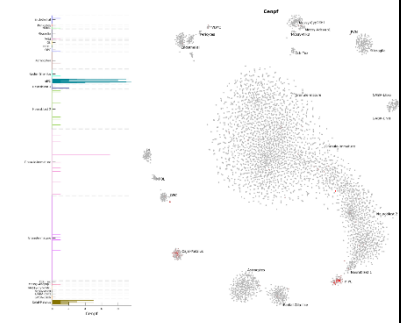
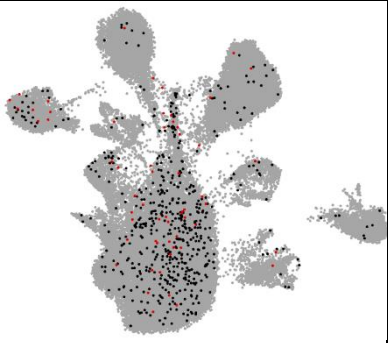
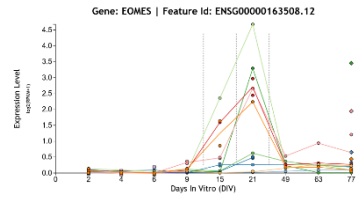
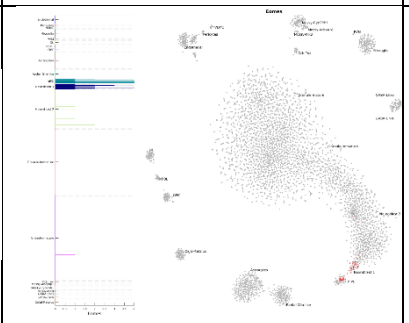
Supplementary figures and tables

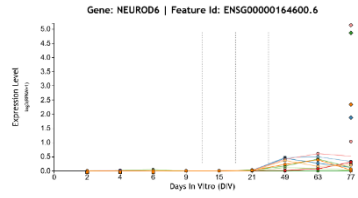
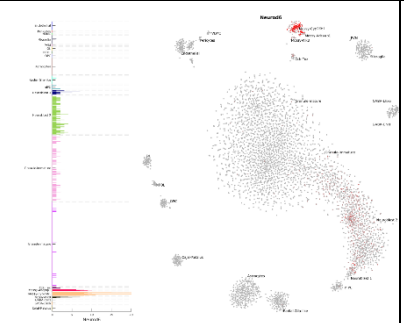
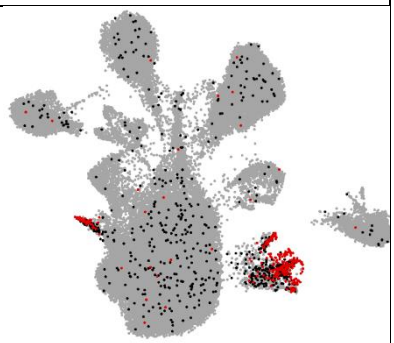
Supplementary Figure 15 – Preliminary data of scRNA sequencing of adult human DG and relative cell population highlighted with different colours – Giorgia Tosoni.

	Human iPSCs differentiation	Mouse DG	Human DG
VIM			
ID4			

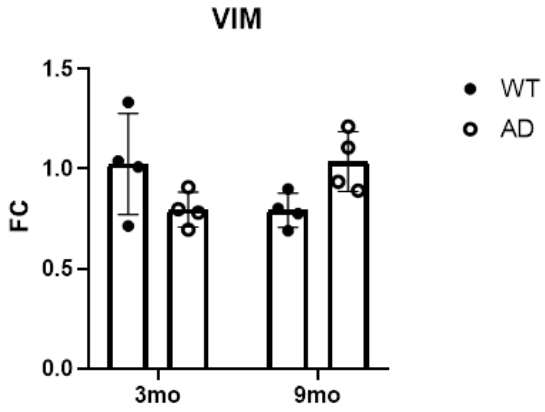
Supplementary Table 116 – NSCs candidate marker VIM and ID4 expression along differentiating human iPSCs (http://stemcell.libd.org), in mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/)

	Human iPSCs differentiation	Mouse DG	Human DG
MKI67			(not detected)
CENPF			
EOMES			(not detected)

Supplementary Table 2 – NPCs candidate markers MKI67, CENPF and EOMES expression along differentiating human iPSCs (http://stemcell.libd.org), in mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/)

	Human iPSCs differentiation	Mouse DG	Human DG
NEUROD6			

Supplementary Table 3 – ImNs candidate marker NEUROD6 expression along differentiating human iPSCs (http://stemcell.libd.org), in mouse DG (http://linnarssonlab.org/dentate/) and human DG (http://scope.aertslab.org/)



Supplementary figure 2 – NSCs candidate marker Vim expression along aging WT and AD mouse DG models