Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

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ABSTRACT

Neural progenitor (NP) cells proliferate in the ventricular zone (VZ) of the neural tube and migrate towards the mantle layer (ML) upon neural differentiation. In order to produce the correct type and number of neurons at the right time, a precise control of the proliferation of neural progenitor (NP) cells and their differentiation is required. The Notch signaling pathway, through lateral inhibition, is involved in this balance, restraining NP differentiation. However, not much is known about Notch activity in single NPs, mainly regarding possible variations on Notch activity in each NP, and how this putative dynamic activity contributes to the maintenance of these cells in the undifferentiated state. In order to answer these questions, I used several ES cell lines expressing different reporters of Notch activity driven by the promoter of the *Hes5* gene. These ES cells can be directed to neural differentiation in adherent monolayer cultures, resulting in the production of neuroepithelial rosettes that mimic their in vivo counterpart, the neural tube. Here, I show that an already described *Hes5::GFP* reporter ES cell line is not suitable to be used as a reporter of Notch activity since the half-life of the reporter protein is much longer than that of the HES5 protein, not allowing the detection of the termination of Notch activity. I also test other ES cell lines, *Hes5::VNP*, that express an unstable reporter protein, which might allow a more precise and accurate monitoring of Notch activity dynamics. Using this cell lines, I could observe that not all cells in neuroepithelial rosettes express the reporter protein, and that the levels of Notch activity are variable between NPs. Further engineering of these cell lines needs to be performed in order to be able to construct a double reporter cell line carrying a reporter of Notch activity together with a reporter of neuronal differentiation to allow visualization of differentiated neurons.

Key-words: Notch signaling, HES5 reporter protein, neuroepithelial rosette, Neural progenitors, Interkinetic nuclear migration.

RESUMO

O tubo neural, estrutura embrionária que no adulto origina o sistema nervoso central, encontra-se organizado em duas zonas: a zona ventricular, onde residem os progenitores neurais, e a zona do manto, para onde migram estas células quando começam a diferenciar em neurónios. Os núcleos dos progenitores neurais movimentam-se entre as regiões apical e basal da zona ventricular num mecanismo designado de movimento intercinético nuclear. Este movimento encontra-se relacionado com as fases do ciclo celular, sendo que a mitose ocorre quando o núcleo dos progenitores neurais se encontra na região apical e a fase S quando este se encontra na região basal. Durante a neurogénese é necessária a existência de um controlo preciso entre a manutenção de células num estado indiferenciado e a diferenciação destes progenitores em células neurais. Sabe-se que a via de sinalização Notch está implicada neste balanço através do processo de inibição lateral, restringindo a diferenciação das células progenitoras. Inicialmente todas as células expressam níveis semelhantes de ligandos Notch e genes pró-neurais. Contudo, devido a variações estocásticas, uma das células começa a expressar níveis mais elevados de ligandos Notch e consequentemente é mais eficiente na activação de Notch nas células vizinhas. Nas células em que Notch é activado há expressão de genes *Hes* que reprimem a expressão de genes pró-neurais e levam à consequente manutenção dessas células como progenitores neurais. Por sua vez, nas células que não activaram Notch ocorre a expressão de genes pró-neurais com consequente diferenciação dos progenitores neurais.

Após a migração dos neurónios nascentes para a zona do manto, estas células deixam de sinalizar para as células adjacentes. Desta forma a actividade de Notch diminui nos progenitores neurais vizinhos e o processo de inibição lateral poderá ser reiniciado. Assim, os níveis de Notch em progenitores neurais não deverão ser constantes e a sinalização Notch será dinâmica. A actividade dinâmica da via de sinalização Notch foi monitorizada na mesoderme pré-somítica e em culturas de progenitores neurais isolados utilizando uma proteína reporter instável sob o controlo do promotor de um gene alvo de Notch: *Hes1*. A utilização deste repórter permitiu observar variações na expressão de *Hes1*. Contudo, foi observado que a expressão de *Hes1* não é apenas dependente da actividade da via Notch, respondendo a outras vias de sinalização como a via Jak/Stat, o que impossibilita a monitorização apenas da actividade da via de sinalização Notch.

Uma outra observação concordante com a actividade dinâmica de Notch é a expressão de componentes da via Notch em gradientes entre as regiões apical e basal da zona ventricular,

sugerindo que nos progenitores neurais a activação de Notch é específica de determinadas fases do ciclo celular. Em retina de peixe zebra foi observado que o receptor Notch activado é expresso em níveis elevados na região apical, onde os progenitores se encontram nas fases G2/M/G1 do ciclo celular. Contudo, em cérebro de ratinho o receptor Notch activado foi detectado em células em fase S do ciclo celular e não em mitose. No tubo neural de galinha o mRNA de Notch foi detectado na região apical da zona ventricular, onde ocorre a mitose. Estes resultados contraditórios podem indicar regulações diferentes da actividade Notch em diferentes organismos e tecidos. Desta forma, uma análise mais profunda é necessária para determinar se a activação de Notch ocorre especificamente em determinadas fases do ciclo celular. Por outro lado é necessária a análise de progenitores neurais ao nível de células individuais recorrendo a um repórter que responda apenas a Notch. Apenas desta forma será possível determinar se Notch pode ser activado mais do que uma vez no tempo de vida de cada progenitor neural e se a sua actividade é necessária para manter estes progenitores neurais num estado indiferenciado.

Neste trabalho utilizaram-se várias linhas celulares de células estaminais embrionárias (*Hes5::GFP* and *Hes5::VNP*) que expressam diferentes proteínas repórter sob o controlo do promotor do gene *Hes5*, que se pensa ser o principal alvo da via Notch no sistema nervoso central. Estas células estaminais embrionárias podem ser diferenciadas em tecido neural, através de um protocolo de cultura de células aderentes em monocamada, formando estruturas características designadas rosetas neuroepiteliais, onde a expressão de *Hes5* foi já documentada.

A linha celular *Hes5::GFP* já tinha sido descrita e foi testada relativamente às suas capacidades de auto-renovação e pluripotência tendo-se observado que não diferia da linha celular controlo. Adicionalmente, a sua capacidade de diferenciação neural e a expressão da proteína repórter foram testadas tendo-se confirmado a capacidade de originar rosetas neuroepiteliais em que a expressão da proteína repórter estava presente. O tempo de semi-vida da proteína repórter foi determinado de forma a avaliar se esta linha celular iria permitir a monitorização e determinação do início e terminação da actividade da via Notch. Observou-se que 12 horas após o bloqueio da tradução as células ainda exprimem cerca de 75% do valor inicial de proteína repórter GFP. Dado que o tempo de semi-vida da proteína HES5 é de aproximadamente 1 hora foi possível concluir que esta linha repórter não pode ser usada para monitorizar a actividade Notch uma vez que permanece nas células durante muito tempo após a sua terminação.

Foram geradas no Laboratório linhas celulares que expressam uma proteína repórter instável sob controlo do promotor do gene *Hes5* de forma a possibilitar a detecção do começo e terminação da actividade Notch. Estas linhas foram validadas em termos de capacidade de auto-renovação e pluripotência tendo-se observado que as suas características não diferiam daquelas da linha celular controlo. Adicionalmente, foi confirmada a capacidade de formação de rosetas neuroepiteliais e expressão da proteína repórter. Das linhas geradas a linha Hd foi escolhida para continuar as experiências tendo-se observado que a intensidade de expressão do repórter não é igual em todas as células das rosetas originadas por estas células. Este resultado sugere que a intensidade da actividade Notch varia entre os progenitores neurais. Este facto pode ser explicado pelo facto de a expressão de Notch ser constante em cada célula mas variar entre diferentes células, ou pelo facto de esta expressão ter variações na mesma célula. De realçar ainda que estes resultados sugerem possíveis flutuações na actividade da via Notch, enfatizando a importância da monitorização de Notch ao nível de um único progenitor neural.

A linha celular Hd, possui a sequência do gene de resistência à Neomicina localizada entre a região codificante da proteína VNP e a região 3'UTR do gene *Hes5*, onde se localiza o sinal de poli-adenilação necessário para a produção de um transcrito com todos os sinais de regulação pós-transcricional necessários. Desta forma procedeu-se à remoção do gene de resistência à Neomicina de forma a assegurar a correcta expressão da proteína repórter. Contudo, nas linhas celulares obtidas após este procedimento não se observou expressão da proteína reporter. Após vários testes à sequência promotora e à expressão do repórter ao nível de mRNA e proteína concluiu-se que a falta de expressão do repórter deveria dever-se a problemas a nível da transcrição. De forma a ultrapassar estes constrangimentos novas linhas celulares deverão ser desenhadas e geradas de forma a que o cDNA da proteína reporter possua o seu próprio sinal de poliadenilação antes de se proceder à remoção da cassete de selecção. Desta forma a expressão da proteína repórter poderia ser analisada imediatamente aquando da geração das linhas sem serem necessários procedimentos adicionais de remoção da cassette de selecção, que acarretam a possibilidade de introdução de instabilidade genómica e danos no DNA.

Após a construção de linhas *Hes5::VNP* capazes de monitorizar de forma fiel a actividade da via Notch será possível monitorizar esta actividade em progenitores neurais ao nível de células individuais. Assim será possível determinar se Notch pode ser activado várias vezes ao longo do tempo de vida destas células e especificamente em determinadas fases do ciclo celular e se essa activação está correlacionada com a manutenção das células num estado indiferenciado. Adicionalmente, estas linhas celulares podem ser usadas para a formação de uma dupla linha

repórter que expressa repórteres da actividade Notch e também de diferenciação. Desta forma será possível avaliar não só as variações na actividade da via Notch mas também diferenciação neuronal, correlacionando a actividade Notch com o destino celular dos progenitores neurais.

Palavras-chave: Via de sinalização Notch, Proteína repórter HES5, Roseta neuroepitelial, Progenitores neurais, Movimento Intercinético nuclear.

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LIST OF ABBREVIATIONS

3'UTR	3' Untranslated Region
BAC	Bacterial Artificial Chromosome
bFGF	basic Fibroblast Growth Factor
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Embryoid Body
ES cells	Embryonic Stem cells
FACS	Fluorescence Activated Cell Sorting
FI	Fold Increase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GMEM	Glasgow Minimum Essential Medium
hrs	Hours
HT	*Hes5::GFP* cell line [1]
INM	Interkinetic Nuclear Migration
LIF	Leukemia inhibitory factor
min	Minutes
ML	Mantle Layer
NICD	Notch Intracellular Domain
NN	Cell division originating two Neurons
NP	Neural Progenitor

O/N	Over-Night
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PN	Cell division originating one Neural Progenitor and one Neuron
PP	Cell division originating two Neural Progenitors
RNA	Ribonucleic acid
rpm	rotations per minute
RT	Room Temperature
VNP	Venus-NLS-PEST
VZ	Ventricular Zone

CHAPTER 1

Introduction

1. INTRODUCTION

1.1 The Neural tube and the Neuroepithelium

In vertebrates, the process of neurulation gives rise to the neural tube, a structure that develops into the adult central nervous system (brain and spinal cord). Production of neurons occurs in the neural tube by means of an asynchronous and mitotically active neuroepithelial population [2]. These neural progenitor (NP) cells are located in the ventricular zone (VZ), migrating away to the mantle layer (ML) upon neuronal differentiation (**Figure 1**).

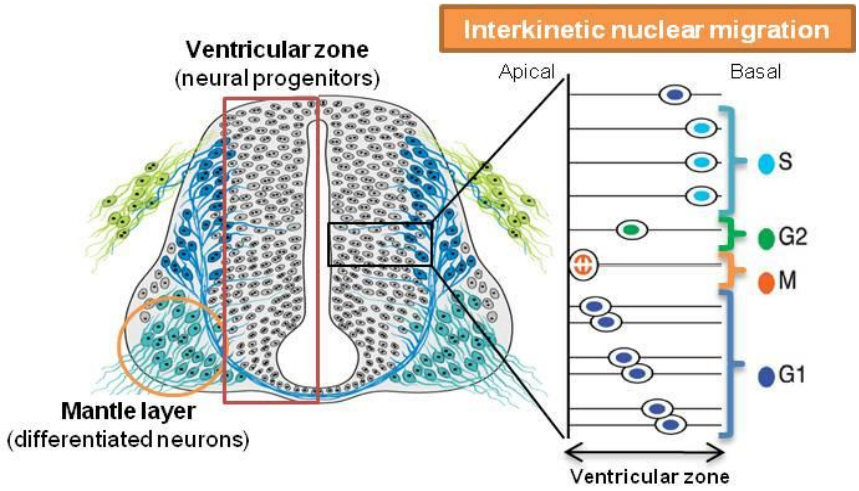

Figure 1: The neural tube and interkinetic nuclear migration. The neural tube is divided into two major regions: the VZ, where NPs reside, displaying the characteristic INM, and the ML, to where cells migrate when they differentiate. The nucleus of a NP migrates within the cytoplasm (INM) according to the phases of the cell cycle being at the basal region of the VZ during S-phase and at the apical region of the VZ during mitosis. Adapted from Kosodo et al, 2011.

The VZ is pseudostratified, with NPs being attached to both the outer basal and inner apical surfaces. The nucleus of a NP migrates within the cytoplasm in a characteristic movement between the apical and basal regions of the VZ (**Figure 1**), called interkinetic nuclear migration (INM), that was first described by Sauer in 1935 (reviewed in [3], [4]).

A key feature of INM is that nuclear migration is correlated with the phases of the cell cycle of NPs, with mitosis occurring when the nucleus is located at the apical region of the VZ, and S-phase when it is at the basal region (**Figure 1**) (reviewed in [4], [5]). However, the mechanisms involved in the coordination between INM and the cell cycle are not clear. It has been reported that inhibition of INM by inhibiting actin or microtubule polymerization does not influence cell cycle progression. In contrast, INM stops when the cell cycle is blocked [2], (reviewed in [3]). These results suggest that the regulators of the cell cycle also regulate the molecular machinery involved in nuclear migration but the contrary does not seem to occur with nuclear migration not being necessary for cell cycle progression. Recently, a molecular mechanism connecting cell cycle to INM was described [6]. In this study, the microtubule-associated protein Tbx2 was

reported to be translocated to the apical process of NPs during G2 phase, ensuring the proper movement of nuclei to the apical region during the appropriate cell cycle phase (S-G2-M transition).

Regarding INM functions, it has been suggested that it might act to increase the density of proliferating NPs while maintaining their apical and basal attachments during mitosis and consequently junction-associated signaling molecules. Another possible function for INM is a role in the determination of cell fate diversification of NPs (reviewed in [3]). Cell fate determinants, such as Notch or Delta1 are expressed specifically in the apical or basal regions of the VZ [7] and consequently INM may generate heterogeneity between NPs, according to the differential nuclear positions (reviewed in [3]).

NPs in the VZ can undergo different types of cell division: proliferative symmetric cell divisions (PP division), expanding the pool of NPs, and asymmetric or symmetric neurogenic cell divisions (PN and NN divisions, respectively), which generate neurons (reviewed in [9], [10]) (**Figure 2**).

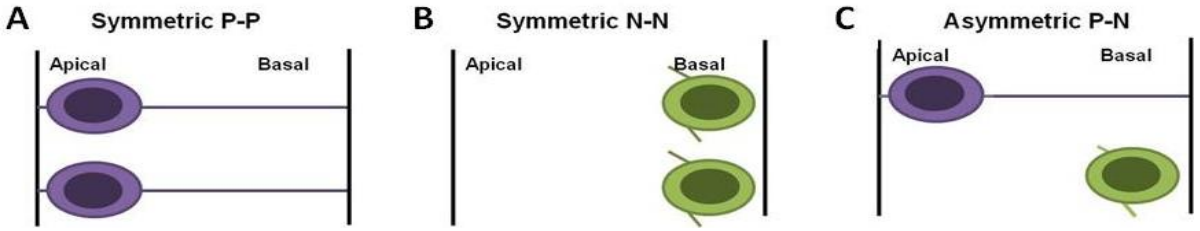

Figure 2: Types of cell division of NP cells. The scheme summarizes the types of cell division that NPs can undergo. **(A)** Symmetric cell division originating two neural progenitors (PP). **(B)** Symmetric cell division originating two neurons (NN). **(C)** Asymmetric cell divisions originating one neural progenitor and one neuron (PN).

It has been observed a correlation between the type of cell division and the angle of the mitotic cleavage plane relative to the lumen of the VZ. NN cell divisions were reported to be generated by vertical cleavage planes, while PP and PN divisions can occur in all orientations, suggesting that cleavage plane orientation would only be important in cells with potential to generate neurons [10]. Regarding PN cell divisions, it has been observed that asymmetric inheritance of the apical membrane is indicative of asymmetric daughter cell fates [8], [11]. It was therefore suggested that the correlation between the angle of mitotic cleavage plane and the type of cell division involves the distribution of the apical elements of the dividing cell between the two daughter cells: vertical cleavage planes would bisect the apical elements (distributing them equally to the daughter cells) while horizontal cleavage planes would bypass them (resulting in an unequal distribution of the apical elements) (reviewed in [9]). Contradictory data has been published regarding the cell fate of the cell that inherits the apical membrane:

some results suggest that the daughter cell that inherits it is going to be maintained as a progenitor cell [8], while others suggest that the cell that inherits the apical membrane is going to differentiate into a post-mitotic neuron [11]. In addition, it has also been reported that the length of the G1 phase of the cell cycle might play an important role in the type of cell division that NPs undergo [6], (reviewed in [12], [13]). NPs that undergo PN cell divisions have a longer G1 phase than the ones that undergo PP cell divisions suggesting that the lengthening of G1 in neuroepithelial cells is correlated with the neurogenic potential [10]. Further analysis of the cell cycle and the type of cells generated will be essential to better elucidate their relation and clarify previous contradictory reports.

1.2 Notch pathway in vertebrate neurogenesis

During neurogenesis, a balance between the proliferation of NPs and their differentiation into neurons needs to be maintained, in order to produce the correct number and types of neurons at the right time. It is known that the Notch pathway, a highly conserved mechanism among metazoans, is implicated in this process, controlling cell fates through cell interactions (reviewed in [14-16]). The Notch receptor gene was first characterized in *Drosophila melanogaster* and encodes a cell surface type I transmembrane protein with an extracellular ligand-binding domain and a cytoplasmic domain that acts in signal transduction. The extracellular domain comprises epidermal growth factor (EGF)–like tandem repeats, thought to be involved in ligand binding, and a cystein-rich conserved region that appears to negatively regulate receptor activation (reviewed in [17]). In the cytoplasmic domain there are 6 tandem ankyrin repeats (that are sufficient for Notch activity), a glutamine-rich domain, and a PEST sequence that is involved in Notch protein turnover (reviewed in [14], [17]). The Notch receptor is subjected to post-translational modifications in the trans-Golgi network by proteases of the Furin-family, and is then translocated to the plasma membrane where it works as an heterodimer that comprises an ectodomain and a membrane tethered intracellular domain (reviewed in [18]). The Notch ligands are also type I transmembrane proteins of the DSL family (invertebrate ligands Delta, Serrate and LAG-2). Similar to the Notch receptors, members of the DSL family of Notch ligands have multiple (EGF)–like tandem repeats in the extracellular domain, but unlike the receptor they have characteristic degenerate N-terminal EGF repeats. The Notch ligands do not share the same functions between themselves, as they seem to regulate different developmental decisions (reviewed in [17]).

Upon ligand-receptor interaction, the Notch receptor undergoes a series of proteolytic cleavages mediated by a γ-secretase complex, resulting in the release of the Notch intracellular domain (NICD) to the cytoplasm. NICD is then translocated to the nucleus, where it binds CSL

(a DNA-binding protein) and recruits Mastermind, forming a ternary complex. In the absence of NICD, CSL binds to specific regulatory regions, acting as a repressor. Upon Notch activation, the complex suffers conformational changes replacing the co-repressors by transcriptional co-activators (such as Mastermind), which activate the expression of Notch target genes (reviewed in [15], [18], [19]). The main Notch targets are the *HES* genes that encode basic helix-loop-helix (bHLH) transcriptional repressors. The HES proteins repress the expression of proneural bHLH genes (like Mash1 or Neurogenin2) that are responsible for the induction of the neural differentiation program (reviewed in [15], [20]) (**Figure 3**).

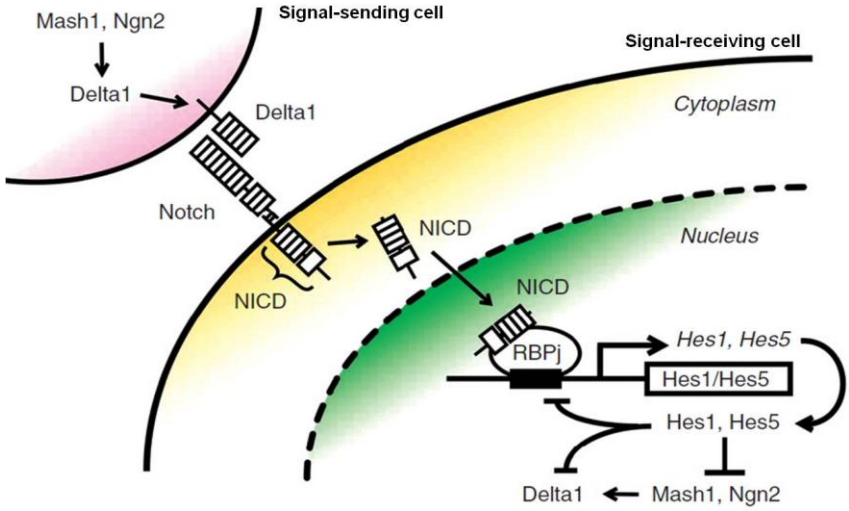

Figure 3: A scheme representing the Notch signaling pathway. The proneural genes (like Mash1 and Ngn2) induce the expression of Notch ligands (like Delta1), activating Notch in the neighboring cells. In these cells, the receptor-ligand interactions trigger a series of proteolytic cleavages that result in the release of NICD. NICD is then translocated into the nucleus where it forms a complex with RBPj (CSL). NICD/RBPj complex functions as a transcriptional activator of Notch target genes (like the *Hes* genes). The *Hes* genes, in turn, inhibit their own expression and the expression of proneural genes. As a result, cells where Notch is not activated express proneural genes and differentiate into neurons while in cells that have activated Notch, proneural gene expression is repressed and therefore these cells are maintained as progenitors. Adapted from Kageyama 2008.

The Notch pathway ensures the generation of cells with different fates in a population of cells that have similar developmental potential by the mechanism of lateral inhibition (reviewed in [18], [19], [21]). By this mechanism, one cell is chosen from a group of equivalent cells to acquire one determined cell fate and by competing with its neighbors inhibits them from acquiring the same fate (reviewed in [16], [19]). Initially, in the vertebrate neural tube, all cells are equivalent, expressing similar levels of both Notch ligands and proneural genes (**Figure 3**). At a certain point and due to stochastic variations, one cell starts to express higher levels of Notch ligands (signal sending cell) and consequently becomes more effective at activating Notch in neighboring cells. The signal receiving cells activate the expression of Notch target genes (like the *Hes* genes) that are going to repress the expression of proneural genes and Notch ligands. Consequently, signal receiving cells have lower levels of proneural gene expression and are

maintained as progenitor cells. In contrast, signal sending cells up-regulate the expression of proneural genes, differentiating into post-mitotic neurons (reviewed in [15], [20-22]).

In differentiating neurons ligand expression is transient, being down-regulated upon cell migration from the VZ to the ML. As a consequence, the levels of Notch activity in the neighboring cells are reduced, the lateral inhibition process is reset and NPs are able to respond to new signals and to decide again if they enter differentiation or are maintained as NPs. This suggests that Notch activity levels in NPs are not constant and that Notch signaling might be dynamic. The dynamic nature of Notch signaling has already been observed in different systems by the detection of fluctuations or oscillations in Notch target genes. It was observed that the bHLH component of Notch pathway, *Hes1*, is cyclically expressed in the presomitic mesoderm [23]. *Hes1* expression fluctuations were also observed in isolated NP cultures [24]. However, it is known that other signaling pathways, like the Jak/Stat pathway, are implicated in *Hes1* fluctuations [24], (reviewed in [20], [25]), and therefore *Hes1* expression is not a specific readout of Notch activity.

In agreement with the dynamic activity of Notch signaling, Notch components were reported to be expressed in apical-basal gradients in the VZ of the neural tube, suggesting that NPs are able to activate Notch only in specific phases of the cell cycle. In the developing zebrafish retina, the activated form of the Notch receptor is expressed at higher levels in the apical region of the neuroepithelium, where NPs are at G2/M/G1 phases of the cell cycle, while Delta1 mRNA is expressed at higher levels at the basal region [26]. It was suggested that the INM regulates the duration and level of exposure of NPs to Notch signaling in the apical region of the VZ: in retinas of zebrafish mutants for the microtubule motor associated protein Dynactin-1, NPs nuclei move more quickly and deeply to the basal region of the VZ and more slowly to the apical region, resulting in less exposure to Notch signaling and a consequent premature exit of NPs from the cell cycle, leading to massive neuronal differentiation [26]. In the VZ of the developing chick neural tube similar patterns of expression of Notch components were also observed, with Notch1 mRNA being expressed specifically in the apical region [7]. In the developing chicken brain it was observed that Notch mRNA expression is reduced in S-phase [7], [27]. These results suggest that Notch activity occurs distinctively in a specific region of the neuroepithelium where cells undergo mitosis. However, in the developing mouse brain, the activated form of the Notch receptor is not detected in the apical region of the VZ. Instead, it is detected in S-phase cells, suggesting that Notch is not activated in mitosis [28]. These contradictory results might indicate different regulations of Notch activity in different tissues and organisms.

Further analysis of Notch activity in NPs is thus necessary to determine whether activation occurs in specific cell cycle phases. Also, analysis of single NPs using a bona fine readout that responds only to Notch signaling is essential to assess if the Notch pathway can be activated more than once in the NPs life-time and if Notch activation is necessary to maintain these NPs in an undifferentiated state.

1.3 Embryonic Stem cells

Embryonic Stem (ES) cells are derived from the inner cell mass of embryos in the blastocyst stage (reviewed in [29]) and are characterized by their self-renewal capacity (being able to proliferate indefinitely under the appropriate conditions) and their pluripotency capacity (giving rise to all cell types from the three germ layers - ectoderm, endoderm and mesoderm) (reviewed [30]). Initially, the maintenance of mouse ES cells in culture was dependent on a proper medium, containing the necessary metabolites, nutrients and serum and also on the presence of feeder cells. Currently, it is known that feeder cells can be replaced by a specific cytokine, leukaemia inhibitory factor (LIF) and serum by BMP4 (reviewed in [31], [32]). Upon withdrawal of LIF, cells lose their undifferentiated characteristics and rapidly differentiate (reviewed in [32]). Due to their unique properties, ES cells have been used as a powerful tool for the development of cell-based therapies, generation of cellular disease models, test of new drugs and also to unravel the molecular mechanisms and pathways involved in the differentiation fate of a pluripotent cell (reviewed in [30], [34]).

ES cells can be driven into neural differentiation by the formation of multicellular aggregates, embryoid bodies (EBs), or by adherent monolayer cultures [35-37]. However, neural differentiation of ES cells mediated by EBs formation is very inefficient, occurring only in a small fraction of cells [36]. A more efficient method to drive neural differentiation of ES cells relies upon adherent monolayer cultures and was firstly described in 2003 by Ying and co-workers [36]. In this protocol, ES cells are cultured in feeder-free conditions in the presence of a serum-free medium that does not contain LIF or BMPs, which are known to inhibit neural differentiation. Therefore, cells leave the undifferentiated state, passing through successive stages from an identity of ES cells to NPs and organize in rosette-like structures [35] (**Figure 4**). These rosettes have been shown to mimic the *in vivo* neural tube as they show: (i) proper apico-basal polarity, with apical markers localizing at the centre of the rosette and differentiating neurons migrating to the outer surface of the rosette; (ii) proper cell cycle stage localization, with mitosis occurring at the apical surface (centre) of the rosette and S-phase at the periphery, reproducing the characteristic INM observed in the VZ of the vertebrate neural tube; (iii) active Notch signaling in

NPs but not in differentiated cells; and (iv) proper timing of production of neurons and glia. Additionally, as rosettes are two-dimensional structures, they are much easier to analyze and to image than their embryonic counterpart, being a useful tool to study Notch activity in single cells.

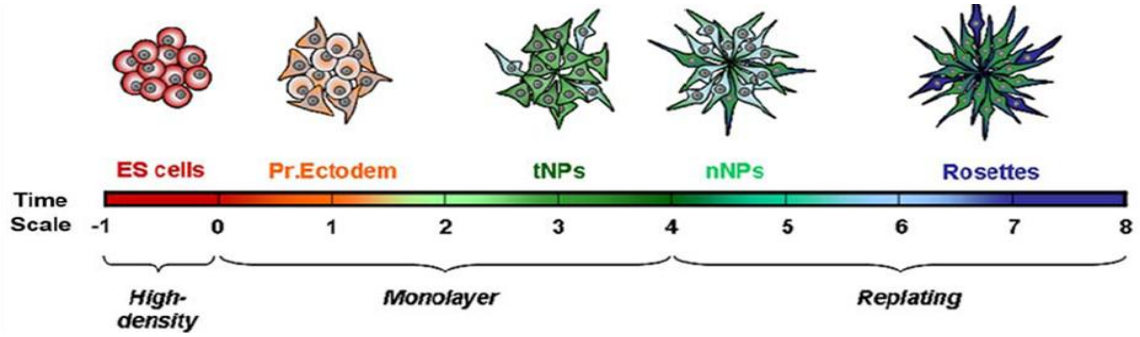

Figure 4: Schematic representation of the successive competence states acquired along the monolayer neural differentiation protocol. During neural differentiation ES cells pass through successive states of competence. ES cells initially acquire characteristics of Primitive Ectoderm (Pr.Ectoderm). Then, a transient population of NPs (tNPs) emerges and gives rise to a NPs population that is able to originate neurons (nNPs). This population of NPs will then originate the final set of NPs organized in rosette-like structures having proper Notch activity, INM and production of differentiated neurons. Adapted from Abranches et al 2009.

ES cells can be modified to generate ES cell lines expressing reporter proteins (like GFP) under the control of specific promoters, allowing to monitor gene expression in live ES cells [1], (reviewed in [37]). To generate reporter ES cell lines there are several aspects to consider: (i) the promoter used to drive the expression of the reporter protein; (ii) the type of reporter protein that is going to be used; and (iii) the strategy to engineer the desired cell line.

Reporters of Notch signaling have been described to use either synthetic promoters (repeats of CSL binding-boxes) [39] or promoters of Notch target genes (like *Hes1* or *Hes5*) [1], [23], [24], [40] to drive the expression of the reporter proteins. Expression of reporter proteins driven by synthetic promoters does not reflect all Notch activity [39]. Therefore, the use of Notch target genes seems to be a better choice. Among the Notch target genes, it has been shown that *Hes1* expression is not affected by the inactivation of the Notch pathway in the developing embryo [41] and does not respond specifically to Notch signaling [24]. In contrast, *Hes5* expression is dramatically reduced in Notch mutants [41], suggesting that *Hes5* is a specific Notch target gene in the developing nervous system. Therefore, *Hes5* promoter would be the most appropriate to use as readout of Notch activity.

The characteristics of the reporter protein to be used in the generation of this reporter cell line are very important. One of the most relevant features of the reporter protein is its half-life. Ideally, the reporter protein should have the same half-life as that of the protein coded by the gene that is going to be monitored, so that the onset and termination of expression can be observed. This is particularly important for proteins with short half-lives, such as the HES5

protein that has a half-life shorter than 1hr [42]. In this case an unstable reporter protein should be used to visualize its expression. The reporter should be unstable both at the protein and mRNA levels so that it allows the precise monitoring of Notch activity.

Transgenic ES cells can be generated by different methods: (i) site-specific targeting of the cDNA encoding a reporter protein into the locus of the gene of interest or (ii) random integration into the genome of the potential regulatory regions of the gene of interest linked to a cDNA encoding a reporter protein. The first method has the advantage of normally resulting in a correct expression of the reporter. However, the process is very inefficient and results in a cell line with only one functional allele of the targeted gene. In the second method, in which a potential regulatory region linked to a cDNA encoding a reporter protein is randomly integrated into the genome, the expression of the reporter is frequently influenced by the chromosome integration site. Furthermore, this strategy normally requires that the promoter and other regulatory regions of the gene of interest are known.

One method that allows overcoming these limitations is the use of Bacterial Artificial Chromosome (BAC) vectors expressing the reporter protein. A BAC is a DNA construct based on the conjugation plasmid (F plasmid) of *E. coli* that can be electroporated into ES cells. These vectors carry long genomic regions, containing entire genes as well as distant flanking cis-regulatory regions that are necessary for proper gene regulation and expression (reviewed in [43]). BACs can be modified to express a reporter protein with all the regulatory sequences of the gene of interest, allowing the monitor of gene expression. This is very important because reporters of Notch activity driven by a 0.76-Kbp promoter of *Hes5* showed not to fully mimic the expression of the endogenous *Hes5* gene [44]. The large size of these BAC vectors also diminishes the effects of the integration site on reporter expression [43]. Besides these advantages, as BAC vectors integrate randomly into the genome they do not disrupt the endogenous copies of the gene of interest.

The generation of a reporter using a BAC vector comprises several successive steps: (i) choice of a BAC vector that comprises the coding region of the gene of interest and the largest possible flanking regions; (ii) generation of the reporter cassette (in a plasmid) containing the cDNA encoding the reporter protein, appropriate selectable markers and homology regions that are needed for a step of homologous recombination in *E.coli*; (iii) BAC recombineering (homologous recombination) performed in bacteria, replacing the coding region of the gene of interest in the BAC by the reporter cassette DNA, present in the plasmid; (iv) purification of the modified BAC; and (v) its electroporation into ES cells and the creation of a stable cell line (reviewed in [45], [46]). This strategy has already been used to generate reporter systems of

Notch signaling [1], [40]. Specifically, Tomishima and co-workers engineered an ES cell line (*Hes5::GFP*, from here on referred as HT cell line) expressing a stable reporter protein (GFP) under the control of the *Hes5* promoter [1].

A reporter of Notch activity has also previously been generated in the Laboratory using the promoter of the *Hes5* gene and BAC recombineering strategy (*Hes5::VNP* cell line). In contrast with the reporter protein employed in the HT cell line, the reporter protein of the Hes5::VNP cell line is unstable, comprising Venus (a yellow fluorescent reporter protein), NLS (a nuclear localization signal that targets the reporter protein to the nucleus) and PEST (a peptide sequence that reduces the half-life of the protein to which it is fused). This reporter protein is based on an already described Venus-PEST unstable reporter [47] that allows monitoring of 2hrs periodic waves of *Lfng* reporter expression. Also, *Hes5::VNP* cells express an unstable reporter mRNA due to the presence of post-transcriptional regulatory signals in the 3'UTR, which decreases the time of reporter activity.

Thesis Outline

The objective of this thesis is to monitor Notch activity in live neural progenitors, in order to investigate the role of Notch signaling during vertebrate neural development. The specific aims were:

- Determine if Notch is activated more than once in a life of a NP;
- Determine if Notch activation occurs preferentially in a specific phase of the cell cycle;
- Define the timing of commitment to neurogenesis;
- Assess if Notch activity is correlated with the cell fate of NPs.

In order to do so, a previously described ES cell line expressing a fluorescent reporter protein under the control of the *Hes5* promoter (HT) [1] was analyzed to assess its suitability to perform these experiments. Specifically, the stability of the reporter protein (GFP) was investigated by determination of its half-life. In addition, the novel ES cell lines generated in the Laboratory were also investigated for their suitability as reporters of Notch activity. These cell lines, (*Hes5::VNP*), express an unstable reporter protein upon Notch activation, which might allow the precise monitoring of Notch activation and termination in NPs, in real-time and with single cell resolution.

CHAPTER 2

Materials and Methods

2. MATERIALS AND METHODS

2.1 Materials /Reagents

2.1.1 Embryonic stem cell lines

The mouse ES cell lines used in this project are listed in **Table S2** (Chapter 6).

2.2.2 Reagents

The reagents, solutions/media, primers and antibodies used in this study are listed in **Table S3**, **Table S4**, **Table S5** and **Table S6**, respectively (Chapter 6).

2.2 Methods

2.2.1 ES cell culture

All steps involved in the manipulation of ES cells were performed in a sterile laminar flow hood class II, type A/B3.

2.2.1.1 Expansion of ES cells

ES cells were thawed in pre-heated Glasgow Modified Eagles Medium 1x (GMEM) and plated on 0.1% (v/v) gelatin-coated dishes in supplemented GMEM (with 2ng/ml LIF). Medium was changed 6hrs later to eliminate DMSO residues. ES cells were grown at 37ºC in a 5% (v/v) CO_2 incubator on gelatin-coated dishes in supplemented GMEM. The morphology and health of the cells were assessed daily by direct visualization on a bright field microscope and cells were passaged every other day, at a constant plating density of $3x10^4$ cells/cm^2. For each passage, cells were washed twice with PBS and dissociated with 0,025% trypsin for 2-3min at 37ºC. Cells were immediately resuspended in GMEM (in order to neutralize trypsin), centrifuged at 1200 rpm for 4min and again resuspended in GMEM. Cells were counted using trypan blue dye exclusion method and the required amount of cells was then plated on gelatin-coated dishes in supplemented GMEM. To prepare ES cell stocks, $3x10^6$ cells were frozen in GMEM 1x with 10% Dimethyl sulphoxide (DMSO) and stored in liquid N_2. Every time cells were frozen, a sample was collected to test for Mycoplasma contamination (see section 2.2.5.2).

2.2.1.2 ES cell differentiation

2.2.1.2.1 Embryoid Bodies formation

To generate EBs, ES cells were plated at $2x10^4$ cells/cm^2 in GMEM 1x on bacterial grade dishes. EB formation was checked on day 1 and the morphology was assessed every day, until day 8, to confirm the normal differentiation pattern of the cultures.

2.2.1.2.2 Neural differentiation in adherent monolayer cultures

The protocol described in Abranches et al. was used (**Figure 4**) [35]. Briefly, 24hrs before the beginning of the monolayer differentiation protocol (day -1), cells were plated at high density ($1x10^5$ cells/cm^2) on gelatin-coated dishes in serum-free medium (ESGRO Complete Clonal Grade medium) supplemented with 2ng/ml of LIF. After 24hrs (day 0), cells were observed on a bright field microscope to assess morphology and confluence of the cultures. Cells were then dissociated, counted and plated on fresh gelatin-coated dishes in RHB-A medium at a cell density of $3x10^4$ cells/cm^2. Medium was changed at day 2 and cells were replated at day 4. For the replating, cells were dissociated, counted and plated at a cell density of $2x10^4$ cells/cm^2 on PDL-Laminin coated tissue culture plastics or coverslips in RHB-A supplemented with 5ng/mL of murine bFGF and medium was changed at day 6. According to the analysis to be made, at the end of the neural differentiation protocol (day 8) different cell treatments were performed (see sections 2.2.3.2, 2.2.4.1, 2.2.3.3).

2.2.1.2.2.1 PDL-Laminin Coating

The tissue culture dishes or coverslips were covered with PDL solution (10µg/mL in PBS) and were left for 1h at room temperature (RT). The dishes were then washed twice with PBS, covered with Laminin solution (2,5 µg/mL in PBS) and left overnight (O/N) at 37ºC and 5% CO_2. Laminin was removed immediately before plating the cells.

2.2.2 ES cell line generation

2.2.2.1 BAC electroporation into ES cells

In this work were used ES cell lines that had been previously generated in the Laboratory. These cell lines were engineered, by the insertion of a BAC vector, to express a reporter protein driven by the promoter of the *Hes5* gene. The BAC was chosen from the CHORI database (REF. RP24-345H15), comprising the *Hes5* coding region and the largest possible flanking regions. The BAC was manipulated through recombinogenic engineering (reviewed in [46]), replacing the coding region of the *Hes5* gene by the reporter cassette DNA. This reporter cassette contains the cDNA of the reporter protein followed by a Neomycin resistance cassette flanked by loxP sites. The reporter protein (VNP) has unstable kinetics, comprising Venus, a yellow fluorescent reporter protein with bright signal and fast maturation, NLS, a nuclear localization signal that targets the reporter protein to the nucleus and PEST, a peptide sequence that reduces the half-life of the proteins to which it is fused. The modified BAC vector was electroporated into E14tg2a ES cells, integrating randomly and in a stable manner in the genome. After electroporation, clonal ES cell lines were generated.

2.2.2.2 Selection cassette removal from ES cells

Cells were dissociated (section 2.2.1.1) and $5x10^6$ cells were resuspended in ice cold PBS. Cells were electroporated with 0.5µg of pCAGGsPuromycin and 50µg of pTurboCre, at 400V, 250µF. After the electroporation procedure, cells were left for 10min at RT and plated in GMEM on gelatin-coated dishes. Medium was changed 6hrs later to supplemented GMEM. Selection started in the next day, by adding 2µg/µL of Puromycin to the medium, and lasted for two days. After that, medium was changed every day for 9 days. Subsequently, 24 individual colonies of cells were manually picked and grown clonally. Genomic DNA from each clone was extracted (section 2.2.5.1) to perform screening PCRs. As a control for the electroporation, $2.5x10^6$ cells were treated as described previously being electroporated with no DNA.

2.2.3 Protein expression analysis

2.2.3.1 Fluorescence Activated Cell Sorting (FACS)

To monitor the expression of GFP/VNP reporters, FACS analysis was performed on a FACS Calibur cytometer (Becton Dickinson). Cells were dissociated and $5x10^5$ cells were resuspended in FACS buffer. Live cells were gated based on forward and side scatter and by propidium iodide dye exclusion. In each data acquisition 10000 gated events were recorded and the data obtained was subsequently analyzed using the *FlowJo* software.

2.2.3.2 Immunocytochemistry

Cells in coverslips were washed twice for 5min in PBS and fixed for 15min at 4ºC with 4% (w/v) paraformaldehyde (PFA) in PBS. Cells were washed twice for 5 min in PBS and residual PFA was washed with 0.1M Glycine in PBS for 10min at RT. Cells were permeabilized by incubation with 0.1% Triton in PBS for 10min at RT and blocked with blocking solution for 30 min at RT. Primary antibodies (see **Table S5**, Chapter 6) were diluted in blocking solution and incubated O/N at 4ºC. Cells were then washed three times with TBST for 5min. Appropriate secondary antibodies (see **Table S5**, Chapter 6) were diluted in blocking solution and incubated for 30min at RT. Cells were washed three times for 5min with TBST and counterstained with 4',6-diamidino-2- phenylindole (DAPI) for 5min. After washing three times in PBS for 5min, cells in coverslips were mounted with Mowiol mounting medium.

2.2.3.3 Determination of protein half-life

At day 7 or day 8 of the neural differentiation protocol, cells in coverslips were treated with 100 µg/mL of cycloheximide, an inhibitor of translation. Cells were then harvested after 30min, 1h, 3h and 6h and immunocytochemistry was performed to detect expression of the GFP reporter (section 2.2.3.2). Alternatively, day 4 NPs were plated in MatTek dishes and rosettes were allowed to form. Cells were treated with cycloheximide at days 7 or 8 of the neural

differentiation protocol and time-lapse movies were performed during 6 or 12hrs. Cells were imaged on an inverted fluorescence Zeiss Axiovert 200M microscope in a chamber kept at 38 ºC with a humidified 5% CO_2/95% air mix. Images were captured using a 40x/0.75 NA objective lens (Zeiss EC Plan-Neofluar) with an Hg-arc lamp and acquired with Metamorph software. Multiple points in the dish were chosen and cells were imaged with 30min or 1hr intervals. Data was analyzed using ImageJ software by measuring the average intensity of a maximum intensity projection of three Z planes of selected cells in the different time-points. The background was subtracted for each cell at each time-point.

2.2.4 RNA expression analysis

2.2.4.1 Isolation of total RNA from cultured ES cells

To extract total RNA from the cells, 10^6 cells were collected and washed twice in PBS. The RNA was then extracted using the *High Pure RNA Isolation Kit* (Roche), according to the manufacturer's instructions, and quantified (section 6.1.1).

2.2.4.2 cDNA synthesis

To synthesize cDNA from the total RNA extracted it was used the Superscript II Reverse Transcriptase system, with random primers, according to the manufacturer's instructions. The cDNA obtained was used to perform standard PCR (section 6.1.3).

2.2.5 DNA analysis

2.2.5.1 Isolation of genomic DNA for screening PCRs

For the extraction of genomic DNA for ES cell cultures, 10^6 cells were washed twice with ice-cold PBS and immediately lysed with 1mL of SNET containing 400µg/mL of proteinase K. Cells were incubated O/N at 55ºC and then an equal volume of phenol:chlorophorm:isoamyl alcohol was added. The mixture was incubated for 30min at RT. The aqueous phase was collected after centrifugation at 2000rpm for 5min at RT. The DNA was precipitated by adding an equal volume of isopropanol with 1:10 sodium acetate and centrifuging at 13000g for 15min at 4ºC. Pellets were washed with 70% ethanol and resuspended in TE O/N at 4ºC. DNA was quantified (section 2.2.6.1).

2.2.5.2 Mycoplasma detection

2.2.5.2.1 Control DNA extraction

For high quality small-scale preparation of mycoplasma positive control plasmid DNA, 3mL of an O/N bacterial culture of transformed competent cells, in the appropriate selective LB medium, was processed using the *Wizard Plus SV Minipreps DNA Purification System* (Promega), according to the manufacturer's instructions.

2.2.5.2.2 PCR for Mycoplasma detection

To check for the absence of mycoplasma contamination in ES cell cultures, samples were routinely collected using the following procedure. 10^6 cells were centrifuged at 2000rpm for 5min, resuspended in wash buffer and centrifuged again in the same conditions. The pellet was then resuspended in a 1:1 mix of solution A and solution B and incubated for 1h at 60ºC. The suspension was denatured, to inactivate proteinase K, by incubation at 90ºC for 10min.

The PCR for mycoplasma detection was performed using rTaq Polymerase, amplifying a conserved region in the 16S RNA gene. The amplification was performed with an initial step of denaturation at 95ºC for 5min, followed by 30 cycles of denaturation at 95ºC for 30sec, annealing at 58ºC for 1.5min and extension at 72ºC for 1.5min, and a final step of extension at 72ºC for 10min. The reactions were prepared for a final volume of 25µL: 3µL of sample, 1x buffer, 0.2mM dCTP, 0.2mM dGTP, 0.2mM dATP, 0.2mM dTTP, 25pmol of each primer and 2.5U of rTaq Polymerase. The PCR products were analyzed in agarose gel (section 6.1.2) The quality of the DNA preparation was confirmed by performing a PCR to detect GAPDH, a housekeeping gene that functions as an internal control. Also, a plasmid that carries an insertion that corresponds to the amplified fragment was used as a positive control (plasmid DNA was obtained as described in section 2.2.5.2.1).

2.2.5.3 Purification and precipitation of plasmid DNA for electroporation

To obtain high quality large-scale preparations of the plasmids to be electroporated into ES cells, 50 mL of bacterial culture of transformed competent cells, in the appropriate selective LB medium, were processed using the High Speed Plasmid Midi Kit (Quiagen) according to the manufacturer's instructions. Precipitation of the plasmid was done by adding 1/10 of the solution volume of 3M sodium acetate and 9/10 of isopropanol followed by centrifugation for 30 min at 13000 rpm. The pellet was washed with 70% ethanol in sterile water and resuspended in PBS in a sterile laminar flow. DNA was quantified (section 6.1.1) and DNA integrity was confirmed by agarose gel electrophoresis (section 6.1.2).

CHAPTER 3

Results

3. RESULTS

To identify a cell line that allows monitoring of Notch activity and its dynamics, different mouse ES cell lines expressing two different reporter proteins driven by the *Hes5* promoter were tested: (i) HT cell line (*Hes5::GFP*), described by Tomishima and co-workers [1], that expresses a stable reporter protein; and (ii) Ha, Hc, Hd and derivatives (HdB5 and HdB8) and He cell lines (together named *Hes5::VNP*), that were generated in the Laboratory (section 2.2.2.1) and express the unstable reporter protein VNP (**Figure 5**). The 46C cell line, which is a *Sox1*:GFP knock-in [36], was used as a control for the experiments.

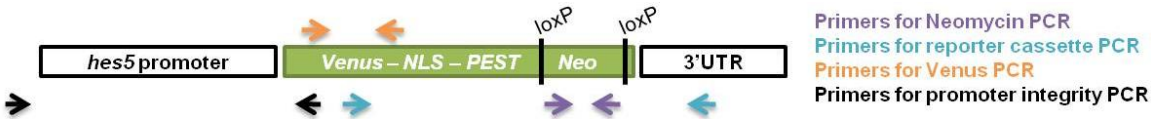

Figure 5: Structure of *Hes5::VNP* reporter cassette and Screening PCRs scheme. The *Hes5* BAC was modified replacing the coding region of *Hes5* by the reporter cassette. The reporter cassette comprises a cDNA encoding the reporter protein Venus-NLS-PEST (VNP) and a Neomycin resistance cassette flanked by loxP sites. To validate the removal of Neomycin cassette several PCRs were performed, which are illustrated in the figure by the arrows.

3.1 HT reporter ES cell line

The HT cell line was already described by Tomishima and co-workers [1] and was further validated in terms of its stemness potential: self-renewal and pluripotency capacity. The half-life of the reporter protein was determined to assess its suitability as reporter of Notch activity.

3.1.1 Self-renewal capacity

The self-renewal capacity of HT cells was analyzed regarding the morphology, viability and fold increase (FI) of cell cultures. The morphology was assessed by direct observation of the cells in an inverted bright field microscope. It was observed that HT cells grew in typical ES morphology with cells being organized in clusters with almost no differentiation (**Figure 6A-F**).

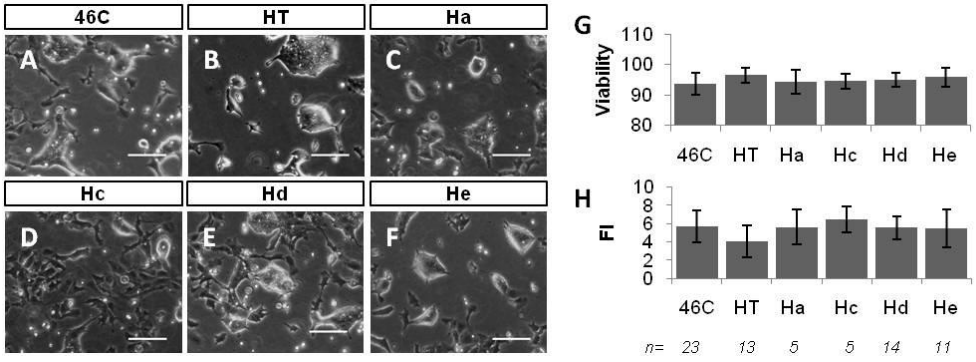

Figure 6: Self-renewal capacity of 46C, HT, Ha, Hc, Hd and He ES cell lines. **(A-F)** Bright field images of ES cells, organized in clusters. **(G)** The viability of the cell lines is not statistically different from that of the control cell line (46C). **(H)** FI values are within the normal range of variation of ES cells and are similar to those of 46C. These values represent the average of *n* passages from several replicates. (Scale-bar=100 µm)

Cells were passaged several times and the values of viability and FI were calculated as the average of several passages in many replicates. For each replica the viability was assessed, using the dye exclusion method by trypan blue and calculated by the ratio between the number of viable cells (that excluded the dye) and the total number of counted cells. The FI was determined by the ratio between the number of cells counted at the end of the passage and the initial number of plated cells. Viabilities and FI were observed to be similar to those of the control cell line (46C), confirming the self-renewal capacity of HT cells (**Figure 6G,H**).

3.1.2 Pluripotency capacity

The pluripotency capacity of HT cells was assessed regarding the ability to generate embryoid bodies (EBs) and, specially, the ability to undergo neural differentiation.

3.1.2.1 Embryoid bodies formation

To confirm the pluripotency capacity of the HT cells, the differentiation method through EBs was performed. HT cells generated EBs in a normal differentiation pattern (**Figure S1**).

3.1.2.2 Neural differentiation in adherent monolayer cultures

To further test the differentiation potential of HT cells into NPs, the neural differentiation protocol in adherent monolayer was performed [35]. Cell morphology was monitored daily on a bright field microscope and the formation of neuroepithelial rosettes was observed at day 8 of the protocol (**Figure 7A**). The values of viability and FI were analyzed at several time-points of the protocol (section 2.2.1.2.2). Both in high density and monolayer cultures, it was observed that HT cells had similar values of FI and viability to those of 46C cell line (**Figure 7B,C**).

The efficiency of the protocol was assessed by FACS analysis using as a control the 46C cell line that expresses GFP under the control of the *Sox1* promoter. *Sox1* expression is activated in proliferating neuroectodermal cells, allowing to monitor the neural commitment efficiency [35], (reviewed in [36], [47]). At day 4, 80% GFP positive cells were observed, a value that was maintained at day 8 (**Figure 7D**), confirming the efficiency of the protocol.

In HT cell line, FACS analysis performed at days 0, 4 and 8 of the protocol showed an increase in the expression of GFP along the protocol, with the reporter protein being expressed in approximately 60% of NPs at day 8 of the protocol. This increase was expected, since Notch starts to be active around day 3 [35] and is active in NPs at day 8 of the protocol. However, the levels of GFP expression are higher than the reported values that point out to 30% of the cells expressing *Hes5* at a certain time-point in E3 chick neural tube [49]. Also, the percentage of GFP-expressing cells is variable between replicates and dependent on the efficiency of neural commitment, in clear contrast with 46C cell line in which this variability is not so evident. These

results were confirmed by immunocytochemistry performed at day 8, labeling the GFP reporter protein of 46C and HT cell lines. Cells were co-labeled with N-cadherin, marking apical adherents junctions, to allow the identification of the centre of each rosette (**Figure 8A,B**).

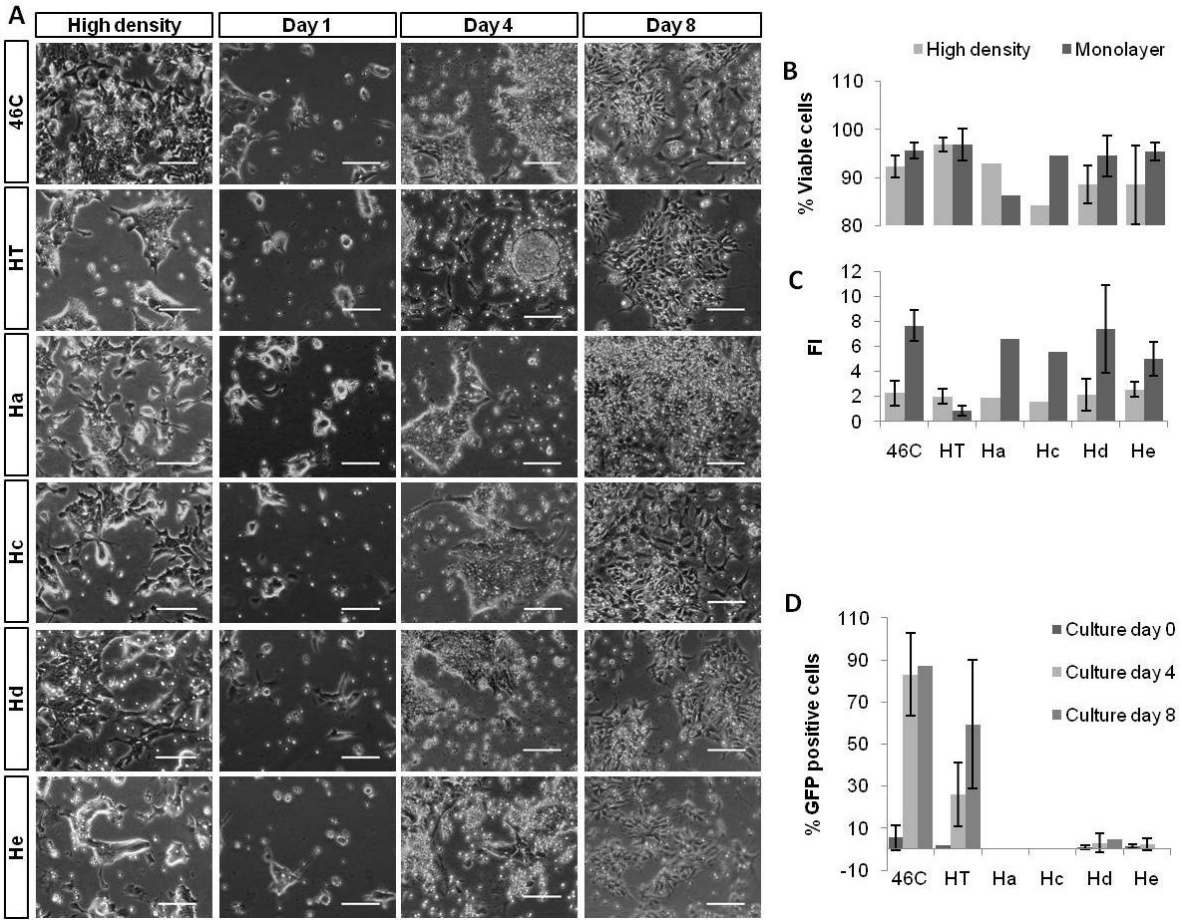

Figure 7: Monolayer neural differentiation of HT and *Hes5::VNP* ES cell lines. **(A)** Bright field image of ES cultures along the monolayer neural differentiation protocol. All cell lines showed typical morphology at each time-point being able to form neuroepithelial rosettes. **(B,C)** Values of viability and FI were similar to the ones of 46C cell line, both in High density and Monolayer cultures. **(D)** Analysis of the reporter proteins by FACS revealed that GFP is being expressed in approximately 60% of HT cells, while VNP is expressed only in Hd and He cell lines in approximately 5% of the cells. (Scale-bar=100µm)

3.1.3 GFP half-life

The suitability of the HT cell line as a good reporter of Notch activity was assessed by measuring the half-life of the reporter protein in neuroepithelial rosettes (section 2.2.3.3) using time-lapse movies and immunocytochemistry to visualize GFP.

Three time-lapse movies were preformed from which 72 cells were analyzed. It was observed that 12hrs after the blockage of translation the expression of GFP in HT cells, as measured by the fluorescence intensity, is still 75% of the initial value (**Figure 9A**). These results were confirmed by immunocytochemistry measuring the fluorescence intensity of 230 cells at several time points. It was observed that 6hrs after inhibition of translation blockage the levels of GFP

expression are very similar to those of the initial time-point (Figure 9B). These results show that the HT cell line cannot be used to monitor Notch activity since the reporter protein perdures in the cell for much longer than the HES5 protein [42], impairing the observation of possible variations in levels of Notch activity. Therefore, a novel cell line that expresses a reporter protein with a half-life closer to that of the HES5 needs to be generated in order to be able to monitor the onset and termination of Notch activity.

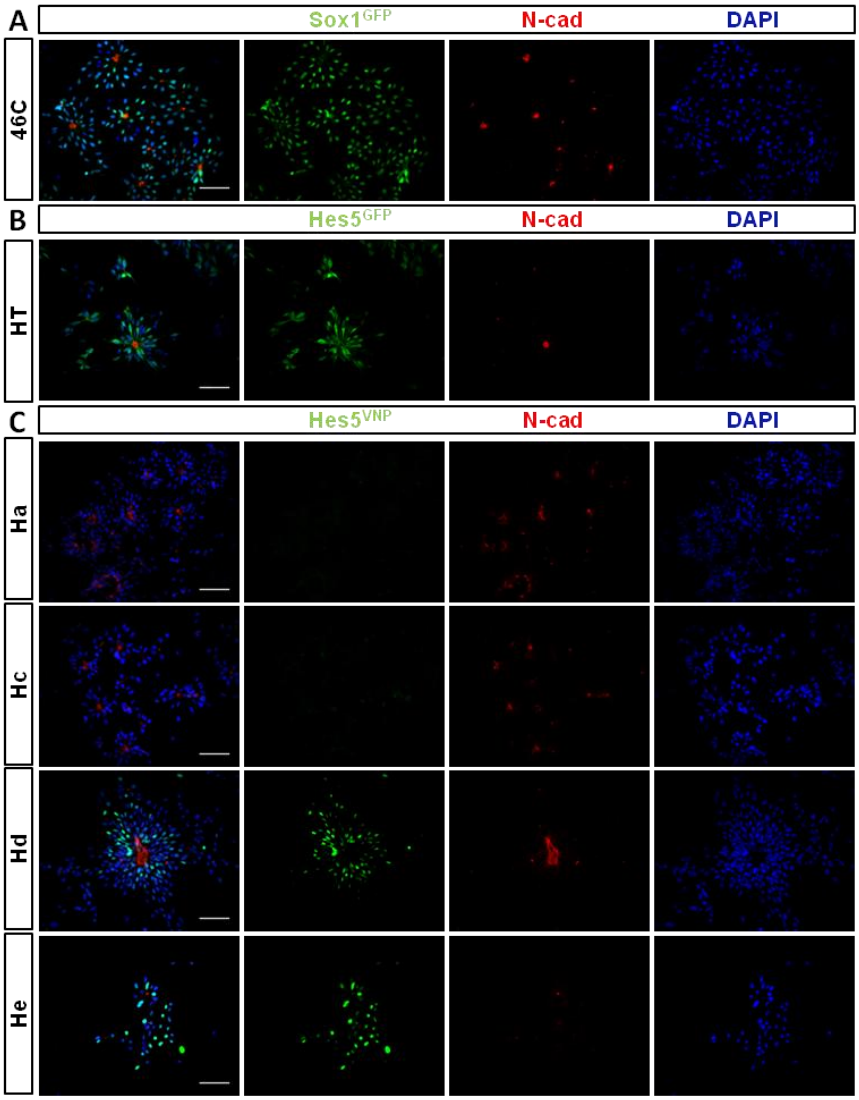

Figure 8: Immunocytochemistry of neuroepithelial rosettes preformed at day 8. Cells were labeled by immunocytochemistry using antibodies against N-cadherin (red), marking the apical junctions of the rosettes, and by GFP (green) marking the respective reporter protein: **(A)** 46C cells expressing GFP driven by the Sox1 promoter, **(B)** HT cells expressing GFP under the control of the *Hes5* promoter **(C)** *Hes5::VNP* cells expressing VNP under the control of the *Hes5* promoter. Nuclei are stained with DAPI. (Scale-bar=50µm)

3.2 Ha, Hc, Hd, He reporter ES cell lines

Four *Hes5::VNP* ES cell lines (Ha, Hc, Hd and He) were generated in the Laboratory by the electroporation of a modified BAC vector, expressing an unstable yellow fluorescent reporter protein that localizes to the nucleus (VNP) under the control of the *Hes5* promoter. Similarly to the analyses performed for the HT cell line (section 3.1), these novel cell lines were validated in terms of their stemness potential.

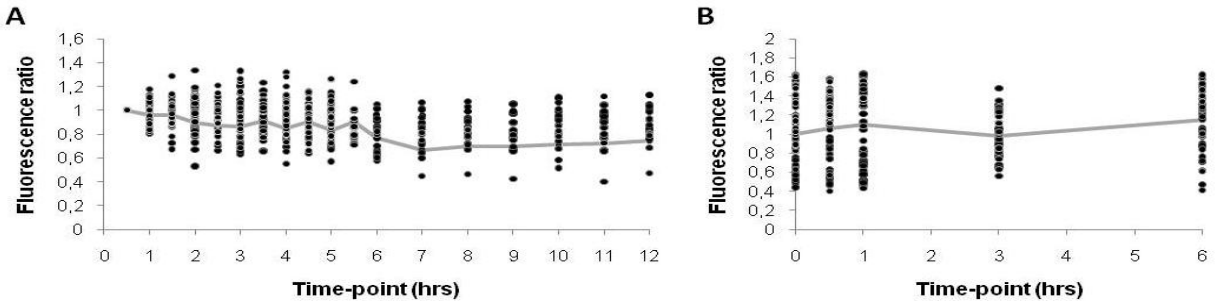

Figure 9: Intensity of GFP expression in HT cells after translation blockage. (A) Fluorescence intensities measured in time-lapse movies of neuroepithelial rosettes: the values were calculated for each cell by the ratio between the fluorescence intensity of each time point and the fluorescence intensity of the initial time point. After 12hrs of blockage of translation, the expression of GFP reporter is still ~75% of the initial value. **(B)** Fluorescence intensities measured in cells labeled with anti-GFP antibody: the values were calculated by the ratio between the fluorescence intensity of one cell at a certain time-point and the average of fluorescence intensities of all cells measured at the initial time-point. After 6hrs of translation inhibition the fluorescent ratio is similar to the initial value. The line represents the average between several replicates and for each time-point each dot represents one cell.

3.2.1 Self-renewal capacity

The morphology of the cells was assessed by their direct observation in an inverted bright field microscope. It was observed that all cell lines had a cluster-like ES morphology with almost no differentiation (**Figure 6C-F**). Furthermore, they all show values of FI and viability that were similar to the ones of 46C, confirming their self-renewal capacity (**Figure 6G,H**).

3.2.2 *Pluripotency capacity*

3.2.2.1 EB formation

To confirm the pluripotency capacity of the cell lines, the differentiation method through EBs was performed. Hd and He generated EBs in a normal differentiation pattern (**Figure S1C,D**).

3.2.2.2 Neural differentiation in adherent monolayer cultures

As done with the HT cell line, the neural differentiation capacity of the four ES cell lines expressing the *Hes5::VNP* reporter was assessed. Cell morphology was observed daily on a bright field microscope and the formation of neuroepithelial rosettes was observed at day 8 of the protocol, confirming that the four cell lines were able to undergo neural commitment (**Figure 7A**). The values of viability and FI were analyzed at several time-points of the protocol (days -1, 0, 4 and 8). Both in the high density and monolayer cultures, it was observed that the four ES cell lines had similar values of FI and viability to those of the 46C cell line (**Figure 7B,C**). The

efficiency of the neural commitment was again confirmed by FACS analysis performed at day 8, using the 46C cell line as a control, which showed 80% of GFP-expressing cells (**Figure 7D**).

FACS analysis at days 0, 4 and 8 showed that only two of the four clones (Hd and He) had a slight increase in VNP expression along the protocol. Hd is the cell line expressing higher levels of VNP at day 8 of the protocol, ~5% (**Figure 7D**), a value that is lower than the expected compared with previously published data in E3 chick, where approximately 30% of cells have been shown to express *Hes5* [49]. This might be due to the presence of the Neomycin resistance cassette before the polyadenylation signal, which could be causing the transcription of an incomplete reporter mRNA lacking a poly(A) tail sequence and that is potentially very unstable. The observed VNP expression levels for these novel cell lines are also lower than those observed for the HT cell line, as expected, since GFP expressed in HT cells is a stable reporter protein and consequently is not degraded as fast as the VNP reporter protein. VNP expression was confirmed by immunocytochemistry performed at day 8 (**Figure 8C**) using the same markers as in HT validation (GFP and N-cad) (section 3.1.2.2). It was also observed that the reporter is not expressed in all Hd and He cells, as expected, since Notch signaling is not active in all NPs at the same time (reviewed in [15]). Additionally, it was observed that the expression levels of the reporter protein are different between cells. This could mean that there are different levels of Notch activity between cells, but constant in each cell, or that Notch activity varies in individual cells and the differences observed result from the harvesting of cells in different phases of that variation. Overall these results suggest that the *Hes5::VNP* reporter cell lines might be adequate to monitor Notch activity, but the presence of Neomycin resistance cassette might be impairing VNP expression.

3.2.3 Removal of Neomycin resistance cassette

To allow the proper expression of the reporter protein, the Neomycin resistance cassette was removed. As this cassette is flanked by loxP sites, the Cre-loxP system was be used. Being Hd the *Hes5::VNP* cell line with the highest levels of expression of the reporter protein, it was chosen to proceed with the experiments.

3.2.3.1 ES cell electroporation

For the removal of the Neomycin selection cassette, Hd cells were electroporated with pTurbo-Cre, which encodes a Cre recombinase expressed under a strong promoter and that is targeted to the nucleus (increasing its efficiency), and pCAGGsPuromycin, which encodes a Puromycin resistance protein to allow for the selection of electroporated cells. The plasmids to be electroporated were isolated (section 2.2.5.3), quantified and the quality of the plasmids was

confirmed by the presence of a single band in an agarose gel (**Figure S2**) (an additional band indicates nicked plasmids, which cannot be used because they are more probable of integrating into the genome of the cells, causing subsequent deleterious effects). After electroporation, selection was performed with Puromycin for 2 days. Almost all cells detached from the plates but some small clusters remained attached and kept growing, in clear contrast with the negative control (cells electroporated without plasmid), where all cells detached (data not shown). When clusters started to be visible by eye (9 days after electroporation), 24 clusters were carefully picked (clones HdA1-A12 and HdB1-B12) and expanded. From the 11 clones that survived to the expansion, genomic DNA was extracted and used for PCR analysis (clones HdA2, HdA3, HdA8, HdA10, HdA11, HdB1, HdB4, HdB5, HdB7, HdB8, HdB11).

3.2.3.2 Validation of Neomycin resistance cassette removal

The removal of the Neomycin cassette was confirmed by the inability of cells to grow in Neomycin supplemented media and by PCR analysis. The self-renewal capacity of the clones was also analyzed to determine if any deleterious genomic effects occurred.

3.2.3.2.1 *Growth in Neomycin*

To identify the ES cell clones where the Neomycin cassette was excised, cells were grown in GMEM supplemented with 4µg/mL of Neomycin, for 4 days. From the 11 expanded clones, 7 of them were not able to grow upon Neomycin addition and, therefore, were good candidates to have excised the cassette (HdA2, HdA11, HdB1, HdB5, HdB7, HdB8, HdB11) (**Table S1**).

3.2.3.2.2 PCR validation

Several PCR reactions were performed in order to confirm the excision of the Neomycin cassette (see annealing regions of the primers in **Figure 5**). Specifically, a PCR to detect the Neomycin resistance gene and a PCR to detect the reporter cassette were used to confirm the results obtained by growth in Neomycin. The PCR to detect the reporter cassette amplifies a DNA fragment of 767 bp when the selection cassette is excised, but when the selection cassette is present the sequence between the annealing region of the primers is too long for PCR amplification. For all the clones, PCRs gave results that are coincident with the data from growth in Neomycin: clones where the selection cassette was removed have amplification of a DNA fragment correspondent to the PCR to detect the reporter cassette and no amplification of a DNA fragment correspondent to the Neomycin resistance gene (**Figure 10**). The results confirm that clones HdA2, HdA11, HdB1, HdB5, HdB7, HdB8, HdB11 excised the selection cassette. In addition, a PCR to detect Venus DNA was performed to ensure that it was not excised together with the Neomycin resistance cassette, due a recombination error. Only in the case of HdB7

clone the PCR did not amplify Venus DNA (**Figure 10**), and this clone was therefore discarded from future analyses. The quality of the DNA was assessed by PCR to detect GAPDH.

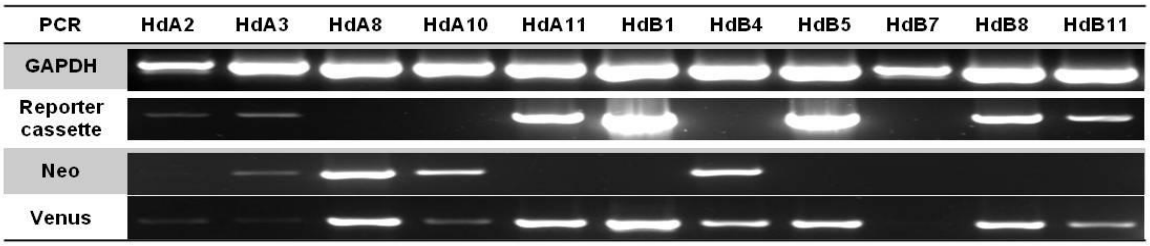

Figure 10: Screening PCRs. The clones HdA2, HdA11, HdB1, HdB5, HdB8 and HdB11 fully respected the PCR selection criteria for Neomycin selection cassette removal: (i) amplification of a DNA fragment with the correct size of the reporter cassette PCR; (ii) no amplification of Neomycin resistance gene DNA; (iii) amplification of *Venus* DNA.

3.2.3.3 Cell line choice

The clones HdA2, HdA11, HdB1, HdB5, HdB8 and HdB11 respected all the criteria of Neomycin resistance cassette removal validation. No significant differences were observed between the clones, regarding the self-renewal capacity (morphology, viability and FI), except for HdA11 and HdB1 that had many differentiated cells (data not shown) and consequently were not chosen to proceed with the experiments. Initial data suggested that the cell lines with higher values of viability and FI were HdB5 and HdB8. These were then chosen to be used in the following experiments and for further validation in terms of self-renewal capacity and reporter protein expression, to assess their suitability as reporters of Notch activity. Also, PCRs were performed to assess the integration of the electroporated plasmids (pTurboCre and pCAGGsPuromycin) into the genome of the cells, which could cause deleterious effects. The absence of the plasmids was confirmed for both ES cell lines, since no DNA bands could be detected (**Figure S3**). The quality of the DNA was confirmed by a PCR to detect GAPDH.

3.3 HdB5 and HdB8 ES cell lines

HdB5 and HdB8, the cell lines generated by the removal of Neomycin cassette, were validated regarding their stemness capacity as described previously (sections 3.1.1 and 3.1.2).

3.3.1 Self-renewal capacity

No significant differences were observed in terms of morphology, viability and FI between HdB5 and HdB8 ES cell lines and the parental cell line (Hd) (**Figure 11A-C**).

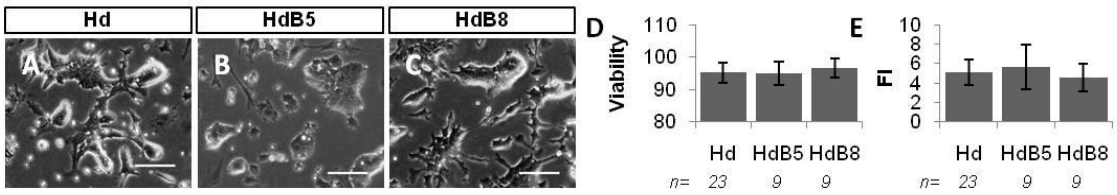

Figure 11: Self-renewal capacity of HdB5 and HdB8 ES cell lines. **(A-C)** All analyzed cell lines show typical morphology of ES cells, organized in clusters. **(D)** The viability of both cell lines is similar to that of the control cell line 46C. **(E)** Values of FI are within the normal range of variation of ES cells. These values represent the average of *n* passages from several replicates. (Scale-bar=100µm)

3.3.2 Pluripotency capacity: neural differentiation in adherent monolayer cultures

The neural differentiation capacity of both ES cell lines was assessed by the ability of the cells to undergo neural commitment, which was confirmed by the observation of neuroepithelial rosettes at day 8 of the protocol (**Figure 12A**). Additionally, analyses of morphology, viability and FI of the cells were performed. No differences were observed in morphology relative to the control cell line. The values of viability and FI were analyzed at several time-points of the protocol (section 2.2.1.2.2) showing similar values to those of the parental cell line both in high density and monolayer cultures (**Figure 12B,C**).

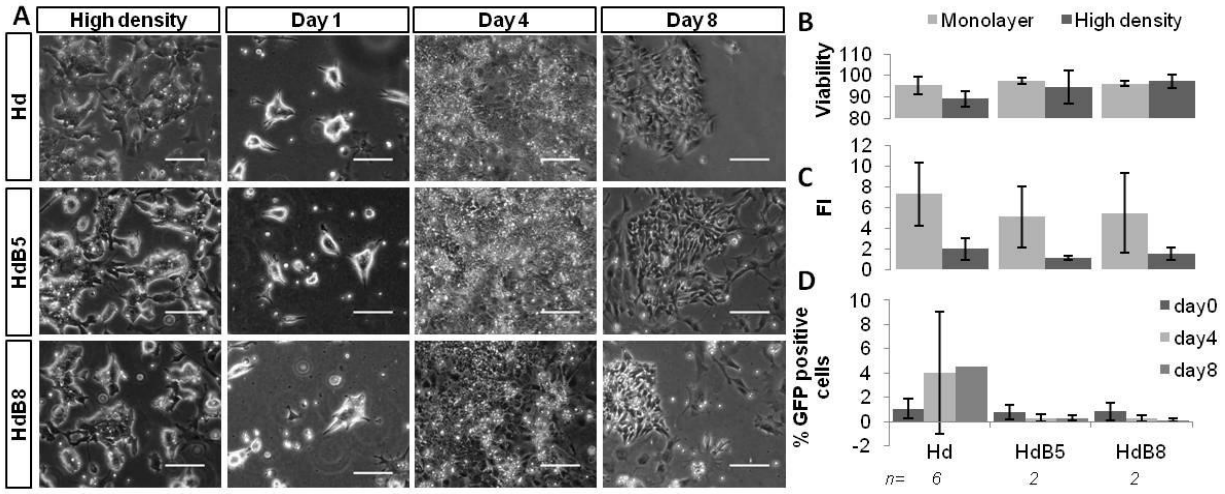

Figure 12: Monolayer neural differentiation of HdB5 and HdB8 ES cell lines. **(A)** Morphology of ES cultures along the monolayer neural differentiation protocol. All cell lines had typical morphology at each time-point and all were able to form neuroepithelial rosettes. **(B,C)** Values of viability and FI are not statistically different from those of the control cell line Hd both in High density and Monolayer cultures. **(D)** Analysis of the expression of the reporter proteins by FACS revealed that VNP is not expressed in any of the HdB5 or HdB8 ES cell lines. These values represent the average of *n* passages from several replicates. (Scale-bar=100µm)

To determine if the HdB5 and HdB8 cell lines show reporter expression in NPs, FACS analysis was performed along the differentiation protocol and immunocytochemistry at day 8. By either method, the analysis of the results showed that none of the clones were expressing the reporter protein (**Figure 12D, Figure 13**), in clear contrast with the parental cell line (Hd), which showed ~5% of the cells expressing the VNP reporter protein. This could be due to the low copy number of the reporter DNA due to multiple copy excision during the removal of Neomycin resistance cassette, coupled to the fast degradation of the reporter mRNA and protein, which might impair the visualization of VNP expression. In addition, damages in the promoter sequence or other signals necessary for transcription or translation might have occurred.

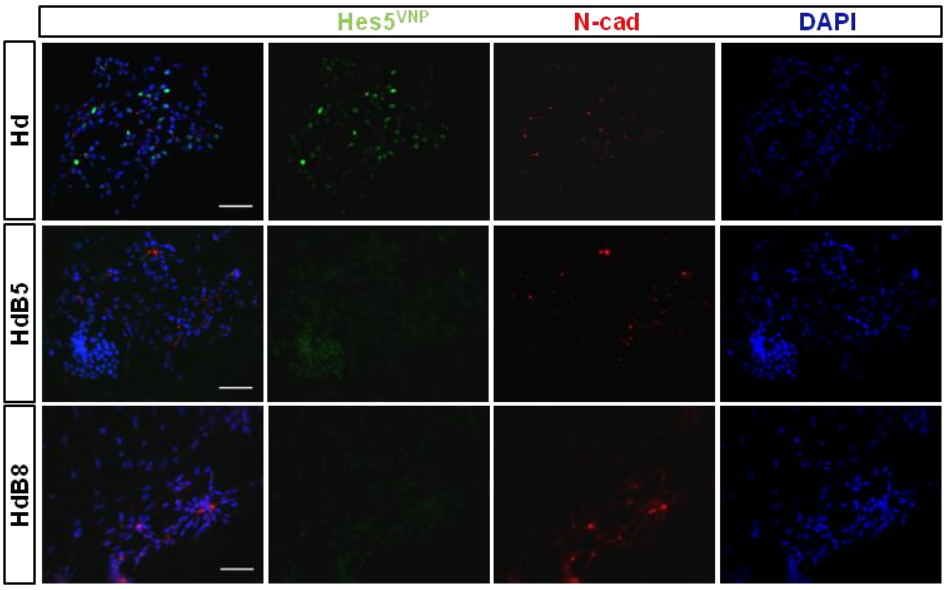

Figure 13: Immunocytochemistry of neuroepithelial rosettes (day 8) of HdB5 and HdB8 cell lines. Cells were labeled using antibodies against N-cadherin (red), marking the apical junctions of the rosettes and GFP (green), marking VNP reporter protein. Nuclei are stained with DAPI. (Scale-bar=50µm)

3.3.3 *Analysis of expression of VNP* mRNA *in Hd*B5 and HdB8 cell lines

To assess if the lack of reporter expression in HdB5 and HdB8 was due to problems in translation or in transcription, the expression of the mRNA encoding the reporter protein was analyzed by RT-PCR for each cell line at days 0, 4 and 8 of the neural differentiation protocol (section 2.2.4). The quality of the cDNA produced was confirmed by a PCR to detect GAPDH and 46C cells were used as controls for the expression of endogenous *Hes5* and reporter protein. In 46C cells it was observed that expression of the *Hes5* increases along the protocol, as occurs also with the expression of reporter mRNA driven by the *Sox1* promoter, as expected [35], [36]. This increase in *Hes5* mRNA expression was also observed for Hd, HdB5 and HdB8 cell lines confirming that cells are expressing the gene and that the lack of reporter expression is not due to deleterious effects on the signals necessary for *Hes5* expression.

In HT cells a precise relation between the levels of expression of endogenous *Hes5* and the ones of the reporter mRNA was observed, with both increasing with the same magnitude along the protocol. In contrast, in HdB5 and HdB8 cells and in the parental cell line Hd, the increase in endogenous *Hes5* expression from day 4 to day 8 is not followed by the same increase in the expression of the reporter mRNA. This shows that in Hd, HdB5 and HdB8 cells, reporter expression is not a good readout of endogenous *Hes5* expression, suggesting that these cell lines might not be suitable to report Notch activity. Altogether these results also show that the lack of reporter expression is caused by impaired mRNA expression. When compared with the levels of expression of GFP mRNA in HT cells, the levels of expression of VNP mRNA in Hd,

HdB5 and HdB8 cells are very low, as expected since the VNP reporter is unstable at the mRNA level, in contrast with GFP mRNA in HT cells. However, the expression levels of VNP were lower in HdB5 and HdB8 than in the parental cell line Hd (**Figure 14**), which suggests that after the removal of Neomycin selection cassette, only one or very few copies of the reporter DNA remained, not being sufficient to drive detectable expression of the very unstable reporter mRNA/protein. In addition, the reduction in the number of copies of the reporter BAC DNA might have reduced the insulator effect on the surrounding chromatin, causing a silencing of reporter expression. Alternatively, the procedure for the removal of the Neomycin resistance cassette might have caused some mutations on the promoter regions that preclude the expression of the reporter protein.

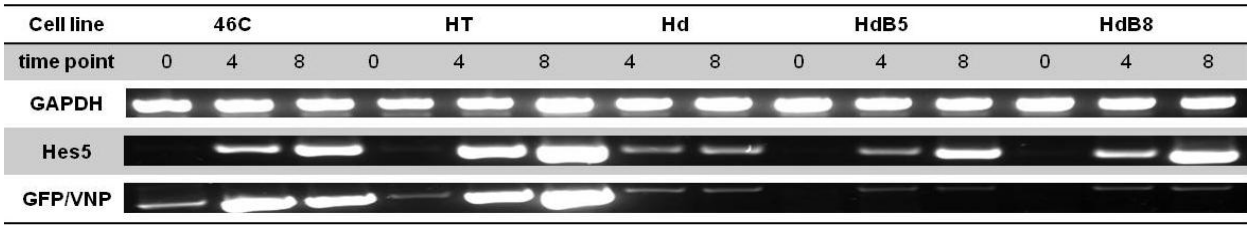

Figure 14: Analysis of *Hes5* and reporter *VNP* mRNA expression at several time points of the neural differentiation protocol. The analysis of the expression of VNP mRNA shows that the levels of its expression were very low when compared to the levels of expression of endogenous *Hes5* mRNA. The 46C and HT cell lines were used as controls, showing an increase in endogenous *Hes5* expression along the protocol and an increase in reporter expression. The parental cell line Hd was also used as a control for the reporter mRNA expression. The quality of cDNA synthesis was assessed by performing a PCR to detect GAPDH.

A PCR to detect the proximal promoter region of 676bp of the reporter DNA was thus performed in order to identify possible damages (like large insertions or deletions) that would impair the expression of the reporter protein (see primer annealing regions on **Figure 5**). It was observed that the DNA bands produced by PCR using DNA from HdB5 and HdB8 cell lines had a similar size to that obtained using DNA from the parental cell line Hd (in contrast with the negative control 46C), therefore confirming the integrity of the proximal promoter region of the reporter (**Figure 15**). These results show that the lack of reporter protein expression is not due to gross damages in the promoter region of the reporter protein cDNA. Overall analysis of the data relative to the expression of the reporter protein in HdB5 and HdB8 led to the conclusion that these cell lines are not good readouts of Notch activity, since the reporter protein is not being expressed in neuroepithelial rosettes, where Notch activity occurs.

Figure 15: *Hes5* promoter integrity. The integrity of the *Hes5* promoter in the HdB5 and HdB8 cell lines was confirmed by PCR, where a fragment with the expected size and coincident with that of the parental cell line was obtained. 46C cell line was used as a negative control, where no band could be detected.

CHAPTER 4

Discussion

4. DISCUSSION

In this work I have analyzed an already described *Hes5::GFP* reporter ES cell line (HT cell line), to test its suitability as a reporter to monitor Notch activity with high accuracy at the single cell level. I found that the HT cells express a reporter protein that perdures in the cell long after Notch activity is extinguished. Therefore, I have started the development of a novel cell line expressing a reporter protein with a short half-life that would allow the monitoring of Notch signaling dynamics.

In the construction of a reporter cell line, one of the most important characteristics of the reporter protein is its half-life, especially when the endogenous protein whose expression is going to be monitored has short half-life, as is the case of HES5. In order to be able to detect the onset and termination of Notch activity the reporter protein must have a half-live close to that of HES5, which has been shown to be shorter that 1hr [42]. However, it should be noted that the experiments to determine the half-life of HES5 were not performed in neural progenitors and therefore the value for this type of cell might differ. I validated HT cells in terms of self-renewal capacity and analyzed their capacity to undergo neural differentiation, confirming the ability of HT cells to form neuroepithelial rosettes expressing the reporter protein. I also tested the half-life of the GFP reporter protein in HT cells to assess its suitability as a Notch activity reporter with fast response kinetics, confirming that it perdures in the cell for a long time after the stop of translation. Actually, 12 hrs after the blockage of translation, HT cells still express 75% of the initial values of GFP, in agreement with previous published data that report a half-life of approximately 26hrs for GFP. Therefore, I showed that HT cells should not be used as reporters to monitor Notch activity dynamics since GFP perdures in the cell after the termination of Notch activity. Additionally, I have observed that the expression levels of *GFP* in HT cell line at day 8 are very variable between replicates and highly dependent on the efficiency of neural commitment and on the culture state, in contrast with the 46C cell line that has a more robust expression of GFP and is less dependent on the state of the culture. Altogether these results show that HT cells cannot be used to monitor dynamics of Notch activity.

Due to the inadequacy of the HT cell line, I proceeded to the establishment of a novel cell line, with an unstable VNP reporter protein, whose expression would faithfully mimic the expression of the endogenous *Hes5* gene. A similar reporter protein, unstable Venus-PEST, has already been used allowing the visualization of oscillations with 2hrs period in the expression of *Lfng* promoter [47]. This suggests that VNP is suitable to detect variations of HES5 expression if it has a similar period [42]. Several ES cell lines expressing the VNP reporter were generated

and validated, confirming no significant differences to the parental cell line in terms of self-renewal and pluripotency capacities. Specifically, the neural differentiation capacity of these cells was confirmed, as well as the expression of the reporter protein.

From the initial generated ES cell lines, Hd was validated and shown to express the reporter protein in neuroepithelial rosettes. However, Hd cells cannot be used as an adequate reporter of Notch activity since the reporter cassette comprises a Neomycin resistance gene that might be interfering with reporter expression because it is located between the coding region of VNP and the polyadenylation signal present at the 3'UTR region. Therefore, in this cell line the reporter mRNA might be produced with the poly(A) signal from Neomycin gene or with no poly(A) signal. This might result in the production of a mRNA without the proper regulation signals or of a highly unstable mRNA impairing the detection of reporter expression. The cell lines in which the Neomycin resistance gene was removed showed self-renewal and pluripotency capacities similar to the control cell line. However, the cell lines selected to continue the experiments, HdB5 and HdB8, were proven not to be suitable to reporter Notch activity since they do not express the reporter protein VNP, as observed by FACS analysis and confirmed by immunocytochemistry.

To investigate whether the cause of lack of reporter protein expression was reduced transcriptional activity, the reporter expression was analyzed at the mRNA level. Analysis of the mRNA reporter expression in HdB5 and HdB8 showed that it was lower than in the parental cell line and not increasing along the differentiation protocol. These results were in clear contrast with the levels of expression of the endogenous *Hes5* gene in the same cell lines that showed an increase from days 0 to 8. These results show that the reporter expression is not mimicking that of the endogenous *Hes5* and suggest that the lack of reporter protein expression is due to problems at the level of reporter transcription and not to some deleterious effects affecting signals necessary for *Hes5* expression. One possible explanation for the lack of reporter protein expression might reside on damages (insertions or deletions) in the reporter promoter region. This hypothesis was tested by the amplification of a DNA fragment from either cell line, comprising the proximal promoter region of the reporter bigger than the 400 bp that were described to be sufficient for transcriptional activation after Notch activity [50]. It was observed that in either case a fragment with the expected size and similar to that of the parental cell line (Hd) could be amplified, suggesting that no major deletion or insertion was made in the promoter region that could impair reporter protein expression. For a detailed analysis of the promoter region, sequencing would have to be performed. Also, these results exclude the hypothesis of

damages in the promoter region but do not exclude the existence of damages in the sequence of other regulatory transcriptional signals.

Alternatively, upon the generation of *Hes5::VNP* cell lines, the modified BAC might have inserted in multiple copies in tandem, allowing the expression of the reporter. However, upon the excision of the selection cassette, some copies of the BAC might have also been excised by the recombination between loxP sites of different BAC copies, remaining only one copy of the construct after recombination. This one copy might have been silenced due to the inhibiting effects of the neighboring chromatin. Also it has been reported that repression at single-copy levels might occur because tandem repeats might amplify the effects of inhibitory or activating sequences present in the repeated sequence [51]. Additionally, the production of a highly unstable reporter mRNA, due to the presence of the Hes5 3'UTR, that is degraded faster than the folding of the reporter protein might account for the inability to detect reporter expression. This would explain the expression of the reporter protein in Hd cells and the lack of expression in the cell lines where the selection cassette was removed.

Despite the already described restrictions in the observation of reporter expression in Hd cells due to the presence of the Neomycin resistance cassette, it was observed by immunocytochemistry that in a neuroepithelial rosette not all cells express the reporter protein at the same levels, which suggests that the intensity of Notch activity varies between cells. This might be the result of cells having different levels of Notch activity between themselves but constant in each cell; also, this could mean that Notch activity is not constant, varying in each cell and that the observed differences are due to the harvesting of cells in different phases of that variation. These results suggest that there might be fluctuations of Notch activity, emphasizing the importance of monitoring Notch activity in single NPs.

In order to overcome the limitations of the previous strategy to generate *Hes5::VNP* reporter cell lines and to engineer a cell line that would allow monitoring of Notch activity some other strategies have to be used. First, as there were generated four initial *Hes5::VNP* cell lines from which two were expressing the reporter protein (Hd and He), He cell line should be tested to assess its suitability as a Notch activity reporter and the Neomycin cassette should be removed. To generate new cell lines, it will be necessary to generate more initial clones, which would have different integration sites, increasing the probability of finding a cell line suitable for monitoring Notch activity. Also, upon the engineering planning of the cell lines, the construct should be designed so that the Neomycin resistance cassette will not need to be removed to allow the proper expression of the reporter and the visualization of the reporter protein expression. In this new strategy, the reporter DNA must have its own polyadenylation signal before the Neomycin

resistance cassette sequence to confer proper expression of the reporter protein, allowing its assessment before the removal of the Neomycin selection cassette. This would diminish the time and handling procedures for the cell line validation process and also the probabilities of genomic instability and damage inherent to ES cell culture.

After the generation of novel ES cell lines expressing the VNP reporter protein under the control of the *Hes5* promoter, it will be possible to monitor Notch activity and follow single NP cells. This will allow determining if Notch can have multiple activations in a life-time of a NP, if its activation occurs specifically in one phase of the cell cycle and if it is correlated with the fate of that NP being necessary to maintain cells in an undifferentiated state. Additionally, other BAC constructs can be electroporated into the *Hes5::VNP* cell line generated in order to create double reporter cell lines. This is the case of a BAC carrying a reporter of Delta1 expression that has already been generated in the Laboratory. The construction of a double reporter cell line in which *Hes5* and *Delta1* expression can be followed in real time and at the single cell level would allow following differentiated cells to determine the timing of commitment to neural differentiation.

CHAPTER 5

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CHAPTER 6

Supplementary Information

6. SUPPLEMENTARY INFORMATION

6.1 Supplementary Methods

6.1.1 DNA/RNA quantification

The concentration of each DNA/RNA preparation was determined by spectrophotometry using the NanoDrop spectrophotometer (Thermo Scientific). The sample concentration in ng/ul was calculated based on absorbance at 260 nm. The purity of the nucleic acid preparation was estimated by the ratio between the readings obtained at 260nm and 280nm (pure preparations of DNA show ratio values of 1.8 and pure RNA preparations have values closer to 2).

6.1.2 Agarose gel electrophoresis

To separate and estimate the size of DNA fragments, agarose gel electrophoresis was performed. Gels were prepared by heating until complete dissolution of agarose in 1x TAE buffer. The final agarose concentration depended on the size of DNA to be resolved and ranged from 1% to 1.5%. Samples were mixed with loading buffer in a 5:1 proportion and DNA was visualized under an ultraviolet light at 260nm or 365nm, by the addition of gel red. The size of the fragments was estimated by comparison with linear DNA strands of known molecular weight (1kb Plus DNA Ladder – Invitrogen).

6.1.3 Screening PCRs

Primers were designed to amplify specific target sequences (**Table S4**, Chapter 8). The reactions were prepared for a final volume of 25µL: 5µL of extracted genomic DNA (section 2.2.5.1) or 5ul of cDNA (section 2.2.4.2), 1x buffer, 0.2mM dCTP, 0.2mM dGTP, 0.2mM dATP, 0.2mM dTTP, 25pmol of each primer and 2.5U of DreamTaq Polymerase. The amplification was performed with an initial denaturation step at 94ºC for 5min, followed by 30 to 35 cycles of denaturation at 94ºC for 30sec, annealing at the specific temperature required for the primer set (**Table S4**) for 1min, extension at 72ºC for 1min, followed by 10min at 72ºC and 5min at 4ºC.

6.1.4 Bioinformatics

DNA, mRNA and Protein sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and Ensemble (http://www.ensembl.org/index.html) databases. Primer sequences were designed and analyzed using: Netprimer (http://www.premierbiosoft.com/netprimer/index.html) and Primer3 (http://frodo.wi.mit.edu/primer3/).

6.2 Supplementary Results

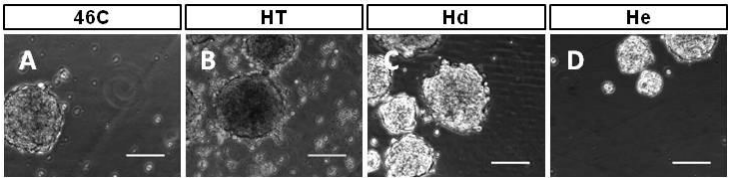

Figure S1: EB formation in 46C, HT, Hd and He ES cell lines. (A-D) Bright field images of EBs from the different cell lines studied, showing normal and typical morphology. (Scale-bar=100µm)

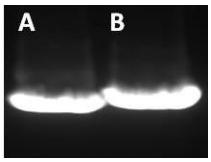

Figure S2: Quality assessment of pTurboCre and pCAGGsPuromycin plasmids. The observed bands correspond to the visualization, in an agarose gel, of the plasmids. **(A)** pTurboCre plasmid. **(B)** pCAGGsPuromycin. Both plasmids were in proper conditions to be electroporated.

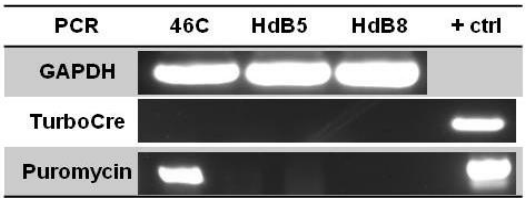

Figure S3: PCRs to test for the integration of pTurboCre or pCAGGsPuromycin plasmids into HdB5 and HdB8 cell lines. The PCRs confirmed that neither plasmid integrated into the genome of either clone. For both PCRs positive controls consisting of plasmids containing the fragments to be amplified were used. In addition, for the PCR to detect the Puromycin resistance gene the 46C cells were used as a positive control since they contain a Puromycin resistance gene inserted into the genome.

Table S1: ES cell growth in Neomycin supplemented media. (+) and **(-)** represent the ability of cells to grow or not in Neomycin supplemented media, respectively.

ES clone	HdA2	HdA3	HdA8	HdA10	HdA11	HdB1	HdB4	HdB5	HdB7	HdB8	HdB11
Growth	-	+	+	+	-	-	+	-	-	-	-

6.2.1 LIF test

To test the activity of new batches of LIF, the stemness potential of ES cells was tested in different conditions. For these studies a modified cell line was used, which expresses an unstable VNP reporter protein under the control of the *Nanog* promoter (a readout of the stemness state in ES cells) (Abranches et al, submitted). These cells can be analyzed by FACS to determine the percentage of cells expressing the *Nanog::VNP* reporter, which is typically around 50% when cells are grown in GMEM supplemented with LIF (data not published). Cells were plated at different cell densities and with different concentrations of LIF. Relatively to the cell density, cells were plated at: (i) normal density (3E+04 cells/cm^2) to see if LIF is being able to maintain cells in an undifferentiated state in “normal” passages; and (ii) clonal density (1E+03

cells/cm^2) to address if LIF is still able to do its function when cells are grown at a lower density and more dependent on the media. At normal density cells were analyzed during three passages in terms of morphology, viability, FI and *Nanog::VNP* expression. At clonal density the same characteristics were analyzed six days later. Relatively to LIF concentrations, three different concentrations were tested: the usual concentration 1/500, that corresponds to 2 ng/mL, and the dilutions 1/250 and 1/1000. As controls, cells were plated without LIF [(-) ctrl], and with 1/500 dilution of the LIF from the previous lot [(+) ctrl]. Analysis of the data showed that, both at clonal and normal density, the three different LIF concentrations tested are able to sustain ES cell stemness state, in clear contrast to the negative control condition, where cells were totally differentiated. Namely, cell morphology, cell viabilities, FI and %*Nanog::VNP* positive cells were similar (**Figure S6**).

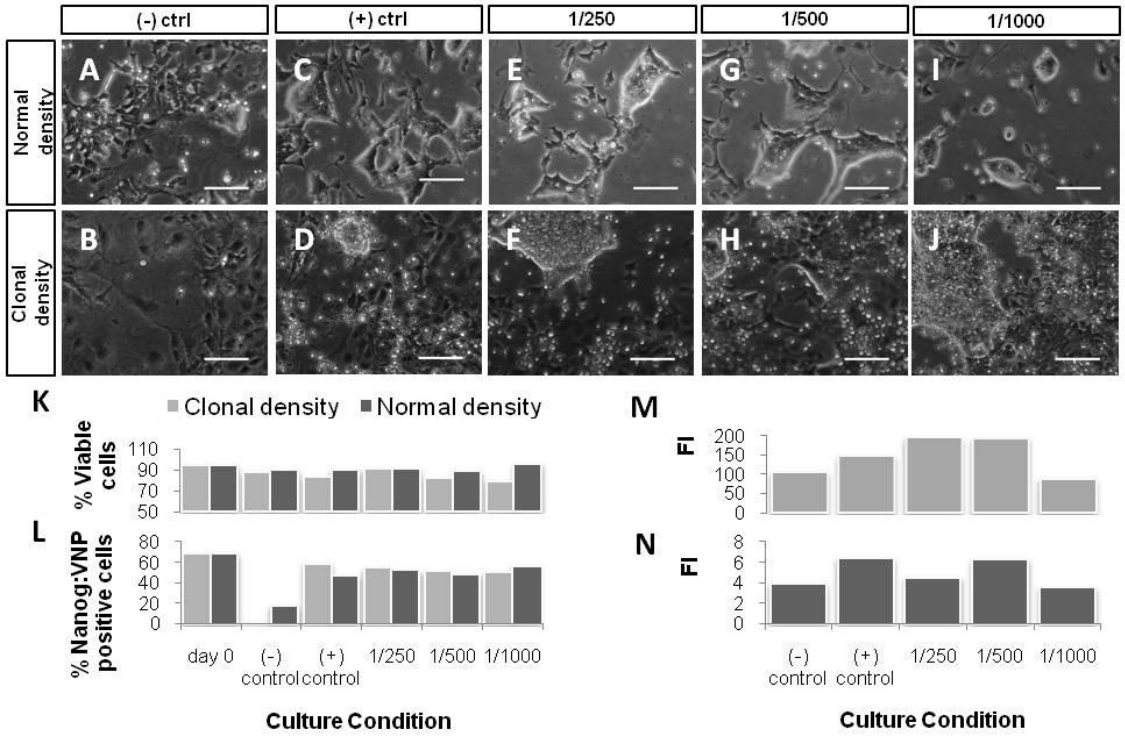

Figure S 4: Lif test. (A-J) Cells were analyzed in terms of morphology, **(K)** viability, (**M-N)** FI and **(L)** *Nanog::VNP* expression, concluding that the new batch was as effective as the previous one.

Table S2: ES cell lines used in the experiments described in this thesis. ES cells used are listed with the description and source.

Cell line	Description	Source
46C	Mouse ES cell line derived from the E14Tg2a ES cell line, expressing the reporter protein GFP under the control of the Sox1 promoter	Meng Li (MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, London, UK) and Austin Smith (Wellcome Trust Centre for Stem Cell Research,

		University of Cambridge, Cambridge UK)
HT	Mouse ES cell line derived from the E14Tg2a ES cell line, engineered by the insertion of a BAC vector, expressing the reporter protein GFP under the control of the *Hes5* promoter	Mark Tomishima (Sloan-Kettering Institute, Rockefeller University, New York, USA).
Ha, Hc, Hd, He	Mouse ES cell lines derived from the E14Tg2a ES cell line, engineered by the insertion of a BAC vector, expressing the unstable reporter protein VNP under the control of the *Hes5* promoter	Cell lines engineered in the Laboratory
HdB5, HdB8	Mouse ES cell lines derived from the Hd ES cell line, expressing the unstable reporter protein VNP under the control of the *Hes5* promoter	Cell lines engineered in the Laboratory
Nd	Mouse ES cell line derived from the E14Tg2a ES cell line, expressing the reporter protein VNP under the control of the *Nanog* promoter	Cell lines engineered in the Laboratory

Table S3: List of relevant reagents used in the experiments described in this thesis. The reagents are listed with information relative to suppliers, catalogue numbers and stock solutions.

Reagent	Supplier	Cat. Number	Stock	Working Stock
2-mercaptoethanol	Sigma	M-7522	RT	0.1 M in H_20, 4ºC
DAPI	Sigma		1 mg/ml in PBS, -20ºC	1.5 µg/ml in PBS, 4ºC
DMSO	Sigma	D-2650		RT
Dream Taq	Fermentas		-20ºC	-20ºC
ESGRO COMPLETE PLUS	Millipore Inc.	SF001-100P	-20ºC	4ºC
Fast Red	Roche	1149644900	-20ºC	
FBS ES-qualified	Invitrogen	10439-024	-20ºC	Heat-inactivate, -20ºC
Gel red	Biotium	41603-01	10000X, RT	500X
Gelatin 2%	Sigma	G-1393	4ºC	0.1% in PBS, 4ºC
Glutamine	GIBCO	25030-123	200mM	100x, -20ºC
Glycine	Sigma	G-7403		RT
GMEM	GIBCO	21710-025		1x, 4ºC
Laminin	Sigma	L-2020	-20ºC	-20ºC
Mowiol	Calbiochem			
murine bFGF	Prepotech	100-18B	-20ºC	4ºC
Non-essential Aminoacids	GIBCO	11140-035		100x, 4ºC
PDL	Sigma	P-7280	-20ºC	-20ºC
Pen-Strep	GIBCO	15140-122		100x, -20ºC
Propidium Iodide	Invitrogen	P-3566	1mg/mL, 4ºC	1ng/mL, 4ºC
RHB-A	StemCell Science Inc	SCS-SF-NB-01	-20ºC	4ºC
rTaq Polymerase	GE Healthcare	27-0798-05	-20ºC	-20ºC
SeaKem LE Agarose	Lonza			
Sodium Pyruvate	GIBCO	11360-039	100mM	100x, -20ºC
SuperScript II Reverse Transcriptase	Invitrogen	18064-014	-20ºC	-20ºC
Trypsin	GIBCO	25090-028	2.5% (v/v), -20ºC	0,25% or 0.025% in PBS,-20ºC/4ºC
Cyclohexamide	Sigma	C-4859	4ºC	100mg/mL, 4ºC

Table S4: List of relevant solutions used in the experiments described in this thesis. The composition of most important solutions is listed. The working stock characteristics of the solutions are listed in Table3.

Solutions	Components

1x TAE Buffer	40mM Tris; 1mM EDTA; 0.35% glacial acetic acid
Blocking solution	10% (w/v) FBS; TBST
FACS Buffer	4% (v/v) FBS; PBS
Gelatin 0.1%	2% gelatin; PBS
GMEM 1x	80% (v/v) GMEM; 1% (v/v) Glutamine; 1% (v/v) Pen-Strep; 1% (v/v) Sodium Pyruvate; 1% (v/v) non-essential Aminoacids; 10% (v/v) FBS; 0,001% (v/v) of 2-mercaptoethanol
Loading buffer	60% (v/v) Glycerol ; 10mM EDTA; 0.2% OrangeG
Mowiol mounting medium	0.1% Mowiol; 33% glycerol; 0.1M Tris, pH 8.5;
SNET	20mM Tris-HCl pH 8; 5mM EDTA pH 8; 0.4M NaCl; 1% (w/v) SDS
Solution A	10mM Tris-HCl pH 8.3 ; KCl 100mM ; $MgCl_2$ 2.5mM
Solution B	10mM Tris-HCl pH 8.3; $MgCl_2$ 2.5mM; 1% (v/v) Tween20; 1% (v/v) TritonX100; 120µg/mL proteinase K
TBST for FISH	150mM NaCl; 0.1%Tween-20; 10mM KCl; 50mM Tris pH 7.5
TBST for Immunocytochemistry	20mM Tris-HCl pH 8; 150mM NaCl; 0.05% Tween-20
TE	10mM Tris; 1mM EDTA pH=8;
Trypsin 0.025%	0.25% Trypsin; PBS
Trypsin 0.25%	2,5% (v/v) Trypsin; 0.01% (v/v) chicken serum; 0.02% (v/v) 0.5M EDTA; PBS
Wash buffer	10mM Tris-HCl pH 8.3 ; KCl 50mM ; MgCl2 1.5mM

Table S5: Oligonucleotide primers that were used in the experiments described in this thesis. The primers are listed with the respective sequences, annealing temperature, size of the amplified product and other observations (primers were provided by Sigma Genosys or Frilabo).

Marker	Sense primer 5' to 3'	Anti-sense primer 5' to 3'	Annealing T. (ºC)	Product size (bp)	Obs
Cre	GCATAACCAGTGAAA CAGCATTGCTG	GGACATGTTCAGGG ATCGCCAGGCG	61	270	
GAPDH	ATTCAACGGCACAGT CAAGG	TGGATGCAGGGATG ATGTTC	60	580	
***Hes5* promoter**	TCCTCTGGAAGTCGC TGTCT	AGATCAGCTTCAGGG TCAGC	60	676	5% formamide
Mycoplasma	TGCACCATCTGTCAC TCTGTTAACCTC	ACTCCTACGGGAGG CAGCAGTA	58	717	
Neomycin	TGAATGAACTGCAGG ACGAG	AATATCACGGGTAGC CAACG	56	515	
Puromycin	GTCACCGAGCTGCAA GAACT	GCTCGTAGAAGGGG AGGTTG	56	382	
Screenning	TGGTCCTGCTGGAGT TCGT	TTAAGGATCATCGTG GAGACC	65	767	
Venus	ATGGTGAGCAAGGG CGAGG	CTTGTACAGCTCGTC CATGCCG	55	720	

Table S6: Antibodies used in the experiments described in this thesis. Antibody dilution, animal in which it was raised and origin are listed.

Antibody anti-	Dilution	Animal	Origin
GFP	1:500	Mouse	Abcam #ab1218
GFP	1:400	Rabbit	Abcam #ab290
N-cadherin	1:200	Mouse	BD Transduction Lab. #610920
Sox2	1:200	Rabbit	Chemicon #ab5603
Tuj1	1:500	Mouse	Covance # MMS-435P