Rett Syndrome Modeling from Human Induced Pluripotent Stem Cells Using a Monolayer Culture System

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Efforts and courage are not enough without purpose and direction.

John F Kennedy

Everything is theoretically impossible, until it is done.

Robert A. Heinlein

ABSTRACT

Induced pluripotent stem cells (iPSCs) have been cataloged with great potential since their discovery, by Yamanaka's group. Since then, much progress has been achieved trying to increase efficiency of the reprogramming method as well as in reaching a completely safe approach.

As iPSCs represent an important tool for drug discovery and disease modeling, the establishment and optimization of culture methods for hiPSCs differentiation urges with the need of revolutionizing not only treatments but also the comprehension of neurodevelopmental diseases, as Rett syndrome.

Here, it is reported an approach that reproduces *in vitro* neurogenesis and gliogenesis from hiPSC, and leads to the achievement of mature neurons, using the dual-SMAD inhibition method, under chemically-defined conditions on a monolayer culture system. Cells were firstly expanded using Vitronectin™ as substrate and mTeSR™1 culture medium and neural identity was obtained using N2B27 enriched with two small molecules, SB-431542 and LDN-193189.

Using this protocol it was possible to achieve mature neurons using three hiPSC lines: two wild-type and one mutated – MeCP2 mutation of Rett syndrome. Thus, it was then possible to compare expansion and neural differentiation behavior of healthy and mutated cell lines. Cells containing Rett syndrome's mutation presented similar proliferation behavior and pluripotency maintenance throughout expansion when compared to healthy cells. However, during differentiation, these cells displayed some alterations, being the most accentuated the lower number of projections and TUJ1 or MAP2-positive cells, being thus possible to conclude that these cells present a lower number of mature neurons and lower complexity of dendritic spines.

Keywords: Induced pluripotent stem cells, Rett syndrome, dual-SMAD inhibition, neural induction

RESUMO

As células estaminais pluripotentes induzidas (iPSCs) têm sido associadas a um enorme potencial desde a sua descoberta. Desde então, tentativas têm sido feitas para melhorar a segurança e a eficiência do método de reprogramação.

As iPSCs representam um papel importante no desenvolvimento de novos fármacos e na modelação de doenças e, assim, surge a necessidade de optimização de métodos de cultura para a diferenciação destas células, para revolucionar tratamentos mas também para melhor se compreenderem doenças do desenvolvimento, como o síndrome de Rett.

Nesta tese de mestrado, foi desenvolvida uma abordagem que reproduz a neurogénese e a gliogénese *in vitro* a partir de hiPSC, e permite obter neurónios maturos, usando o método de inibição dual-SMAD, num sistema de cultura aderente e bem definido. As células foram expandidas em Vitronectina^{TM} usando o meio mTeSR^{TM}1, e a diferenciação neural foi alcançada utilizando N2B27 suplementado com duas moléculas: SB-431542 e LDN-193189.

Assim, foi possível obter neurónios maturos, usando três linhas celulares: duas provenientes de dadores saudáveis e uma com a mutação MeCP2, característica do síndrome de Rett. Foi posteriormente possível comparar os seus comportamentos durante a expansão e a diferenciação neural. As células mutadas apresentaram o mesmo comportamento que as células sem mutação durante a expansão – o mesmo nível de proliferação e manutenção da pluripotência. Todavia, durante a diferenciação, estas células apresentaram alterações, como menos projecções neurais e células positivas para TUJ1 e MAP2, concluindo-se que as células mutadas apresentam um menor número de neurónios maturos e uma menor complexidade dendrítica.

Palavras-chave: Células estaminais pluripotentes induzidas, síndrome de Rett, inibição dual-SMAD, indução neural

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LIST OF ABBREVIATIONS

bFGF - Basic fibroblast growth factor

BMP - Bone-morphogenic protein

BSA- Bovine serum albumin

CNS – Central Nervous System

E8 - Essential 8™

EBs - Embryoid bodies

ECM - Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

ESCs – Embryonic stem cells

FBS - Fetal bovine serum

FGF8 - Fibroblast growth factor 8

FGF2 - Fibroblast growth factor 2

FOXG1 – Forkhead box G1

GDNF - Glial cell line-derived neurotrophic factor

GFAP - Glial fibrillary acidic protein

hiPSCs – Human induced pluripotent stem cells

hPSCs – Human pluripotent stem cells

ICM– Inner cell mass

IFL– Inner fiber layer

iPSCs - Induced Pluripotent Stem Cells

IZ – Intermediate Zone

KLF4 - Kruppel-like factor 4

KO-DMEM - Knockout-Dulbecco's modified Eagle's Medium

KO-DMEM/SR - KnockOut^{TM}-DMEM/SerumReplacement

LDN - LDN-193189

LN-511 - Laminin-511

MEFs - Mouse embryonic fibroblasts

mESCs - Mouse embryonic stem cells

OCT4 - Octamer-binding transcription factor 4

oRGCs – Outer radial glial cells

OTX1/2 – Orthodenticle homoebox 1/2

PAS - Peptide-acrylate surfaces

PAX 6 – Paired box 6

PD – Parkinson's disease

PFA - Paraformaldehyde

PNS – Peripheral Nervous System

PS – Penicillin Streptomycin (PenStrep)

PSCs - Pluripotent stem cells

qRT-PCR – Quantitative real time reverse transcriptase PCR

RA - Retinoic acid

RGs – Radial glial cells

ROCKi - ROCK inhibitor

RTT – Rett Syndrome

SB - SB-431542

SFEB – Serum free culture of embryoid-body-like aggregates

SFEBq – Serum-free floating culture of embryoid-body-like aggregates with quick aggregation

SHH - Sonic hedgehog

Sox1 - Sex determining region Y-box 1

SOX2 - Sex determining region Y-box 2

SSEA-3 - Stage-specific embryonic antigen 3

SSEA-4 - Stage-specific embryonic antigen 4

SVZ – Subventricular Zone

TRA-1-60 - Tumor related antigene 60

TRA-1-81 - Tumor related antigene 81

TGF-β – Tumor/Transforming growth factor beta

VZ- Ventricular zone

ZO1- Tight junction protein 1

I. AIM OF STUDIES

With the rise of induced pluripotent stem cell technology, due to Yamanaka's work, it is now possible to revert the adulthood of cells into an embryonic stem cell like state to induce their subsequent differentiation. This was a major breakthrough in regenerative medicine, thus enabling the study of an enormous spectrum of diseases through the use of patient-specific induced pluripotent stem cells.

For the purposes of the current work, it is particularly important the neural differentiation of patient's iPSCs, as neural cells are very hard to obtain from primary sources (patients), not only due to ethical reasons but also due to obvious safety reasons.

Rett syndrome is a neurodevelopmental disorder in the autism spectrum having extremely disabling symptoms, in which no cure nor effective treatments are available. Having that in mind, the aim of this study is to establish a neural differentiation method on a monolayer culture to mimic neurogenesis and gliogenesis from hiPSCs *in vitro*, and obtain homogeneous populations of cortical neurons to study Rett syndrome.

Patient's derived hiPSCs were expanded in a monolayer-based culture system and differentiated into cortical neurons via dual-SMAD inhibition protocol, using SB-431542 and LDN-193189. During expansion, cells were characterized by immunocytochemistry and flow cytometry. During neural differentiation, cells were characterized by immunocytochemistry and qRT-CR.

Since hiPSCs were derived from Rett syndrome's patients and also from healthy donors, it was possible to compare the neuronal cells obtained from both sources and understand their differences. This work may thus contribute to identify possible targets for the Rett Syndrome disease and identify where to intervene.

II. INTRODUCTION

II.1. Stem Cells

Stem cells were discovered in the early 60s by Till and McCulloch while studying the radiation effects on mice, and their relevance for a number of biomedical applications has been increasing since.
Stem cells are commonly defined by its two main features which are the ability of prolonged self-renewal and differentiation into multiple cell types [1], as it is shown in Figure II.1.

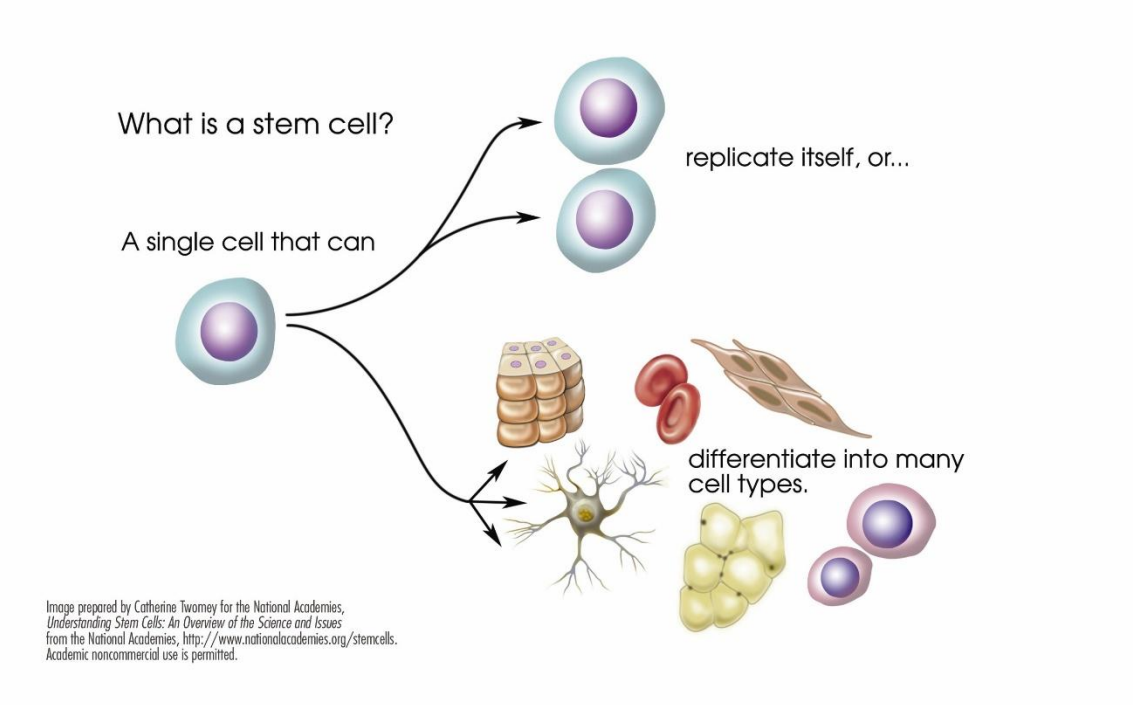


Figure II.1 – Stem cells' hallmarks. Image prepared by Catherine Twoney for the National Academies, Understanding Stem Cells: An Overview of the Science and Issues from the National Academies.

II.1.1. Classification

Stem cells are grouped according to its differentiation potential: totipotent, pluripotent, multipotent and unipotent. During the stages from zygote to morula, the cells are considered totipotent since they can give rise to a full organism, due to their ability to generate the three germ layers of the embryo as well as extra embryonic tissue, such as the placenta. Pluripotent stem cells, which will be the focus of this work, are able to give rise to the three germ layers of the embryo - endoderm, mesoderm and ectoderm - meaning that these cells can originate any cell or tissue of the body but not a full organism.
Multipotent stem cells are more specific cells than the previous ones and have already some level of commitment, therefore being able to generate only a limited type of cells. Finally, unipotent stem cells can only generate one type of cells, such as testis stem cells which give rise to spermatozoon. Another type of unipotent stem cells has been recently discovered: ovarian germline stem cells. This

recent finding establishes that these cells contribute to the renewal of oocytes, thus overcoming the previous belief that adult mammalian ovary was endowed with a finite number of oocytes [2].

In addition to stem cells, there are also progenitor cells which can be misclassified as stem cells but, even though these cells do not have unlimited self-renewal, they are indeed capable of a considerable number of asymmetric divisions and give rise to cells from one lineage only.

Figure II.2 displays the classification of stem cells, according to their potential and it is also possible to foresee the types of cells that can be achieved from each stem cell type.

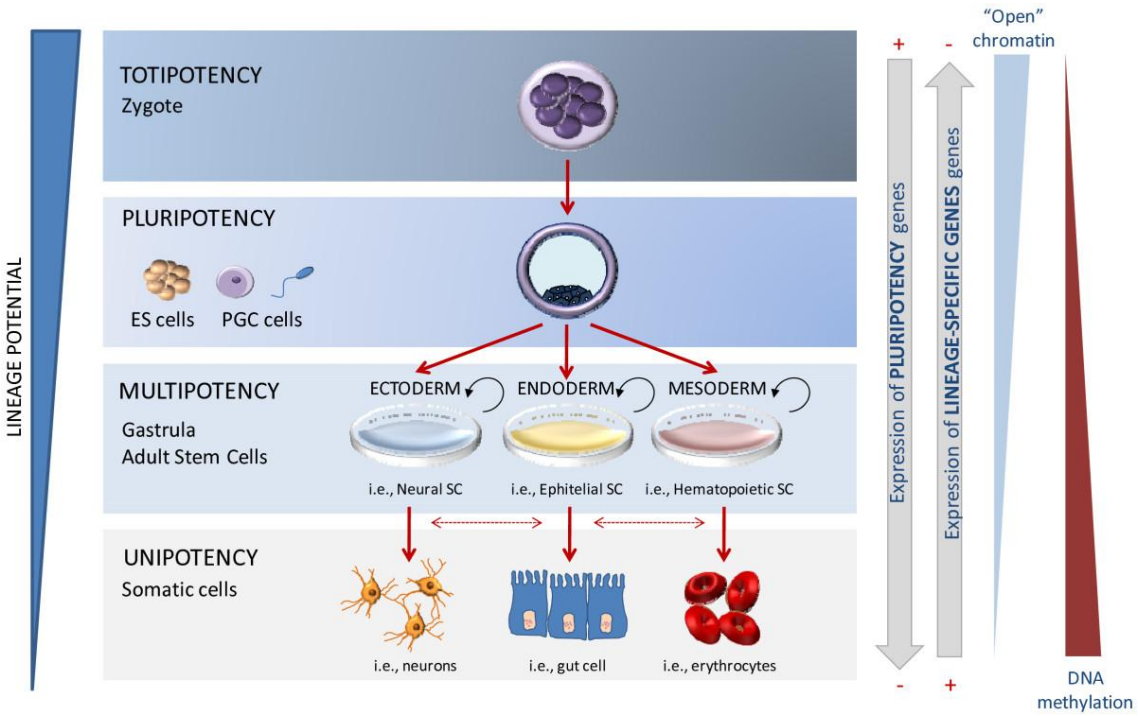


Figure II.2 – Stem cells classification from totipotent to somatic cells. Lineage restriction of human developmental potency [3].

II.1.2. Pluripotent Stem cells

Pluripotent stem cells will be the focus of this work, and, as mentioned earlier, these cells have the ability to give rise to the three germ layers, as it is possible to see in Figure II.3.

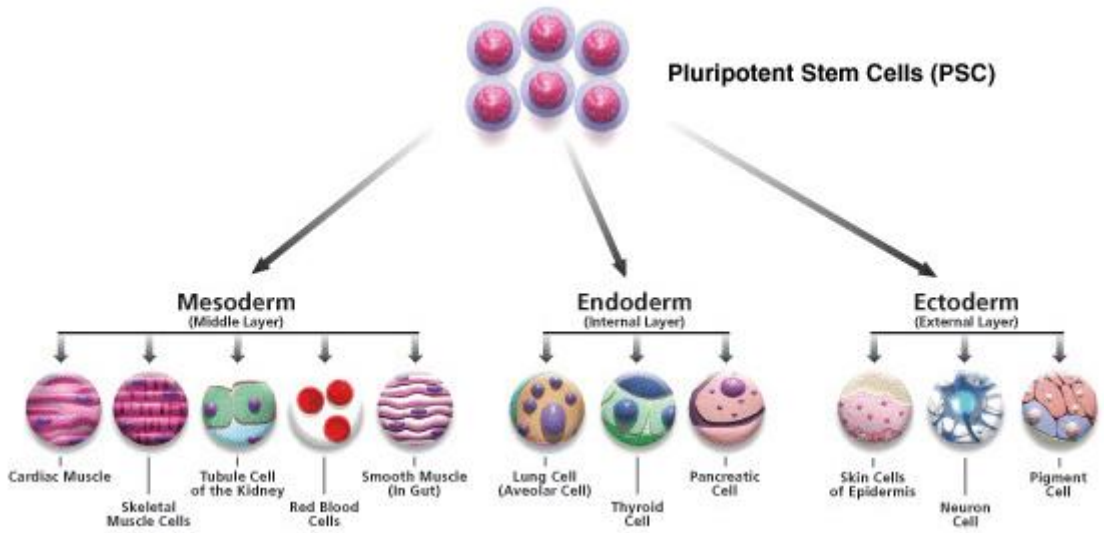


Figure II.3 – Differentiation ability of pluripotent stem cells *from* Styczynski Research Group website.

II.1.2.1. Sources of PSCs

Pluripotent stem cells were, until recently, only isolated from the inner mass of the blastocyst. These cells are called embryonic stem cells (ESCs). ESCs are derived from embryos around day 5 (blastocysts), when the inner cell mass (ICM) and the trophoectoderm, which is the outer layer of the embryo where the cells are committed to become part of the placenta, are separated. Even though ICM cells have the potential to generate any cell type of the body, after implantation they start to differentiate, losing their pluripotency. However, if the ICM cells are isolated from the blastocyst and cultured *in vitro* under specific conditions, they continue to replicate and proliferate indefinitely while maintaining their pluripotency. As ESCs are usually obtained from supernumerary blastocysts that remain from *in vitro* fertilization or from germ cells derived from spontaneously aborted fetuses, there are serious ethical issues associated with ESCs as their derivation implies the destruction of human embryos.

John Gurdon was able to reprogram cells by transfer of somatic nuclei into enucleated oocytes, which do not divide and are not fertilized, showing that the actual DNA of the nucleus of the somatic cell can undergo reprogramming. [4] [5]. Gurdon thus showed that the nuclei of differentiated cells can be fully reprogrammed (by the egg cytoplasm) and then allow expression of all genes needed for the formation of a fertile organism.

However, in 2006, Yamanaka's group was able to reprogram somatic cells into a pluripotent stem cell state using a mixture of transcription factors. As the cells used in this method are somatic cells, which

are highly available, they were able to achieve a highly reproducible method for the generation of induced pluripotent stem cells (iPSCs) [6].

Even though the ethical issues associated with these cells are diminished they present other issues, mostly about safety. Nevertheless, iPSCs brought many expectations into the biomedical field mainly due to their potential applications in disease modeling, drug screening and regenerative medicine.

II.1.3. iPSCs generation

In 2006 Yamanaka's group was able to achieve pluripotent stem cells by reprogramming somatic cells, inducing the activation of pluripotent genes using a mixture of factors.

This mix was composed of four transcription factors - OCT4, SOX2, C-MYC and KLF4 - which were introduced into somatic cells (fibroblasts) using retroviral vectors [6], as it is displayed in Figure II.4.

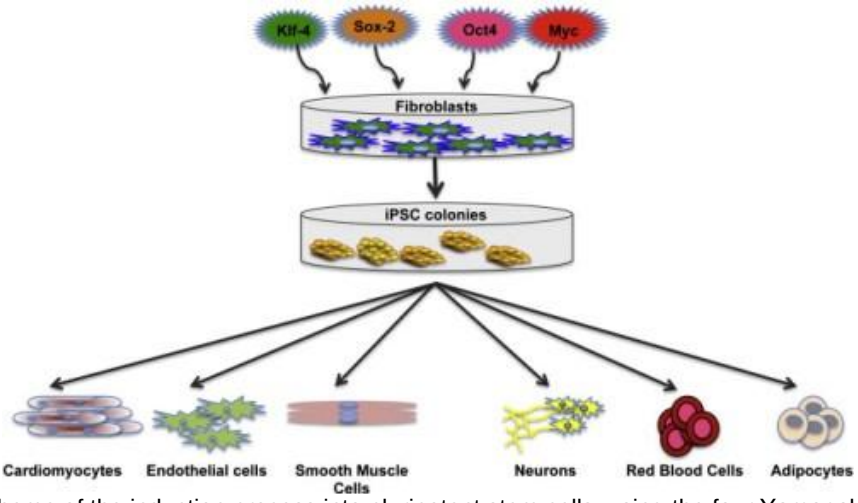


Figure II.4 – Scheme of the induction process into pluripotent stem cells, using the four Yamanaka's transcription factors: KLF4, Sox-2, OCT4 and C-MYC [7].

It was demonstrated in this work that NANOG, which is one of the three important pluripotent markers (along with SOX2 and OCT4), was not vital in reprogramming.

Even though this was a major breakthrough, this method has several drawbacks as some of the factors, C-MYC and KLF4, were discovered to be tumorigenic [6] [8]. Nonetheless, as tumors have a high replication rate, the removal of these factors would also mean a decrease in the efficiency process, which is already below 1%.

In a parallel study, another group showed that OCT4, SOX2, NANOG and LIN28 were sufficient to establish pluripotent stem cells from human somatic cells, discarding the tumorigenic genes and adding NANOG [9].

Also, as the vectors used by Yamanaka were retrovirus, that integrate into the genome, being thus prone to cause mutations, its removal for regenerative medicine applications, even though imperative, would also mean a decrease in the efficiency [8] [10]. Henceforth, many studies have been performed in order to increase safety to the reprogramming method, using different cocktails and transfection methods.

Recent studies have been using integration-free techniques, such as plasmids, recombinant proteins, small molecules [11], Sendai virus (SeV) [12] [13] or mitochondrial deletion [14], which can be alternatives for the generation of safer hiPSC for regenerative medicine.
Meanwhile, the use of retrovirus or tumorigenic factors suits the current purposes of iPSCs such as disease modeling or drug screening.

II.1.4. Properties of hPSCs

Pluripotent stem cells, whether embryonic or induced, can be characterized by molecular markers, which have been identified to verify the pluripotency state. hPSCs express surface proteins, such as tumor related antigens - TRA-1-60 and TRA-1-81 - and stage-specific embryonic antigen 3 and 4 – SSEA-3 and SSEA-4 [15] [16]. Besides these surface markers, they can also be identified by intracellular markers which are also preponderant in pluripotency maintenance, like NANOG, OCT4 and SOX2 [16] .
It is also important to perform karyotype analysis in order to confirm that the cells do not carry chromosomal abnormalities due to *in vitro* culture.
Pluripotency must be verified along expansion in order to ensure that cell proliferation and differentiation are led with homogeneous and well-established populations, without contaminations. These expression studies can be performed using RT-PCR, flow cytometry or fluorescence microscopy.
Besides these markers, to be truly considered as pluripotent, human cells must generate teratomas, which are tumors containing all three germ layers, if transplanted into an immunocompromised mouse, for example. In mice and other animal species, to be considered pluripotent, cells must also be able to originate chimeras capable of germline transmission.

II.1.5. Biomedical applications of hPSCs

Given their unique characteristics and abilities, hPSCs present almost unlimited potential to be used in medicine and treat many diseases such as diabetes, heart diseases or neurodegenerative diseases.
Before cell-based therapies become a tangible reality it is important to overcome all manufacturing hurdles and to fully understand not only the cells' biological and molecular pathways, but also all safety issues that can be associated with transplantation.
These cells have associated safety issues and for that reason multipotent stem cells are the only stem cells with clinical applications so far. Nevertheless, as these cells can differentiate only into a limited type of cells, their use is limited to a narrow scope of diseases.
Despite not being able to be used yet in regenerative medicine, hPSCs, more specifically hiPSCs, present their most promising potential in disease modeling, drug and toxicity screening, patient-tailored therapies and engineered tissues, preventing, moreover, immunosuppression and graft rejection since the tissue would present the same genetic material as the host.

As these cells can be obtained from patients, it is possible to differentiate them into any cell type, as more suitable for the disease to model.

There are many disorders that can benefit from hiPSCs, such as neurological disorders since the retrieval of neural cells is a rather invasive process.

So, the ability of hPSCs, either hESCs or hiPSCs, to give rise to neural cells, under specific conditions, can be used for autologous or allogeneic transplantation in diseases like Parkinson's disease. The following Figure II.5 shows the biomedical applications of hiPSCs.

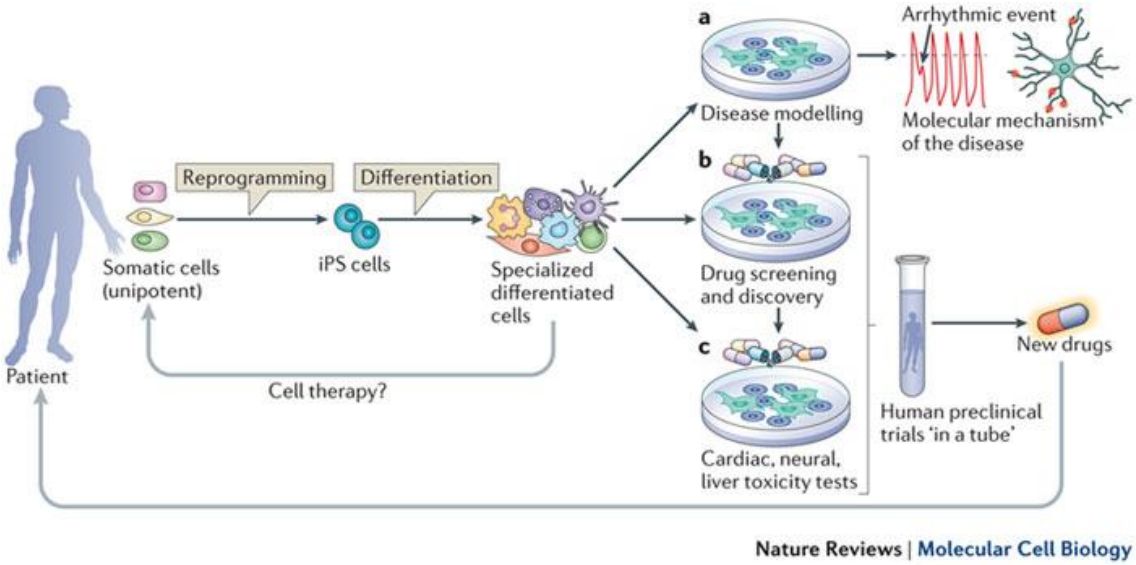


Figure II.5 – Biomedical applications of human induced pluripotent stem cells [17].

II.2. Human Induced Pluripotent Stem Cell Culture

hPSCs have the ability to expand indefinitely *in vitro* as well as differentiate into any cell type. Therefore, these cells are promising for many biomedical applications such as cell replacement therapies, toxicology screens, and tissue engineering. However, since these applications require a large number of high quality cells and, as *in vitro* cell culture yields and subsequent *in vivo* survival of transplanted cells are typically very low, the initial number of cells must be very high, making *in vitro* expansion a very important step [18].

II.2.1. Monolayer culture of hPSCs under adherent conditions

As hPSCs survival depends on cell-cell and cell-matrix interactions, they are commonly cultured under adherent conditions as colonies, using flasks or plates. These interactions are made through myosin

cytoskeleton components, like actin and integrins, which are transmembrane receptors involved in many signaling pathways and they also promote cellular proliferation, survival and motility.
As hPSCs are anchorage-dependent cells, when they are cultured *in vitro,* they must be cultured using supportive substrates and can be maintained in their undifferentiated and proliferative state *in vitro* using feeder-dependent or feeder-free culture systems, which can take advantage of fibronectin, vitronectin, collagen and laminin, as they serve as anchorage points for integrins.

II.2.1.1. Feeder-dependent culture systems (MEFS)

Mouse embryonic fibroblasts (MEFs) are the most common type of feeder cells used in hPSC culture, supporting the growth and proliferation through secretion of growth factors, cytokines and ECM proteins such as activin, laminin or vitronectin [19]. These fibroblasts are irradiated through γ-rays or treated with mitomycin in order to be mitotically inactivated, otherwise MEFs would overgrow the hPSCs culture, over time [20]. However, the use of a culture system with two types of cells increases variability, which also hampers the creation of a standard and reproducible protocol.
Even though this culture system supports the growth and proliferation of hPSCs, it also leads to several issues like the exposure to animal pathogens, which is limiting for clinical applications [19].
These reasons urged the need to develop alternatives to feeder-based culture systems.
Human feeder layers arose as an option and, in these culture systems, human foreskin fibroblasts (HFF) are used as feeder layers as an alternative to animal-derived feeders. Still, the use of this culture system is much more expensive and the risk of contamination by viral and non-viral infectious agents also exists when using human feeder cells [19]. The development of feeder-free culture systems has proved to be a better alternative, with most of all overcoming the problems presented by the feeder-dependent cultures.

II.2.1.2. Feeder-free dependent culture systems – Coating matrices

As mentioned above, the need to develop feeder-free culture systems arose with their possible applications in clinical therapies. Coating matrices aim to mimic ECM through integrin binding, in order to achieve a more efficient adhesion and consequently increase cell survival.

II.2.1.2.1. Matrigel® coating

Matrigel® is a feeder-layer alternative, being the most commonly used substrate for supporting the growth and self-renewal of hPSCs. Matrigel® is composed mainly of laminin, collagen IV, heparin sulfate proteoglycans, entactin, and growth factors. Nonetheless, Matrigel® is derived of Engelbreth-Holm-Swarm mouse sarcomas which leads to lot-to-lot variability and xenogeneic contaminations [19].
Even though Matrigel® presents a barrier for clinical applications, as it is more prone to induce immunological rejection, it remains one of the most used substrates as it fulfills the needs for academic scientific purposes.

II.2.1.2.2. Individual ECM protein coatings

Even though feeder-free culture systems are overtaking feeder-dependent culture systems, it remains urgent to develop culture methods that fit therapeutic needs, adopting non-xenogeneic reagents and reducing non-intrinsic variability. Having that in mind, the use of individual ECM proteins can be an interesting alternative for coating matrices, as cell attachment derives from integrin binding, which are the main surface receptors that mediate cell-surface adhesion. Laminin-511 and Vitronectin XF™ are examples of individual ECM protein coatings.

Laminin-511 is a recombinant human laminin and represents a great step towards defined and xeno-free conditions. Even though it still faces some production cost problems and lot-to-lot variability, it does not compromise hPSCs self-renewal, which occurred when using fibronectin or collagen IV [19].

Vitronectin is an abundant glycoprotein found in ECM and serum, and promotes cell-adhesion and proliferation. Vitronectin XF™ is a defined, xeno-free cell culture matrix composed of human recombinant vitronectin that supports the growth and differentiation of human pluripotent stem cells [21]. It can be used for hPSCs expansion when used with appropriate culture medium (E8™ or TeSR™), providing a completely defined expansion culture system.

II.2.1.2.3. Synthetic matrices

Synthetic matrices are based on proteins, polymers and hybrids of polymers with active biomolecules.

Synthetic matrices are chemically defined, stable and exhibit low lot-to-lot variability, which facilitates scalability. These matrices mimic certain beneficial characteristics of natural matrices and enable tailorability, which means it is possible to modify characteristics as elasticity, strength or rate of biodegradation.

Peptide-acrylate surfaces (PAS) have also proved to support self-renewal of hPSCs in chemically defined, xeno-free medium [22]. Cells cultured on these matrices, which are composed of biologically active peptides conjugated on an acrylate surface, showed similar morphologies and phenotypic marker expression as cells grown on Matrigel®. Cells grown in PAS also maintained a normal karyotype and pluripotency.

Synthemax™ is an example of PAS and being entirely synthetic, the risk of xenogeneic contamination is annulled, being also suitable for sterilization unlike other polymers containing biologic components.

Sythemax™ has proved to be, in combination with defined medium, an adequate defined culture system for hPSCs expansion which can also be used for clinical applications [23].

Figure II.6 displays the timeline of the evolution of pluripotent stem cell culture matrices.

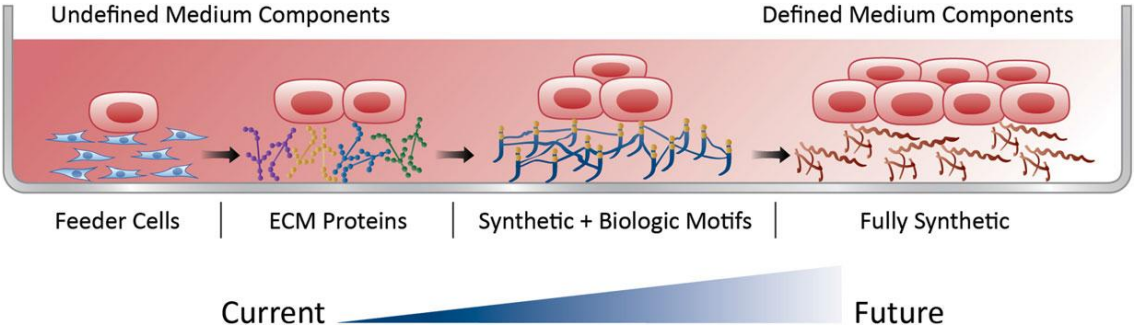


Figure II.6 – Evolution of pluripotent stem cells culture matrices [19].

II.2.2. Human Induced Pluripotent Stem Cell Culture Media

hPSCs culture systems have been optimized throughout the years in order to achieve higher fold increases, without losing pluripotency and karyotype stability. The fundamental molecules that support self-renewal and proliferation have been recently identified, enabling the creation not only of simpler culture media, but also of media that are serum and xeno-free and totally defined.
The main culture media available for hPSC expansion are described next.

II.2.2.1. Knockout-Dulbecco's modified Eagle's Medium (KO-DMEM) supplemented with KO-DMEM/SR

KO-DMEM is a common basal medium that can be used for the growth and maintenance of undifferentiated hPSCs. It can be supplemented with FBS or KO-DMEM/SR (KnockOut-DMEM/SerumReplacement). In addition, KO-DMEM it is also supplemented with bFGF, antibiotics, glutamine, β-mercaptoethanol and non-essential aminoacids. However, as both FBS and KO-DMEM/SR–supplemented media use animal-derived components, they lead to immunogenic responses by human cells. As clinical applications of hPSC depend on culturing hPSCs under serum and xeno-free conditions, other media have been developed to overcome that issue such as mTeSR™ and E8™**.**

II.2.2.2. Serum-free media

II.2.2.2.1. mTeSR™

mTeSR™1 and mTeSR™2 are chemical-defined cell culture media for the maintenance of hPSCs. mTeSR™1 has the components present in DMEM/F12 and other 18 components, such as TGF-β, bFGF and BSA, the only animal component on its constitution [24].

However, the removal of BSA leads to a major decrease in the hESCs viability [21]. Nevertheless, it was also demonstrated that albumin is only necessary to protect the cells from the toxic effect of β-mercaptoethanol. Therefore, if β-mercaptoethanol is not present in the formulation, albumin becomes dispensable [21].

Contrariwise, mTeSR™2 is a xeno-free culture medium since in this case BSA was substituted by human recombinant albumin. However, as human-sourced materials are more expensive than animal-sourced materials, mTeSR™2 is more costly than mTeSR™1.

II.2.2.2.2. Serum-free medium: Essential 8™

The Essential 8™ (E8™) medium is a fully-defined, xeno-free and feeder-free medium that results from the optimization of mTeSR™1, removing all the unnecessary compounds, developed in the laboratory of James Thomson. E8™ is a totally chemically defined medium, being composed by just eight known components: insulin, selenium, transferrin, L-ascorbic acid, FGF2, and TGFβ (or NODAL) in DMEM/F12 with pH adjusted with $NaHCO_3$ [21]. As E8 medium supports proliferation of undifferentiated hPSCs, while maintaining the expression of pluripotency markers and normal karyotypes, it can become a rather appealing alternative for mTeSR™ medium.

II.2.3. hPSCs passaging

As the hPSCs expand, their colonies become denser and, at a certain point, they ought to be split to maintain cell viability, pluripotency and normal karyotype [25] [26]. Cell passaging can be chemical or mechanical.

Mechanical passaging is performed using a tissue chopper or a razor blade to chop the colonies. The cells are then detached from the dish using a cell scraper followed by gentle pipetting [25]. This method presents a clear drawback regarding the homogeneity of the aggregate's size since it is dependent of the operator's sensibility. hPSCs can also be passaged chemically, using enzymes like dispase, trypsin, collagenase IV or accutase or non-enzymatically, using dissociation buffers, such as Ethylenediaminetetraacetic acid (EDTA) buffer.

The use of enzymes leads to an efficient and standardized single-cell passaging. However, the repeated mechanism of enzymatic passaging is associated with genetic instability, since the karyotype is frequently altered [25]. Also, some enzymatic reagents, like trypsin, that can be used for cell passaging are animal derived.

Even though mechanical dissociation requires manual micromanipulation and may be more exhaustive, enzymatic dissociation, even though enabling large-scale expansion, being faster and simpler, still presents some protocol reproducibility issues such as the aggregates' size and there are evidences suggesting increased rates of karyotypic abnormalities [25]. Also, as hPSCs are very sensitive to apoptosis upon dissociation being contact-dependent cells, single-cell passaging must be complemented with the use of ROCK inhibitor (ROCKi) [27].

The Rho/ROCK pathway is activated when hPSCs are isolated, leading to gene transcription and apoptosis.

One example of a cell-permeable and selective inhibitor of Rho-dependent protein kinase is Y-27632, displayed in Figure II.8, allowing hPSCs survival upon dissociation.

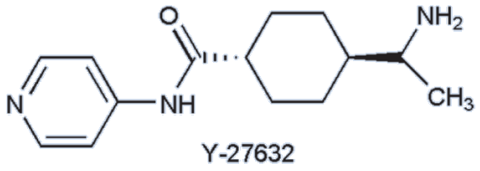


Figure II.7- Y-27632: Selective inhibitor of the ROCK p160ROCK (Sigma) *in http://www.abmgood.com/StemCell/images/Y-27632.jpg.*

As for the non-enzymatic passaging using EDTA, it is an enzyme-free procedure which enables maximum cell survival without enzyme neutralization, centrifugation or drug treatment. In this protocol, EDTA sequesters Ca^{2+}, leading to cadherin's inactivation and consequently cells' detachment from the plate. EDTA-passaging has proved to be an excellent alternative for enzymatic passaging since it requires minimal material, limits contamination and its protocol is simple, reproducible and safer for clinical applications [28].

II.3. Neural Development *in vivo*

Neural induction in mammalian embryos, or neurulation, leads to the formation of the neural tube, which gives rise to both the spinal cord and the brain. This process is presented in Figure II.8.

During gastrulation, which is the organization of the embryo into a multi-layered structure, a distinct cylinder of mesodermal cells called notochord is formed. The notochord is vital in neurulation and the ectoderm that lies above is called neuroectoderm, and gives rise to the entire nervous system. The notochord sends inductive signals to the neuroectoderm, causing the differentiation of a subset of neuroectodermal cells into neural precursor cells.

During neurulation, there is thickening of the midline ectoderm, forming the neural plate. Afterwards, the peripheries of the neural plate suffer invagination and the neural tube is formed. The neural tube's progenitor cells are neural precursors and will eventually originate neurons.

The cells at the ventral midline of the neural tube, due to their proximity to the notochord, will differentiate into epithelial-like cells called floorplate. The floorplate will send signals that lead to the differentiation of cells in the ventral portion of the neural tube into hindbrain motor neurons.

Precursor cells farther away from the ventral midline give rise to sensory neurons within the spinal cord and hindbrain. At the most dorsal limit of the neural tube, a third population of cells emerges in the region where the edges of the folded neural plate join together called the neural crest. Neural crest cells migrate away from the neural tube and give rise to a variety of cell types including neurons and glia but also non-neural structures such as pigment cells, cartilage, and bone.

The neural tube gives rise to distinct regions of the central nervous system as the prosencephalon, the mesencephalon, the rhombencephalon and the spinal cord with the division of neuroepithelial cells.

The cerebral cortex is a multilayer structure and its development starts with the expansion of the neuroepithelium of the dorsal telencephalon, originated from the prosencephalon, and generates radial glial stem cells (RGs), which will divide at the apical surface within the ventricular zone (VZ) to generate neurons and intermediate progenitors. These progenitors will then populate the adjacent subventricular zone (SVZ), while neurons migrate through the intermediate zone to populate specific layers within the cortical plate. The SVZ is split by an inner fiber layer (IFL) into an inner SVZ and an outer SVZ (oSVZ). The oSVZ represents an entirely separate progenitor layer populated by intermediate progenitors and a unique stem cell subset termed outer radial glia (oRGs) and are abundant in the neocortex [29].

The early steps in neural induction are a result of the inhibition of mesoderm and endoderm promoting signals such as Wnts, nodal, and BMPs [30]. Neural inducers, such as noggin, dorsalize the ectoderm and drive it into neural fate by binding to and inactivating the endogenous epidermalizing factor BMP-4 [31]. There are also evidences that retinoic acid (RA) signaling along with FGF play a role in the developing of the spinal cord [30].

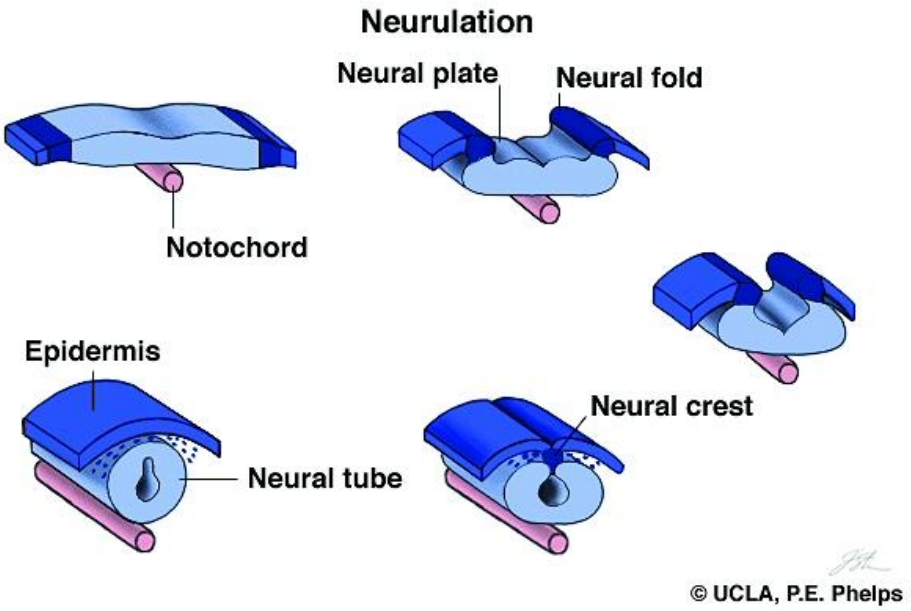


Figure II.8 – Neurulation process *from Neuroscience,* 2nd edition, Purves D, Augustine GJ, Fitzpatrick D, et al.; 2001).

II.4. Signaling pathways in hPSCs differentiation towards neuroectoderm

The comprehension of the subjacent mechanisms of cell differentiation is vital to the neural conversion *in vitro* as hPSCs are cells with high plasticity properties. Therefore, it is extremely important to understand the signaling pathways that are active during the generation of the human cortex *in vivo* in order to modulate them *in vitro*.

Transforming growth factor (TGF-β) signaling controls a wide range of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate, during embryogenesis [32]. The TGF-β family is therefore essential for the maintenance of hPSCs pluripotency and self-renewal as well as it is responsible for the differentiation into definitive mesodermal and endodermal lineages during gastrulation. The TGF-β superfamily includes some TGF-β proteins, activin/nodal growth factors, and BMPs, all of which are involved in maintaining stem-cell homeostasis and differentiation.

II.4.1. Activin/nodal signaling pathway

Activin and nodal, members of the TGF-β superfamily, are also responsible for inducing mesendoderm, the precursor of the definitive mesodermal and endodermal lineages during gastrulation [33] [34].

Nodal signaling is regulated by cripto, an extracellular GPI-linked protein that acts as a co-factor. Lefty1, lefty2 and cerberus are endogenous inhibitors of nodal signaling and can modulate the pathway signaling by binding to cripto and preventing the formation of the nodal/cripto/receptor complex, thus inhibiting nodal signaling.

Activin/nodal signaling occurs by SMAD signaling with the ligands joining type I (ALK 1-7) and type II (TβR-II, ActR-II, ActR-IIB, BMPR-II and AMHR-II) receptors, which are later activated through phosphorylation. Activin pathway signals through combinations of ActR-II or ActR-IIB and ALK4, while nodal signals through a complex of ActR-IIB and ALK7.

The signals are then transduced to the nucleus after activation of SMAD complexes where they modulate gene expression [35].

It has been shown that nodal is required to maintain the pluripotent status of the epiblast layer before gastrulation as well in mesendoderm induction, through phosphorylation of smad2 and 3 [34].

As mentioned, activin/nodal pathway leads to mesodermal and endodermal differentiation, thereby making its inhibition an interesting way to drive an *in vitro* differentiation towards the ectodermal lineage, and consequently, neuroectoderm.

In fact, lefty expression has also been implicated in ectoderm specification and overexpression of either lefty or cerberus resulted in a marked increase in neuroectoderm development. Moreover, similar effects could be achieved with the specific pharmacological inhibitor of type I activin/nodal receptor signaling, SB-431542.

SB-431542 is a potent inhibitor of ALK5 and acts as a competitive ATP binding site kinase inhibitor and inhibits the *in vitro* phosphorylation of smad3. It was also found that SB-431542 also inhibits ALK4 and ALK7, inhibiting thus activin-induced phosphorylation of smad2 [35].

In fact, using SB-431542 lead to significant higher levels of expression of neuroectoderm markers, when compared to overexpression of lefty [33].
Therefore, it can be concluded that nodal signaling inhibits neuroectoderm specification during early hPSCs differentiation, and its inhibition can provide a robust method in the achievement of neuronal pathway from hPSCs.

II.4.2. BMP signaling pathway

Bone morphogenetic proteins (BMPs) were initially described as bone morphogens, but it is now clear their role in many processes during embryogenesis, tissue development and tissue repair.
BMPs bind to two types of transmembrane kinase receptors (type I and type II receptors), which activate downstream SMAD and non-SMAD signaling cascades upon phosphorylation.
As the ligand binds to the receptor complex, the constitutive active kinase domain of the type II receptor phosphorylates and thereby activates the type I receptor which then phosphorylate regulatory SMADs (SMAD 1/5/8) [36].
Secreted antagonists control the accessibility of the ligands for the receptors and as for co-receptors, they modulate the transmission of the extracellular signal into the cytoplasm while cytoplasmic and nuclear proteins bind and therefore regulate the activity of downstream signaling components.
BMP signaling can inhibit the expression of neuroectodermal markers in hPSCs and in fact, BMP-4 inhibits the neuroectodermal path by promoting differentiation towards trophoblast.
So, the inhibition of BMP signaling leads the cells into the neuroectoderm path, and this inhibition can be achieved through genetic approaches using gene silencing or protein overexpression as well as using specific proteins or small molecules [30] [36] [37].
One protein that can be used to inhibit BMP signaling is noggin, which is known to take part in inducing the early nervous system in several vertebrate model systems by dorsalizing the ectoderm and driving it into neural fate by binding to and inactivating the endogenous epidermalizing factor BMP-4 [31]. Also, dorsomorphin, a small molecule, can inhibit BMP signaling by inhibiting the BMP type I receptors ALK2, ALK3 and ALK6 blocking BMP-mediated SMAD 1/5/8 phosphorylation [36].
More recently, it was discovered a molecule with similar outcome as dorsomorphin, LDN-193189 [36], which inhibits ALK2 and ALK3. Figure II.9 shows the mechanism of generation of neuroectodermal cells by inhibiting signaling pathways BMP and TGF-β.

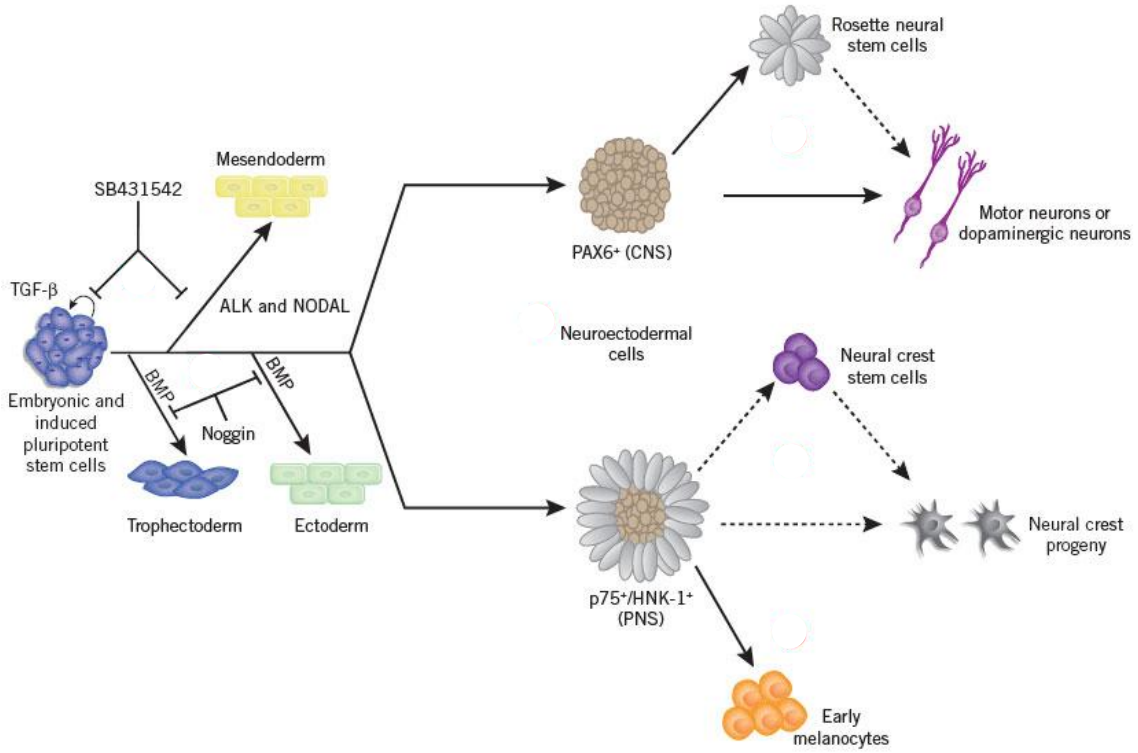


Figure II.9 –Model for generating neuroectodermal cells through dual-SMAD inhibition [38].

II.5. Neural differentiation of hPSCs *in vitro*

Neural differentiation can be achieved under adherent culture conditions, without the use of feeder-layers, through the dual inhibition of SMAD signaling [15], or through embryoid bodies (EBs) which are 3D aggregates grown in suspension. The latter mimics the process that occurs *in vivo,* which do not occur in a monolayer culture, but EBs also present some drawbacks as poor environmental control, formation of heterogeneous structures and difficulties in cell characterization through microscopy or electrophysiology.

Neuronal differentiation has been improved in its efficiency due to efforts taken by several groups that take advantage of inductive signals found in the embryonic development. These signals increase the efficiency of generating neuroectoderm cells *in vitro* and reduce the presence of non-neural cell types [30]. The generation of early neuroectoderm, based on Sox1 expression, can be achieved by inhibiting Wnt and nodal signaling.

More recently, it has been shown that inhibiting the SMAD signaling with a synthetic TGF-β antagonist can effectively produce neural progenitors [15].

II.5.1. Adherent culture conditions – Dual-SMAD inhibition

After expansion of hPSCs, it is possible to differentiate the cells into a neural lineage in flasks or plates through the dual inhibition of SMAD signaling.

The dual-SMAD inhibition protocol was developed by Chambers and colleagues [15] and it is a very straightforward method to obtain a complete differentiation of hPSCs into the neural lineage.

SMADs are intracellular proteins that transduce extracellular signals to the nucleus, where they activate gene transcription.

The dual-SMAD inhibition protocol is based on the inhibition of the SMAD signaling pathway, blocking the two signaling pathways that use SMADs for transduction: BMP and activin/nodal, combining two potent inhibitors - noggin and SB-431542.

As mentioned previously, SB-431542 leads to pluripotency loss by differentiation towards trophoblast lineage and also mediates mesodermal lineage suppression (Brachyury) [15]. As for noggin, it inhibits BMP endogenous signals that drive trophoblast fates upon differentiation as well as suppress the endodermal lineage.

Nonetheless, this protocol requires the combined used of these molecules as they work synergistically at multiple stages of differentiation to achieve efficient neural conversion of hPSCs and when used alone, neither one is able to efficiently convert the cells [15].

This protocol was proved to be sufficient to achieve a full neural conversion, in monolayer and feeder-free cultures, generating a population of neural progenitors expressing Sox1, PAX6, and NESTIN. Finally, the same results were achieved later, by other research group that replaced Noggin, a bioactive protein, by a small molecule, LDN-193189, a derivative of Dorsomorphin, which is also a BMP signaling inhibitor, contributing to a less expensive protocol [36].

The monolayer culture system, which relies on the cell-adhesion to the substrate coating or feeder cells, has been shown to support proliferation and decreases apoptosis when compared with EB strategy. Moreover, it also supports neural differentiation of hPSCs allowing the immediate formation of neural rosettes and producing a highly enriched population of desired neural cell types.

Even though adherent culture methods present some attractive aspects they also present some drawbacks, such as limited scalability or the fact that 2D culture is a poor mimic of *in vivo* development [39].

II.5.2. Embryoid Bodies

When hPSCs are cultured in suspension, without any factors blocking differentiation, they spontaneously form 3D multicellular aggregates called embryoid bodies (EBs) which recapitulate many aspects of cell differentiation during early embryogenesis [40] and consists of ectodermal, mesodermal and endodermal tissues.

Many protocols for hPSCs neural differentiation start with an embryoid body stage and combined with RA and serum-free defined media can maximize neural differentiation [30].

Also, using a SFEBq culture method, early steps of corticogenesis were recapitulated and it was possible to achieve aggregates positive for FOXG1 (a neural marker) using a TGF-β inhibitor and a Wnt inhibitor, promoting telencephalic differentiation [41].

Even though EBs constitute a 3D system, and thus mimic cell-cell interactions that occur *in vivo*, EBs are also heterogeneous structures containing both differentiated and undifferentiated cells, which introduces a microenvironment difficult to control, contributing to high variability. Moreover, its 3D structure makes cell characterization by microscopy and electrophysiology techniques more difficult.

II.5.3. Cerebral Organoids

More recently, it was possible to achieve a 3D organoid culture system, cerebral organoids, that developed various discrete but interdependent brain regions, like cerebral cortex containing progenitor populations that organize and produce mature cortical neuron subtypes [29]. Lancaster et al. built a protocol based on hPSCs self-organization and without patterning growth factors, enhancing growth conditions and the necessary environment for intrinsic cues for development. They started with embryoid bodies' formation to achieve neuroectoderm which was then encapsulated on Matrigel® droplets as a scaffold, maintaining its 3D structure. The droplets were transferred to a spinning bioreactor in order to improve nutrient and oxygen transport. By doing so, they obtained brain tissues, which they denominated as cerebral organoids. Neural identity took 8-10 days to appear and after 30 days they were able to identify defined brain regions. These steps are displayed in Figure II.10. They also tried a stationary culture method, which lead to an aggregate of several small rosette-like neuroepithelia, and the tissues formed were also smaller than the ones obtained using the stirred bioreactor. Nevertheless, the tissues reached maximal size after 2 months but could be maintained in culture indefinitely in a spinning bioreactor. The 3D organoids showed reminiscences of cerebral cortex, choroid plexus, retina and meninges. Also, the authors were able to model microcephaly using patient-specific hiPSCs which means this culture system can be suitable to study other neurodevelopmental diseases like RTT, providing novel insights.

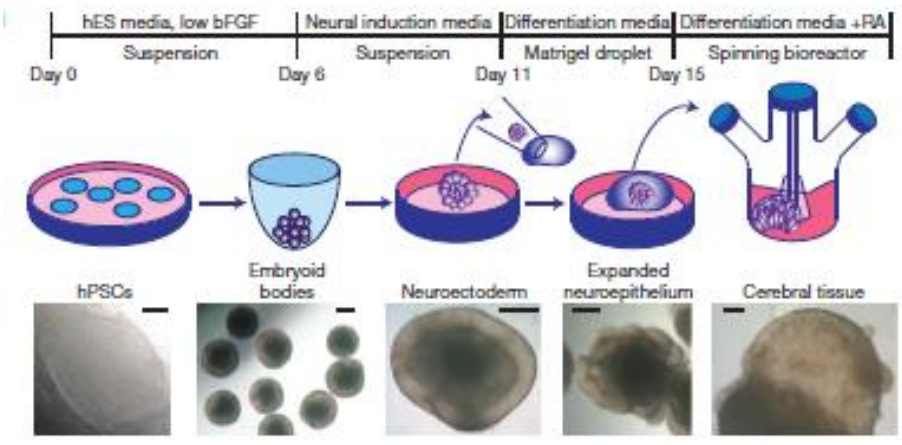


Figure II.10 – Cerebral organoid culture system description [29].

II.5.4. Neural markers

When hPSCs are cultured *in vitro* under neural induction conditions, the process begins with the formation of neuroectoderm, which is characterized by the formation of neural rosette-like structures by primary neural progenitors, resembling the neural plate (OCT4-, SOX2+, PAX6+, FOXG1+, OTX1/2+ and NESTIN+).

These primary neural progenitors will then evolve to secondary neural progenitors (TBR2+) and finally into neurons (TUJ1+ and MAP2+) and early glial-like cells (GFAP+) [42].

The deletion of the OTX1/2 gene leads to an embryonic lethal phenotype in mice and this gene specifies the anterior neuroectoderm during gastrulation. During embryo development, OTX1/2 is expressed throughout the forebrain and midbrain.

NESTIN is an intermediate filament protein type VI and is expressed in dividing cells during the development of the CNS and PNS.

Besides being important for pluripotency maintenance, SOX2, or sex determining region Y box 2, is also expressed by developing cells from the neural tube and in neural progenitors in the CNS, being inactivated in the end of neural differentiation.

FOXG1, or forkhead box protein G1, plays an important role during early neural embryonic development, particularly in the telencephalon [59].

PAX6 is an essential marker for neural stem cell multipotency and proliferation. PAX6-expressing human neuroectoderm cells are able to differentiate into any region-specific neural progenitor. Also, PAX6 is considered to be a key in the onset of human neuroectoderm differentiation [42].

TBR2, or T-box brain protein 2, intervenes in neural differentiation and axonal growth. It is expressed in basal neural progenitors.

As for TUJ1 and MAP2, these proteins belong to the tubulin protein family and are exclusively expressed in neurons.

The following Figure II.11 displays the evolution of marker expression along differentiation day, *in vitro*.

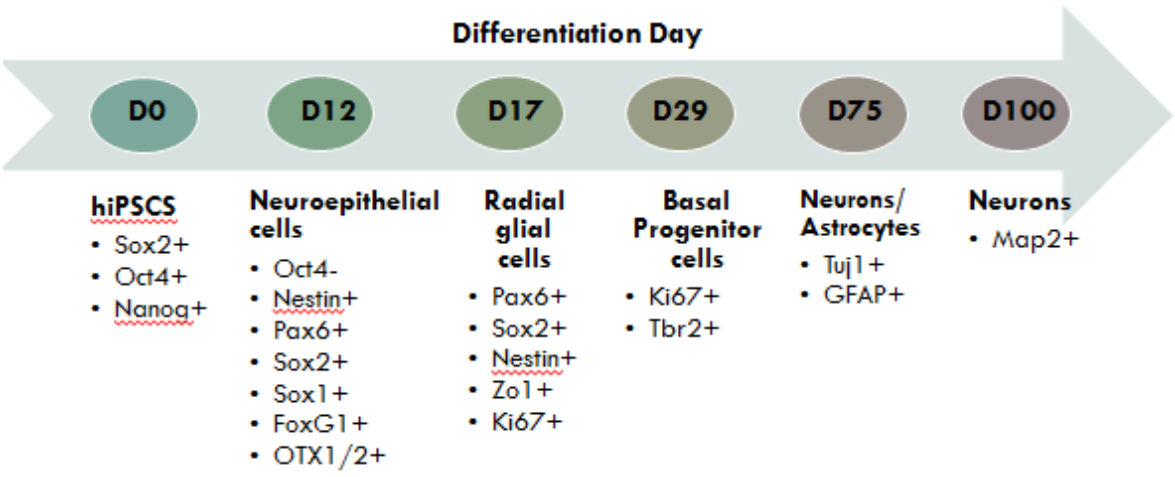


Figure II.11 – Markers' expression with the evolution of neural induction *in vitro*

II.5.5. Applications of hiPSC neural differentiation *in vitro*

Due to their localization, it is difficult to use neural cells as main tools in biomedical applications. However, and as mentioned previously, the discovery of hiPSCs has made possible the use of neural cells obtained *in vitro* as a research element with high potential.

Neural differentiation in vitro allows drug and toxicity screening, and disease modeling [8].

Disease modeling is one of the major applications for these cells obtained *in vitro,* and is crucial in the study of neurodevelopmental disorders, as it is possible to follow the stages of differentiation and the disease progress. One example of neurodevelopmental disease is Rett syndrome, which will be the focus of this work and, for that reason, this disorder will be explored in the following sections.

II.5.5.1. Rett Syndrome

Rett Syndrome (RTT) is a neurodevelopmental disorder that was first identified and described in 1966 by an Austrian physician, Dr. Andreas Rett, but only acknowledged in 1983, due to Dr. Bengt Hagberg's work. It is estimated that this disorder affects 1 out of 15.000 live female births [43] in all racial and ethnic groups worldwide. As children with RTT exhibit autistic-like behaviors in the early stages, this disease is often misdiagnosed as autism, cerebral palsy or non-specific developmental delay. RTT is in fact the most physically disabling disease in the entire autism spectrum.

II.5.5.1.1. What causes Rett Syndrome?

The cause of Rett Syndrome is a mutation in the methyl CpG binding protein 2 gene (*MECP2*) of the X chromosome. The protein binds to the methylated cytosine, acting as one of the many biochemical switches for gene expression [44] [43]. Thus, an abnormality in this gene prevents the brain's nerve cells from working properly and RTT's patients present insufficient amounts or structurally abnormal forms of the protein, causing other genes to be atypically expressed.

This disease affects mainly girls since the *MECP2* gene is found on the X chromosome, one of the two sexual chromosomes. For girls, as they present two X chromosomes and as only one is active in any given cell, there is only a portion of the cells with the defective protein, mosaicism. The remaining cells present the healthy gene of the other X chromosome, expressing thus normal amounts of the protein.

As for male patients with a *MECP2* mutation, since they have only one X chromosome (and one Y chromosome) there is always expression of the mutated protein. For that reason, male patients presenting this defect frequently do not show clinical features of RTT, experiencing, though, severe problems at birth and dying shortly after. Even though male patients are not as common as female patients, there are cases due to different *MECP2* mutations, due to less aggressive mutations, sporadic mutations such as the existence of an extra X chromosome (XXY, known as Klinefelter's syndrome), or even mosaicism that occurs when the change is only found in some cells [45] [46].

The severity of RTT depends on the percentage of cells expressing the normal copy of the *MECP2* gene. If the X chromosome that is carrying the defective gene is turned off in a large portion of cells,

the symptoms will be mild. However, if this percentage is higher, the onset of the disorder may occur earlier and the symptoms may be more severe.

RTT is not limited to a mutation in the gene M*ECP2* as mutations in the *CDKL5* and *FOXG1* genes have also been identified as causing RTT as well. For these mutations the disease appears to be even more severe.

As mentioned, RTT is a genetic disorder but the mutation occurs randomly and spontaneously, being the percentage of inherited cases recorded rather low. However, some RTT patients present other female family members with a *MECP2* mutation but with no clinical symptoms. These females are known as “asymptomatic female carriers” and in these cases it is possible that the patients have mild mutations or that mutated cells are located in non-crucial spots.

There are more than 300 different mutations found on the *MECP2* gene, like insertions or deletions of DNA in the gene, being the most common mutations found in eight different locations.

II.5.5.1.2. Clinical Symptoms

As mentioned before, RTT shows large similarities with common autism due to its phenotype. This disorder is therefore characterized by a normal early growth till 6 to 18 months when the child’s development starts slowing down and other symptoms start to appear. These include loss of functional hand use and start of compulsive hand movements, such as wringing and washing, slowed brain and head growth, gait and other motor impairments and intellectual disabilities. These children show general lack of communication skills and severe dystonia, which is considered to be the main disabling feature of this disorder. This disorder is also associated with scoliosis, seizures and impaired cardiac, circulatory and respiratory function [45] [47].

II.5.5.1.3. Diagnosis and Disease’s Stages

RTT can be diagnosed with a simple blood test to identify the mutation but as this mutation can occur in other disorders it is also important to perform a clinical diagnosis based on signs and symptoms, fulfilling the diagnostic criteria.

There are usually four stages described for RTT [45]. The stage I, or early onset, typically begins between 6 and 18 months of age and lasts a few months or can continue for more than a year. As the symptoms at this point are imprecise, this stage may not be properly diagnosed, being missed by parents or physicians. The child may begin to lose eye contact as well as interest in toys. There can also be a delay in motor skills such as sitting or crawling. Stage II, also named rapid destructive stage, usually begins between ages 1 and 4 and may last for weeks or months and is characterized by the loss of purposeful hand skills and spoken language, but also characteristic hand movements such as wringing or washing. Likewise, breathing irregularities such as episodes of apnea and hyperventilation may occur, although breathing usually improves during sleep and walking may be unsteady and initiating motor movements can be difficult. It is at this stage that the slowed head growth is noticed.

Stage III, or the plateau or pseudo-stationary stage, usually begins between ages 2 and 10 and can last for years. Dystonia and seizures are prominent during this stage even though there can be an improvement in the child's behavior. The child can stay in this stage for most of her life.
Stage IV, or the late motor deterioration stage, can last for years or decades and noticeable features include reduced mobility, curvature of the spine (scoliosis) and muscle weakness, rigidity, spasticity, and increased muscle tone with abnormal posturing. Girls who were previously able to walk may stop walking. Cognition, communication, or hand skills generally do not decline in stage IV. Repetitive hand movements may decrease and eye gaze usually improves.

II.5.5.1.4. Treatment

Rett Syndrome has no cure and the current available treatments are not effective since they are symptomatic treatments that focus on diminishing the symptoms. According to each patient it may be needed specific medication like anti-epileptic drugs to control the seizures such as sodium valproate, or anxiolytics such as diazepam to prevent collateral damages of dystonia and repetitive behavior as mutilations. Specific physiotherapy to treat scoliosis or ventilators for respiratory impairment can also be options. These patients need special equipment and constant care and follow up [45].

II.5.5.1.5. Prospects

Having in mind the severity of this disease and the lack of permanent cure or even efficient treatments, the present study expects to help in the development of a robust differentiation method in order to obtain homogeneous populations of cortical neurons to a better understanding of the disease.
Understanding the disease pathway is essential for developing new therapies to manage specific symptoms and better control the disease, knowing that even the smallest improvement can be a huge step for these patients.
Animal models are well established in the science community and even though each model can be of great help, it is also well known that animal models can be rather poor. In fact, for neurodevelopmental diseases this poverty is more evident due to the human brain's complexity and divergence comparing, for instance, with guinea pig's brain.
As RTT is in fact a neurodevelopmental disorder, the available models do not fit the requirements and Yamanaka's new approach can be of major help for this type of diseases.
So, as explained previously, hiPSCs are an important breakthrough to better understand disease's pathology since they can be derived from patients. So, in this case, using hiPSCs derived from Rett Syndrome's patients, it becomes possible to expand and differentiate cells into specific neurons. These neurons will then be used to foresee differences between patient's neurons and healthy neurons, not only for morphologic and functional (electrophysiological) analysis but also analysis through flow cytometry and immunofluorescence.

This approach enables the possibility to test drugs or other approaches to correct or improve the phenotype of these patients.

Also, the newly cerebral organoids culture method can also be used for RTT as it was already used for microcephaly. In this case it is possible to generate organoids that would be a rough comparison to the brain and mimic neurodevelopment in some ways. Being RTT a neurodevelopmental disorder it can be possible to understand the crucial steps for the development of this disease and know where to intervene. It is also important to be aware that being a rare disease the available knowledge is still very scarce.

II.5.5.1.6. Deriving neuronal and glial subtypes from RTT patient specific iPSCs

As mentioned, RTT is a severe neurodevelopmental disorder caused by mutations in the X-linked gene *MECP2* and it has already been proved that neural precursors derived from RTT-hiPSCs did not show distinct anomalies during expansion or survival [44] [43] [48]. These cells have fewer glutamatergic synapses, morphological alterations and deficits in spontaneous activity.

Marchetto and colleagues [44] were also able to obtain the same alterations for RTT-hiPSCs derived neurons regardless the site mutation or differentiation methods, which can indicate these phenotypes as biomarkers for RTT.

More recently, it was also investigated molecular and cellular features of RTT by establishing hESC lines with a *MECP2* mutation and comparing mutant neurons to isogenic neurons from the parental hESC [49].

MAP2-positive neuronal cells differentiated using the dual SMAD-inhibition method were mainly VGluT1-positive excitatory neurons, and displayed many typical deficits of RTT neurons previously shown in mouse models and neurons from RTT-specific hiPSCs, such as smaller soma and nuclei, reduced neurite complexity, and electrophysiological deficits. Furthermore, it was also detected a global translational deficit due to reduced AKT/mTOR activity which is an important signaling pathway in apoptosis as well as mitochondrial defects [49] [48].

As mentioned before, some RTT patients may present other mutations such as in *CDKL5* gene but the subjacent mechanism is still unknown [48].

Also, it was studied the function of *CDKL5* in mouse hippocampal neurons using short-hairpin RNA to knock out *CDKL5* and it was demonstrated that *CDKL5* is essential for proper dendritic spine structure and for activity of excitatory synapses, thus explaining some key features in RTT [50]. Afterwards the group generated hiPSCs lines from two patients with *CDKL5* mutations and differentiated them into cortical neurons, achieving similar results as the obtained in a mouse model.

II.5.5.1.7. RTT neuronal phenotype rescue

Marchetto et al. [44] have already confirmed *MeCP2*'s role in regulating synapse formation, showing that the overexpression of *MeCP2* in control hiPSCs lead to an increase in glutamatergic synapse numbers. This data is supported by results obtained in mouse models lead by Chao et al. [51] in which

the loss or overexpression of MeCP2 lead to a decrease or increase of glutamatergic synapses, respectively [52] [53].

Not only neurons derived from RTT iPSCs have smaller soma and nuclei, and have fewer dendritic spines when compared to control iPSC and ESC neurons, as they are also functionally impaired and exhibit a transient rise of intracellular calcium levels reduction. They also show diminished frequency and amplitude of spontaneous excitatory and inhibitory post-synaptic currents when compared to control cells.

Studies have shown that re-activation of *MeCP2* expression in knockout mice led to a prolonged life span and delayed onset or reversal of certain neurological symptoms [54]. Given the specific phenotype presented in RTT neurons, Marchetto et al. [44] tried to rescue the *in vitro* phenotype using specific drugs, such as aminoglycosides antibiotics (gentamicin). They have also observed the effects of insulin-like growth factor 1 (IGF-1), which had previously been shown to partially reverse some RTT symptoms in *MeCP2* -deficient mice [55].

It is important to understand that as most of *MeCP2* mutations are random and create premature stop codons in the gene [56], the use of gentamicin, for instance, enables ribosomal read-through of stop codons, as it binds to the 16S rRNA [44].

Hence, they found that the administration of IGF1 in RTT neurons, with different *MeCP2* mutations, lead to an increase in *MeCP2* protein levels and in glutamatergic synapse numbers after 1 week.

These studies have therefore showed that RTT syndrome can benefit from Yamanaka's revolutionary method through drug discovery and screening, as it has already been proved useful in the discovery of novel drugs that can partially rescue RTT phenotype, and also through disease modeling for a better understanding of RTT pathology.

III. MATERIALS AND METHODS

III.1. hiPSCs expansion

III.1.1. Substrate preparation

III.1.1.1. Matrigel®

BD Matrigel® matrix growth factor reduced was stored in liquid aliquots at -20ºC and was thawed, overnight, on ice, at 4ºC. Afterwards, it was diluted in a proportion of 1:30 on cold DMEM/F12 with PenStrep (1:100 on DMEM/F12). The diluted solution was then used to coat the wells of a 6-well plate, which was incubated for at least two hours, at room temperature. The plate was coated with 1ml of the diluted solution per well and stored at 4ºC up to two weeks. Matrigel® was removed prior to cell seeding and replaced by cell culture medium.

III.1.1.2. Vitronectin XF™

An aliquot of Vitronectin XF™ (Life Technologies) containing 60µl was diluted in 6ml of sterile PBS, and the diluted solution was ready to coat the wells. Vitronectin™ was removed prior to cell seeding and replaced by cell culture medium.

III.1.2. Culture media preparation

III.1.2.1. mTeSR™1 (STEMCELL™ Technologies)

mTeSR™1 (STEMCELL™ Technologies) is a complete and defined serum-free medium, containing in its composition high concentrations of bFGF, TGF-β, GABA, pipecolic acid, lithium chloride. mTeSR™1 is not xeno-free as it contains bovine serum albumin. To prepare 500ml of mTeSR1, 100ml of thawed mTeSR™1 5X supplement was mixed with 400ml of mTeSR™1 basal medium. mTeSR™1 was then frozen in 50ml aliquots.

III.1.3. Other Cell Culture Reagents

III.1.3.1. EDTA (Life Technologies)

EDTA solution (Life Technologies) was prepared by mixing 500µl of 0.5M of EDTA stock, 500ml of sterile PBS and 0.9g of NaCl (Sigma®). This mixture was then filtered using a filtration unit and stored at 4°C.

III.1.3.2. Accutase (Sigma®)

Accutase (Sigma®) is a ready-to-use solution consisting of a mixture of proteolytic and collagenolytic enzymes used for the detachment of cells and tissues. It was used to perform single cell passaging of hiPSCs-derived neural precursors.

III.1.3.3. Penicillin Streptomycin

PenStrep (P/S) mixture contains 5,000 units of penicillin and 5,000µg/ml of streptomycin utilizing penicillin G (sodium salt) and streptomycin sulfate. It is an antibiotic mixture used in culture to prevent bacterial contaminations.

III.1.3.4. Washing Medium

To prepare 250ml of washing medium, 218,5ml of KO-DMEM (Gibco®) were mixed with 25ml of KO-SR (Gibco®), 2,5ml of MEM non-essential aminoacids (1%), 1,25ml of L-Glutamine (1mM), 250µl of β-Mercaptoethanol (0,1mM) (all from Life Technologies) and 2,5ml of P/S (1%).

III.1.3.5. PBS (Sigma®)

Phosphate buffered saline is a buffer solution containing sodium phosphate, sodium chloride and, in some formulations, potassium chloride and potassium phosphate. The osmolarity and ion concentrations of the solutions match those of the human body.

III.2. Cell culture

III.2.1. Cell lines

During this work four hiPSC lines were used, being two of them wild type cell lines and the other two derived from Rett syndrome patients.

EMC23i, commonly designed by R1, was kindly provided by Erasmus Medical School, in Rotterdam, and was derived from a female patient with a MeCP2 mutation.

Rett-male R855X3, provided by Professor Alysson Muotri, at UCSD, in USA, was derived from a male patient, also with a MeCP2 mutation.

Two hiPSCs lines obtained from healthy donors were also used: WT-Évora F0000B13 and iLB-C1-30 m-r12. WT-Évora was kindly provided by TCLab, in Évora, derived from a male donor. iLB-C1-30 m-r12 cells, commonly designed by Bonn cells along this work, and were provided by Professor Oliver Brüstle from University of Bonn, in Germany. These cells were derived from fibroblasts and reprogrammed through retroviral transduction of the four human genes OCT4, SOX2, C-MYC and KLF4. These cells were used as wild type control to increase the robustness of the method.

III.2.2. Thawing of hiPSCs

A cryovial containing hiPSCs was quickly withdrawn from the liquid nitrogen and covered in gaze containing ethanol, and then put into a water bath at 37ºC for a few seconds.
Then, drop-by-drop, 1ml of washing medium was added to the cryovial to softly thaw and mix the cells by up and down mixing. All the content of the cryovial was transferred to a previously prepared Falcon tube containing 3ml of washing medium. The Falcon tube was centrifuged for 3 minutes at 1000 rpm and the supernatant was discarded.
The cells were resuspended with 1ml of culture medium (mTeSR™1+P/S) and all the content was uniformly distributed in culture plates pre-coated with Matrigel®.

III.2.3. hiPSCs expansion

Once thawed, cells were plated on Matrigel®-coated plates, with mTeSR™1 supplemented with P/S. Cells were expanded during a few passages, with a splitting ratio of 1:3, in order to create cell banks, for each cell line.
Once all lines were cryopreserved for future use, cells were passaged onto Vitronectin™-coated plates, using mTeSR™1 medium supplemented with P/S. Cells were expanded for a few more passages, using the EDTA-passaging method with a splitting ratio of 1:3. Once confluence was attained, neural commitment protocol was initiated.

III.2.4. hiPSCs passaging

III.2.4.1. Enzyme-free method: using EDTA

To perform cell passaging using EDTA, the culture medium was firstly aspirated from the culture wells and cells were then washed twice with 0,5mM EDTA. After this, the wells were incubated for 3 minutes with 1,5ml of EDTA, at room temperature.
After these 3 minutes, the EDTA was removed and 1ml of medium (mTeSR™1 for hiPSCs) was added per well. Using the pipette, the cells were scraped from the well and collected into a collection tube. This process was performed twice. Finally, the total needed volume was added into the tube and the cells were ready to be plated.

III.2.5. Cryopreservation (using EDTA)

To freeze the cells, firstly the cells were washed with 1ml of EDTA per well and then incubated with 1mL of EDTA for 3 minutes. Afterwards, the cells were resuspended using 1ml of washing medium and scratched carefully from the well. The cells were then recovered into a Falcon tube which was centrifuged at 1000 rpm, for 3 minutes, and the supernatant was discarded. Then, the cells were resuspended with freezing medium (10% DMSO in KO-SR replacement) and transferred to a cryovial.

III.3. Neural differentiation of hiPSCs

III.3.1. Substrate preparation

III.3.1.1. Laminin (Sigma®)

The wells were pre-coated with poly-L-ornithine (Sigma®) and incubated at 37ºC, overnight.
Laminin was slowly thawed at 4ºC and then diluted in sterile PBS (Sigma®), to a final concentration of 20 µg/ml of laminin coating solution. Afterwards, the laminin was distributed onto the wells and the plates were incubated at 37ºC, for 2 hours. Before cell plating, laminin was aspirated.

III.3.2. Culture media preparation

III.3.2.1. N2B27

N2B27 medium is a chemically-defined and serum-free medium composed by 50% (v/v) of N2 medium and 50% (v/v) of B27 medium and is generally used for neural differentiation.
500ml of N2 medium is composed of 481.6ml of DMEM/F12 (Gibco®)+Glutamax, 5ml of N2 supplement (1%), 8ml of Glucose (1,6g/l), 400µl of insulin (20µg/ml) and 5ml of P/S (1%) (all reagents from Life Technologies).
250ml of B27 medium is composed of 241,25ml of Neurobasal, 5ml of B27 supplement (2%), 2,5ml of L-Glutamine (2mM) and 1,25ml of P/S (0,5%) (all reagents from Life Technologies).

III.3.3. Single-cell passaging using accutase

The medium in the wells was aspirated and 1 ml of accutase (Sigma®) was added per well. The plates were then incubated for 5 minutes at 37˚C. Washing medium (1ml) was used to resuspend and detach the cells from the wells. The cells were then transferred to a collection tube which was centrifuged at 1000 rpm for 3 minutes, at room temperature. The supernatant was discarded and the pellet resuspended in culture medium, N2B27. Finally, the cell suspension was distributed in new culture plates coated with culture substrate, laminin.

III.3.4. Neural commitment via dual-SMAD inhibition

The protocol used for neural induction of hiPSCs was the same for all cell lines.
Cells were plated on 1 well of a 6 well plate and on 4 wells of a 12 well plate. The latter wells were used for immunostaining at checkpoints: day 12, 17 and 29.
Cells were cultured on culture plates coated with Vitronectin™ and neural commitment started when the cells achieved full confluence. On day 1, mTeSR™1 medium was changed for N2B27 medium supplemented with small molecules, SB-431542 and LDN-193189 (Life Technologies). Until day 12, medium was changed every day and at day 12 the cells were passaged using EDTA and replated

onto laminin-coated plates, again in 1 well of a 6 well plate and on 4 wells of a 12 well plate. Once again the 4 wells were used for immunostaining using day 12 markers. After day 12 the medium was changed every day without supplementing the small molecules.

Around day 14, when neural rosettes started to appear, the medium was supplemented with 20ng/ml of FGF2 and the cells stayed 1 day without changing the medium. FGF is added when neural rosettes appear in order to promote the proliferation of neural progenitors in the rosettes, maintaining the neural markers expression [57]. The size of the rosettes influences cell fate and the smaller the clumps, the fewer the cortical cells are generated.

At day 17, the cells were passaged using EDTA and replated onto laminin-coated wells. Also, the 4 wells were used for immunostaining using appropriate markers. The medium was changed every day until day 29 when they were passaged with accutase and replated onto laminin-coated plates, at a density of $\sim 50.000\ cells/cm^2$. Also, immunostaining was performed using appropriate markers. After day 29, no more passages were performed and the medium was changed every other day. Immunostaining for days 75 and 100 were performed, using the appropriate markers. Also, around day 120 the cells were recovered for electrophysiology. The several stages of the neural differentiation protocol are presented in a schematic way, in the following Figure III.1.

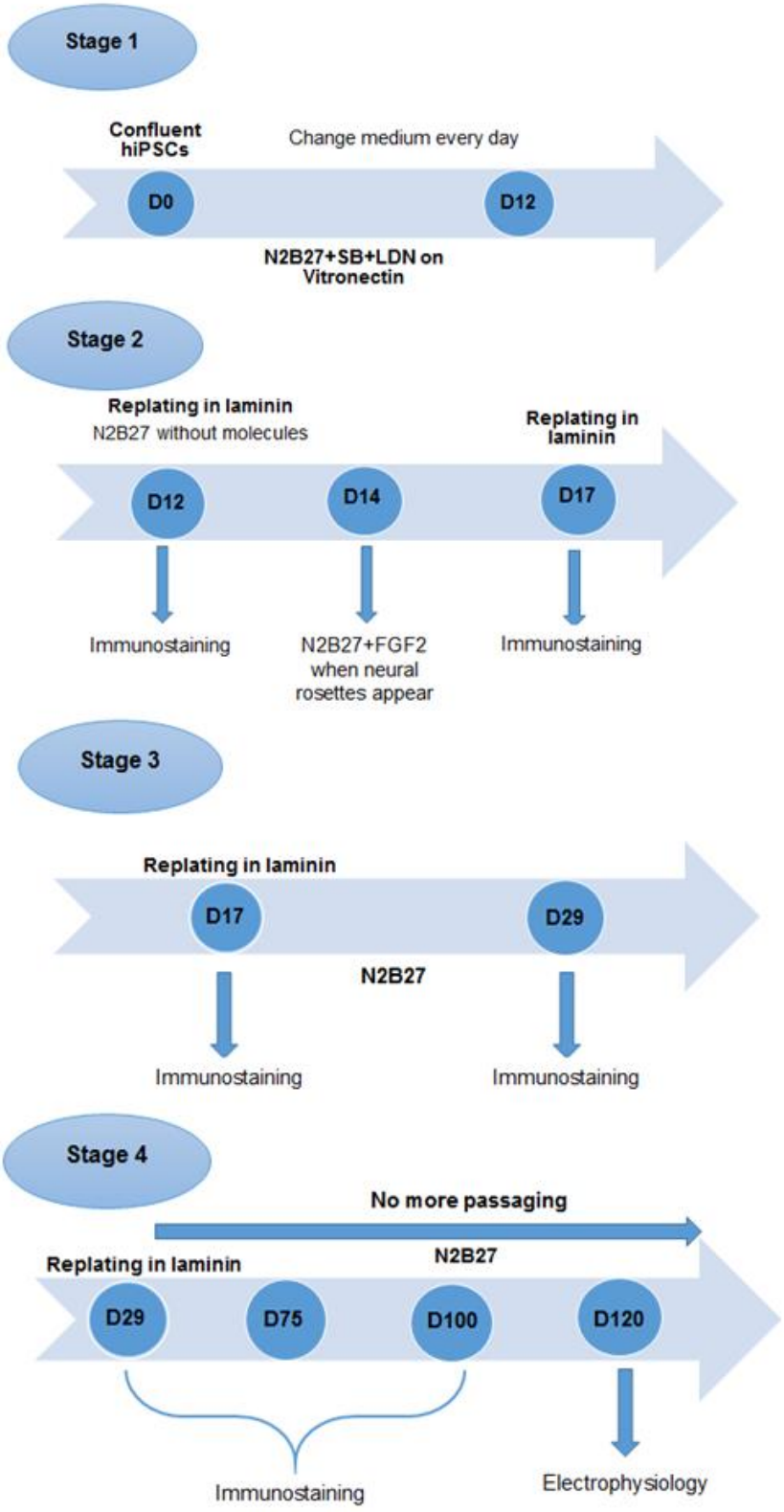


Figure III.1 - Different stages of the protocol used for neural induction of hiPSCs – from day 0 to day 120.

III.4. Cell characterization

III.4.1. Immunofluorescence microscopy

In order to analyze the phenotype of hiPSCs, immunostaining was performed, using specific fluorescently labeled antibodies. The used antibodies bind specifically to surface or intracellular antigens expressed by the cells and are fluorescently labeled.

III.4.1.1. Extracellular immunostaining protocol

The culture medium was replaced by medium containing the primary antibodies, previously prepared. Then, cells were incubated for 30 minutes at 37ºC. Afterwards cells were washed 3 times with 1ml of washing medium. The medium was substituted by culture medium containing the secondary antibodies and incubated for more 30 minutes at 37ºC. Then, cells were washed 3 times with 1ml of culture medium and were then ready to be examined using fluorescence microscopy.
All primary (Stem Gent) and secondary (Invitrogen) antibodies, as well as respective dilutions used in immunocytochemistry for extracellular staining are displayed in Table III-1.

Table III-1 - Primary and secondary antibodies used in immunocytochemistry analysis for surface markers in hiPSC cultures.

	Markers	Primary Antibodies	Dilution	Secondary Antibodies	Dilution
Pluripotency Markers	Tra1-81	Anti-Tra1-81	1:100	Goat anti-mouse IgM	1:400
	Tra1-60	Anti-Tra1-60		Goat anti-mouse IgM	
	SSEA4	Anti-SSEA4		Goat anti-mouse IgG	

III.4.1.2. Intracellular immunostaining protocol

The medium on the wells was aspirated and replaced by PFA 4% (Gibco®), for 30 minutes. Then, PFA was replaced with blocking solution and left at room temperature for 60 minutes. Afterwards, blocking solution (10% (v/v) NGS and 0.1% (v/v) Triton-X in PBS, all from Sigma®) was changed for the previously prepared staining solution (5% NGS (v/v) and 0.1% (v/v) Triton-X in PBS, all from Sigma®) with the primary antibodies and the plate was incubated at 4ºC overnight.
The following day, the primary antibodies were removed and cells washed 3 times using TBS (Tris Buffered Saline, Sigma®). The cells were then incubated in the dark, for 60 minutes, with the secondary antibodies in staining solution. After that, cells were washed 3 times with TBS and incubated with DAPI for 3 minutes. After 3 more washes with TBS, PBS was added to check staining at the microscope.

In the following Table III-2 are shown all primary antibodies, secondary antibodies (all secondary antibodies are from Invitrogen) and respective dilutions, used in immunocytochemistry for intracellular staining.

Table III-2 - Primary and secondary antibodies used in immunocytochemistry analysis for intracellular markers in hiPSC cultures.

	Primary Antibodies	Supplier	Dilution	Secondary Antibodies	Dilution
Pluripotency Markers	Anti-NANOG	Millipore	1:5000	Goat anti-rabbit IgG-546	1:400
	Anti-OCT4	Millipore	1:500	Goat anti-mouse IgG-546	
	Anti-SOX2	R&D Systems	1:200	Goat anti-mouse IgG-488	
	Anti-NESTIN	R&D Systems	1:400	Goat anti-mouse IgG-488	
Neural Differentiation Markers	Anti-PAX6	Covance	1:400	Goat anti-rabbit IgG-546	
	Anti-FOXG1	Abcam	1:100	Goat anti-rabbit IgG-546	
	Anti-OTX1/2	Millipore	1:100	Goat anti-rabbit IgG-546	
	Anti-ZO1	Zymed	1:100	Goat anti-rabbit IgG-546	
	Anti-KI67	BD	1:100	Goat anti-mouse IgG-488	
	Anti-TBR2	Abcam	1:400	Goat anti-rabbit IgG-546	
	Anti-TUJ1	Covance	1:4000	Goat anti-mouse IgG-546	
	Anti-GFAP	Millipore	1:100	Goat anti-mouse IgG-488	
	Anti-MAP2	Abcam	1:400	Goat anti-mouse IgG-546	

III.4.2. Flow cytometry

III.4.2.1. Extracellular staining for flow cytometry

To perform the extracellular staining for flow cytometry, 200.000 cells/condition (including negative control, which were resuspended in 3% NGS) were needed. Cells were washed twice with PBS (3ml) and centrifuged at 1000 rpm for 5 minutes. Then, cells were resuspended in 100µl of FACS buffer and the primary antibodies were added to the FACS tube. The tubes were incubated for 15 minutes in the dark, at room temperature, and then cells were washed twice with 2ml of PBS and centrifuged for 5 minutes at 1000 rpm. Afterwards, cells were resuspended in FACS buffer with the appropriate antibodies and incubated for 15 minutes at room temperature in the dark. Cells were then washed with 2ml of PBS and centrifuged for 5 minutes at 1000 rpm. This washing step was repeated twice and then cells were resuspended in 500µl of PBS and were ready to be analyzed.

The following Table III-3 presents all primary antibodies (from mouse host, Stem Gent) and dilutions, used in flow cytometry for extracellular staining. As primary antibodies were associated with fluorophores, secondary antibodies were not needed.

Table III-3 - Primary and secondary antibodies used in flow cytometry for analysis of surface markers in hiPSC.

Marker	Primary antibody	Dilution
TRA-1-60	Anti-Tra1-60-PE	1:10
TRA-1-81	Anti-Tra1-81-PE	1:10
SSEA4	Anti-SSEA4-PE	1:10

III.4.2.2. Intracellular staining for flow cytometry

Cells were fixed in 2% PFA and the Eppendorf tubes were coated with 400µl of BSA solution (1%) for 15 minutes. Then, cells were washed twice with 1% NGS and centrifuged at 1250 rpm for 5 minutes, each time. After that, cells were resuspended in 1ml of 3% NGS and 500µl of cell suspension was distributed in each Eppendorf tube (minimum of 5×10^5cells/condition), after BSA was removed. The tubes were then centrifuged at 1000 rpm for 3 minutes. The supernatant was removed and the pellet resuspended in 150µl of 3% NGS and 150µl of 1% saponin. The tubes were incubated at room temperature for 15 minutes and then centrifuged at 1000 rpm for 3 minutes. The supernatant was removed and the pellet resuspended with 300µl of 3% NGS and incubated at room temperature, for more 15 minutes. After this, the tubes were centrifuged at 1000 rpm for 3 minutes and the supernatant removed. The tubes were then resuspended with 300µl of 3% NGS for the negative control, and for the samples the antibody was diluted in 3% NGS. The tubes were incubated for 90 minutes in the dark and after that period, they were centrifuged at 1000 rpm, for 3 minutes. Afterwards, the tubes were washed twice with 1% NGS and then centrifuged at 1000 rpm for 3 minutes. The negative control was resuspended in 300µl of 3% NGS and the samples in 298µl of 3% NGS and 2µl of secondary antibody.

The tubes were incubated for 45 minutes in the dark and then centrifuged ate 1000 rpm for 3 minutes. Then the tubes were washed twice with 1% NGS and centrifuged again at 1000 rpm for 3 minutes. Finally, cells were resuspended in 500µl of PBS and transferred to FACS tubes and were ready for the analysis.

All primary and secondary antibodies, as well as respective dilutions, used in intracellular staining for flow cytometry, are displayed next, in Table III-4.

Table III-4 - Primary and secondary antibodies used in flow cytometry for analysis of intracellular markers in hiPSC.

Marker	Primary antibody	Dilution	Secondary antibody	Dilution
Oct 4	Anti-OCT4	1:300	Mouse Ig-G	1:400
Sox 2	Anti-SOX2	1:300		
NANOG	Anti-NANOG	1:5000	Rabbit Ig-G	

III.4.3. Quantitative real time polymerase chain reaction (qRT-PCR)

The expression of pluripotency and neural markers in Bonn hiPSCs, cultured on Vitronectin^{TM} with N2B27, was evaluated after 9 days of neural commitment using qRT-PCR.

This method was used for quantification of the pluripotency marker transcripts NANOG and OCT4 and for the neural markers PAX6 and Sox1. The housekeeping gene used was GAPDH.

First, the RNA was isolated from the collected samples at each day and used to synthesize cDNA templates. RT-PCR reactions were run in duplicate, following manufacturer instructions.

III.4.3.1. Isolation of total RNA from cultured cells

Total RNA was isolated from cell pellets previously stored at -80ºC at different culture stages (days 0, 3, 6 and 9) of neural commitment of hiPSCs using a high pure RNA isolation kit (Roche) - suited for 1×10^6 cells, according to manufacturer's instructions.

To resuspend the pellets, 200µl of PBS was added followed by 400µl of lysis-binding buffer. The content was put to vortex for 15 seconds. Then, the samples were transferred to a high-pure filter tube previously assembled to a collection tube and the assembly tube was placed in a standard tabletop and centrifuged for 15 seconds at 8000 rpm. The flow through liquid was discarded and the filter tube was once again combined with the collection tube. Afterwards, 90µl of DNAse incubation buffer was pipetted, per sample, into a sterile reaction tube with 10µl of DNAse I. The mix was pipetted on the glass filter fleece in the upper reservoir of the filter tube. The solution was left to incubate for 15 minutes at room temperature and then 500µl of washing buffer I was added to the upper reservoir of the filter tube assembly and centrifuged for 15 seconds at 8000 rpm.

After that, the flow through was discarded and, after recombining once again the filter tube with the collection tube, 500µl of washing buffer II was added and centrifuged for 15 seconds at 8000 rpm. Again, 200µl of washing buffer II was added to the upper reservoir of the filter tube, centrifuged for 2 minutes at 13000 rpm, in order to remove any residual washing buffer. Then, the collection tube was discarded and the filter tube was inserted into a clear, sterile microcentrifuge tube of 1,5ml.

To elute the RNA, 50µl of elution buffer was added to the upper reservoir of the filter tube and centrifuged for 1 minute at 8000 rpm. The eluted RNA was then quantified.

III.4.3.2. cDNA synthesis

cDNA was synthetized from RNA using a transcriptor first strand cDNA synthesis kit (Roche). For cDNA synthesis, 1µg of total RNA was used for each condition.

All the reagents were thawed on ice before being used and briefly centrifuged at 4000 rpm for 15 seconds. Using a sterile, nuclease-free, thin-walled PCR tube, the template-primer mixture was prepared by mixing the total RNA (1µg), anchored-oligo dT primer 50pmol/µl (1µl) and water PCR-grade (was added enough to make 13µl of final volume).

Afterwards, it was added 4µl of reverse transcriptase buffer, 0.5µl of RNAse inhibitor, 2µl of deoxynucleotides and 0,5µl of reverse transcriptase.

In the following Table III-5 are displayed all the reagents and the quantity used, by order of addition.

Table III-5 - Reagents added to the PCR tubes for cDNA synthesis and their amount, by order of addition.

Reagents	Quantity
Total RNA	1 µg
Anchored-oligo dT primer	1 µl
Water PCR-grade	Enough to complete 13 µl
Reverse transcriptase buffer	4 µl
RNAse inhibitor	0,5 µl
Deoxynucleotides	2 µl
Reverse transcriptase	0,5 µl

All the reagents were mixed carefully in the tube and centrifuged for 5 seconds at 4000 rpm and the thermal block cycler was pre-heated and the program was chosen. Then, the sample tubes were placed into the machine and ready to start the cDNA synthesis.

III.4.3.3. qRT-PCR protocol

To perform the qRT-PCR it is important to use the same amount of cDNA for all samples. It was pipetted, into a 1,5ml nuclease-free microcentrifuge tube 20x TaqMan Gene Expression Assay, 2x TaqMan Gene Expression MasterMix, cDNA template and RNAse free water. The tubes were capped and centrifuged briefly. Then, 20µl of PCR reaction mix was transferred into each well of a 48-well reaction plate according to the previously prepared scheme, as shown in Figure III.5. The plate was finally sealed and placed into the equipment.

Table III-6 shows the compounds and volumes present in the prepared PCR-reaction tubes.

Table III-6 – PCR reaction mix preparation.

PCR reaction-mix components	Volume needed per sample (single reaction)
2x TaqMan Gene Expression Master Mix	10 µl
20x TaqMan Gene Expression Assay Mix (Target Gene)	1 µl
Sample (cDNA template)	4 µl
RNAse free H_2O	5 µl
Total	20 µl

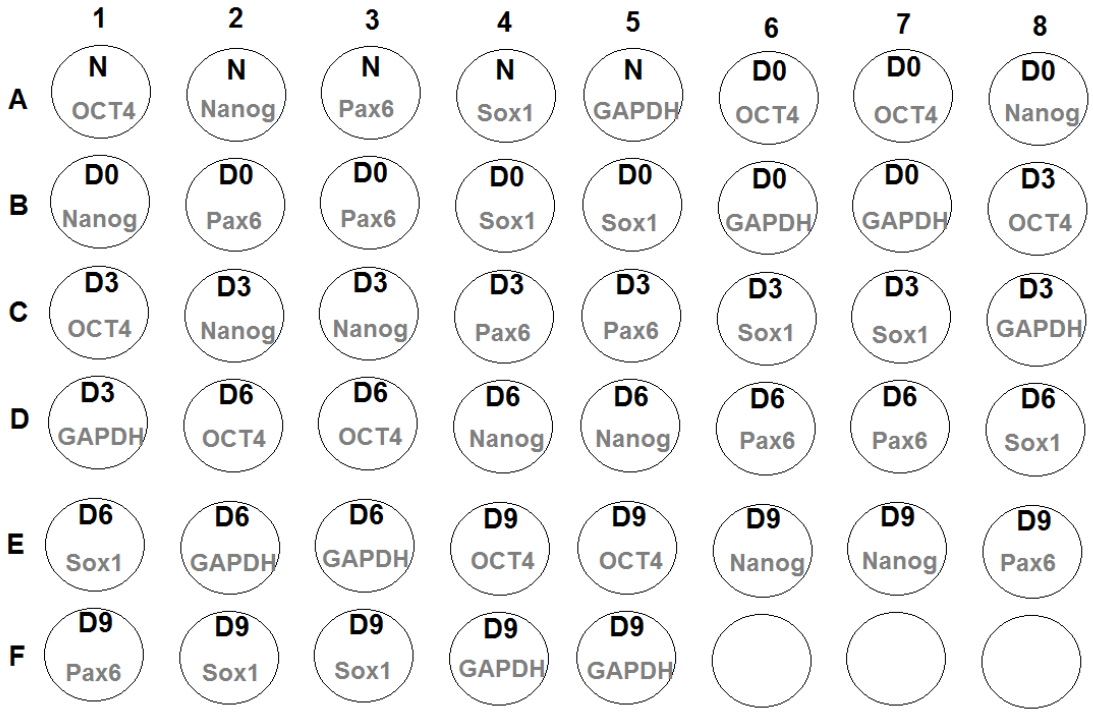


Figure III.2 – Schematic arrangement for qRT-PCR analysis

IV. Results and Discussion

IV.1. Characterization of hiPSCs-derived cells during expansion

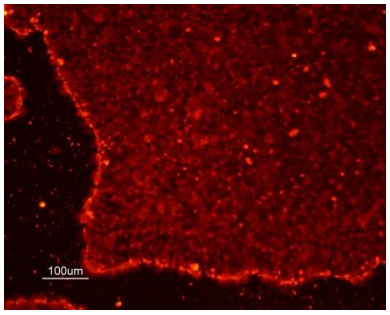
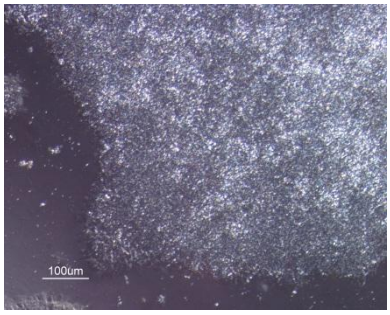
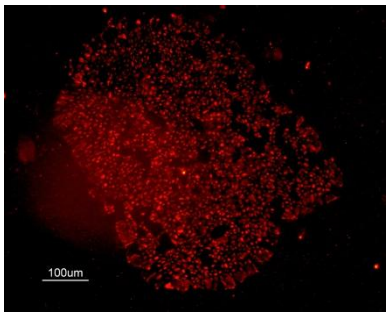
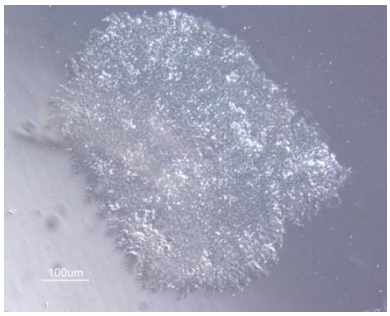
IV.1.1. Phenotype characterization – Immunofluorescence microscopy

IV.1.1.1. Extracellular Markers

To assess the pluripotency of hiPSC after *in vitro* expansion, immunostaining against extracellular markers as well as intracellular markers was performed. Figure IV.1 and Figure IV.2 present the results obtained for R1 and R-Male cells, respectively.

TRA-1-81, TRA-1-60 and SSEA4 are antigens expressed on the surface of undifferentiated cells as iPSCs or ESCs. Immunostaining results for these markers are presented in the left column of the tables; in the right column are presented images obtained through bright field microscopy.

The images only contain results for R1 and R-male as it was not possible to achieve results with WT-Évora cells, since they detached from the plates. As for Bonn cells, as they are a well-established lineage in the laboratory, these results have been already collected and these cells present high levels of pluripotency marker expression (both intracellular and extracellular).

R1 cells	Extracellular Marker	Bright-Field
SSEA4		
TRA-1-60		

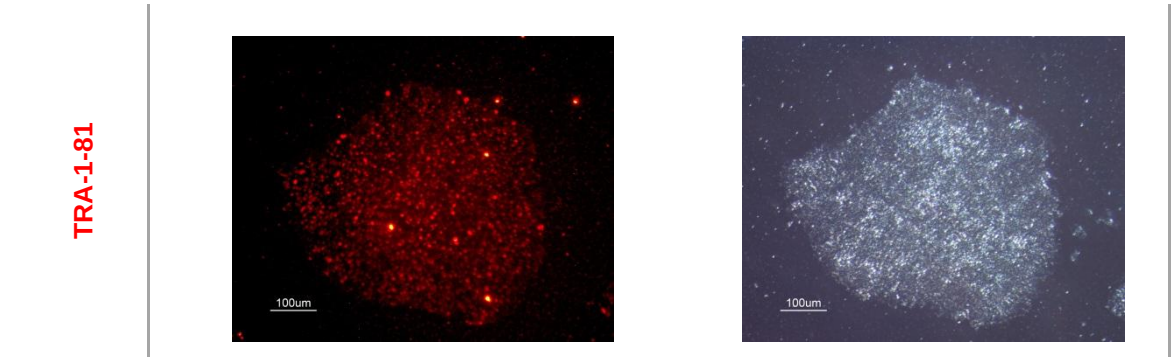


Figure IV.1 – Immunostaining analysis of hiPSC for the pluripotency extracellular markers TRA-1-60, TRA-1-81 and SSEA4 for R1 and the corresponding bright field microscopy images.

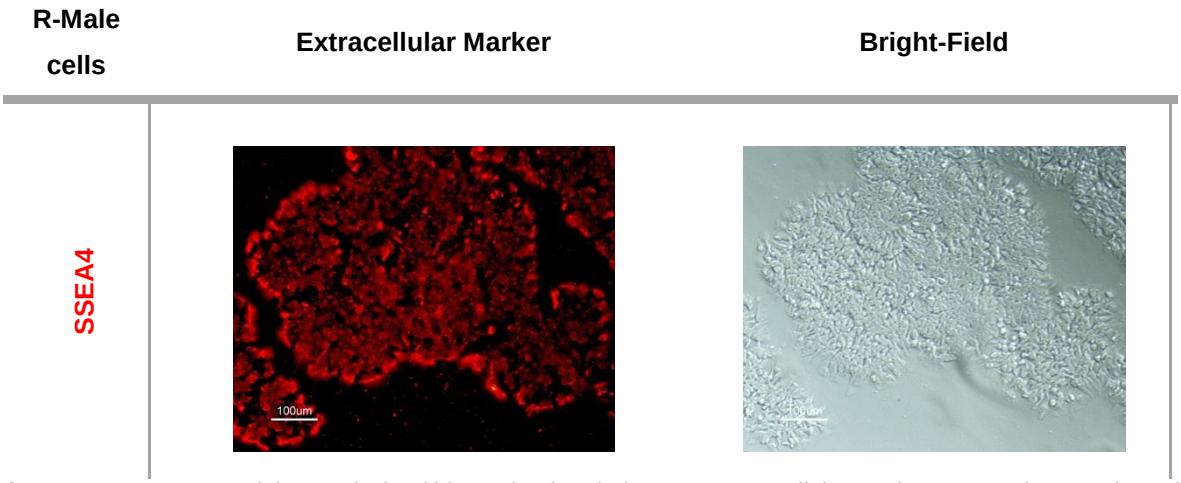


Figure IV.2 - Immunostaining analysis of hiPSC for the pluripotency extracellular marker SSEA4 for R-Male and the corresponding bright field microscopy images.

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It is possible to verify, by analysis of the Figure IV.1 and Figure IV.2, that both cell lines presented high levels of extracellular pluripotency markers' expression.

Next, it will be presented the results for intracellular markers.

IV.1.1.2. Intracellular Markers

The following Figure IV.3 presents the results obtained for immunostaining against intracellular markers of pluripotency NANOG, OCT4 and SOX2, for both R1 and R-Male cells. These results were merged with the results obtained for DAPI (nucleus marker) in order to better evaluate pluripotency.

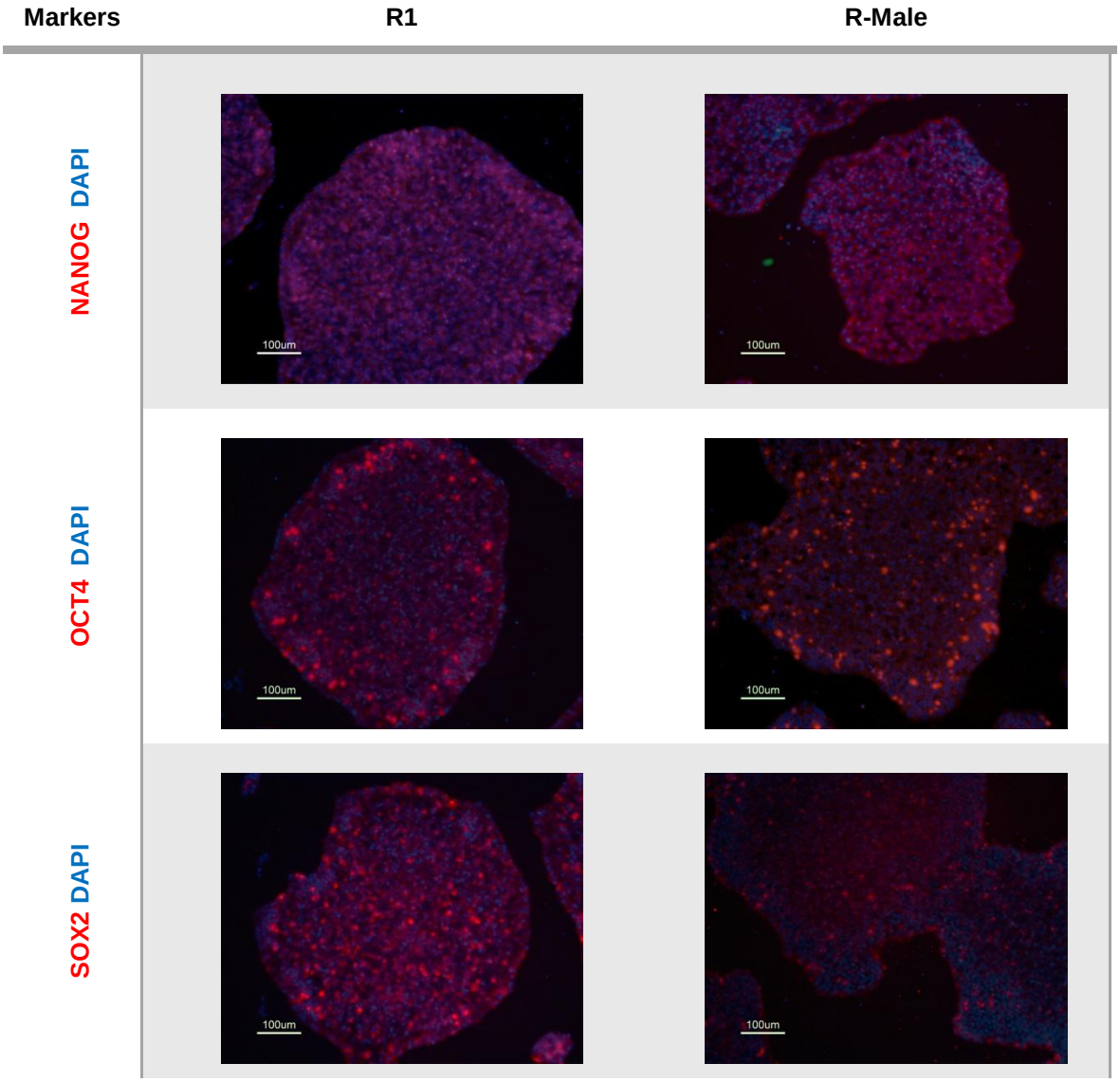

Figure IV.3 - Immunostaining analysis of hiPSC for the pluripotency intracellular markers NANOG, OCT4 and SOX2, all merged with DAPI results, for R1 and R-Male.

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It is possible to conclude that both mutated lines present high levels of expression for pluripotency markers, being therefore possible to conclude that these cells were able to maintain their pluripotency after 6 passages *in vitro.*

Also, no relevant differences in terms of pluripotency were observed between the two mutated cell lines and the wild-type cell line (results obtained for Bonn cells are not presented in this work).

IV.1.2. Quantitative characterization – Flow cytometry

IV.1.2.1. Extracellular Markers

Flow cytometry was also performed in R1, R-Male and WT-Évora cells to increase the robustness of the results obtained by immunocytochemistry for pluripotency analysis of the expression of TRA-1-60 and SSEA4 extracellular markers.

For this analysis, debris and cell aggregates were excluded through side and forward scattering, which measure the cytoplasmic complexity and the cell size, respectively.

These results for the surface markers analysis using flow cytometry are presented next on Figure IV.4:

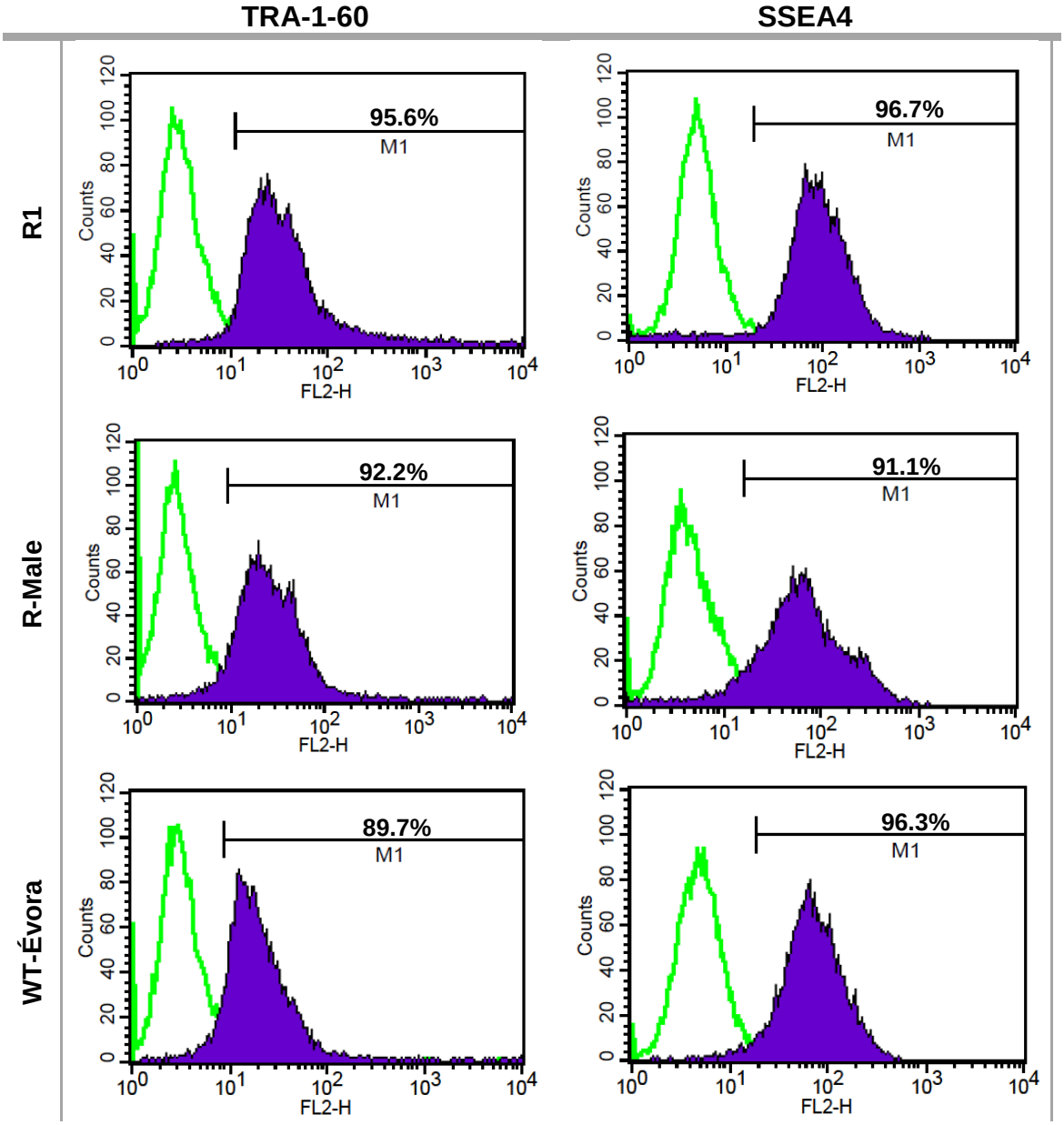

Figure IV.4 – Flow cytometry analysis for the expression of extracellular markers TRA-1-60 and SSEA4 for R1, R-Male and WT-Évora cells.

IV.1.2.2. Intracellular Markers

Besides using surface markers, the expression of intracellular markers was also analyzed as a measure of cell pluripotency. OCT4, NANOG and SOX2, which are the main pluripotency genes, were analyzed by flow cytometry on R1, R-Male and WT-Évora cells.

The following Table IV-1 displays the results for intracellular markers analysis using flow cytometry, for all three cell lines.

Table IV-1 - Flow cytometry analysis for the expression of intracellular markers OCT4, SOX2 and NANOG for R1, R-Male and WT-Évora cells

Cell Line	OCT4	SOX2	NANOG
R1	97.9%	97.8%	98.6%
R-Male	97.4%	97.7%	98.3%
WT-Évora	97.1%	93.4%	97.8%

The results presented in Table IV-1 show the expression percentage for each marker and it is possible to see that all three lines show high levels of expression for all pluripotency markers, therefore corroborating the results obtained for immunocytochemistry. In fact, all three hiPSC lines presented high expression of pluripotency markers, both surface and intracellular. At this point it was possible to settle that all lines were able to maintain their pluripotency, after expansion during six consecutive passages *in vitro*. Moreover, there were not relevant differences between patient and wild type hiPSCs.

IV.2. Characterization of hiPSCs-derived cells after 12 days of neural commitment using the dual-SMAD inhibition protocol using Vitronectin™

The neural commitment protocol started when cells grown on Vitronectin™ with mTeSR™1 were confluent. At this point, the dual-SMAD inhibition protocol was initiated and mTeSR™1 was switched for N2B27 medium supplemented with small molecules (LDN-193189 and SB-431542).

This methodology aimed the achievement of a high yield of early neuroepithelial cells and consequently mature neurons, in a robust and reproducible way. By day 12, cells were passaged onto laminin and the N2B27 medium was no longer supplemented with SB-431542 and LDN-193189.

IV.2.1. Phenotype characterization - Immunofluorescence microscopy

Immunostaining was performed to characterize hiPSCs-derived cells after starting the dual-SMAD inhibition protocol. After the first 12 days, cells were stained against primary progenitors markers PAX6, NESTIN, SOX2, FoxG1 and OTX1/2; and also against OCT4 pluripotency marker, to check whether cells lost their pluripotent state [58].

PAX6, or paired box 6 protein, is coded by the *PAX6* gene and is expressed during embryonic development in neural and epidermal tissues and sensory organs. It is an important gene during neurogenesis and oculogenesis.

NESTIN is an intermediate filament protein type VI and is expressed in dividing cells during the development of the CNS and PNS.

Besides being important for pluripotency maintenance, SOX2, or sex determining region Y box 2, is also expressed by developing cells from the neural tube and in neural progenitors in the CNS, being inactivated in the end of neural differentiation.

FoxG1, or forkhead box protein G1, plays an important role during early neural embryonic development, particularly in the telencephalon [59]. OTX1/2, homeobox protein OTX1/2, is used as a forebrain/midbrain marker [29]. It is also widely accepted that the OTX1/2 and FoxG1 are some of the first markers to be expressed in neuroectodermal cells during neural differentiation [48].

The following Figure IV.5 shows the immunostaining analysis obtained after 12 days of neural differentiation for both mutated lineages, R1 and R-Male. As for Figure IV.6, it the immunostaining analysis obtained after 12 days of neural differentiation for wild-type lineages, WT-Évora and Bonn.

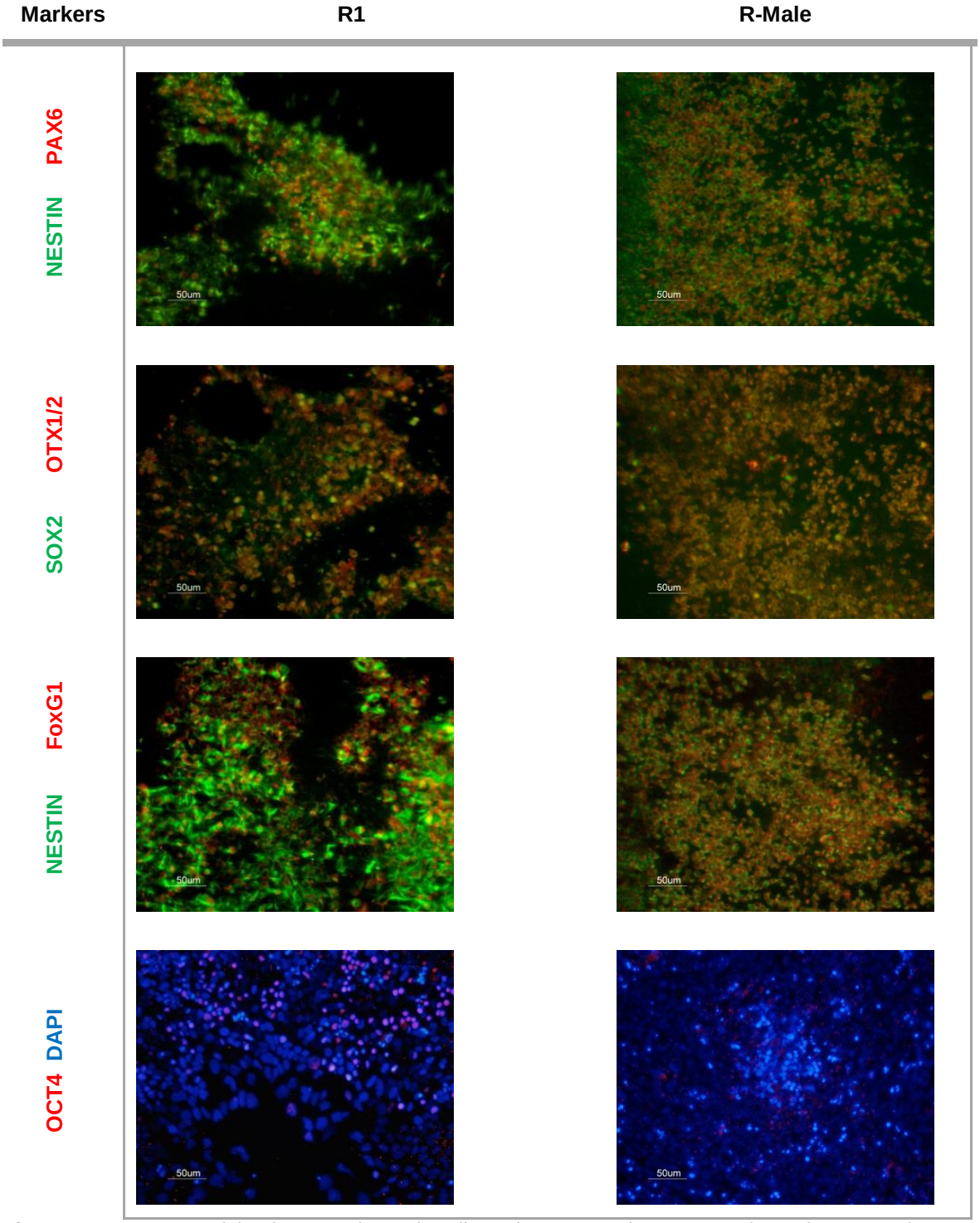

Figure IV.5 - Immunostaining for R1 and R-Male cells against NESTIN/PAX6, SOX2/OTX1/2, NESTIN/FoxG1 and OCT4/DAPI after 12 days of neural differentiation using the dual-SMAD inhibition method.

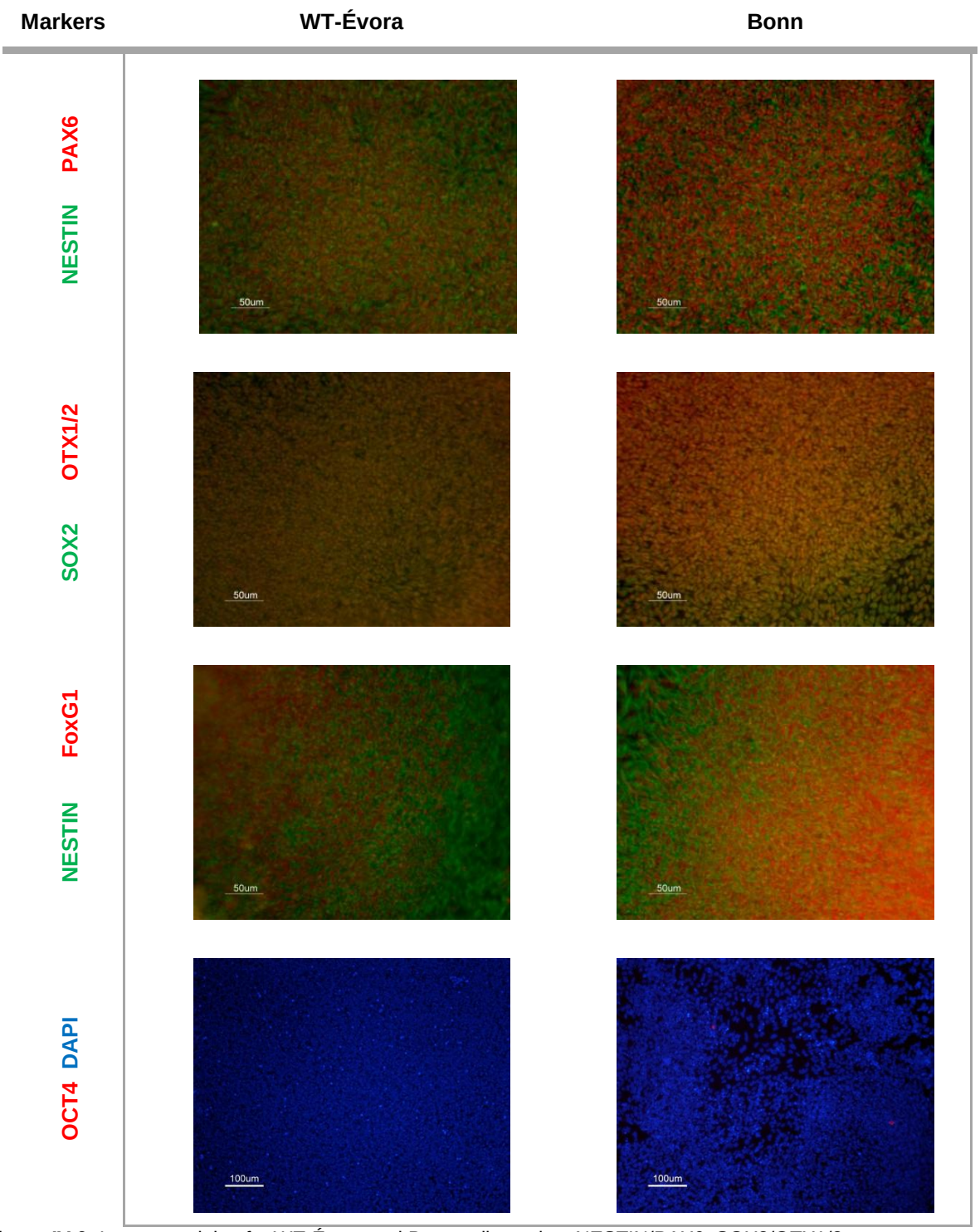

Figure IV.6- Immunostaining for WT-Évora and Bonn cells against NESTIN/PAX6, SOX2/OTX1/2, NESTIN/FoxG1 and OCT4/DAPI after 12 days of neural differentiation using the dual-SMAD inhibition method

Figures IV.5 and IV.6, presented above, show the results for immunostaining at day 12 of differentiation. At this day, cells expressed characteristic neuroectodermal cell markers by the 12th day of differentiation. Also, along with the increase of these neural markers, OCT4 expression decreased when compared with the results obtained during expansion, as it is possible to validate by observation of Figure IV.3 (immunostaining results for pluripotency markers during expansion).

At this point, wild type cells showed more homogeneity in marker expression that can be related to a faster achievement of neural identity, when compared to mutated cells.
As R-Male cells detached from the wells at day 12, from this day on, it was not possible to gather results for this cell line, due to time restrictions.

IV.2.2. Quantitative real time PCR

Gene expression profiling was also performed for a better characterization of Bonn hiPSCs-derived cells, at different time points of neural induction: day 0, day 3, day 6 and day 9.
Initially, it was extracted total RNA from the cells at those time points, using a high pure RNA isolation kit (ROCHE).
After performing the protocol for RNA extraction, it was performed RNA quantification and the results obtained are displayed in the following Table IV-2:

Table IV-2 – RNA quantification (ng/µl) after RNA extraction from hiPSCs (Bonn cells) for gene expression profiling during neural differentiation, using quantitative real time PCR. Two measurements were performed and the average was used for further calculations

Quantification	D_0	D_3	D_6	D_9
#1	292	1605	3715	563,6
#2	281,2	1495	3509	418
Average	286,6	1550	3612	490,8

After quantifying the RNA, using a transcriptor first strand cDNA synthesis kit (ROCHE), cDNA was synthetized. Once synthetized the cDNA it was then possible to perform the quantitative RT-PCR. The genes used are displayed in Table IV-3.

Table IV-3 – Target genes used in quantitative real time PCR analysis for hiPSCs (Bonn cells) during neural differentiation.

Genes	
Pluripotency	OCT4
	NANOG
Neural Induction	Sox1
	PAX6
Housekeeping	GAPDH

As GAPDH was used as endogenous control, its expression was used to normalize the results as its expression does not diverge between samples. Since GAPDH is involved in glycolysis, it is a high level expressed gene in most cells being thus an appropriate choice for housekeeping gene in qRT-PCR. As this technique gives relative expression, all expressions for days 3, 6 and 9 are comparatively to day 0. The results obtained using the qRT-PCR are presented next in Figure IV.7.

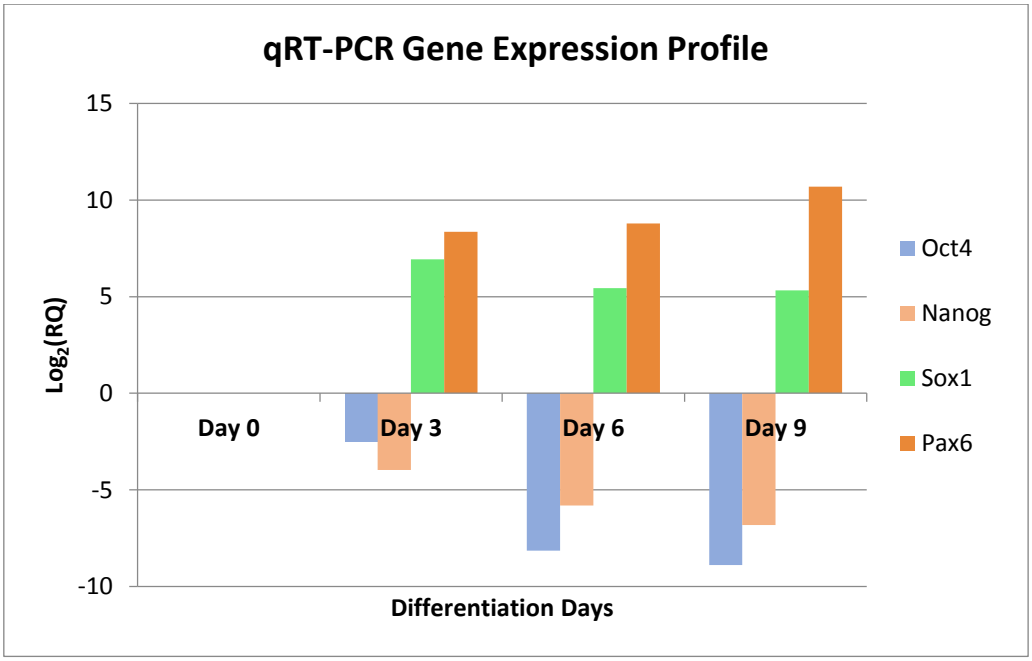

Figure IV.7 - Gene expression profile by qRT-PCR for the characterization of neural differentiation of hiPSCs (Bonn). Expression of pluripotency (OCT4 and NANOG) and neural differentiation (Sox1 and PAX6) genes was evaluated comparatively to the expression of the housekeeping gene GAPDH.

Figure IV.7 represents the expression of specific markers (NANOG, OCT4, Sox1 and PAX6) throughout 9 days of neural differentiation. These expressions are presented in terms of ΔΔCt which is in fact ΔCt sample – ΔCt calibrator. Also, as RQ, relative quantification, is equal to $2^{-\Delta\Delta Ct}$ and RQ is the sample fold change compared to the calibrator (day 0) which has a RQ value of 1, this is a comparative analysis, being all samples compared to the calibrator. If a sample has a RQ of 10 for a specific gene, it means that gene is 10 times more expressed in that sample then in the calibrator sample which is day 0. However, if RQ is 0.1, that gene is 10 times less expressed. The results for day 3 are presented in the following Table IV-4.

Table IV-4 – Values obtained using qRT-PCR for ΔΔCt and RQ for day 3, for OCT4, NANOG, PAX6 and Sox1, using hiPSCs (Bonn cells).

Gene	ΔΔCt	RQ
OCT4	2.49	0.18
NANOG	3.96	0.06
PAX6	-8.36	327.80
Sox1	-6.94	122.46

According to the Table IV-4, NANOG was expressed approximately 17 times less in day 3 and PAX6 was expressed approximately 300 times more.

First of all, as it was expected throughout differentiation, OCT4 and NANOG expression decreased as a signal of pluripotency loss. By day 9, OCT4 and NANOG expression decreased 200 and 900 times, respectively. As for Sox1 and PAX6, by day 9 their expression was 40 and almost 2000 times higher, respectively, when compared to day 0, suggesting an efficient neural induction.

After analyzing Figure IV.7 it is possible to observe that Sox1 expression is higher at day 3 of neural differentiation (120 times higher than in day 0) and by day 9 its expression it is only 40 times higher. This was expected as Sox1 is in fact one of the earliest transcription factors to be expressed in ectodermal cells committed to the neural fate and its expression decreases alongside the increase of neural differentiation. The onset of this gene coincides with the induction of neuroectoderm.
As for PAX6 expression, it is 300 times higher in day 3 and by day 9 almost 2000 times higher. Thus, PAX6 expression increases with differentiation progress, which was expected, as this gene is an important transcription factor throughout neurogenesis. qRT-PCR analysis is an important tool as it provides quantitative results that complement qualitative results obtained through other tools as immunostaining. In fact, these results were able to confirm immunostaining results, and it is possible to conclude that neural induction was successful, with almost none expression of pluripotency markers after 12 days and strong expression of neural markers along differentiation according to *in vivo* neurogenesis.

IV.3. Neural stem cell expansion and long-term differentiation

The purpose of this work, besides the reproduction of *in vitro* neurogenesis and gliogenesis, is the achievement of functional neurons from hiPSCs to understand the hallmarks of RTT and to compare their behavior with wild-type cell behavior. So, the cells had to be maintained in culture until ~day 120, when they could be used for electrophysiological studies to confirm whether or not they could generate action potentials. So, along the process of neural differentiation, the cells were characterized by immunofluorescence at days 17, 29, 75 and 100.

IV.3.1. Day 14

By day 14, as cells presented some neural rosettes, N2B27 was supplemented with 20ng/ml FGF2, and the medium was changed after 2 days. As previously mentioned, FGF is added when neural rosettes appear in order to promote the proliferation of neural progenitors in the rosettes, maintaining the neural markers expression [57]. Also, the size of the rosettes influences cell fate and the smaller the clumps the fewer the cortical cells are generated. Thus, after adding FGF, neural rosettes presented show of growth with neural progenitor cell expansion.

IV.3.2. Day 17

At the 17th day of neural commitment, cells were passaged, once again, onto laminin plates, using the EDTA^{TM}-based passaging method. Also, immunostaining was performed at this day and cells were stained against neuroectodermal characteristic markers PAX6, NESTIN, SOX2 and ZO1. ZO1, tight junction protein 1, is used as a marker in neural differentiation as it shows the cellular polarization

through ZO1 distribution. ZO1 is uniformly distributed in undifferentiated cells and its apical localization is a typical indicator of neural induction.

The results obtained after immunochemistry at day 17 are presented next, on Table IV.8.

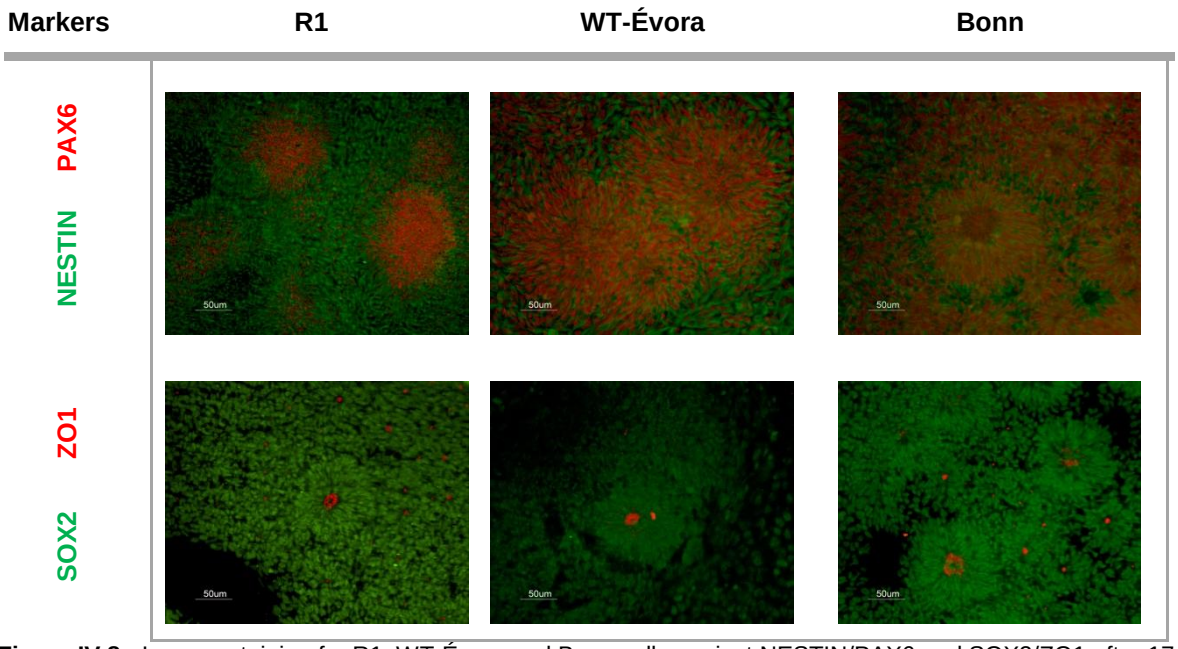

Figure IV.8 - Immunostaining for R1, WT-Évora and Bonn cells against NESTIN/PAX6 and SOX2/ZO1 after 17 days of neural differentiation using the dual-SMAD inhibition method.

At day 17 it was possible to observe some rosette structures and these cells presented apical expression of ZO1 on the rosette centers surrounded by the presence of SOX2-positive cells. These cells were also still positive for NESTIN and PAX6, which can indicate the presence of radial glial cells. This neural rosette organization mimics *in vivo* neurogenesis, as it is also possible to observe a radial display of the cells as they migrate to the rosette periphery.

With these results it is possible to confirm that neural commitment was successful in all tested cell lines and that R1 cells do not present marked differences when compared with wild-type cells at this point of the differentiation. However, it can be inferred by analysis of Figure IV.8 that PAX6 expression appears to be lower in R1 cells which can indicate less neuroepithelial cells comparatively with wild-type cells.

IV.3.3. Day 29

At the 29th day of neural commitment, cells were split into single cells, using accutase, and were quantified. Cells were plated at the ideal cell density of 50,000 cells/cm^2 [15]. After this day, no more passages were performed as neurons are sensitive to cell passaging. However, as culture time increased, cells begin to detach from laminin substrate and, for that reason, laminin was periodically added to the medium upon medium change, which occurred every other day. Also, immunocytochemistry analysis was performed using the following markers: PAX6, NESTIN, KI67, ZO1

and TUJ1. KI67 is a nuclear protein, which is associated with cellular proliferation and TUJ1, a neuronal class III tubulin, is expressed exclusively by neurons in development and is used to identify neural progenitors. The results for immunostaining at day 29 are presented next in Figure IV.9.

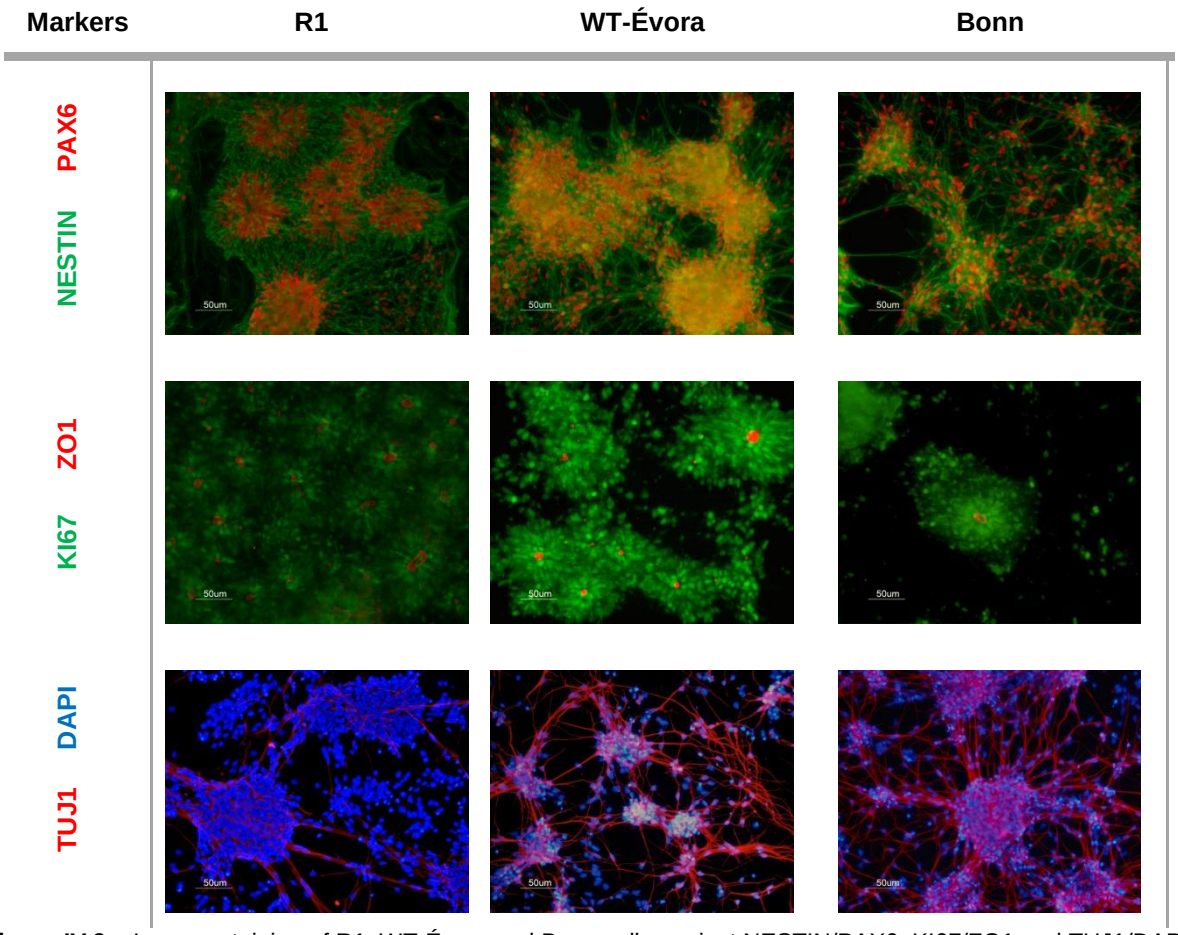

Figure IV.9 – Immunostaining of R1, WT-Évora and Bonn cells against NESTIN/PAX6, KI67/ZO1 and TUJ1/DAPI after 29 days of neural differentiation using the dual-SMAD inhibition method.

As it is possible to verify in Figure IV.9, all lines began to show some morphological changes at day 29 as some projections started to appear. PAX6 is still expressed at this time point of the differentiation, which indicates that there are still some neural progenitor cells in culture. The expression of TUJ1 is also present, since some immature neurons started to appear in rosettes' periphery as they remain present at day 29 (presence of rosette confirmed by KI67/ZO1 staining). Moreover, at this day, primary neural progenitor population started the transition to secondary neural progenitor population, as TBR2 expression was positive in a significant number of cells, especially for wild-type cells (results not shown). TBR2 expression indicates the presence of basal progenitors, confirming thus the presence of immature neurons. So, as PAX6 expression decreased on day 29, when compared to day 17, it suggests a transition from neuroepithelial cells and radial glial cells (primary cell population) to basal progenitors (secondary cell population), confirmed by TBR2 expression. Finally, it was possible to observe that wild-type and R1 cells presented similar results for PAX6 and NESTIN expression

markers as well as for KI67, ZO1 and TBR2. However, R1 cells present considerably less TUJ1-positive cell projections.

IV.3.4. Day 75

At the 75th day of neural differentiation cell cultures were again characterized by immunocytochemistry to evaluate the generation of neurons and astrocytes, using TUJ1 and GFAP which is an astroglial cell marker. The results for immunostaining after 75 days of neural differentiation are presented next on Figure IV.10:

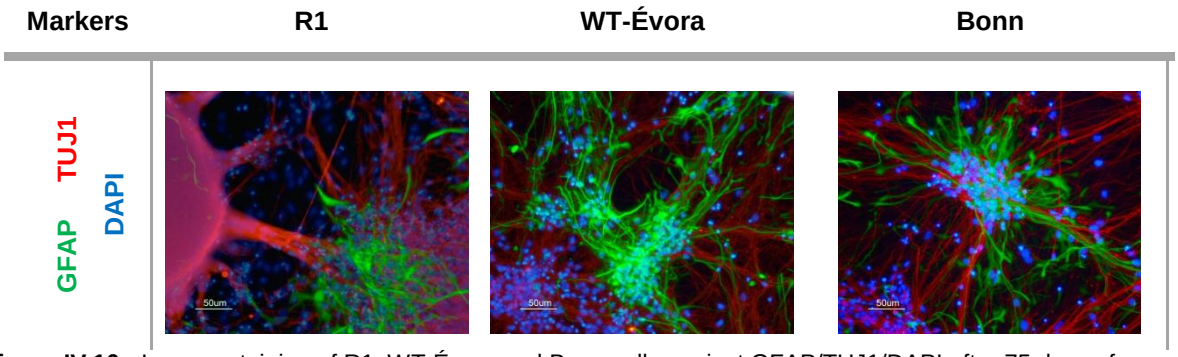

Figure IV.10 - Immunostaining of R1, WT-Évora and Bonn cells against GFAP/TUJ1/DAPI after 75 days of neural differentiation using the dual-SMAD inhibition method.

At this point, and after 75 days on culture, neural progenitor cells were able to give rise to cells with long projections (dendrites). These cells were positive for the TUJ1 marker, suggesting a neuron-rich population. Also, there were cells positive for GFAP, suggesting a mixed population of neurons and astrocytes. Comparing wild-type cells (WT-Évora and Bonn) with mutated cells (R1), it is possible to conclude that mutated cells present a population containing a lower number of GFAP-positive cells as well as Tuj-1 positive cells. Moreover, and even though it is not possible to verify a lower total number of cells in R1, it is clear that these cells present not only a lower number of Tuj-1-positive cells but also fewer projections and lower neurite complexity when compared either to WT-Évora or Bonn cells.
These results are validated by previous studies performed by other groups as Marchetto's group [44], as mentioned previously in this report.

IV.3.5. Day 100

At the 100th day of neural differentiation cell cultures were characterized by immunocytochemistry, stained against MAP2, microtubule-associated protein 2, which is a microtubular protein as TUJ1. MAP2 intervenes in the microtubular association essential in neurogenesis, stabilizing the microtubules. MAP2 is expressed in more advanced stages of neurogenesis, marking, therefore, more mature progenitors. The results for immunostaining at day 100 are presented next on Figure IV.11:

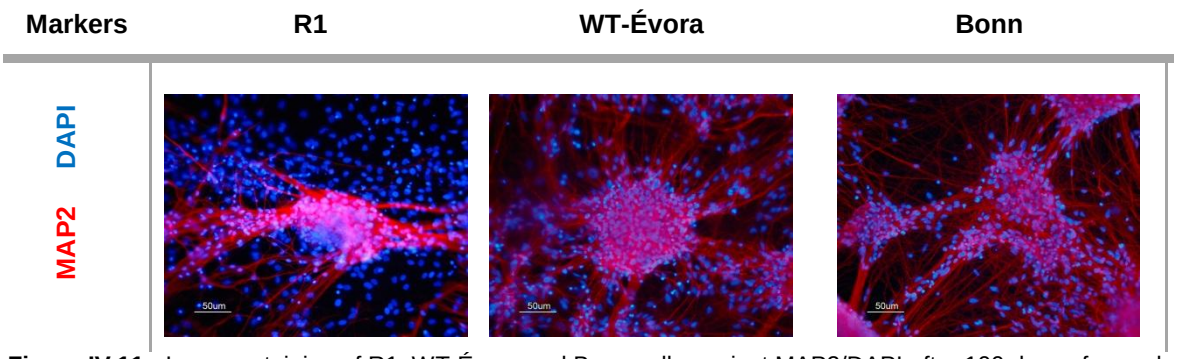

Figure IV.11 - Immunostaining of R1, WT-Évora and Bonn cells against MAP2/DAPI after 100 days of neural differentiation using the dual-SMAD inhibition method.

By analysis of the Figure IV.11 it is possible to verify that in all three hiPSC lines, by day 100, there was already an enriched population of mature neurons expressing MAP2. However, once again, it is possible to see that, when compared to wild type lineages, R1 presents considerably fewer dendritic spines. Regarding the number of cells, it appears that R1 differentiating cultures do not present fewer cells when compared to wild-type cells. Therefore, R1 cultures may present, along with fewer projections, fewer neurons.

V. Conclusions and Future Work

The culture system developed in this work, consisting on a monolayer-adhesion system, using Vitronectin^{TM} as substrate, and using a modified dual-SMAD inhibition protocol was successful in mimicking neurogenesis and gliogenesis from hiPSCs *in vitro,* when using three distinct hiPSC lines (R1, R-Male and WT-Évora). In particular, in previous work performed in this lab, it was demonstrated that this method was successful in generating functional glutamatergic neurons from hiPSCs (Bonn hiPSC line), after ~130 days in culture.

During the first 12 days of differentiation, two small molecules, SB-431542 and LDN-193189, were added to the N2B27 medium. After these 12 days, neural precursors were already present in all 4 lineages used: R1, R-Male, WT-Évora and Bonn. After these 12 days, small molecules were withdrawn and after a short period of FGF treatment, neural progenitors matured only in the presence of N2B27, on laminin-coated culture plates.

As this protocol revealed to be a robust system for the achievement of mature neurons from hiPSCs, it was possible to compare the behavior of mutated cells and wild-type cells and understand characteristic hallmarks of Rett syndrome.

During expansion, as it is possible to confirm by analysis of the results obtained from flow cytometry and immunofluorescence microscopy, hiPSC cultures did not exhibit any significant differences, presenting similar rates of proliferation and similar percentages of pluripotent cells.

However, during neural differentiation, some differences started to be visible between mutated and wild type lines, as determined by immunofluorescence microscopy. In fact, after 12 days of differentiation, wild type cells displayed more homogeneous populations of PAX6 and NESTIN-positive cells and also seemed to have fully lost pluripotency. As for mutated cells, they still presented some OCT4-positive cells at this point and appear to be expressing lower levels of neuroectodermal markers.

After 17 days of differentiation, R1 presented many rosette structures, but fewer PAX6-positive cells. This difference of expression may lead to the conclusion that mutated cells present deficits in expansion of neuroepithelial cells or that progenitor cells are maturing faster.

At the 29th day of differentiation, the cell structure started to rearrange as cells matured and some projections were already visible. Once again, important differences were detectable between lines as R1 presented fewer Tuj-1-positive cells.

By day 75, it was still possible to verify that wild-type cell culture displayed higher numbers of mature cells, thus more TUJ1-positive cells than R1 cell culture. At this day, cells were also stained against GFAP, which is glial marker and all three hiPSC lineages showed a similar expression of this marker, presenting R1 cultures slightly lower expression of GFAP. However, these results indicate that R1 cells do not show relevant deficits in maturation into astrocytes.

The last immunocytochemistry analysis was performed at day 100 of hiPSC neural differentiation and it was possible to conclude that at this point R1 cells show considerably less projections than wild-type cells as well as less MAP2-positive cells.

As it was possible to achieve electrophysiological results from R1 cells, it can be concluded that it was possible to obtain R1-mature cells, even though that when compared to wild-type lines (WT-Évora and

Bonn cells) R1 cultures presented less MAP2-positive cells by day 100. These results can lead to the conclusion that R1 cultures presented a lower mature/immature cell ratio or that R1 cultures presented a lower number of neural cells, when compared to wild type lines.

Even though the main goals of this work were achieved, there are still many issues that should be focused in future work. First of all, since R-Male cells were lost during differentiation, important data were not collected during this work. As this protocol is very time consuming, it was not possible to re-start R-Male differentiation in this work lifetime. However, it would be very important to have two more mutated lineages to corroborate all the results obtained from R1 cells and assure very robust results.

In general, to achieve more tangible results, it would be important to repeat all the experiments, using the same lines, to verify the reproducibility of the results.

As mentioned, this is a very time consuming protocol, and the final results were achieved only 4 months after starting the differentiation, without including the time period for hiPSC expansion. Therefore, as all cells detached from the plates at day 120, it was not possible to perform the last immunocytochemistry analysis, to confirm the achievement of glutamatergic neurons. However, and as mentioned, this protocol had already been performed before this thesis, in the laboratory, using Bonn cells, and glutamatergic neurons were achieved. Thus, it is possible to make the assumption that glutamatergic neurons were also achieved during this thesis as well, as all the steps were reproduced.

Regarding the culture system, a monolayer culture system was chosen due to its easiness and control ability. Nevertheless, it would be interesting to use a 3D culture system, as cerebral organoids [29], as it could provide novel insights since cells are grown similarly as *in vivo* and it is possible to follow the process of brain's development. Also, it is a great model to study neurodevelopmental diseases, like RTT. However, this protocol has many issues as lack of control of cells or core death, as organoids increase its size.

As for the future, and as this is a rather disabling disorder, worsen by the fact that there is neither cure nor effective treatment, it would be very interesting to use this culture system to test drugs, growth factors or other soluble factors that could revert some of the hallmarks of RTT. In fact, one of the most promising applications for patient specific hiPSCs is the screening of drugs and other factors. For example, it was already proved that IGF1 can partially reverse some symptoms of RTT [55].

VI. References

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VII. Attachments

VII.1. Solutions for flow cytometry

BSA (1%):

Bovine Serum Albumin is composed of 670µL of 30% BSA stock solution and 19,3mL of PBS.

Saponin (1%)

Composed of 0,2g of saponin and 20mL of PBS.

NGS (3%)

Normal goat serum (3%) is composed of 1mL of NGS previously thawed and then dissolved in 32mL of PBS.

NGS (1%)

Normal goat serum (1%) is composed of 16,7mL of 3% NGS dissolved in 33,3mL of PBS.