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

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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^{TM} as substrate and mTeSR^{TM}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

Introduction

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] and since their discovery in the early 60s, by Till and McCulloch, their biomedical relevance has been increasing.

Pluripotent stem cells (PSCs) can give rise to any cell type as they can originate all of the three germ layers of the embryo – endoderm, mesoderm and ectoderm.

PSCs were, until recently, only isolated from the inner mass of the blastocyst, being called embryonic stem cells (ESCs). As ESCs are derived from embryos, their isolation leads to embryos' destruction, which associates these cells with severe ethical issues.

However, in 2006, Yamanaka's group was able to reprogram somatic cells into the pluripotent stem cell state using a mixture of four transcription factors, and established a highly reproducible method for the generation of induced pluripotent stem cells (iPSCs) [2]. Yamanaka used four transcription factors - OCT4, SOX2, c-MYC and KLF4 - which were introduced into somatic cells (fibroblasts) using a retroviral vector [2].

Even though the ethical issues associated with these cells are diminished, they present other issues, mostly about safety.

Nevertheless, human induced pluripotent stem cells (hiPSCs) brought many expectations into the biomedical field due to their potential applications in disease modelling, 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.

hPSCs, whether embryonic or induced, can be characterized by surface molecular markers related with the pluripotency state, as tumor related antigens - TRA-1-60 and TRA-1-81 - and stage-specific embryonic antigen 3 and 4 – SSEA-3 and SSEA-4 [3] [4]. Besides these surface markers, they can also be identified by intracellular markers, which are also preponderant in pluripotency maintenance, as NANOG, OCT4 and SOX2 [4] .

Culture of hPSCs

hPSCs survival depends on cell-cell and cell-matrix interactions and so they are commonly cultured under adherent conditions as colonies, using flasks or plates.
hPSCs can be maintained in their undifferentiated and proliferative state *in vitro*, using feeder-dependent or feeder-free culture systems. Feeder-dependent culture systems such as MEFs (mouse embryonic fibroblasts) are far from ideal as the use of a culture system with two types of cells increases variability, hampering the creation of a standard and reproducible protocol [5].
Coating matrices aim to overcome those impairments and to mimic ECM through integrin binding in order to achieve more efficient adhesion and consequently increase cell survival. Examples of feeder-free culture systems are Matrigel®, Vitronectin™ or Synthemax®.

Neural commitment via dual-SMAD inhibition

The dual-SMAD inhibition protocol was developed by Chambers and colleagues [3] and it is a very straightforward method to obtain a rapid and complete commitment of hPSCs into the neural lineage.
SMADs are intracellular proteins that transduce extracellular signals to the nucleus, where they activate gene transcription. This protocol is based on the inhibition of two signaling pathways that use SMADs for transduction: BMP and activin/nodal, combining two potent inhibitors - noggin and SB-431542.
SB-431542 leads to a fast loss of NANOG expression which results in pluripotency loss as well as prominent increase of CDX2 expression, meaning that the pluripotency loss is associated with differentiation towards trophoblast lineage [3]. SB-431542 also mediates mesodermal lineage suppression (Brachyury), by inhibiting activin/nodal pathway [3].
As for Noggin, its use leads to a CDX2 repression, thus inhibiting BMP endogenous signals that drive trophoblast fates upon differentiation. It has been used in many neural induction protocols. Also, there are evidences of Sox17 suppression – endodermal lineage - with Noggin use [3]. Thus, SB-431542 and Noggin work synergistically at multiple stages of differentiation to achieve efficient neural conversion of PSCs [3]. 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. It was later discovered that Noggin can be switched by another small molecule, LDN-193189, a derivative of Dorsomorphin, which is also a BMP signaling inhibitor, contributing to a less expensive protocol [6].

Rett Syndrome

Rett Syndrome (RTT) is a neurodevelopmental disorder characterized by autistic-like behaviors and is commonly caused by a mutation in the methyl CpG binding protein 2 gene (*MECP2*) of the X chromosome. This gene intervenes in the synthesis of the methyl cytosine binding protein 2 (MeCP2), which is preponderant for brain development and acts as one of the many biochemical switches for gene expression [7] [8]. Thus, an abnormality in this gene prevents the brain's nerve cells from working properly and RTT patients present insufficient amounts or structurally abnormal forms of the protein, causing other genes to be atypically expressed [9].
This disease affects mainly girls, since the *MECP2* gene is found on the X chromosome, one of the two sexual chromosomes. Therefore, as female patients present two X chromosomes, and being only one active in any given cell, there is only a portion of the cells with the defective gene. The remaining cells use the healthy gene of the other X chromosome, expressing normal amounts of the protein. In cases of male patients, since they have only one X chromosome (and one Y chromosome), they lack a back-up copy that would compensate for the defective one. For that reason, male patients are rarer but the existing are related with less aggressive mutations or sporadic mutations [9] [10]. The severity of RTT depends on the percentage of cells expressing the normal copy of the *MECP2* gene.
RTT symptoms appear around 6 to 18 months of birth and 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 [10] [11].
Rett Syndrome has no cure and currently available treatments are not effective since they are symptomatic treatments.
Having in mind the severity of this disease and the lack of permanent cure or even efficient treatments, the present thesis expects to help in the development of a robust differentiation method, able to mimic neurogenesis and gliogenesis *in vitro* from hiPSC, and 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. In fact, even the smallest improvement can be a huge step for these patients.

Materials and Methods

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, 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.

Feeder-free culture of hiPSCs

Human iPSCs were quickly thawed and cultured in mTeSR™1 medium (STEMCELL™ Technologies) with PenStrep and Rocki (Millipore), on Matrigel® (BD)-coated plates, prepared on DMEM/F12 (Gibco®) and PenStrep. Medium was changed daily only with mTeSR™1 and PenStrep, and cells were passaged every 3-4 days, after reaching around 80% confluence, using EDTA™ buffer 0,5mM (Life Technologies) with a split-ratio of 1:3.

Neural commitment of hiPSCs

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™ (Life Technologies) and neural commitment started when the cells achieved full confluence. On day 1, mTeSR™1 medium was changed for N2B27 medium (Life Technologies) 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 (Sigma®), 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 appropriate 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 (Life Technologies) 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 [12]. 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 (Sigma®) 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 now presented in Figure1:

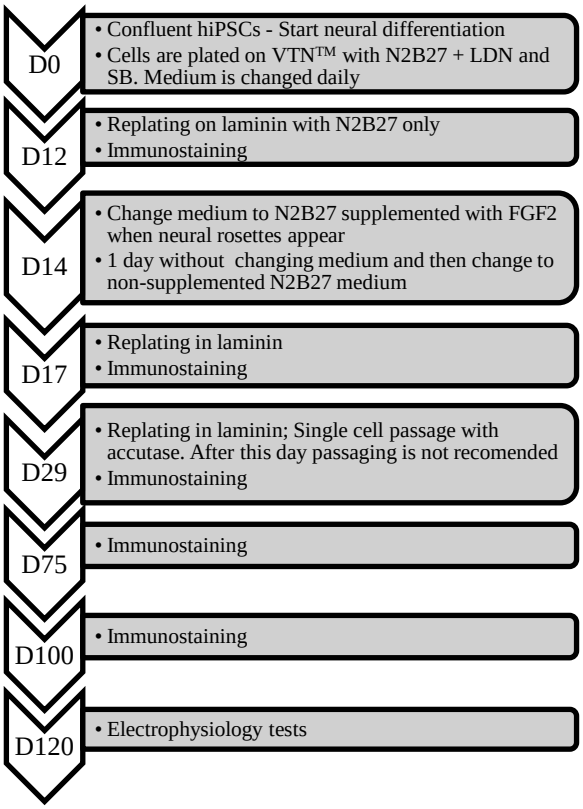


Figure 1 – Different stages of the protocol used for neural induction of hiPSCs – from day 0 to day 120.

Intracellular immunostaining

Cells were fixed with 4% paraformaldehyde (PFA, Gibco®) for 30 minutes at room temperature. Then, PFA was substituted for blocking solution (10% (v/v) Normal Goat Serum (NGS, Sigma®)), 0.1% Triton (Sigma®) in PBS) and incubated at room temperature for 60 minutes. After 60 minutes, blocking solution was aspirated and cells were incubated, at 4 ºC overnight, with primary antibodies in staining solution (5% NGS with 0.1% Triton in PBS). The following day, cells were washed three times with PBS and incubated with the secondary antibodies in staining solution, for 60 minutes at room temperature, in the dark. Cells were then washed three times with PBS and then incubated for 3 minutes, with DAPI (4',6-diamidino-2-phenylindole nucleic acid stain, 1:10000, Sigma®). After nuclear staining, the cells were washed twice with TBS and PBS was then added to check staining under a fluorescence optical microscope.
Table 1 shows primary and secondary (Invitrogen) antibodies and their dilutions used for intracellular staining.

Table 1 - Primary and secondary antibodies used in immunocytochemistry analysis for intracellular markers in hiPSC cultures. Dilution for all secondary antibodies is 1:400. Suppliers: 1-Millipore, 2-R&D Systems, 3-Covance, 4-Abcam, 5-Zymed, 6-BD

	Primary Antibodies	Dilution	Secondary Antibodies
Pluripotency Markers	Anti-NANOG[1]	1:5000	Goat anti-rabbit IgG-546
	Anti-OCT4[1]	1:500	Goat anti-mouse IgG-546
	Anti-SOX2[2]	1:200	Goat anti-mouse IgG-488
Neural Commitment Markers	Anti-NESTIN[2]	1:400	Goat anti-mouse IgG-488
	Anti-PAX6[3]	1:400	Goat anti-rabbit IgG-546
	Anti-FOXG1[4]	1:100	Goat anti-rabbit IgG-546
	Anti-OTX1/2[1]	1:100	Goat anti-rabbit IgG-546
	Anti-ZO1[5]	1:100	Goat anti-rabbit IgG-546
	Anti-KI67[6]	1:100	Goat anti-mouse IgG-488
	Anti-TBR2[4]	1:400	Goat anti-rabbit IgG-546
	Anti-TUJ1[3]	1:4000	Goat anti-mouse IgG-546
	Anti-GFAP[1]	1:100	Goat anti-mouse IgG-488
	Anti-MAP2[4]	1:400	Goat anti-mouse IgG-546

Extracellular immunostaining

The culture medium was replaced by medium containing the primary antibodies and incubated for 30 minutes at 37ºC. Then, the cells were washed 3 times with 1ml of washing medium and incubated with culture medium containing the secondary antibodies for more 30 minutes at 37ºC.
Afterwards, cells were washed 3 times with 1ml of culture medium and then were ready to be examined using fluorescence microscopy. Table 2 shows all primary (Stem Gent) and secondary (Invitrogen) antibodies and their dilutions used for extracellular staining.

Table 2 - Primary and secondary antibodies used in immunocytochemistry analysis for surface markers in hiPSC cultures

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

Flow cytometry analysis

Extracellular staining

Cells were washed twice with PBS and centrifuged at 1000 rpm for 5 minutes. Next, they were resuspended in 100µl of FACS buffer and the primary antibodies were added to the FACS tubes. 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. Cells were resuspended in FACS buffer with the appropriate antibodies and incubated for 15 minutes at room temperature in the dark, and then washed with 2ml of PBS and centrifuged for 5 minutes at 1000 rpm. The washing step was repeated twice and cells were resuspended in 500µl of PBS. Finally, cells were ready to be analyzed.

Intracellular staining

Cells were fixed in 2% PFA, washed twice with 1 % NGS and centrifuged at 1250 rpm for 5 minutes. Cell pellets were then resuspended in 1ml of 3% NGS and the cell suspension was distributed on Eppendorf tubes previously coated with 1% BSA (Life Technologies™). The tubes were centrifuged at 1000 rpm for 3 minutes and the pellet was resuspended in 150µl of 3% NGS and 150µl of 1% saponin (Sigma®), to permeabilize cell membranes. Cells were incubated, at room temperature, for 15 minutes and then centrifuged at 1000 rpm for 3 minutes, being the pellet resuspended in 300µL of 3% NGS and incubated at room temperature for 15 minutes. Once again, cells were centrifuged and resuspended in 300µL of 3 % NGS with the primary antibody. Negative controls were resuspended only in 3% NGS. Cells were incubated at room temperature for 90 minutes. Afterwards, cells were centrifuged and washed twice with 1 % NGS solution to remove unattached primary antibodies and pellets were resuspended in staining solution containing the secondary antibodies. Cells were incubated for 45 minutes at room temperature, in the dark. Finally, cells were washed twice with 1% NGS and resuspended in 500µL of PBS, on FACS tubes. Analysis was performed using the FACSCalibur flow cytometer (BD Biosciences).
All primary and secondary antibodies and respective dilutions used for both intracellular and extracellular staining for flow cytometry are displayed in Table 3.

Table 3 – Extracellular and intracellular markers and dilutions used for flow cytometry analysis in hiPSC cultures. Dilution for all secondary antibodies is 1:400.

	Primary antibody	Dilution	Secondary antibody
Extracellular Markers	Anti-TRA-1-60	1:10	-
	Anti-TRA-1-81	1:10	
	Anti-SSEA4	1:10	-
Intracellular Markers	Anti-OCT4	1:300	Goat anti-mouse IgG
	Anti-SOX2	1:300	Goat anti-mouse IgG
	Anti-NANOG	1:5000	Goat anti-rabbit IgG

Quantitative real-time PCR (qRT-PCR)

The expression of pluripotency and neural markers in Bonn hiPSCs, cultured on Vitronectin™ 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 the neural markers PAX6 and Sox1. The housekeeping gene used was GAPDH.
First, total RNA was isolated from cells 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. cDNA was synthetized from RNA using a transcriptor first strand cDNA synthesis kit (Roche).
For cDNA synthesis 1µg of total RNA was used in each condition. Taqman Gene Expression Assays (20X) (NANOG, OCT4, PAX6, Sox1 and GAPDH) and cDNA samples were thawed on ice and briefly centrifuged. The PCR-reactions were run in duplicate, using the StepOneTM RT-PCR System (Applied Byosistems). Reactions were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, endogenous control). Results were analysed with the StepOne software.

Results and Discussion

Human iPSCs were expanded and differentiated for 120 days and characterized at specific check points. All lines were submitted to equal conditions, being therefore possible to compare the behaviour of wild-type cells and mutated cells.

hiPSCs pluripotency characterization: flow cytometry analysis

Table 3 - Flow cytometry analysis for hiPSCs after expansion for surface (SSEA4 and TRA-1-60) and intracellular (OCT4, SOX2 and NANOG) pluripotency markers for R1, R-Male and WT-Évora cells.

	Cell Lines	R1	R-Male	WT-Évora
Surface Markers	TRA-1-60	95.6%	92.2%	89.7%
	SSEA4	96.7%	91.1%	96.3%
Intracellular Markers	OCT4	97.9%	97.4%	97.1%
	SOX2	97.8%	97.7%	93.4%
	NANOG	98.6%	98.3%	97.8%

The results displayed on Table 3 were obtained by flow cytometry and show the pluripotency markers' expression, both extracellular and intracellular, for hiPSCs during expansion.
All hiPSC lines presented high expression of pluripotency markers (above ~90%), both surface and intracellular, for flow cytometry. So, along with the results obtained by immunocytochemistry (results not shown), it is possible to conclude that all hiPSC lines presented similar behavior during expansion. At this point, it was also possible to settle that all hiPSC lines, expanded during six passages *in vitro*, were able to keep pluripotency. There were no relevant differences between mutated and wild type cells.

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

Quantitative RT-PCR analysis

Gene expression profiling was performed on Bonn hiPSCs-derived cells, at different time points of neural induction: day 0, day 3, day 6 and day 9.
The results obtained using the qRT-PCR are presented next, on Figure 2.

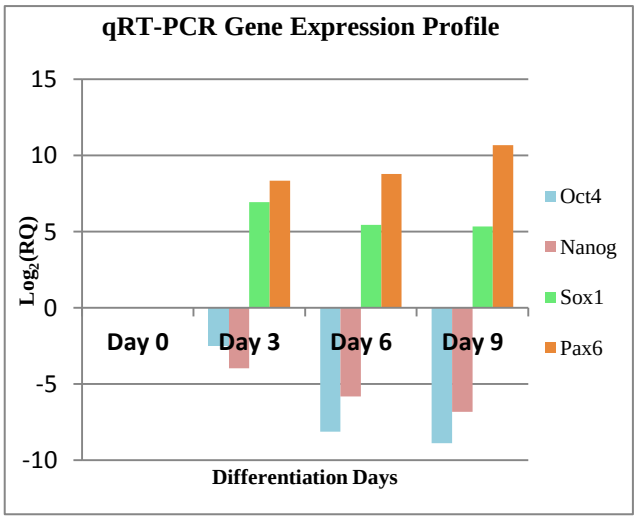


Figure 2 - Gene expression profile by qRT-PCR for the characterization of neural differentiation of hiPSCs (Bonn). Expression of pluripotency (OCT4 and NANOG) and neural differentiation (Sox1 and PAX6) genes was evaluated comparatively to the expression of the housekeeping gene GAPDH.

Figure 2 shows 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 Table 4

Table 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 4, OCT4 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 approximately 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.
Analyzing Figure 2 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.

Immunocytochemistry analysis – phenotype characterization

Immunostaining was also performed to characterize hiPSCs-derived cells after starting the dual-SMAD inhibition protocol. After the first 12 days, cells were stained against primary progenitor's markers PAX6, NESTIN, SOX2, FOXG1 and OTX1/2 and also against
OCT4 pluripotency marker, to check whether cells lost their pluripotent state [13].
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 in the 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 SNC, 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 [14].
OTX1/2, homeobox protein OTX1/2, is used as a forebrain/midbrain marker [15]. 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 [16].

The following Figure 3 shows the immunostaining analysis obtained after 12 days of neural differentiation for mutated lines, R1 and R-Male cells, and wild-type lines, WT-Évora and Bonn cells.

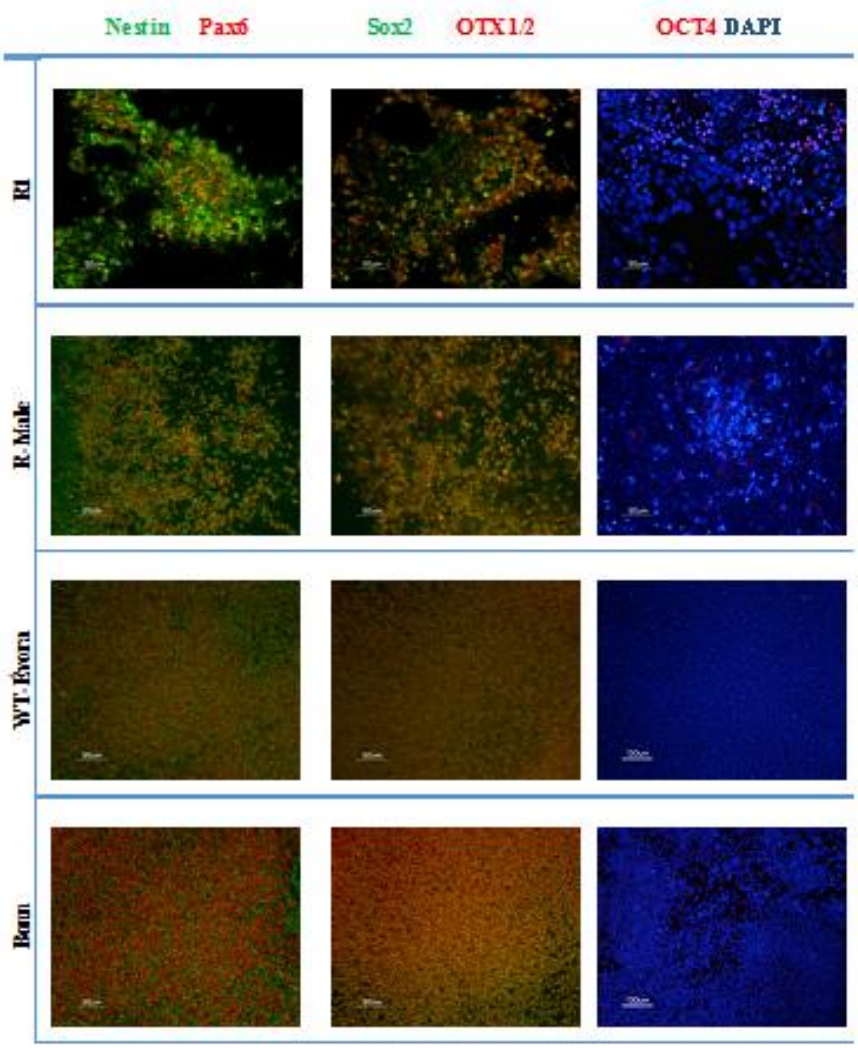


Figure 3 – Immunostaining analysis for all four lines (R1, R-Male, WT-Évora and Bonn) against NESTIN/PAX6, Sox2/OTX1/2, NESTIN/FOXG1 and Oct4/DAPI after 12 days of neural differentiation using the dual-SMAD inhibition method.

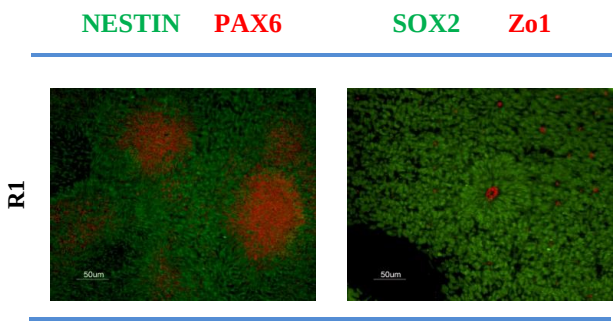
At day 12 cells expressed characteristic neuroectodermal cell markers and it is possible to verify that along with the increase of these neural markers, OCT4 expression decreased when compared with the results obtained during expansion. As all early neural markers were expressed in all lines, it was successfully achieved patterning of neural lineage in all three lines.
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.

<u>Neural stem cell expansion and long-term differentiation</u>

- <u>Day 17</u>

At the 17th day of neural commitment, cells were passaged onto laminin-coated plates, using the same protocol with EDTA^{TM}. Also, immunostaining was performed at day 17 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 Figure 4:



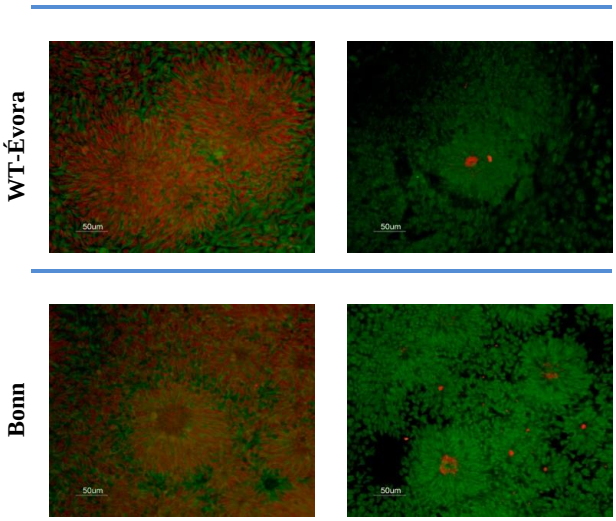


Figure 4- Immunostaining for R1, WT-Évora and Bonn cells against NESTIN/PAX6 and SOX2/ZO1 after 17 days of neural differentiation using the dual-SMAD inhibition method.

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 4 that PAX6 expression appears to be lower in R1 cells which can indicate less neuroepithelial cells comparatively with wild-type cells.

- 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 [3]. 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 5.

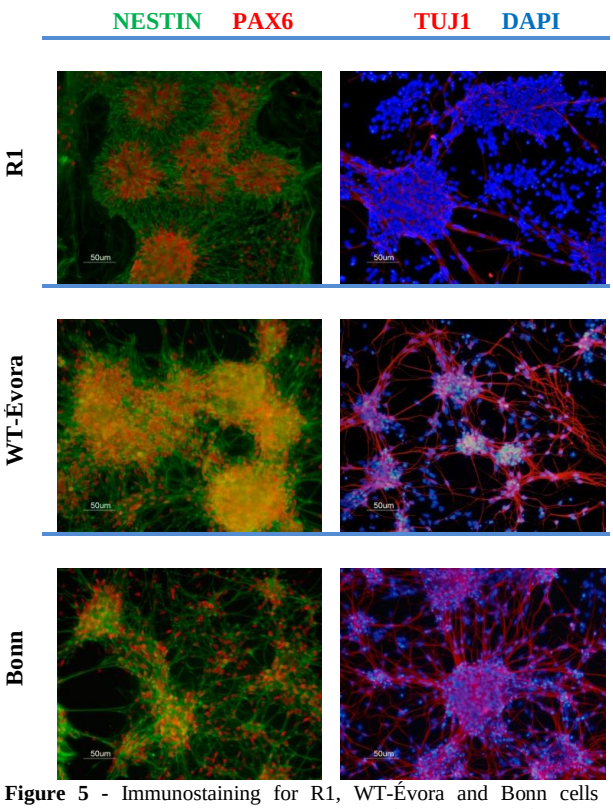


Figure 5 - Immunostaining for R1, WT-Évora and Bonn cells against NESTIN/PAX6 and TUJ1/DAPI after 29 days of neural differentiation using dual-SMAD inhibition method.

As it is possible to verify in Figure 5, 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 – results not shown). 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

- 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 6:

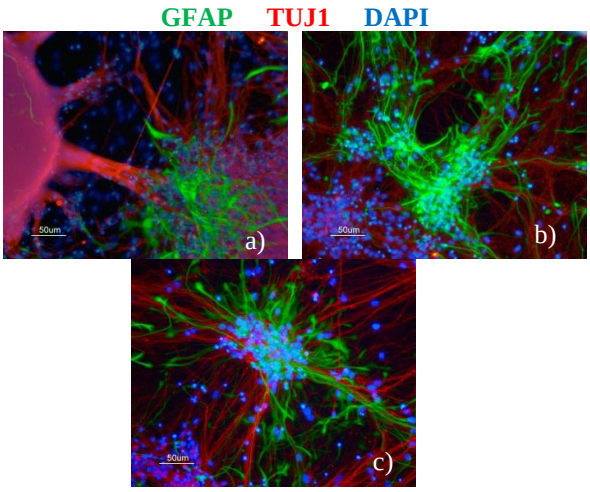


Figure 6 - Immunostaining results after 75 days of neural differentiation, using the dual-SMAD inhibition method, on hiPSCs against GFAP/TUJ1/DAPI. a) R1 cells; b) WT-Évora cells; c) Bonn cells.

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.

- Day 100

At the 100th day of neural commitment, cells 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 7:

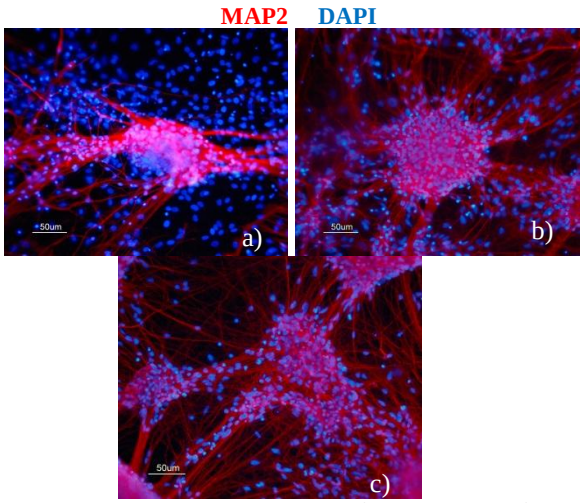


Figure 7 - Immunostaining results after 100 days of neural differentiation, using the dual-SMAD inhibition method, on hiPSCs against MAP2/DAPI. a) R1 cells; b) WT-Évora cells; c) Bonn cells.

By analysis of the Figure 7 it is possible to verify that in all three lines, by day 100, there was already an enriched population of mature neurons as they were MAP2-positive.
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.

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 glutamatergic neurons from hiPSCs (Bonn hiPSC line). Thus, and even though it was not possible to confirm by immunocytochemistry, it can be inferred that it was possible to achieve functional glutamatergic neurons with the lines used in this work as well.
This protocol revealed to be a robust system and it allowed the comparison between mutated and wild-type cells behavior as well as to understand characteristic hallmarks of Rett syndrome.
During expansion, as it is possible to confirm from 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.

Even though the main goals of this work were achieved, there are still many issues that should be focused in future work. It would also be important to repeat this protocol using the same lines and new lines to corroborate and achieve more robust results.

Also, it would be interesting to use a 3D culture system, as cerebral organoids [15], 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

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 [17].

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References

[1] O. Lindvall, Z. Kokaia, and A. Martinez-Serrano, "Stem cell therapy for human neurodegenerative disorders-how to make it work.," *Nat. Med.*, vol. 10 Suppl, no. July, pp. S42–50, Jul. 2004.

[2] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells.," *Nature*, vol. 448, no. 7151, pp. 313–7, Jul. 2007.

[3] S. M. Chambers, C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain, and L. Studer, "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling," *Nat. Biotechnol.*, vol. 27, no. 3, pp. 275–280, 2009.

[4] W. Zhao, X. Ji, F. Zhang, L. Li, and L. Ma, "Embryonic stem cell markers.," *Molecules*, vol. 17, no. 6, pp. 6196–236, Jan. 2012.

[5] L. G. Villa-Diaz, A. M. Ross, J. Lahann, P. Krebsbacha, and H., "Concise Review : The Evolution of Human Pluripotent Stem Cell Culture : From Feeder Cells to Synthetic Coatings," *Stem Cells*, no. 734, pp. 1–7, 2013.

[6] J. H. Boergermann, J. Kopf, P. B. Yu, and P. Knaus, "Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells.," *Int. J. Biochem. Cell Biol.*, vol. 42, no. 11, pp. 1802–7, Nov. 2010.

[7] R. M. Walsh and K. Hochedlinger, "Modeling Rett Syndrome with Stem Cells," *Cell*, vol. 143, no. 4, pp. 499–500, 2013.

[8] M. C. N. Marchetto, C. Carromeu, A. Acab, D. Yu, G. Yeo, Y. Mu, G. Chen, F. H. Gage, and A. R. Muotri, "A model for neural development and treatment of Rett Syndrome using human induced pluripotent cells," *Cell*, vol. 143, no. 4, pp. 527–539, 2010.

[9] "Gene MECP2." [Online]. Available: http://ghr.nlm.nih.gov/gene/MECP2.

[10] "Condition: Rett Syndrome." [Online]. Available: http://www.nhs.uk/conditions/rett-syndrome/Pages/Introduction.aspx.

[11] "Rett Syndrome Research Trust." [Online]. Available: http://www.rsrt.org/rett-and-mecp2-disorders/rett-syndrome/.

[12] D.-S. Kim, D. R. Lee, H.-S. Kim, J.-E. Yoo, S. J. Jung, B. Y. Lim, J. Jang, H.-C. Kang, S. You, D.-Y. Hwang, J. W. Leem, T. S. Nam, S.-R. Cho, and D.-W. Kim, "Highly pure and expandable PSA-NCAM-positive neural precursors from human ESC and iPSC-derived neural rosettes.," *PLoS One*, vol. 7, no. 7, p. e39715, Jan. 2012.

[13] Y. Shi, P. Kirwan, and F. J. Livesey, "Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks.," *Nat. Protoc.*, vol. 7, no. 10, pp. 1836–46, Oct. 2012.

[14] D. Kamiya, S. Banno, N. Sasai, M. Ohgushi, H. Inomata, K. Watanabe, M. Kawada, R. Yakura, H. Kiyonari, K. Nakao, L. M. Jakt, S. Nishikawa, and Y. Sasai, "Intrinsic transition of embryonic stem-cell differentiation into neural progenitors.," *Nature*, vol. 470, no. 7335, pp. 503–9, Feb. 2011.

[15] M. a Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L. S. Bicknell, M. E. Hurles, T. Homfray, J. M. Penninger, A. P. Jackson, and J. a Knoblich, "Cerebral organoids model human brain development and microcephaly.," *Nature*, vol. 501, no. 7467, pp. 373–9, Sep. 2013.

[16] D.-S. Kim, P. J. Ross, K. Zaslavsky, and J. Ellis, "Optimizing neuronal differentiation from induced pluripotent stem cells to model ASD.," *Front. Cell. Neurosci.*, vol. 8, no. April, p. 109, Jan. 2014.

[17] D. Tropea, E. Giacometti, N. R. Wilson, C. Beard, C. McCurry, D. D. Fu, R. Flannery, R. Jaenisch, and M. Sur, "Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 6, pp. 2029–34, Feb. 2009.