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Research Article

Neural commitment of human pluripotent stem cells under defined conditions recapitulates neural development and generates patient-specific neural cells

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Standardization of culture methods for human pluripotent stem cell (PSC) neural differentiation can greatly contribute to the development of novel clinical advancements through the comprehension of neurodevelopmental diseases. Here, we report an approach that reproduces neural commitment from human induced pluripotent stem cells using dual-SMAD inhibition under defined conditions in a vitronectin-based monolayer system. By employing this method it was possible to obtain neurons derived from both control and Rett syndrome patients’ pluripotent cells. During differentiation mutated cells displayed alterations in the number of neuronal projections, and production of Tuj1 and MAP2-positive neurons. Although investigation of a broader number of patients would be required, these observations are in accordance with previous studies showing impaired differentiation of these cells. Consequently, our experimental methodology was proved useful not only for the generation of neural cells, but also made possible to compare neural differentiation behavior of different cell lines under defined culture conditions. This study thus expects to contribute with an optimized approach to study the neural commitment of human PSCs, and to produce patient-specific neural cells that can be used to gain a better understanding of disease mechanisms.

Keywords: Defined culture conditions · Dual-SMAD inhibition · Induced pluripotent stem cells · Neurodevelopment modeling · Rett syndrome

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Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; KO, knock Out; LDN, LDN-193189; MECP2, methyl CpG binding protein 2; NPs, neural precursors; PSCs, pluripotent stem cells; RTT, Rett syndrome; SB 5B-431542; SR, serum replacement; VTN, vitronectin

1 Introduction

Pluripotent stem cells (PSCs) are cells capable to differentiate and give rise to every tissue cell in the human body [1]. Until recently, these cells could only be isolated from the inner cell mass of the blastocyst, being designated

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embryonic stem cells (ESCs) [2, 3]. However, in 2007, Shinya Yamanaka and co-workers were able to reprogram human somatic cells into the pluripotent stem cell state using transfection of four transcription factors (OCT4, SOX2, KLF4, c-MYC). These human induced pluripotent stem cells (iPSCs) brought many expectations into the biomedical field due to their potential applications in disease modeling, drug and toxicity screening, patient-tailored therapies and engineered tissues [4], potentially preventing immunosuppression and graft rejection, and paving the way for the next generation of personalized medicine [5, 6].

Countless protocols for in vitro expansion and differentiation of human PSCs have been developed since then, which typically comprise the use of undefined culture components [7–12]. Problematically, this presents many difficulties to the translation of these protocols from basic research to tangible applications [13]. For example, the use of products from animal origin renders the produced cells unsuitable for clinical applications due to the risk of cross-species contamination and immunogenic reactions [14, 15]. Furthermore, the use of undefined culture conditions poses barriers to the understanding of the biological processes involved in pluripotency and lineage specification and compromises the reproducibility of these culture systems. Therefore, there is an urgent need to develop methods and protocols that use defined culture conditions and allow reproducibility when generating specific cell populations from PSCs.

In particular, neural specification can be induced by the synergistic inhibition of both Activin/Nodal and BMP signaling pathways with small molecules resulting in blockage of mesoderm, endoderm and trophoblast lineages while allowing neuroectodermal specification [7, 16]. Despite being relatively efficient and straightforward, this method uses substrates and medium components of animal origin and of undefined nature (e.g. matrigel and serum replacements). Chemically defined substrates based on proteins, polymers and hybrids of polymers with active biomolecules have been developed, exhibiting low lot-to-lot variability and presenting high reproducibility [17–19]. Truncated, recombinant vitronectin (VTN) conjugates, in particular, have been used as a support for robust expansion and differentiation of human iPSCs under standardized and totally defined culture conditions [19–22]. Such system would greatly benefit neural commitment protocols, allowing the foundation of defined settings for in vitro studies of neural development and ensuring reliable methods for the anticipated biomedical applications.

In this study, we have validated the use of defined culture conditions to achieve an efficient and reproducible neural commitment of human PSCs. A defined culture medium supplemented with chemical inhibitors of BMP and TGFβ signaling, together with vitronectin for adherent growth, was shown to be sufficient to generate neural precursors (NPs) expressing SOX1, PAX6, and NESTIN.

As a proof-of-concept, we have validated the neural commitment protocol using different cell lines, including patient-specific Rett syndrome-derived iPSCs. This neurological disorder is characterized by autistic-like behaviors and is commonly caused by mutations in the X-linked methyl CpG binding protein 2 (MECP2) gene, which is vital for proper functioning of the brain and acts as one of the many biochemical switches for gene expression [23, 24]. Consequently, abnormal expression of this gene causes atypical brain function, leading to many disease processes that are mostly uncharacterized [25, 26]. Thus, this study also expects to contribute with a novel methodology capable to provide patient-specific neural cells that could be used to gain a better understanding of the disease. Furthermore, the ability to recapitulate the development of the human nervous system in vitro will provide important insights on the mechanisms involved in the maturation of specific neural cell types, making this approach transversal to other related areas in neurodevelopmental research.

2 Materials and methods

2.1 Cell lines

Two human iPSC lines obtained from healthy donors (46, XY) were used: WT-Évora F0000B13 and iLB-C1-30 m-r12. WT-Évora was kindly provided by TCLab (Évora, Portugal), and iLB-C1-30 m-r12 cells were derived at the University of Bonn, Germany. These cell lines were reprogrammed from fibroblasts through retroviral transduction of human genes OCT4, SOX2, c-MYC and KLF4 [4], and were used as wild type controls to verify the robustness of the neural commitment method. The EMC23i cell line was generated at the iPSC Facility, Erasmus Medical Center, Rotterdam, using engineered color-coded lentiviral vectors (as described by Warlich and coworkers [27]), and was derived from a patient (46, XX) with a MECP2 mutation (R306C). Cells were routinely evaluated for karyotype abnormalities by conventional cytogenetics using the services of Genomed SA (Lisbon, Portugal). Biological samples collected for this work were obtained following rigorous national and European ethical guidelines and informed consent from donor or patient’s legal guardian.

2.2 Feeder-free culture of human iPSCs

Human iPSCs were thawed and cultured in mTeSR™1 medium (StemCell Technologies™), on Matrigel™ (BD)-coated, Synthemax™ (Corning®)-coated, or Vitronectin (VTN-N, Gibco®)-coated plates. Medium was changed daily and cells were passaged every three to four days, using EDTA dissociation buffer 0.5 mM (Gibco®, diluted in sterile PBS) with a split-ratio of 1:4 [21].
2.3 Neural induction of human iPSCs

When human iPSC cultures were nearly confluent, two different strategies were followed for neural induction, based on Chambers et al [7] and Shi et al [28]. The first protocol was performed using DMEM/serum replacement (SR)-based differentiation medium (KO-DMEM medium (Gibco®) supplemented with 20% v/v KO-SR (Gibco®), 1% v/v non-essential aminoacids (Gibco®), 1 mM L-glutamine (Gibco®), 0.1 mM β-mercaptoethanol (Sigma®) and 1% v/v Penicillin/Streptomycin [PenStrep, Gibco®]), while the other was performed in N2B27 medium (50% v/v DMEM/F12/N2 medium (DMEM-F12 (Gibco®), supplemented with 1% v/v N2 (Gibco®), 1 mM L-glutamine, 1.6 g/L Glucose (Sigma®), 1% v/v PenStrep, and 20 μg/mL Insulin [Sigma®]) and 50% v/v of B27 medium (Neurobasal (Gibco®) supplemented with 2% v/v B27-supplement (Gibco®), 1 mM glutamine and 1% v/v [PenStrep]). In both cases, media formulation was supplemented with 10 μM SB-431542 (SB) and 100 nM LDN-193189 (LDN) (both from Stemgent®), as outlined in Fig. 1A.

2.4 Neuronal differentiation

At day 12 of differentiation, cells were passaged with EDTA dissociation buffer (0.5 mM) and re-plated onto laminin (Sigma®)-coated plates (split ratio of 1:3) [28]. When neural rosettes were observable (around day 14), N2B27 was supplemented with 10 ng/mL of bFGF (Peprotech®) for two to four days. At day 17, cells were passaged using EDTA to new laminin-coated plates (split ratio of 1:3). Cells were then cultured until day 28–30 in N2B27 medium without the addition of small molecules or any other factors. Finally, cells were split with accutase (Sigma®) and plated at a density of 100 000 cells/cm². Cells were further cultured until day 150 with medium replacement every other day (Supporting information, Fig. S1).

2.5 Immunofluorescence staining of intracellular markers

We performed immunofluorescence staining as previously described [29]. Briefly, cells were fixed in paraformaldehyde (PFA, Gibco®) 4% for 30 min, followed by blocking for

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**Figure 1.** Neural commitment of human PSCs. (A) Neural induction was initiated when cells reached confluence and two strategies were tested in parallel. The first strategy involved the use of serum replacement-based medium (KO-DMEM/SR) for the first four days and a gradual increase in N2 medium thereafter. In the second case, N2B27 defined medium was used for 12 days. Small molecule inhibitors of SMAD signaling (SB and LDN) were used as described in the scheme. (B) Human PSCs were cultured in adherent conditions (i) until confluence (ii). A neuroepithelial sheet of cells could be observed after neural induction (iii). (C) and (D) PSC colonies stained for pluripotency markers OCT4 and SOX2 (scale bars: 100 μm). (E) Quantification of immunofluorescence images for pluripotency markers OCT4 and SOX2 at the start of neural commitment. (F) Immunofluorescence staining for pluripotency marker OCT4 after 12 days of differentiation in N2B27 medium and in serum replacement (KO-SR) conditions (scale bars: 100 μm). (G) PAX6 and NESTIN immunostaining for differentiated cells in N2B27 or KOSR-based medium (scale bars: 50 μm). (H) Quantification of OCT4- and PAX6-positive cells for the two medium compositions (KOSR or N2B27) used for neural commitment. *p-value<0.05. Results are presented in this figure as mean ± SEM of three independent experiments, and were obtained using iLB-C1-30 m-r12 PSCs and matrigel coating.
60 minutes at room temperature. Primary antibodies were then added and left at 4°C overnight. Secondary antibodies (goat anti-mouse or goat anti-rabbit IgG, Alexa Fluor® 488 or 564, 1:500 v/v dilution, Molecular Probes®) were prepared and incubated with cells for 1 h at room temperature. DAPI (Sigma®) was used to counterstain cell nuclei. Primary antibodies used were FoxG1 (1:100, Abcam®), GFAP (1:100, Millipore), Ki-67 (1:100, BD), MAP2 (1:400, Abcam®), NANOG (1:5000, Millipore), NESTIN (1:400, R&D®), OCT4 (1:500, Millipore), OTX1/2 (1:100, Millipore), PAX6 (1:400, Covance®), SOX2 (1:200, R&D®), Tuj1 (1:5000, Covance®), VGLUT1 (1:300, Synaptic Systems) and ZO-1 (1:100, Novex®).

2.6 Quantification of immunofluorescence images

Quantification of OCT4, SOX2 and PAX6-expressing cells was performed using Fiji® software (for ImageJ). A macro was developed to perform conversion of the original image to eight bits, adjustment of a threshold, watershed treatment, and definition of parameters for particle analysis. Firstly, total cell nuclei were counted using DAPI images. Then, PAX6, SOX2 and OCT4 positive nuclei were counted in the same way and the ratio between positive cells and the total number of cells was calculated.

2.7 Flow cytometry analysis

For staining, approximately 500,000 cells were resuspended in FACS buffer with the diluted primary antibody, and incubated for 15 min at room temperature in the dark. Cells were then washed and resuspended in PBS for later analysis by flow cytometry (FACSCalibur, Becton Dickinson). Antibodies used for flow cytometry were SSEA-4-PE (1:10), and Tra-1-60-PE (1:10) (Stemgent®).

2.8 Quantitative real-time PCR

Total RNA was isolated from cells at different stages of neural commitment (day 0, 3, 6, 9 and 12) using a high pure RNA isolation kit (Roche) according to manufacturer’s instructions. cDNA was synthesized from RNA, starting amount of 1 μg, using a transcriptor first strand cDNA synthesis kit (Roche). Taqman® Gene Expression Assays (20X) were selected for PAX6, SOX1, NANOG, OCT4/POU5F1 and GAPDH (Applied Biosystems, Supporting information, Table S2). All other genes (FGF5, NESTIN, β-Tubulin III) were analyzed using SYBR® green chemistry (primers are presented in Supporting information, Table S1). PCR-reactions were run in duplicate, using the StepOne™ RT-PCR System (Applied BioSystems). Reactions were normalized to the housekeeping gene GAPDH and results analyzed with StepOne software.

2.9 Electrophysiology

Whole-cell recordings were performed at room temperature in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 1 MgCl2, 2 CaCl2, 10 Glucose, pH 7.4 (gassed with 95% O2, 5% CO2). Patch pipettes (4–9 MΩ) were pulled from borosilicate glass electrodes (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus) with PC-10 Puller (Narishige Group) and filled with an intracellular solution containing (in mM): 125 K-glucuronate, 11 KCl, 0.1 CaCl2, 2 MgCl2, 1 EGTA, 10 HEPES, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine, pH 7.3, adjusted with KOH (1 M), 280–290 mosm. Cells were viewed using a Carl Zeiss Axioskop 2FS upright microscope equipped with a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics) and screen and recorded with an EPC-7 electrical amplifier (List Biologic). Signals were low-pass filtered using a 3- and 10-kHz three-pole Bessel filter of an EPC-7 amplifier, digitized at 10 kHz using a Digidata 1322A board, and registered by the Clampex software version 10.2 (Molecular Devices). Series resistance was not compensated during voltage-clamp recordings, but was regularly monitored throughout each experiment with a -5 mV, 50 ms pulse, and cells with >20% change in series resistance were excluded from the data. Data were not corrected for junction potentials. GABA-evoked postsynaptic currents (GABA-PSCs) were evoked through a micropipette (2–4 MO) containing GABA (30 μM in aCSF) coupled to a pressure application system (Picopump PV820, World Precision Instruments) and positioned close to the soma of the recorded cell. Single pulses of 100–150 ms and 6–8 psi were applied every 2 min. Action potential firing was systematically evoked in current-clamp mode by injecting current pulses of –50 to +300 pA, in 50 pA increments for 1000 ms from an initial holding potential of -70 mV.

2.10 Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Comparisons between experimental results were determined by Mann-Whitney test for independent samples. Unless stated otherwise, three replicates (n = 3) were performed and a p-value less than 0.05 was considered significant.

Additional methods and practical information can be found in the Supporting information section of the paper.
3 Results

3.1 Human PSCs efficiently commit to the neuroectoderm lineage without the need for serum replacement-based medium

The dual-SMAD inhibition protocol is a procedure for the rapid commitment of confluent human PSCs into early PSC-derived neural precursors (NPs) [7, 16, 28, 29] (Fig. 1A). Cells are first plated as cell colonies in conditions that support pluripotency, and allowed to reach confluence (Fig. 1Bi and ii). Culture conditions are then changed to include chemical inhibitors of BMP and Activin/Nodal signaling pathways, causing the emergence of a neuroepithelial cell sheet (Fig. 1Bii). This swift induction is due to the blocking of SMAD signaling transduction by SB and LDN small molecules [30, 31]. When combined, these inhibitors repress mesoendodermal fates, directing the differentiation towards neuroectoderm [32].

In this work we first compared different strategies to achieve human NPs based on two different methods [7, 16] (Fig. 1A). In both cases human PSCs were first plated in matrigel-coated plates as clumps using a non-enzymatic passaging procedure [33]. This yielded compact colonies of cells (Fig. 1Bii) that stained positively for pluripotency markers OCT4 and SOX2 (Fig. 1C and 1D, respectively). At this stage, a high percentage (>95%) of cells were positive for these markers (Fig. 1E). Cells were then allowed to reach confluence (Fig. 1Bii), at which point the two neural induction strategies were tested.

The first consisted in the use of serum replacement (SR)-based medium for the first four days of commitment supplemented with both SB and LDN. From day 5 to 11, N2 supplementation was gradually added to the medium, while LDN-mediated inhibition of BMP was maintained [7]. In the second protocol, neural commitment was induced in N2B27 medium supplemented with SB and LDN for 12 days [28]. In both cases a neuroepithelial sheet of cells was obtained, but some qualitative differences in the efficiency of the neural commitment process were immediately visualized using immunofluorescence (Fig. 1F and 1G). While in the case of SR-based medium OCT4-positive cells still persisted in culture 12 days after neural induction with chemical inhibitors, for N2B27-based induction these cells were virtually absent (Fig. 1F).

Additionally, the distribution of PAX6- and NESTIN-positive cells was more homogeneous in the case of N2B27 when compared with SR-based medium, which presented islands of PAX6-positive cells surrounded by NESTIN expressing cells instead of a homogeneous monolayer (Fig. 1G). Using image-processing software we then quantified OCT4- and PAX6-expressing cells for both protocols (Fig. 1H). Neural commitment in N2B27 medium resulted in more than 80% of NPs positive for PAX6 and very low numbers of OCT4-positive PSCs (<2%), while in KO-SR medium, an average of 40% PAX6-positive cells was obtained, with more than 20% of OCT4-positive cells still in culture (Mann-Whitney test, p-value <0.05).

These results highlight the importance of medium formulation in maximizing differentiation yield [22]. By comparing data from both differentiation media it is possible to conclude that the chemically defined, serum-free composition of N2B27 yields a more efficient neural conversion into NPs. This medium was particularly optimized for neural cell culture, and contains insulin and retinoids, which have been shown to be crucial for the neuroepithelial induction of human PSCs [16]. The relatively high percentage of pluripotent cells and potential non-neural differentiated cells obtained in SR-based medium (Supporting information, Fig. S2) is not desirable since these cells may interfere with the outcome of in vitro differentiation protocols [29].

3.2 Human PSCs generate neural precursor cells using dual SMAD signaling inhibition under defined culture conditions

In addition to medium formulation, the composition of the extracellular matrix to which cells adhere is also a potential source of ill-defined components. This is the case of matrigel, whose constituents include an heterogeneous mixture of structural proteins of animal origin [34]. However, non-xenogeneic, defined substrates that support the culture of human PSCs have also been proposed in the literature [17]. In particular, vitronectin (VTN) peptide sequences have shown to support both expansion and differentiation of human PSCs under totally defined culture conditions [19–22]. Our next goal was to compare two different extracellular matrices in supporting neural commitment from human iPSCs in combination with a fully defined culture medium, allowing the foundation of defined settings for in vitro studies of neural development.

A side-by-side comparison was made using N2B27 medium together with matrigel or VTN (Fig. 2). The dual SMAD inhibition yielded similar outcomes in both cases, and cells presented typical neuroectodermal markers (Fig. 2A). These included the neuroectoderm fate determinant PAX6 [35], the forebrain marker FoxG1 [7], and the midbrain/forebrain marker OTX1/2 [36], in addition to SOX2 and NESTIN. Cells differentiated for 12 days in both substrates showed up-regulation of PAX6 and SOX1 mRNA, with corresponding down-regulation of pluripotency transcription factors (OCT4/POUSF1 and NANOG) (Fig. 2B). Additionally, real-time PCR analysis indicated that for both substrates expression of the pluripotency markers sharply decreased in the first three days of neural induction (Fig. 2C). After this point, both OCT4/POUSF1 and NANOG retain minor levels of expression when compared to day 0. Also, PAX6 and SOX1 expression increased rapidly from day 0, reaching maximum levels after day 3 in both matrigel and VTN (Fig. 2D). At the
same time, when human PSCs entered differentiation, the presence of cell surface markers characteristic of the pluripotent state (Tra-1-60 and SSEA-4) was reduced, and the percentage of cells positive for these markers dropped progressively during the 12 days of neural commitment (Fig. 3A). The simultaneous up-regulation of FGF5 expression (Fig. 3B), together with down-regulation of pluripotency genes (Fig. 2C) and up-regulation of PAX6 and SOX1 (Fig. 2D), most likely resulted from the emergence of an initial population of NPs after neural induction [37]. Taken together, these outcomes demonstrate that human iPSCs can effectively differentiate to NPs using the dual SMAD inhibition protocol under defined culture conditions composed of N2B27 and VTN as adhesion substrate.

To further confirm the generation of competent NPs after 12 days of dual SMAD inhibition, cells were re-plated onto laminin-coated plates as small clumps, and cultured until day 28–30 without addition of small molecules or any other factors. Substantial maturation of NPs within rosettes was observed (Fig. 3D and 3E). As expected, after peaking during the initial stage of commitment, expression of typical neural markers (PAX6, SOX1 and NESTIN) declined during further differentiation (Fig. 3D). This was followed by up-regulation of the neuronal markers β-Tubulin III. Using immunofluorescence staining we were able to see a proliferative (Ki67-positive) population of progenitors within polarized rosettes (apical expression of ZO1), and to confirm a reduction of the number of PAX6-positive cells. At this stage of differentiation, TuJ1-positive neurons outgrew from neural rosettes (Fig. 3E).

Taken together, these results demonstrate and validate the capacity to generate functional NPs using the dual SMAD inhibition protocol under defined culture conditions. Our results show efficient (>80%) generation of PAX6-positive NPs, and the capacity of these cells to self-organize into neural rosettes upon passaging. Further differentiation into more mature cells was achieved within rosettes, which can be correlated with events leading to the neural tube formation in vivo [7, 16]. As previously demonstrated in similar systems [19–22, 38], peptide conjugates can support robust expansion and differentiation of human PSCs under defined conditions, and in our case VTN supported neural commitment of human PSCs in conjugation with N2B27 medium supplemented with SMAD inhibitors.
3.3 Generation of patient-specific neural precursors and further neural maturation

At this stage we have established conditions that allowed the study of neural specification of human PSCs without interference of undetermined components resulting from serum replacements [7] or extracellular matrix protein mixtures [28, 34]. This is an important feature of our system since undisclosed constituents may interfere with different signaling pathways, and thus affect cell fate decisions. Therefore, to further explore and demonstrate the usefulness of our method for neural specification studies, we have taken advantage of the potential of iPSC technology to investigate the neural commitment of patient-specific iPSCs, in particular Rett syndrome (RTT) patient-derived cells [39]. This disorder is caused by mutations in the MECP2 gene of the X chromosome and affects 1 in 10 000–20 000 girls worldwide [23]. Hallmarks of this condition include impaired motor function, seizures, autistic behavior [40], and changes in neuronal density and in brain size [24, 41].

Control and RTT-derived fibroblasts were reprogrammed to a pluripotent state as described elsewhere [4]. The iPSC lines used in this work showed typical characteristics of pluripotent stem cells, and could be maintained in culture for dozens of passages without signs of differentiation or atypical karyotype (Supporting information, Table S3 and Fig. S3). These cells were then adapted to our culturing conditions using a non-enzymatic passaging procedure and plated to VTN-coated surfaces [21]. Both RTT and control-derived iPSCs were cultured in pluripotency maintenance conditions using mTESR, and stained for typical pluripotency markers (Fig. 4A and 4C, and Supporting information, Fig. S3). After reaching confluence, medium was changed to N2B27 and small molecule inhibitors of SMAD signaling (LDN and SB) were added to culture for 12 days. Both normal and patient-derived cells were able to differentiate and presented typical neuroectodermal markers such as PAX6, NESTIN, FoxG1, OTX1/2 and SOX2 (Fig. 4B and 4D). At day 28 of differentiation, Ki67 and ZO1 immunostaining were used to identify polarized rosettes (ZO1-centered). At this stage cells were also marked for neuronal markers (PAX6, NESTIN), and TuJ1-positive neuronal projections (scale bars: 50 μm). Results are presented in this figure as mean ± SEM of three independent experiments and were obtained using iLB-C1-30 m-r12 PSCs.
uration of NPs within rosettes was observed with cells expressing neural markers (NESTIN, PAX6, SOX2) within polarized structures (ZO1). At this stage, cells were again re-plated onto laminin to allow further neural differentiation. Proliferative, Ki67-positive cells within rosettes could still be identified in both cultures, and polarization could also be seen with apical localization of ZO1 (Fig. 4F and 4H). Nevertheless, while in normal cells Tuj1-positive neurons started to migrate outwards of rosettes, forming neuronal projections and connections, RTT derived-neurons were substantially less frequent, and neurites less complex (Fig. 4F and 4H).

To further assess the neuronal maturation state, cells were finally passed as single-cells at day 30 onto laminin-coated plates and maintained in N2B27 medium. This procedure was expected to generate cortical projection neurons in about 80 days [28]. At day 75 of differentiation, we evaluated not only neuronal maturation, but also differentiation into glial cells, like astrocytes, in two non-affected ‘wild type’ human iPSCs and one Rett syndrome-derived mutant line (Fig. 5A). A substantial number of widespread neuronal projections was observed in both normal cell lines. Furthermore, extensive gliogenesis had occurred in such cultures and GFAP-positive astrocytes could be seen interspersed with Tuj1-positive neurons. This process was minimally reproduced in affected cells, showing less neurons, similarly to what had been visualized at day 28 of differentiation. Similar conclusions could be taken from MAP2 staining at day 100 (Fig. 5B). These observations are in accordance with other reports in the literature [24, 41], and did not seem to result from reduced proliferation, since the number of DAPI stained nuclei within the microscope field of view was similar among samples. By day 120, control neurons developed to glutamatergic neurons presenting the vesicular transporter VGLUT1 (Fig. 5C), which could not be observed in mutated cells.

Finally, electrophysiological recordings performed at day 120 of differentiation, revealed neuronal functional properties characteristic of mature neurons. Values of neuronal resting membrane potential, determined immediately after establishing whole-cell configuration, averaged $-45.0 \pm 2.2$ mV ($n = 17$). Action-potentials were then evoked in current-clamp mode by injecting depolarizing current pulses. Most cells tested showed firing of at least one action potential during current injection steps, revealing a mature neuronal state ($n = 10$, Fig. 5D). In addition, GABA-evoked postsynaptic currents were observed (volt-
age-clamp mode, $V_h = -70 \text{ mV}$) in response to pressure application of GABA (30 μM) directly to the soma of the cell, indicating the expression of functional neurotransmitter receptors in these cells ($n = 11$, Fig. 5E).

4 Discussion

We herein identify defined culture conditions to achieve efficient and reproducible neural commitment of human PSCs. A defined neural maintenance medium supplemented with chemical inhibitors of both BMP and TGFβ signaling pathways, together with VTN for adherent growth, was shown to be sufficient to induce neuronal commitment, generating a homogeneous population of NPs expressing typical neural markers (Fig. 1, 2 and 3). The results obtained herein are comparable with other reported data in terms of neural conversion efficiency [16], when using N2B27 medium, demonstrating that VTN-coating does not affect neural commitment negatively.

In fact, VTN-based conjugates have been previously used for PSC derivation, expansion [20, 21] and neural specification studies [22, 42], and our results also validate the usage of VTN as a defined substrate for neural commitment. Another important feature of this system is the use of retinoid rich medium, which have been shown to increase NPs output [16]. When combined with VTN adhesion motifs, the use of defined medium was thought to facilitate progression through neural maturation, particularly during re-plating of NPs to laminin surfaces, by promoting survival and functionality of differentiated cells [43]. In fact, when compared with serum replacement-based medium, we have been able to significantly increase the number of PAX6-positive NPs in N2B27 (Fig. 1). However, other studies have reported minimally constituted monolayer systems that could achieve similar efficiencies in generating PAX6-positive neuroectoderm [22], questioning the role of retinoids in neural commitment. Nevertheless, monolayer neural commitment protocols still require a high local cell density for generation of NPs with high efficiencies [7]. It would be interesting to engage other methods [44, 45], such as micropatterning or forced aggregation of cells, to evaluate the local dynamics of high-density cell-to-cell contacts in the neural specification of human PSCs. Still, one shortcoming of monolayer systems is the fact that for later stages of neural development, NPs require laminin to adhere and differentiate [46]. Laminins are extracellular matrix trimeric gly-
coproteins and at least a dozen of different isoforms have been identified in nature. Commonly used laminin is isolated from a murine source and represents an undisclosed mixture of several of these natural isoforms. To fully define neural commitment, neural maturation, and neuronal differentiation of human PSCs in adherent conditions, specific laminin isoforms should be investigated and subsequently used. The Tryggvason’s group has been able to produce recombinant laminins and evaluate their specific role in several biological functions [47], but the exact isoforms involved in neural differentiation remain unknown. However, potential future therapeutic strategies for neurological disorders might implicate the use of progenitors [48], since mature cells are less capable of establishing the adequate connections without being continuously exposed to the combination of growth factor and signaling molecules that orientate synaptogenesis and neuronal wiring. Obtaining NPs in xeno-free conditions can be a relevant first step to establish a safe approach for future transplant based therapies.

As a proof-of-concept, we have validated our neural commitment protocol using different cell lines, including patient-specific, Rett syndrome-derived iPSCs. Although investigation of a broader number of patients would be required, we were able to replicate some of the reported Rett syndrome defects in NPs derived in our defined system (Fig. 4 and 5). Interestingly, given the fact that MECP2 is located on the X-chromosome and that Rett syndrome patients present mosaicism [23], X-chromosome activation/inactivation during reprogramming, expansion and differentiation could lead to difficulties in reproducing the pathology of the disease in vitro. We have not confirmed these events during reprogramming of our cells, but Marchetto and coworkers have previously shown the capacity to recapitulate X-inactivation during neural differentiation, producing mosaic neuronal cultures with different MECP2 expression levels [24]. Isogenic human iPSC lines from Rett syndrome patients can therefore be used as in vitro models of the disease [49], and our results (Fig. 5) are in accordance with the published literature [24, 41, 49]. Since several studies have reported similar findings, and taking into consideration the different levels of MECP2 expression in mutated cells, the contribution of non-neural cells, like astrocytes, to the onset of the disease should not be excluded. In fact, this influence was already described, indicating that glial cells carrying MECP2 mutations can also distress healthy neurons [50, 51].

In conclusion, our defined culture system provides a way to recapitulate some of the temporal and regional patterning events that occur during in vivo cortical neurogenesis [52]. Also, by deconstructing the natural complexity of neural development into a simpler experimental approach, we could mimic several aspects of Rett syndrome pathology potentially contributing to a better understanding of cortical development and disease. T.G.F., G.M.C.R. and D.M.R. acknowledge support from Fundação para a Ciência e a Tecnologia (SFRH/BD/86316/2012, SFRH/BD/89374/2012 and SFRH/BD/60386/2009, respectively). S.T.D. integrates the Program for Advanced Medical Education of Calouste Gulbenkian Foundation and Fundação para a Ciência e Tecnologia (SFRH/BD/NM/151548/2011) and D.M.R. the PhD Program of Centro Académico de Medicina (Neurosciences) of the Faculty of Medicine, University of Lisbon. D.M.R is also the recipient of a grant from Fondo BioRett. This work was further financially supported by the European Community’s 7th Framework Programme through projects Neurostemcell (#22943) and NeuroStemcellRepair (#602278), by FCT, through the MIT-Portugal Program, Bioengineering Systems Focus Area and Grants PTDC/SAU-NMC/110838/2008, PTDC/EBB-BIO/122054/2010, EXPL/BBB-EBI/0294/2013 and EXPL/bim-mec/0009/2013, and the government of the federal state of North Rhine-Westphalia (StemCellFactory II; #005-1403-0102). All co-authors have agreed to the submission of the manuscript. Oliver Brüstle is co-founder of and has stock in LIFE & BRAIN GmbH. All other authors declare no financial or commercial conflict of interest.

5 References


