Role of Tauroursodeoxycholic Acid during Expansion and Neural Commitment of Human Induced Pluripotent Stem Cells under Chemically-defined Conditions

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Abstract

Neural stem cells (NSCs) decrease their capability, along the time, of generating new functional neurons in vivo. Regarding that, neurogenesis can be studied using human pluripotent stem cells (hiPSC) as model. Previous reported data revealed that tauroursodeoxycholic acid (TUDCA) increases proliferation and self-renewal potential of NSC, is neuroprotective, anti-apoptotic and differentiation-modulator, being this behaviour dependent on mitochondrial modulation. However, the effect of this molecule towards expansion, neural induction and differentiation of hiPSC has never been reported before, being that the main focus of this work. A significant increase in hiPSCs expansion was observed in the presence of TUDCA, with a higher fold increase (FI) for the 5 µM of TUDCA condition when compared with the control. To further explore TUDCA role in the generation of hiPSCs-derived neural progenitor (NPs) cells, this molecule was added during 12 days to hiPSCs subjected to the dual-SMAD inhibition protocol. Neural rosette structures were quantified and for 10 µM of TUDCA, an increase of 76.6% was verified. Next, it was investigated if the positive effect of TUDCA in rosette generation could be related with an increase in mitochondrial mass and activity. The results revealed that TUDCA treatment induces an increase in mitochondrial DNA (mtDNA) and adenosine triphosphate (ATP) generation, regulates mitochondria-related proteins and assures higher protection against mitochondrial oxidative stress at latter times of neural induction. The results suggest that TUDCA has a positive effect during expansion and in neural commitment of hiPSCs, which was demonstrated to be mostly related to modulation of mitochondrial integrity.

Keywords: Human induced pluripotent stem cells - Tauroursodeoxycholic acid - Expansion - Neural commitment - Neural progenitor cells - Mitochondria
Resumo

As células estaminais neurais (NSC) vão-se incapacitando de, ao longo do tempo, gerar neurónios funcionais in vivo. Assim, recorrendo às células humanas pluripotentes induzidas (hiPSCs) como modelo, é possível estudar o processo de neurogénesese e distúrbios associados. Estudos previamente reportados mostram que o ácido taouroursodesoxicólico (TUDCA) influenciou o aumento da proliferação e auto-renovação das NSC, actuando como neuroprotector, anti-apoptótico, e regulador do processo de diferenciação, comportamentos estes relacionados com a modelação da dinâmica da mitocôndria. Contudo, o efeito desta molécula na expansão, comprometimento neural e diferenciação das hiPSC nunca foi reportado, sendo assim o principal objectivo deste trabalho. Na expansão das hiPSCs, para o tratamento com 5 µM de TUDCA, obteve-se um maior número final de células comparativamente com o controlo. Por outro lado, para estudar o efeito do ácido biliar na geração de progenitores neurais (NPs) seguiu-se o protocolo de inibição dual-SMAD e contabilizou-se o número de rosetas neurais, tendo-se verificado um aumento de 76.6% para a concentração de 10 µM TUDCA. Investigou-se, assim, se este aumento de rosetas reportado estaria relacionado com o aumento da estabilidade da mitocôndria. Nos últimos dias de comprometimento neural, para o tratamento com TUDCA, verificou-se um aumento do ADN mitocondrial, dos níveis de ATP gerados, da estabilidade na geração de espécies reactivas de oxigénio e da estabilidade de proteínas envolvidas na dinâmica da mitocôndria. Assim, os resultados obtidos sugerem um efeito positivo do TUDCA aquando da expansão e do comprometimento neural das hiPSCs, estando potencialmente relacionado com a modelação da integridade mitocondrial.

Keywords: Células humanas pluripotentes induzidas - Ácido taouroursodesoxicólico - Expansão - Comprometimento neural - Progenitores neurais - Mitocôndria
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<tr>
<td>APAF-1</td>
<td>Apoptotic protease activating factor 1</td>
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<td>AIF</td>
<td>Apoptosis inducing factor</td>
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<td>Central nervous system</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CFI</td>
<td>Cumulative fold increase</td>
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<td>DAPI</td>
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<td>Drp1</td>
<td>Dynamic-related protein 1</td>
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<td>Dulbecco’s Modified Eagle Medium</td>
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<td>Essential 8™</td>
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<td>GUDCA</td>
<td>Glycoursodeoxycolic acid</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>Abbreviation</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GMP</td>
<td>Good Manufacturing Practices</td>
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<td>HSC</td>
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<td>hiPSCs</td>
<td>Human induced pluripotent stem cells</td>
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<td>ICH</td>
<td>Intracerebral haemorrhage</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
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<td>KO-SR</td>
<td>KnockOutTM -DMEM/SerumReplacement</td>
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<tr>
<td>Mfn</td>
<td>Mitofusin</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
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<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
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<tr>
<td>MPT</td>
<td>Mitochondria permeability transition</td>
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<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<tr>
<td>miRNA</td>
<td>Micro-RNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Map2</td>
<td>Microtubule-associated protein 2</td>
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<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<td>NGS</td>
<td>Normal goat serum</td>
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<td>NSC</td>
<td>Neural stem cell</td>
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<td>NP</td>
<td>Neural progenitor</td>
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<td>NeuN</td>
<td>Neuronal Nuclei</td>
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<tr>
<td>Oct4</td>
<td>Octamer-binding transcription factor 4</td>
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<td>OXPHOS</td>
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<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PARP</td>
<td>Poly(ADPribose) polymerase</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PSC</td>
<td>Pluripotent stem cell</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RNA</td>
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<td>ROCK</td>
<td>Rho-associated kinase</td>
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<tr>
<td>SSEA</td>
<td>Stage Specific embryonic antigen</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide electrophoresis gel</td>
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<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
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<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<td>Sox2</td>
<td>Sex determining region Y-box 2</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<td>-------------</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TFAM</td>
<td>Mitochondria transcriptor factor A</td>
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<td>TUDCA</td>
<td>Tauroursodeoxycholic acid</td>
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<td>Transforming growth factor</td>
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<td>TRA</td>
<td>Tumour rejection</td>
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<tr>
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I. Introduction

I.1. Stem cells: general principals

The many millions of cells that compose the human body are organized into tissues which, in turn form the organs and the systems that undertake the vital functions of the organism. The tissues are constantly renewed by a daily production of millions of new cells, which is a specific population of cells: Stem Cells. The discovery of stem cells took place in 1963, in mice’s bonne marrow, by Becker et all (Becker et al. 1963). Since that time, stem cells are the most promising and special cell types with two fundamental properties: cell population can keep dividing and maintain themselves in an undifferentiated state (self-renewal) and they can differentiate into cell lineages, giving rise to specialized cell types (Gearhart et al. 2009). As stem cells are gradually differentiated into more specialized cell types, they are restricting their potential. For instance, the fertilized oocyte (Fig.I.1) and cells after the first cleavage are considered to be totipotent, with the capacity of giving rise to the entire organism. With the formation of blastocyst, the cells from the inner cell mass (ICM) are considered pluripotent and are able to form the three germ layers - endoderm, mesoderm and ectoderm – which in turn develop into all the somatic cell types, but not the extraembryonic tissue. Once the three germ layers are formed, stem cells are considered multipotent, generating a number of different cell types, however only tissue-specific cells. Lastly, unipotent stem cells are the more restrictive ones, with the capability of differentiating into only one cell type (Gearhart et al. 2009).

Figure I. 1 - Stem cell differentiation potential, origin and experimental isolation. Adapted from Jen Philpott (scq.ubc.ca).
Besides being classified according to their differentiation potential, human stem cells can also be associated with their tissue source. The embryonic stem cells, the more primitive stem cells type, are isolated from the inner cell mass of blastocyst and collected during embryo development. Also belonging to the pluripotent type of cells, the embryonic germ cells could be collected from Gonadal Ridge. In addition, the majority of multipotent stem cells could be collected from fetus and neonatal tissue, as umbilical cord and placenta. Otherwise, adult stem cells are found in basically every tissue of human body and they only have the capability of producing cell types from the tissue in which they reside. These somatic cells multiply by mitotic cell division and they could be obtained directly from donors. Finally, the unipotent stem cells could be obtained in the germline tissues (Fernandes et al. 2013), (Kirschstein 2001).

I.1.1. Human pluripotent stem cells (hPSCs)

As previously mentioned, a single pluripotent stem cell is capable of differentiating into cells arising from the three germ layers that give rise to somatic cells of the human body. Pluripotent stem cells have indefinite self-renewal capacity, associated with high telomerase activity and undergo symmetric divisions in culture without differentiating. There are several types of PSCs, mainly embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), embryonal carcinoma cells (ECCs) and embryonic germ cells (EGCs) (Kirschstein 2001).

I.1.1.1. Human embryonic stem cells (hESCs)

In spite of ESCs that have been firstly isolated from mouse embryos in 1981 (Evans & Kaufman 1981), human ESCs were isolated and grown in culture by Thomson’s group, in 1998 (Thomson et al. 1998). In order to confirm the pluripotency of these cells, specific pluripotency markers must be expressed, including intracellular Oct-3/4, Sox2 and Nanog and cell surface markers such as tumor rejection antigens (TRA): Tra-1-60, Tra-1-81, and stage specific embryonic antigens (SSEA): SSEA-3 and SSEA-4 (Draper et al. 2002). Furthermore, potency characterization could be made through in vitro differentiation protocols as well as by knowing that human ESCs were able to form teratomas in vivo, when injected in immunodeficient mice. hESCs were derived from the developing embryo at 5th day, from de ICM of the blastocyst, so the sources of hESCs could be supernumerary embryos from in vitro fertilization clinics, or more recently embryos derived from the technology of somatic cell nuclear transfer (Fernandes et al. 2013). The ability of generate cells arising from the three germ layers makes these cells ideal for various clinical applications. Despite that, ethical concerns are still an issue and if these cells are used for possibly transplantation therapeutics, they can end up in immune rejection by the host (Allegrucci & Young 2007).
I.1.1.2. Human induced pluripotent stem cells (hiPSCs)

Some of the ethical concerns associated to hESCs were avoided by a revolutionary scientific discovery, in 2006, when Takahashi and Yamanaka successfully obtained PSCs from mouse adult fibroblasts by using retroviruses and four transcription factors: Oct3/4, Sox2, c-Myc and Klf4 (Takahashi & Yamanaka 2006). These cells were very similar to mouse ESCs (mESCs) in terms of morphology, growth and gene expression. The so-called induced pluripotent stem cells (iPSCs) which were later derived from human fibroblasts, by the same team (Takahashi et al. 2007). Later on, successful reprogramming of mouse and human fibroblasts was achieved without c-Myc, which is potentially tumorigenic (Nakagawa et al. 2008). One year later, Thomson and co-workers were also able to obtain iPSCs from adult human fibroblasts, using a different set of factors, namely Oct3/4, Sox2, Nanog and Lin28 (Yu et al. 2007).

hiPSCs exhibit a similar methylation pattern to hESCs, which suggests that iPSCs reactivate the autoregulatory pluripotency-related transcription network. The cell behavior is influenced by the epigenetic memory, as cells maintain residual methylation from the cell of origin. Importantly, residual transgene expression and genetic background previously mentioned have both been found by comprehensive transcriptional analysis of different hiPSC lines to perturb the global gene expression profile of human pluripotent stem cells (Ghosh et al. 2010). However, the expression of SSEA-3 and SSEA-4, TRA-1-60 and TRA-1-81, as well as the transcription factors Oct3/4, Sox2 and Nanog is also verified in hiPSCs (Takahashi et al. 2007).

In addition to the previous issues, current methods for reprogramming human cells preclude its therapeutic utility, since they rely on delivery of the reprogramming factors by retroviral transduction. Cells that are not fully reprogrammed may have their differentiation capacity altered. The delivery of the reprogramming factors by retroviruses and lentiviruses, may integrate their genetic information in the DNA of the host cell, which can possibly lead to tumor formation through virus-mediated oncogene activation, such as with c-Myc. To avoid those reprogrammed deficiencies, minicircles, episomal plasmids and fewer transcription factors combined with the use of small molecules have been explored (Okita et al. 2007) (Guo et al. 2008) (Tian et al. 2016). Regarding reprogramming concerns, the efficiency had to be improved, and thus a more efficient and safer way of producing integration-free iPSCs may be the introduction into somatic cells of modified RNA molecules encoding for the reprogramming factors. So, the administration of synthetic messenger RNA incorporating modifications in the design to avoid antiviral responses is able to reprogram differentiated human cells into pluripotent ones at higher efficiencies and has superior kinetics compared to established viral protocols (Warren et al. 2010).

However, the aforementioned aspects must be optimized and in addition to that, more well-established and efficient expansion protocols must be achieved as hiPSCs are a huge promise in the field of stem cell engineering.
I.1.1.3. Culture Systems for hiPSC Expansion

Different protocols for culturing hESC have also been applied for ensuring hiPSCs proliferation, survival and differentiation. Culture medium formulation and substrate composition were typically composed of non-defined components. Furthermore, the use of compounds from animal origin seems to be unsuitable for possible clinical applications due to the risk of cross-species contaminations and immunogenic reactions (Fernandes 2015). PSCs require a substrate to adhere and support cell growth, whose composition is important regarding extracellular matrix molecules (ECM), cell-to-cell interactions, soluble factors and culture architecture (Discher et al. 2009).

The first reprogramming procedure of ESCs was made with mouse embryonic fibroblasts (MEFs) (Takahashi & Yamanaka 2006). Those feeder layer cells secret soluble factors and cytokines into the culture medium. However, these feeder cells keep the drawback of having two types of cells in culture, being xenogeneic, which introduces variability to the system (Discher et al. 2009). Feeder-free culture came to replace the previous described ones, and consequently gets the culture close to be acceptable for eventual clinical applications. Matrigel, a basement membrane substrate extracted from Engelbreth-Holm-Swarm mouse tumors that consist of a gelatinous mixture of proteins including ECM components that promote cell adhesion, such as laminin, collagen IV and entactin, and several growth factors (Kleinman et al. 1982). Despite that, the animal origin and variability between batches still prevents the use of these cell cultures for clinical purposes. Other substrates, such as human recombinant laminin have been shown to be efficient in expanding hiPSCs in an undifferentiated state (Rodin et al. 2010) or alternatives as recombinant protein coated surfaces (Nakagawa et al. 2014) or synthetic polymeric matrices (Melkoumian et al. 2010). However, although being a great promise, these alternative substrates are still expensive and under-optimized.

Cell-cell interactions are determinant for cell survival, as hiPSCs cannot survive as single cells. For the regular cell culture procedures, during passaging, cells are individualized. However, hiPSCs are prone to cell death if they are not in colonies, as interaction occurs through membrane integrins, which in turn are associated with signalling pathways associated with cell proliferation and pluripotency maintenance. By detaching cells, the cell-matrix interaction is loose and programmed cell death, named anoikis, can take place (Wang et al. 2009). As individualized cells, they typically die from actomyosin contractions. A procedure that ensures cell individualization survival is through the inhibition of ROCK-actin-myosin pathway components, by incubating cells with rho-associated kinase (ROCK) inhibitor (Discher et al. 2009). As cell could be passaged as small aggregates and survive through re-aggregation, having a maximization of cell survival, EDTA passaging procedure could be used. Cell harvested through EDTA method uses a fast enzyme-free method that achieves high throughput in terms of cell survival, without drug treatment, but requiring direct cell-cell adhesion. EDTA is an alternative to enzymes like trypsin or acutase, being gentler to the cells. The cadherins, cell adhesion molecules located on the cell surface, are calcium-dependent so they require calcium to establish cell adhesion interactions (Hirano et al. 1987). In order to that, EDTA is acting as a chelating agent that firstly breaks calcium dependent adhesion and then binds to calcium and prevents cadherins from different cells from binding (Thomson & Chen 2012).
The pluripotency and expansion maintenance must be retained by providing a culture medium to the cells which includes soluble factors and must be as defined as possible. The most current used medium is mTeSR, containing a total of 18 components, such as glucose, glutamine, basic fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), bovine serum albumin (BSA) and others (Chen, D. Gulbranson, et al. 2011). The role of bFGF has been critical to prevent cells from differentiating, as it is an antagonist of the bone morphogenic protein (BMP) signalling pathway. In addition, TGFβ also maintains pluripotency, as a cytokine that activates transcription factors. Another component, BSA, is a nutritive protein that can introduce batch variability, being from animal origin. Glucose is the preferred carbon and energy source, whereas glutamine may also constitute an energy source in the case of glucose depletion or could also be a nitrogen source (Chen, D. Gulbranson, et al. 2011). This medium is not xeno-free and it’s composition is not fully defined but can be replaced by E8 medium, since Chen and colleagues discovered the redundant role of BSA and β-mercaptoethanol in TeSR (Chen, D. Gulbranson, et al. 2011). Notably, this commercially available culture medium is composed of eight essential components – insulin, selenium, transferrin, L-ascorbic acid, bFGF and TGFβ in DMEM/F12 and NaHCO₃. This completely defined formulation, E8, is an alternative to mTeSR, as it is a Good Manufacturing Practices (GMP)-consistent product that achieves the same results in culture expansion and pluripotency maintenance (Wang et al. 2013).

In order to improve the monolayer culture system, other alternative culture systems have been developed such as 3D culture systems of PSC aggregates in suspension (Miranda et al. 2015). Despite eventual diffusional issues, this system mimics better the architecture structure of biological systems and facilitates the scale-up since avoids the use of microcarriers. Another field in development is the development of engineered biomaterial surfaces, as ultraviolet/ozone-modified tissue culture polystyrene or Synthemax™ (a biologically active peptide-functionalized acrylate polymer), bringing a huge promise in terms of efficiency, scalability and in terms of definition of their components, potentially facilitating the translation for clinical therapeutics (Tong et al. 2015). Other promising platforms are microfluidic devices that potentially enable cell cultivation and cell analysis in parallel. Many advantages are associated with this culturing platforms, including low reagent volumes, in a wide range of applications including differentiation assays and screening of reprogramming factors, drug screening, among others (Fernandes et al. 2009).

I.1.1.4. hiPSCs applications

In spite of the ESCs breakthrough in the fields of research and clinical therapeutics, immune rejection after transplantation and ethical concerns are still the main drawbacks associated to this type of cells, being thus hiPSCs as a promising alternative (Yamanaka 2007). One of the main improvements when using hiPSCs is the capacity of reprogramming patient-specific cells, which is a step-forward in avoiding immune rejection. However, hurdles related to reprogramming technologies, such as the capacity of forming teratomas upon transplantation, maintenance of epigenomic integrity, chromosomal aberrations resulting from the parent somatic cell or adaptation to culture conditions have also been reported (Laurent et al. 2008). Regarding that, the
possibility of treating patients with autologous and personalized cell transplantation therapy with hiPSCs should be put aside until ensuring the safeness and enforcement of GMP conditions.

Besides that, other fields of research make use of iPSC technology, as represented on Figure I.2, including drug discovery, toxicology screening, disease modeling, being a huge breakthrough in medicine investigation. Patient-specific hiPSCs could be generated from patients with neurological pathologies, as Parkinson’s disease (Soldner et al. 2009). After disease-specific cell lineage generation, drugs could be safely tested. Furthermore, using patient-specific cells from a specific disease might allow to mimic the disease in vitro, enabling the characterization of disease mechanisms or phenotypic alterations and also correcting the disease-associated mutated genes (Sterneckert et al. 2014).

To sum up, hiPSCs model appears to have different applications as they might be a source of cells to be used for different fields, such as regenerative medicine, tissue engineering, disease modeling, drug discovery and toxicological screening, as well as for fundamental research.

![Figure I.2 – hiPSCs generated by somatic cells reprogramming, followed by differentiation and possible applications.](image)

Adult somatic cells can be reprogrammed into hiPSCs that could be differentiated into the three germ layers. Several applications cold be noticed: a) disease modelling to understand the molecular mechanisms underlying disease phenotypes, as neurological associated pathologies; b) drug screening and discovery, to determine the effects of candidate drugs and new compounds and identify target pathways; c) toxicity essays that allow the introduction of ‘the patient’ in early stages of the drug discovery process. (Bellin et al. 2012)
I.2. Human brain

The mammalian brain development has been continuously investigated in order to understand the basic stages and mechanisms of the formation of this complex structure. Brain development is mainly related with molecular events from gene expression and by environmental input. The brain developing process begins during the third gestational week, starting with the differentiation in neural progenitor (NPs) cells, and is extended at least through adolescence, with physiological alteration throughout the lifespan (Stiles & Jernigan 2010).

I.2.1. Physiological development of human brain – in vivo neural development

The first event in human development comprises the formation of a single totipotent cell – the zygote – which has the capability of producing all the differentiated cells in the organism. The zygote divides and develops into a blastocyst, which is composed by the outer cells (trophoblast) that will form the placenta and by the ICM (Kirschstein 2001). After the second week, the ICM of blastocyst forms two layered structures which consist of epiblast cells on the upper layer and hypoblast cells (primitive endoderm) at the lower layer. As gastrulation begins, the primitive streak in the middle generates a mesoderm layer that separates the epiblast and the primitive endoderm. So, by that time, the epiblast cells are differentiating into the three layered structures, mesoderm, endoderm and ectoderm (Nichols & Smith 2012) (Niakan & Eggan 2013). Among the previous primary stem cell lines, the neuroectodermal stem cells are capable of producing all different cells that are on the central nervous system (CNS) (Pankratz et al. 2007). The first developing step comprises the neural tube formation, which is the first well-defined neural structure formed. As consequence of neural induction, the ectoderm becomes divided into three distinct regions (represented on Fig.I.3): the neural ectoderm or neural plate, which will give rise to the CNS, the non-neural ectoderm, which will form epidermis and also cells at the border between the neural and non-neural ectoderm, which will end up in the neural crest cells.

During the neurulation, the neural tissue folds in on itself in order to form the neural tube, as well as the neural plate border cells that elevate, causing the neural plate to roll into the neural tube. Since the neural tube formation is complete, the neural progenitor cells form a single layer of cells that lines the center of the neural tube immediately adjacent to its hallow center. The neural progenitor cells located in the most rostral region of neural tube will give rise to the brain, and on the other hand, the more caudally positioned cells end out in the hindbrain and spinal column (Gammill & Bronner-Fraser 2003).

The previous scientific knowledge about neural development as well as neurodegenerative associated defaults has been highly improved in consequence to the focus that has been given to the stem cell field. The recent improvements in all modeling regarding pluripotent stem cells, whether of embryonic origin or induced pluripotent stem cells are providing new prospects for the study of human brain development and related disease modeling, by the use of human cells and tissues.
I.2.1.1. Neural stem and progenitor cells

The adult brain was known for failing cell proliferation capacity, though Galli et al. published that cell replacement occurs within specific brain regions (Galli et al. 2003). In vivo, the neurogenesis process has been reported in the subgranular layer (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles, from where the neural stem cells (NSCs) have been firstly isolated. This self-renewal process is sustained by the life-long persistence of NSCs within the restricted CNS area, and also the capability of this multipotent stem cells of clonally originate the CNS lineages – neurons, astrocytes and oligodendrocytes (Elkabetz et al. 2008). In vitro culture of NSCs still remains a challenge, as the differentiation potential into region-specific neuronal subtypes appears to have an increase in gliogenic bias and concomitant loss of neurogenic potential. Otherwise, neural NPs are slightly more committed than NSCs, and so their ability to self-renewal is more limited (Neural et al. 2009).

I.2.1.2. Neuronal differentiation of hiPSCs – in vitro neuronal development

Human embryonic stem cell (hESC) derivation into region-specific neuronal fate is possible in response to appropriate signals, as well as hiPSCs cultures, which are capable of resembling neuronal differentiation (Fig. I.4). Starting in a pluripotent state, these cells first differentiate into NP cells and then undergo a morphogenetic event of formation of a structure similar to the neural tube, the neural rosettes (Zhang et al. 2001). These structures are characterized by the radial arrangement of columnar epithelial cells, which expressed early neuroectodermal markers, such as Pax6 and Sox1,
and also show a positive immunostaining for Sox2, Nestin and a tight junction protein Zo-1 (Elkabetz et al. 2008). During rosettes formation, the acquisition of cell polarity in a laminin matrix coating could be illustrated by the asymmetric distribution of Zo-1, which is co-localized with the neuroepithelial marker N-cadherin (N-cad) at adherent junction (Ma et al. 2008), (Zetterström et al. 2009) (Elkabetz et al. 2008). The rosette structures not only express proteins from neuroepithelial cells in neural tube, but also could differentiate into various region-specific neuronal and glial cell types in response to appropriate developmental factors.

**Figure I. 4 – Equivalent stages of neural differentiation *in vitro* and *in vivo*.** The differentiation of hiPSCs into neurons transit through intermediate stages that resemble the neural stem cells (NSCs) during neurogenesis process *in vivo*. hiPSCs correspond to the inner cell mass (ICM), from where the embryonic pluripotent stem cells (ESC) could be isolated. hiPSCs differentiate into neural progenitor/neuroepithelial stem cells that corresponds to the NSCs that forms the neural plane *in vivo*. During *in vivo* neurulation, the neural tube closes, patterning along the developmental axes occurs and the first types of neurons are generated. The correspondent step *in vitro* is the formation of neural rosettes that will give rise to neurons and glia (Mertens et al. 2016).
I.2.1.3. Signalling pathways for hiPSCs differentiation into neuroectoderm

The use of hiPSCs for applications in neuronal differentiation protocols implicates the understanding and the triggering of the signaling pathways involved on that specific approach. A key factor for understanding the respective pathway involved in which type of differentiation could be related with the understanding of the mechanisms responsible for pluripotency state maintenance – TGF-β, a growth factor that signals through receptor tyrosine kinases RTKs and WNTs (Pera & Tam 2010).

Several developed neuronal induction protocols reveal a crucial role of bone morphogenetic proteins (BMP) and activin/nodal signaling inhibition, which belong to the TFG-β superfamily (Chambers et al. 2009). The TGF-β family is therefore crucial for the maintenance of hiPSCs pluripotency and self-renewal as well as for the differentiation into definitive mesodermal and endodermal lineages during gastrulation (Pera & Tam 2010). The structural related proteins belonging to the TFG-β superfamily mediate the cells events through binding cell surface receptors.

I.2.1.3.1. Dual-SMAD inhibition protocol

The activin and nodal proteins mediate an important role inducing mesendoderm, which is a precursor of endodermal and mesodermal lineages during the gastrulation process. On the other hand, the BMP mediates the differentiation into ectoderm, specifically the differentiation towards the trophoblast lineage (Chambers et al. 2009).

The molecular control of the previous signaling pathways is crucial to achieve full neuroectodermal differentiation on the in vitro cell cultures. Small molecules inhibitors of type I receptor – activin receptor-like kinases, ALK1-7 – have proved to be an essential pharmacological tool to characterize and understand TGF-β and BMP pathway and derivate cell lineage for neuroectodermal differentiation (Sanvitale et al. 2013).

By using a small molecule SB-431542, the activin/nodal pathway is inhibited. This molecule is an inhibitor of Lefty/Activin/TGFβ pathways that blocks the phosphorylation of the ALK4, ALK5 and ALK7 receptors and inhibits the in vitro phosphorylation of SMAD3 as well as thus inhibiting activin-induced phosphorylation of SMAD2 (Alk et al. 2002). The SMAD2 and SMAD3 complexes are intracellular proteins, whose role is due to the binding into the promoter of genes, regulating gene expression by activating TGF-β pathways, mainly the maintenance of undifferentiated state of hiPSCs (Greber et al. 2007).

In order to inhibit the BMP signaling pathway, the addition of an antagonist, as dorsomorphin or the derivate LDN-193189 with higher specificity for BMP receptors, is crucial (Boergermann et al. 2010). This last mentioned small molecule is more recently used for inhibiting BMP type I receptors ALK2 and ALK3, as BMP-4 pathway inhibits the neuroectodermal path by promoting differentiation towards trophoblast (Pera & Tam 2010).

The neuronal differentiation protocol could be achieved by using the dual-SMAD inhibition, combining both inhibiting small molecules with N2B27 medium, which has been proven to be an efficient protocol for inducing hiPSCs into Pax6+ NPs (Fig. I.5), generating cells characteristic of CNS (Fernandes 2015). The initial cell density has been proven to be an important variable in lineage
specification. When dual-SMAD inhibition protocol is applied to cells at a lower density, the generated cells tend to be characteristic of peripheral nervous system (PNS), as neural crest stem cells (Chambers et al. 2009).

Figure I. 5 - Schematic view of dual-SMAD inhibition protocol for hiPSCs. Model for the mechanism of action of small molecules SB-431542, an inhibitor of mesendoderm lineage, and LDN-193189, an inhibitor of extraembryonic tissue.
Mitochondria

Mitochondria are highly specialized cytoplasmic organelles, presented in most of eukaryotic cells. These dynamic double-membrane organelles are involved in many important cellular processes including generation of ATP through oxidative phosphorylation (OXPHOS), fatty acid oxidation, calcium homeostasis, cell signaling, generation of reactive oxygen species (ROS) and key regulators of apoptosis. Mitochondria contain their only genome, circular mitochondrial DNA (mtDNA), encoding 13 essential protein subunits of complexes I, III, IV and V of the electron respiratory chain (Xu et al. 2013). Being a dynamic organelle, mitochondria undergoes in cycles of fusion and fission, as well as in regulated turnover events, crucial for the maintenance and quality control (Westermann 2010). More recently, it has been revealed the role of mitochondria in regulating NSC proliferation, cell cycle progression, and apoptosis-associated events (Xavier (a) et al. 2014).

I.3.1. Mitochondrial role in hiPSCs neural differentiation

The process of reprogrammed somatic cells in pluripotent ones, is highly associated with mitochondrial alterations, including the transition from OXPHOS to glycolysis through transcriptional and epigenetic regulation of gene expression (Folmes et al. 2011). Besides de upregulation of glycolic genes and downregulation of mitochondrial respiratory chain complexes, PSCs also reveals lower ROS levels generation and decrease in mitochondrial DNA (mtDNA) content and ATP levels (Prigione et al. 2010). In this regard, decreasing mitochondrial activity is one of the common key factors between ESCs and hiPSCs, both types maintaining their proliferative potential. On the other hand, upon NSCs differentiation or hiPSCs neural induction, higher amounts of energy, are required to sustain more specialized functions (Varum et al. 2011). In adult brain, the neurogenesis process occurs in residual population of multipotent stem cells in SGZ of dentate gyrus in hippocampus and in SVZ of the lateral ventricles. In these regions, NSCs or NPCs still have the potential to give rise to neurons, astrocytes and oligodendrocytes. Associated with higher mitochondrial bioenergetics requirements by differentiation, there is an increase in ROS levels, a by-product of OXPHOS. Thus, in this cellular context, many enzymes act as ROS detoxifiers. Among the antioxidants, the manganese superoxide dismutase (MnSOD), localized in mitochondrial matrix, convert superoxide (O$_2^-$) into hydrogen peroxide, which is largely removed by catalase (Candas & Li 2014). The mtDNA content was also shown to increase during differentiation stages, however the mitochondrial genome is susceptible to damage in consequence of previous mentioned high levels of ROS. The oxidative damage of mitochondria may also affect mitochondrial transcriptor factor, mtDNA replication and consequently impact the activity of electron transport chain (ECT). In addition, it has been shown that damages in mtDNA in NSCs were shown to facilitate astroglial differentiation rather neurogenesis (Wang et al. 2011). Furthermore, Finkel and others also demonstrated that high levels of ROS induce upregulation of the cell-cycle regulator p27, associated with NSCs cell cycle exit at G1, which in turn guide cells preferable for glial differentiation (Finkel & Hwang 2009). Curiously, Xavier and co-authors revealed that mitochondrial localization of p53, a tumour suppression protein, interferes positively with neuronal differentiation by reducing mtROS levels (Fig I.6) (Xavier (b) et al. 2014).
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I.3.2. Mitochondria-related mechanisms in neural apoptosis

A range of mitochondrial perturbation events could be associated with programmed cell death. These events include the collapse in mitochondria membrane potential, ROS production, opening of mitochondrial permeability transition (MPT) pore and release of cytochrome c from intermembrane space (Xavier et al. 2015). In fact, deregulation of apoptosis is a major cause associated with ischemia and neurodegeneration. Cell apoptosis play a key role in neuronal cell death from ischemia stroke (Linnik et al. 2002), while many other studies have specifically associated the ROS production with oxidative stress in CNS. In particular, oxidative stress could damage lipids, proteins, and DNA being one of the main consequences of several neurological disorders and cell death associated, as Parkinson’s disease (PD) (Fiskum et al. 2003) and Schizophrenia (Bitanihirwe & TU 2011). In many neurodegenerative disorders, including PD pathologically characterized by decrease of dopamine release and associated death of dopaminergic neurons, the excessive ROS produced triggers the apoptotic cell death, due to mitochondria dysfunction (Fiskum et al. 2003).

The caspase dependent cell death may also occur through the mitochondrial or intrinsic pathway (Fig I.7), which involves the permeabilization of the outer mitochondrial membrane that triggers a chain of reactions and a release of proteins from the mitochondrial intermembranar space (Xavier et al. 2015). This pathway is mainly activated upon intracellular stresses and leads to conformational changes in pro-apoptotic proteins situated in the cytosol. The pro-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family, such as Bax and Bak, migrate from the cytosol to the mitochondria, bind the outer membrane, induce permeabilization and allow the release of apoptogenic factors from mitochondria to the cytosol, such as cytochrome c (Polster & Fiskum 2004) (Xavier et al. 2015).

Apoptosis then occurs as consequence of cytochrome c release, being triggered by the association between cytochrome c, the apoptotic protease-activating factor 1 (APAF-1), and procaspase-9, leading to formation of the apoptosome. The activation of the initiator caspase 9 then leads to the...
activation of a cascade of effector caspases, which sometimes also involves the release of additional apoptosis factors from the mitochondria. For example, apoptosis inducing factor (AIF) and Endonuclease G (EndoG) are released by the mitochondria and are responsible for DNA fragmentation (Smith et al. 2008).

**Figure I. 7 – Mitochondrial apoptotic events.** Bax and Bak migrate to the mitochondria, inducing permeabilization and the release of cytochrome c. Then, the association between cytochrome c with the apoptotic protease-activating factor 1 (APAF-1) and procaspase-9 leads to formation and activation of the apoptosome, which in turn will trigger a cascade of caspase activation that culminates in apoptosis (Xavier et al. 2015).

### I.3.3. Mitochondrial dynamic regulators in neural differentiation

Besides the direct involvement of mitochondria into apoptotic pathways and neural differentiation, a dynamic behaviour could be also noticed in this organelle. This is particularly relevant to allow cells response to its ever-changing physiological conditions. The neural development and survival relies on mitochondrial biogenesis, integrity and function, triggering the quality control pathways (Xavier et al. 2015) (Westermann 2010). On the other hand, the peroxisome proliferator-activated receptor Y coactivator 1 (PGC-1α), a key mitochondrial biogenesis inducer, is also an pivotal in regulating oxidative metabolism and the expression of ROS-detoxifying enzymes (Austin & St-pierre 2012).

There are two main mitochondrial control pathways: the intra-mitochondrial and organellar pathways (Xavier et al. 2015). The influence of ROS increase in mitochondrial DNA (mtDNA) damage was previous described, where the first previous mentioned pathway has the main role in maintaining mtDNA integrity. In turn, Kang and colleagues have demonstrated that the mitochondrial transcription factor A (TFAM) increases mtDNA content, as it binds preferentially to damage DNA, promoting its repair (Kang & Hamasaki 2007). Indeed, Wang and colleagues showed that 8-oxoguanine DNA glycosylase (OGG1) overexpression is associated with increase in neurogenesis, being essential for repairing damage mtDNA and NSCs viability during exposure to mitochondrial oxidative stress (Wang et al. 2011). In addition, the regulation of mitochondria by chaperones and proteases were shown to be related with intra-mitochondrial pathways (Xavier et al. 2015). On the other hand, the organellar pathway is related with fusion, fission and mitophagy processes, collectively termed mitochondrial dynamics (Fig. I.8). Mitochondrial fusion could be described as a dynamic process that enables
genetic material exchange, ensuring the mixing of mitochondrial components while fission process ensures partitioning of organelles during cell division to release of pro-apoptotic factors into intermembrane space and to turnover damage organelles by mitophagy (Twig et al. 2008) (Westermann 2010) (Xavier et al. 2015). The rates of fusion and fission must be controlled in order to keep the dynamic balance of mitochondria. GTPases, in turn, acts as the mediators of the previous described processes, being mitofusin (Mnf) 1 and 2 mitochondrial fusion mediators at the outer mitochondrial membrane (Westermann 2010) (Westermann 2012). In fact, Tondera and colleagues have demonstrated that, under cellular stress conditions, mitochondria forms a hyperfused complex which prevents damage and results in an increase of ATP content, thus ensuring protection against mitophagy (Tondera et al. 2009). Additionally, Mnf2 overexpression on NPs cells was shown to direct differentiation and maturation of these cells into neurons, certainly associated with enhanced mitochondrial functions that are crucial in the maintenance of morphology and operation of the mitochondrial network and metabolism (Fang et al. 2016). In contrast, fission process is mainly mediated by dynamic-related protein 1 (Drp1) and the fission protein 1 (Fis-1). These mitochondrial dynamic-related factors are recruited from the cytosol, and form spirals around mitochondria to split and cleave them (Ashrafi & Schwarz 2012). The solitaries mitochondria generated, in turn, might maintain an intact membrane potential and re-fuse with others. While damaged mitochondria are easy removed from the cell through mitophagy. The instability of genes involved in mitochondria quality control have been associated with neurodegenerative diseases as well as with neural differentiation disturbst. Therefore, a deeper knowledge on mitochondrial dynamic behaviour could allow the screening of novel drugs that might strategically manipulate these pathways and contribute to the treatment of these neurological disorders.

**Figure 1.8 - Mitochondria fusion, fission and mitophagy.** Fusion process involves outer membrane proteins Mnf 1 and 2 and results in mitochondrial networks. Fission generates heterogeneous mitochondria, mediated by Drp1, and dysfunctional mitochondria ends with degradation of organelles by mitophagy. Adapted from (Westermann 2010).
I.4. Tauroursodeoxycholic acid regulatory potential

I.4.1. Bile acids

Bile acids (BAs), a group of molecular species of acidic steroids synthetized from cholesterol in liver and secreted into the intestine, play crucial roles in solubilization of lipids in the intestinal lumen. BAs are one of the major constituents of bile, representing the major pathway of catabolism and removal of cholesterol from the body. The end product obtained has amphiphilic properties which enables the interaction of these water soluble compounds with proteins and the insertion into lipid bilayers. Certain hydrophobic BAs, at high concentrations, become cytotoxic, being already associated with increased cell proliferation and cancer development in the intestinal tract. Curiously, more hydrophilic BAs may also display a cytoprotective effect (Xavier et al. 2015).

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid presented in human bile at a low percentage (4%), while in American black bears UDCA represents the major biliary bile acid (Hagey et al. 1993). UDCA has been used for the treatment of liver disorders in traditional Chinese medicine, thereby, on conventional medicine it has been proven clinical efficacy in the treatment of hepatobiliary disorders (Lazaridis et al. 2001). After administration, this bile acid is conjugated with glycine or taurine forming glycoursoseoxycolic (GUDCA) and tauroursodeoxycholic (TUDCA) acids, respectively. Hydrophilic acids like UDCA or TUDCA might show also positive anti-apoptotic effect, particularly TUDCA which is tolerated in higher doses of administration and targets other tissues, such as brain (Keene et al. 2002).

Further, UDCA is a Food and Drug Administration (FDA) approved molecule and the last century published studies concluded that treatment with UDCA represents a safe and effective option for patients with primary biliary cirrhosis (Lotterer et al. 1989). Later on, TUDCA was also shown to be an orally available and central nervous system penetrating agent as well as the capacity of inhibit apoptosis (Keene et al. 2002) (Xavier (a) et al. 2014).

I.4.1.1. Anti-apoptotic role of TUDCA

UDCA and TUDCA were shown to prevent a wide range of apoptotic events, including mitochondrial release of cytochrome c, cytosolic caspase activation, depolarization of mitochondrial membrane and pore formation (Rodrigues et al. 1999) (Amaral et al. 2009). Programmed cell death is associated with the maintenance of homeostasis but also with the occurrence of many diseases and most of the times is associated with mitochondrial integrity (Polster & Fiskum 2004). It was also reported that TUDCA prevents cleavage of the nuclear enzyme substrate poly (ADPribose) polymerase (PARP) which is induced by hydrophobic deoxycholic acid (DCA). On that previous cytotoxic bile acid has been reported to induce apoptosis in hepatocytes at low concentrations, while UDCA and TUDCA were also shown to inhibit the toxicity of this agent, protecting hepatocytes from membrane-bound Fas-L-induced apoptosis (Sousa et al. 2015) (Castro et al. 2007) (Azzaroli et al. 2002). In addition, Rodrigues and others have also demonstrated that TUDCA inhibits the
mitochondrial perturbation associated with apoptosis induction. While apoptosis induced by 3-nitropropionic acid triggers mitochondrial cytochrome c release, cytosolic caspase-3 and nuclear PARP cleavage, TUDCA treatment inhibits all these apoptotic events by preventing depolarization of mitochondrial membrane as well as translocation of Bax from the cytosol to the mitochondrial (Rodrigues et al. 2000). On the other hand, Castro and others reported that glutamate induces apoptosis in rat cortical neurons, and that indeed TUDCA treatment mediates a reduction in apoptotic threshold induced by glutamate. As glutamate mediates down-regulation of anti-apoptotic members of the Bcl-2 family and dephosphorylation of the serine/threonine protein kinase Akt, TUDCA also induced marked phosphorylation and translocation of Bcl-2 associated death promoter (Bad) from mitochondria to the cytosol, preventing its interaction with anti-apoptotic proteins and letting them promote cell survival (Castro et al. 2004).

In addition, TUDCA was shown to prevent cell death by also acting on endoplasmic reticulum (ER), reducing oxidative stress and preventing protein unfolding (Malo et al. 2010). Sigurdsson and others have reported that hematopoietic stem cells (HSCs) undergo a rapid expansion during fetal liver stages, being very vulnerable to ER stress due to the elevation and accumulation of aggregated proteins. Therefore, BAs may act as chaperones by inhibiting protein aggregation, reducing cell death and enhancing proliferation (Sigurdsson et al. 2016).

Taken together, the evidence that TUDCA has cytoprotective effects results, in part, from its ability to reduce the apoptotic threshold in several cell types through modulation of classical mitochondrial pathways.

**I.4.1.2. Neural protective effect of TUDCA**

The neuroprotective role of TUDCA is indeed mainly related with prevention and modulation of mitochondrial apoptotic events. The potential role of TUDCA in improving several neurological disorders has been demonstrated over the years. In fact, a wide range of in vivo and in vitro animal models with neurodegenerative disorders have been studied, including Huntington’s disease (HD), Parkinson’s disease (PD), Alzheimer’s disease (AD), acute ischemia and hemorrhagic stroke, as well as clinical trials for amyotrophic lateral sclerosis (ALS) (Care et al. 2016).

HD is a neurological pathology that results from the selective death of neurons, mediated by mitochondrial dysfunctions. Mitochondrial compromise in HD demonstrates abnormal energy metabolite concentrations, impairs striatal mitochondrial respiratory chain complex II_III activity, increases of stress induced mitochondrial depolarization and also increases free radical production, being associated to oxidative damage. Mice transgenic for huntingtin protein mutation also exhibits reduced striatal aconitase and mitochondrial complex IV activity, which was reposted to be improved with TUDCA treatment. A marked reduction in striatal cell apoptosis and degeneration were observed, followed by an improvement in locomotors and sensorimotor abilities (Keene et al. 2002).

ALS is, in turn, a neurodegenerative disease characterize by the impairment of upper and lower motor neurons, caused by the interaction of a complex pathways that end up with selective cell death and apoptosis. Through that scenario, TUDCA properties of cytoprotection which may include anti-
apoptotic, immunomodulatory and antioxidant effects were analysed in a series of patients with ALS. The results have shown that TUDCA treatment may slow ALS deterioration, being a potential therapeutic candidate (Elia et al. 2015).

Regarding AD, which is characterized by neuronal dysfunction and massive neuronal loss, in specific areas of the brain, including the hippocampus and the cortex, the anti-apoptotic effect of TUDCA was also tested. Ramalho and colleagues demonstrated that Amyloid-β (Aβ) peptide modulates this pathology and is strongly associated with neuronal loss through apoptosis, namely with the a translocation of pro-apoptotic Bax factor to the mitochondria. In this context, TUDCA has been revealed to inhibit Aβ peptide induced apoptosis by the regulation of E2F-1/p53/Bax pathway. TUDCA was shown to interact with mineralocorticoid receptor (MR) to decrease p53 levels and consequently to inhibit apoptosis (Ramalho et al. 2004). The neural protection conferred by TUDCA was also extended to the synaptic level, as this bile acid increases the capacity of neurons to tolerate the toxic effect of Aβ, in vivo and in vitro (Ramalho et al. 2013).

Another neurological study was in rat model of intracerebral hemorrhage (ICH), which is an acute neurological disorder with a significant loss of neuronal cells thought apoptosis, specifically with caspase-3 activation and DNA fragmentation, both events characteristics of apoptosis (Gong et al. 2001). It has been shown that administration of TUDCA before or up to 6 h after stereotaxic collagenase injection into the striatum reduced lesion volumes at 2 days by as much as 50%. In fact, apoptosis was 50% decreased in the area immediately surrounding the hematoma and was associated with a similar inhibition of caspase activity. So, this bile acid reduce neuronal cell death by apoptosis, by maintaining the membrane stability and inhibiting caspase activation (Rodrigues et al. 2003).

More recently, the potential of this bile acid has been studied at cellular levels. In fact, mitochondrial integrity and apoptotic mechanisms as well as cell survival are again the main focus. For instance, Xavier et al have used NSCs as research model to demonstrate that TUDCA was able to prevent differentiation-induced mitochondrial alterations, including ATP levels, cytochrome c release, membrane depolarization and mtROS production. As TUDCA demonstrates a relieve on mitochondrial stress, NSCs seems to be able of reenter on cell cycle, enhancing cells in S (synthesis) and M (mitotic) phase and reducing the number of cells in G0 and G1 (gap phases), which was associated with augmenting the NSC proliferation. It was also evidence, that longer cell cycles, mediated by bile acid modulation of mtROS and ATP levels, elicit a shift from glial to neurons differentiation of NSCs, indicating that mitochondria damage predicts NSC lineage fate determination. In addition, it was also demonstrated that TUDCA alone was capable of controlling differentiation stress events, such as the p53 mitochondrial translocation and mtDNA damage during early-stages of neural differentiation (Xavier (a) et al. 2014).

That range of performed studies associated with the nontoxic characteristics of hydrophilic bile acids makes them resilient candidates for the treatment of neurological diseases and also useful in fields of mitochondrial-related pathologies and clinical setting (Xavier et al. 2015).
II. Aim of the studies

One of the major breakthroughs in regenerative medicine was the arise of hiPSCs, which are pluripotent cells reprogrammed from somatic cells, with the capability of differentiating into the three germ layers: mesoderm, endoderm and ectoderm. hiPSCs resembles hESCs, sharing the same pluripotent characteristics. Moreover, hiPSCs avoid certain ethical issues related with hESCs isolation and manipulation, letting cells more accessible for therapeutics, drug screening, disease modeling and research. The improvement of culture systems is indeed necessary to enhance proliferation rates and to better control stem cell fate. Neural commitment of hiPSCs allows mimicking of the neurogenesis process as well as to develop models of neurodegenerative diseases. Unfortunately, the nervous system has a limited capacity of self-renewal and repair, and NSCs, along the time, lack the capability of generating new functional neurons.

Regarding that, the recent data by Xavier et al. showed that the bile acid, TUDCA, increases NSCs proliferation and self-renewal potential, by modulating mtROS and ATP levels. In addition, TUDCA also prevents differentiation-induced mitochondrial stress, modulating mitochondria integrity and function at early stages of differentiation (Xavier (a) et al. 2014). Other scientific findings performed by Rodrigues and colleagues directs TUDCA activity in modulating neural apoptosis, more specifically mitochondrial-apoptosis pathways, behaving as potent neuroprotective agent (Rodrigues et al. 2003).

Based on this previous reported data, the first goal of this work was attended to investigate TUDCA effect on hiPSCs expansion and on neural commitment protocol of hiPSCs. Firstly, it was decided to explore the TUDCA potential involvement in proliferation, of both hiPSCs and NPs cells derived from neural differentiation of hiPSCs. Next, it was investigated whether TUDCA influences any neural commitment pathway, namely if the bile acid effect interferes on mitochondrial bioenergetics and dynamics modulation during neural differentiation.

The general aims of these thesis were:

- Investigate whether TUDCA increases hiPSCs number during expansion by using a chemically-defined protocol.
- Evaluate whether TUDCA interferes in neural commitment of hiPSCs also using a chemically-defined protocol.
- Clarify whether the mitochondria is the main target of TUDCA during neural induction of hiPSCs.

The main goal of this thesis was to examine a potential role of TUDCA in modulating expansion and neural differentiation in hiPSCs, envisaging the future application of this cell model for neurological disease modeling and for testing the therapeutic potential of TUDCA.
III. Materials and Methods

III.1. Expansion of hiPSCs

III.1.1. Cell line

The iPSC line used in this work was WT-F002.1A.13 (TCLab - Tecnologias Celulares para Aplicação Médica, Unipessoal, Lda.), reprogrammed from fibroblasts obtained from a skin biopsy on an adult female. By using a retroviral system, the hiPSCs have been generated through ectopic expression of a defined set of reprogramming factors, Oct4, Sox2, Klf4 and c-Myc. The cell line has been tested in terms of differentiation potential towards the three germ layers, while the analysis revealed a normal karyotype.

III.1.2. Adhesion substrate

III.1.2.1. Matrigel preparation

The gelatinous protein mixture Matrigel® (Corning®), extracted from the Engelbreth-Holm-Swarm mouse sarcoma, is rich in ECM molecules as laminin, collagen IV and entactin. Matrigel was stored in aliquots at -20ºC. The first step for Matrigel preparation comprises the thaw of an aliquot on ice, overnight at 4ºC, or at room temperature during approximately 60 minutes. Afterwards, it was diluted in a proportion of 1:100 (v/v) of cold Dulbecco’s Modified Eagle Medium; Nutrient Mixture F-12 (DMEM/F12, Gibco®). The previous diluted solution is then used to coat multiwell tissue culture plates (Falcon®). The prepared plates, if they were for immediate use, were left at least for two hours at room temperature, otherwise they were stored at 4ºC for up to two weeks.

III.1.3. Culture media

III.1.3.1. Essential 8 medium (E8)

The essential 8™ medium (Gibco®) is a xeno-free and feeder free medium formulated for being used in growth and expansion of human pluripotent stem cells. E8 medium was used for hiPSCs expansion procedures, during expansion experiments and for expanded cells before inducing differentiation. This medium contains only 8 essential components needed for stem cell culture. The DMEM/F12 (Gibco®) is the basal medium which is supplemented with insulin, transferrin, selenium, L-ascorbic acid, FGF2 and TGFβ, as well as NaHCO3 for pH adjustment. Insulin and FGF2 are important compounds for cell survival and proliferation, as well as L-ascorbic acid. Otherwise, TGFβ increases NANOG expression levels, which leads to a consistent long term cell culture stability. In contrast to the TeSR medium, which has 18 components added to DMEM/F12 base medium, E8 is a completely defined medium, without bovine serum albumin (BSA), but having a similar cell expression pattern when compared to TeSR medium (Chen, D. R. Gulbranson, et al. 2011).
In addition to E8 medium components, 1:200 (v/v) dilution of penicillin/streptomycin (PenStrep, Gibco®) is added in order to prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria.

III.1.3.2. Washing medium

The washing medium formulation is used when cell processing in suspension occurs or for the inactivation of enzymatic activity. The medium is composed by DMEM/F12 (Gibco®) with L-glutamine, 2.44 g/L of sodium bicarbonate (SigmaAldrich) and was supplemented with 10% KO-SR (Gibco®), 1% minimum essential medium (MEM) non essential amino acids (Gibco®) and 1%Pen/Strep. The formulation was stored at 4ºC, and should be pre-warmed at room temperature before being used.

III.1.4. hiPSCs culture

III.1.4.1. Thawing hiPSCs

The cryovials (Thermo Scientific™) containing hiPSCs are cryopreserved in liquid nitrogen. In order to be thawed, the cryovial was covered in gaze containing ethanol and placed in a 37ºC water bath. A 15mL Falcon tube with 4mL of washing medium pre-warmed has been prepared, from which 1mL was used for resuspend the cells in cryovial. The content of the cryovial was transferred for the previous Falcon tube of washing medium, which was then centrifuged for 3 min at 1,500xg. The supernatant was removed and the pellet was resuspended with E8+P/S medium. The content was uniformly distributed in 9.6 cm² culture wells pre-coated with Matrigel® and finally the plate was incubated inside a humidified incubator (Memmert), at 37ºC with 5% of CO₂ and 20% of O₂.

III.1.4.2. Cryopreservation of hiPSCs

In order to cryopreserve cells in liquid nitrogen, it is necessary to follow some steps. First it was necessary to made a quickly wash with EDTA (Invitrogen™) on the cultured cells, and then incubate with EDTA during 5 min. With a pipette of 1000µL and by using washing medium, the cells have been scratched from the well and collected for a Falcon tube. The previous Falcon tube was centrifuged during 3 min at 1,500xg, and then the supernatant is removed. The pellet is resuspended with the freezing medium, which was composed of 10⁶cells/250µL of KO-SR with 10% v/v of dimethylsulfoxide (DMSO, Gibco®). The mix is collected for the cryovial and placed at -80ºC before being transferred to liquid nitrogen.

III.1.4.3. hiPSCs passaging with EDTA

The cell passaging using EDTA is an enzyme-free dissociation method that allows the detachment of hiPSCs colonies in small aggregates. The culture medium was firstly aspirated, followed by a rapid wash with EDTA in each well. After this procedure, the wells were incubated with EDTA (0.5mM) for 5 min, which allowed the cells to be scraped immediately after. The cells were then released with E8
medium, by using a pipette of 1000µL and were collected for a Falcon tube. The cells should be plated at an approximate cell density of 50,000 cells/cm², in Matrigel-coated wells.

III.1.4.4. TUDCA solution preparation and addition

The stock solution of 100mM of TUDCA was prepared from 26.1 mg of TUDCA powder by adding 500.3 µL of MilliQ water. The powder of TUDCA (Sigma) was provided by Faculdade de Farmácia from Universidade de Lisboa (ULisboa). The stock solution should be stored at 4ºC. For the proliferation assays in hiPSCs, TUDCA was added everyday directly to each test well, in the desired concentration (5 µM and 10 µM), after fresh medium addition.

III.1.5. Cell counting

In order to measure the cell expansion with different concentrations of bile acid and without TUDCA (control) it is mandatory to perform cell counting. For the cell viability quantification, and to distinguish between dead and viable cells, the sample of cell suspension was diluted with a particular stain, Trypan Blue (Gibco®). This staining method uses a dye that selectively penetrates the membranes of dead cells, coloring them blue, which is not observed in the case of live cells.

After washing the cells with EDTA and scraping them with E8, a sample was diluted 1:20 (v/v) with the dye (Trypan Blue Stain 0.4%) in a 96-well plate. From the previous mixture, 10 µL has been taken and placed in a hemocytometer and the viable cells could be visualized and counted under the optical microscope.

Once the total cell count was obtained, cell concentration could be calculated from the following equation:

\[
\text{Total cells/mL} = \text{total cells counted} \cdot \frac{\text{dilution factor}}{\text{# of squares}} \cdot 10^4 \text{cells/mL}
\]  

(1)

Where each square of hemocytometer represents a total volume of 10⁻⁴ cm³.

In order no know how many cells were in original sample, the cell concentration had to be multiplied by the total sample volume.

III.1.6. Kinetic analysis of hiPSC expansion

The results for hiPSC expansion were obtained based on cell counts at each cell passaging time point, when cells were near the confluence. At each cell passaging, cells were re-plated at an initial seeding density of 50,000 cells/cm². The number of cells, \( x \), was plotted against time, \( t \), assuming exponential growth. The apparent specific growth rate, \( \mu \) (day⁻¹) is the slope of a linear trend line adjusted to the plot and was compute by the following equation (Rodrigues et al. 2011):

\[
\ln x = \ln x_0 + \mu t \quad \text{day}^{-1}
\]

(2)

Where \( x_0 \) was the initial viable cell numbers seeding. The average doubling time (Td) for each condition was also determined, taking into account the following equation (Rodrigues et al. 2011):
The results were represented as the fold increase (FI) in total cell number, being determined as the ratio between the final and the initial viable cell number at each cell passage. The cumulative fold increase (CFI) was calculated as the product of fold increase values obtain at all time point passages.

### III.2. Proliferation analysis using PKH67 fluorescent dye on hiPSCs

In order to study the effect of TUDCA in hiPSCs expansion procedure, a cell division analysis using PKH fluorescent dye was used. The PKH67 molecule, a green fluorescent dye with aliphatic tails, is stably incorporated into lipid regions of the cell membrane. When the cell division takes part, the molecule is equally distributed between daughter cells, being the fluorescence distributed among generations of cells. The fluorescence intensity is reduced to one-half in the resulting daughter cells and the number of cells divisions could be measured at any time on the cell culture in proliferation (Da Silva et al. 2009).

#### III.2.1. PKH67 assay protocol

Cells were collected after the second expansion passaging, day 5, when the peak of proliferation was reached and stained according to the manufacturer’s instructions (PKH67 green fluorescent cell linker kit for cells membrane labeling; Sigma). Approximately 1h before the cells had been collected, they were incubated with ROCK inhibitor (1:1000 (v/v)), as cells were stained as single cells. Prior to staining, cells were incubated with accutase (SigmaAldrich®) during 5 min at 37°C. The staining procedure has been made by incubating cells with a dye solution - for $1 \times 10^7$ cells/mL was used 1mL of Diluent C with PKH67 ethanolic dye solution (1:250 (v/v)) - during 4 min. In order to stop the staining, 2mL of 1% of BSA/PBS solution was added and cell incubated during 2 min. After that, 4mL of IMDM-10%FBS was also added. Cells were then centrifuged 3min at 1,500xg and $1 \times 10^6$ cells were collected from each condition for the first flow cytometry analysis, and fixed at 4°C in 2%of paraformaldehyde (PFA, Sigma). The remaining cells were resuspended with E8 medium supplemented with ROCK inhibitor and plated at the desired cell density. The culture medium was changed after 24h of incubation with ROCK inhibitor and during the remaining 3 days in culture, cells were collected and fixed with 2% PFA. The samples were analyzed in FACSCalibur™ flow cytometer (BD Biosciences®) and the proliferation analysis was performed using Proliferation Wizard of ModFit software. The results appear as the percentage of cells in each generation, starting with parental.
III.3. hiPSCs neural commitment

III.3.1. Neural induction of human iPSCs

When hiPSCs cultures were nearly to 90-100% of confluence the neural induction was performed by using N2B27 medium. The formulated medium consists of 50% (v/v) of N2 medium and 50% v/v of B27 medium. N2 medium was DMEM/F1(1:1)+Glutamax (Gibco®) supplemented with 1% (v/v) N-2 Supplement (Gibco®), 1.6 g/L of glucose (Sigma), 1% v/v PenStrep and 20 µg/mL Insulin (Sigma). B27 medium was formulated with Neurobasal® Medium (Gibco®) supplemented with 2% of B-27® Supplement (Gibco®), 2 mM of L-glutamine (Gibco®) and 0.5% of PenStrep. During 12 days of neural commitment, the previous described medium formulation was supplemented with 10 µM of SB-431542 (StemMACS™) and 100 nM of LDN-193189 (StemMACS™).

The bile acid tested, TUDCA, was also added into the medium formulation daily, during the 12 days, in concentrations between 0-10 µM, as schematized on Figure III.1.

III.3.2. Neuronal differentiation of human iPSCs

Human neural progenitor cultures were passaged at day 12 by using EDTA dissociation buffer (0.5 mM), and were re-plated in a split ratio of 1:1 into poly-L-ornithine (15 µg/mL; Sigma)-treated and Laminin (20 µg/mL, Sigma) coated plates. At day 14, when structures like neural rosettes were observable, N2B27 medium was supplemented with bFGF-Basic fibroblast growth factor (10 ng/mL, Peprotech) during 48h. At day 16, cells were again passaged by using EDTA, into new laminin-coated wells, in a split ration of 1:3. The medium was changed daily, without the addiction of any small molecule or any factor. At day 28, cells were split with accutase and plated into laminin-coated wells at a density of 100.000 cells/cm². The N2B27 medium was replaced every two days until day 70.

Figure III. 1 - Schematic view of neural and neuronal differentiation steps: dual-SMAD inhibition protocol by adding LDN – LDN193189, SB – SB431542 and TUDCA in N2B27 neural commitment medium. During neuronal commitment, cells were re-plated into laminin-coated plates at determined time points, where different neuronal structures and markers have been observed.
III.4. Immunocytochemistry and confocal microscopy

III.4.1. Immunofluorescence staining of intracellular markers

The culture wells were washed with PBS and cells were fixed with 4% (v/v) PFA during 30 min. Then, it was incubated with blocking solution (10% FBS and 1% Triton, in PBS) overnight or left during 60 min at room temperature. Primary antibodies were diluted in staining solution (5% NGS and 0.1% Triton, in PBS), added to the culture wells and left at 4°C overnight. Secondary antibodies were also diluted in staining solution and left to incubate with cells during 1h in the dark, at room temperature. The cells were washed with PBS in order to remove the excess of secondary antibody and were left with 4',6-diamidino-2-phenylindole (DAPI), the fluorescent stain that binds to DNA, (diluted 1:10000 in NaHCO3; Sigma) during 2 min at room temperature. Finally, cells were washed to remove any DAPI crystals and left with PBS for further observation under fluorescence optical microscope (Leica Microsystems CMS GmbH, model DMI3000 B) or confocal microscopy (Leica TCS SP5 laser scanning microscope). For confocal observation, the lamellas were taken and by using moviol, a mounting medium, they were assembled on blades.

III.4.1.1. Antibodies for intracellular immunocytochemistry

Primary antibodies: Pax6 (Covance, 1:400); Nestin (R&D, 1:400); ZO-1 (Novex, 1:100); Sox2 (R&D, 1:100); β-III-tubulin (Tuj1, Covance, 1:4000); Glial Fibrillary Acidic Protein (GFAP, abcam, 1:100); Map2 (Sigma, 1:400); NeuN (Cell Signaling Technology®). Secondary antibodies: Goat anti-mouse IgG Alexa Fluor – 488/546 (1:400); Goat anti-rabbit IgG Alexa Fluor – 488/546 (1:400).

III.4.2. MitoTracker_ Red CMXRos

In order to observe and stain mitochondria in neuroepithelial cells, at day 12 of neural commitment, mitochondrion-selective probes have been used. MitoTracker_ Red CMXRos probes (M7512, Invitrogen) passively diffuse across the plasma membrane of living cells and accumulate in active mitochondrias. The Mitotracker stock solution (1mM) was diluted for a final working concentration of 0.5 µM in the growth medium of the living cells and incubated during 30 min at 37°C. After staining live cells, the culture was washed twice with PBS and the cells were fixed with 4% of PFA during 20 min at room temperature. After the fixation procedure the cells were washed twice with PBS and incubated 3 min at room temperature with Hoechst 33258 (861405; Sigma-Aldrich Corp.) at 50 µg/ml in PBS, (1:1000), a fluorescent stain that also binds to DNA. For confocal analysis, the lamellas were taken and by using moviol, a mounting medium, they were assembled on blades for further observation.
III.4.3. Confocal microscopy

Images of hiPSC-derived neural rosettes and mitochondrial structures were acquired using a Leica TCS SP5 laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany), an inverted microscope (DMI6000) and a HCX PL APO CS 1.20W 63.3x water-immersion apochromatic objective (63.3x magnification and 1.2 numerical aperture). Alexa Fluor – 488 (green for Pax6 and Sox2 detection) excitation was performed using 476 nm line of an Argon ion laser as well as Alexa Fluor – 546 (red for Nestin and Zo-1 detection) excitation was performed using 514 nm. DAPI and Hoechst excitation was made by using a multiphoton obtained by a Ti:sapphire laser (Spectra-Physics Mai Tai BB, 710–990 nm). Fluorescence emission was collected at selected wavelengths, using the tunable system and beam splitter of Leica TCS SPC5. Fluorescence emission of Alexa Fluor – 488 was collected at 488–560 nm while Alexa Fluor – 546 was at 566-671 nm and DAPI at 400-478 nm. Images were obtained at a resolution of 512x512 pixels. To record serial optical sections without recording photo-damaged areas, random images were acquired at different x- and y-locations, and the z-plane was advanced from the bottom of the culture to the highest focal plane in steps of 1.5 μm. The image treatment and merge was performed with the Fiji software (for ImageJ).

III.5. Flow cytometry

The samples were collected and singularized by incubated 5 min at 37ºC with accutase. The inactivation of accutase was performed by the addition of washing medium, being all the cells collected in a Falcon tube. The samples were then centrifuge for 3 min at 1,500xg, the supernatant was discarded and the pellet was resuspended with 2% PFA in PBS. The samples were stored at 4ºC.

III.5.1. Intracellular staining

The samples stored in 2% PFA were first centrifuge 3 min at 1,500xg and washed twice with 1% normal goat serum (NGS, Sigma). Eppendorf tubes were coated with 1% (v/v) of BSA (Invitrogen) in PBS for at least 15 min. Then, cells were resuspended in 3% NGS and at least 5 x 10⁵ cells per condition were transferred for pre-coated eppendorf tubes (BSA was first removed). After transferring the same number of cells per condition, eppendorf tubes were centrifuged at 1,500xg during 3 min. For cell membrane permeabilization, incubation with 1:1 of 3% NGS and 1% saponin (Sigma) were performed during 15 min at room temperature. Afterwards, cells were washed with 3% NGS and then pellets of negative control were resuspended in 3% NGS only while the others were resuspended with the primary antibody (in 3% NGS), during 1h at room temperature. Cells were then washed twice with 1% NGS and incubated in the dark, during 30 min, with the secondary antibody. The last washing procedure was applied after incubation, and the cells were resuspended in PBS, transferred for FACS tubes and analyzed in FACSCalibur™ flow cytometer (BD Biosciences®). The results obtained were analyzed using CellQuest software.
III.5.1.1. Antibodies for flow cytometry

Ki-67 (BD Pharmingen™, 1:40), Goat anti-rabbit IgG Alexa Fluor – 488 (1:300)

III.6. Quantitative real-time PCR analysis

By using qRT-PCR it was possible to evaluate the expression of pluripotency and neural markers and also the analysis of relative mtDNA copy numbers. The hiPSCs were cultured in N2B27 medium in the presence of the bile acid, TUDCA, and were collected at different stages of neural commitment – days 0, 1, 3, 6, 9 and 12.

In order to quantify the pluripotency markers transcripts, NANOG and OCT4 genes were used while the neural markers quantified were PAX6 and NESTIN. The housekeeping gene used for control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For the analysis of relative mtDNA, the gene of mitochondrial encoded cytochrome c oxidase I (CO-I) was used, as it is one of the three mitochondrial DNA subunits of the respiratory complex IV. The housekeeping gene used was 18S ribosomal DNA, which is highly conserved among species and it hardly modifies under different tested conditions.

First, for the pluripotency and neural markers, the RNA was isolated from the collected samples at each day and used for synthesize complementary deoxyribonucleic acid (cDNA) templates, while relative mtDNA analysis was performed by using extracted total DNA. Then, both types of qRT-PCR reactions were run in duplicate, following manufacturer instructions.

III.6.1. Isolation of total RNA from cultured cells

The total RNA was isolated by using PureLink™ RNA Mini Link (Ambion®) according to manufacturer's instructions. RNA amount for each sample was quantified using a NanoVue™ Plus spectrophotometer (GE Healthcare®). cDNA was obtain from RNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®), by starting with the amount of 1 µg. The normalization was made with the housekeeping gene GAPDH (HS02758991-g1), and RT-PCR was performed in 48-well plates, by using Taqman® Gene Analysis Assays (Life Technologies) by using a StepOne Real-Time PCR Thermal Cycling System (Applied Biosystems®). The primers used for the experiment were Pax6 (HS00240871-m1), Sox1 (HS01057642-s1), Oct4 (HS009999634-sh), Nanog (HS02387400-g1) all of them from Thermo Fisher.

The threshold cycles (C_T) obtained for each sample were compared with C_T from the housekeeping gene, resulting in ΔC_T. The previous values were normalized with C_T achieved at day 0, resulting in ΔΔC_T, being the final results of gene expression shown as 2^{−ΔΔC_T}.

III.6.2. Quantification of mtDNA copy number

The total cellular DNA was isolated by using the QiaAmp DNA Mini Kit (51304; Qiagen, Hilden, Germany), following the manufacturer’s protocols. The qRT-PCR was run in the ABI7300 (Applied Biosystem®, Life Technologies Corp.) sequence detection system. The 18S rDNA primer sequence
used during amplification was: 5′TAG AGG GAC AAG TGG CGT TC 3′ (forward) and 5′CGC TGA GCC AGT CAG TGT 3′ (reverse). For human cytochrome oxidase-1 (Co-1) mitochondrial gene the primer sequence was: 5′CTA TCC GGA ATG CCC CGA 3′ (forward) and 5′TCT TCT ACT ATT AGG ACT TTT CGC T 3′ (reverse).

For each primer set, two independent reactions were made in 25 µl of total volume with 2xPower SYBR Green PCR master mix and 0.5 µM of each primer. The relative mtDNA copy number was determined based on the standard curve and the ratio of the amount of mtDNA versus 18S for each sample. The values were first expressed as percentage of total input and converted to fold change over control.

III.7. ATP measurements

ATP is a molecule found only in and around living cells and could be detected and measured by using the instructions of the Mitochondrial ToxGlo™ assay Kit (G8001; Promega Co.). Cells were cultured in 96-well plated and the neural commitment protocol was followed, being analyzed at day 0 and 12. Cell lysis was performed by incubating the cells during 1h with MilliQ water. Then, cell lysate was diluted 1:10. By adding the ATP Detection Reagent (1:2 (v/v)) which was composed of luciferin, ATPase inhibitors and thermostable UltraGloTM luciferase, it was possible to measure the intensity of a luminescent signal, proportional to the amount of ATP present in each condition. The reaction produces a flash of yellow-green light, with a peak emission at 560 nm, which was measured by FB12 Luminometer (Berthold detection system). For normalization, total proteins were measured using a colorimetric protein assay, based on an absorbance shift of the dye Coomassie Blue. The absorbance was read at 595 nm using a GloMax Multi+ spectrophotometer (Promega Co.). Data was presented by the fold change over day 0.

III.8. Immunoblotting

III.8.1. Protein isolation and quantification

The cultured cells were incubated with accutase during 5 min at 37°C, and the enzymatic activity was stopped by using washing medium. Cells were then collected for Falcon tube, centrifuged at 1,500xg during 3 min. The supernatant was taken and the pellet was washed with PBS, centrifuged at 1,500xg during 3 min and stored at -80°C for further total protein extraction.

The pellet was then thawing on ice and resuspended with an ice-cold lysis buffer (10mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1.5 mM potassium acetate, 1% Nonidet P-40 and 2 mM dithiothreitol) and protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc., Rockford, IL). Each sample was then vortexed for 5 min and incubated on ice during 30 min, followed by 30 sec. of sonication. The lysate was centrifuged at 3,200xg at 4°C during 10 min, and the supernatant was recovered.

For protein quantification, the samples solutions were diluted 1:800 (v/v) in MilliQ water and measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Using Bradford assay
dye, protein content was measured in microplate reader spectrophotometer using 595 nm wavelength and as standard, BSA has been used.

### III.8.2. SDS-PAGE preparation and transfer to blot

A 12.5 mL separating gel - sodium dodecyl sulfate–polyacrylamide electrophoresis gels (SDS-PAGE) - was made at the percentage of 7.5% and 12%. Each gel was composed of acrylamide/bis (Protogel), 4x lower buffer (0.5 M Tris-HCl, pH 8.8, 0.4% SDS), H₂O, ammonium persulfate (APS) and tetramethylethlenedianine (TEMED).

Samples were denatured in the presence of a 5x denaturating/loading buffer (0.2M Tris-HCl, pH6.8, 20% glycerol, 2% SDS, 10mM β-mercaptoethanol, H₂O, bromophenol blue) at 95°C during 5 min. 60 µg of total protein was loaded, as well as protein size marker (Precision Plus Protein™ Standard Dual Color, BioRad). The gel was run at 140v for approximately 1h. After electrophoresis, samples were transferred to a Hybond-C nitrocellulose membrane 8.5 cm x 6.5 cm by electroblotting. The gel transfer was made in a cold room packed in ice, at 0.2A limit, for 1h.

### III.8.3. Western blot analysis

First of all, the membrane was stained with 1% Ponceou S in order to check equal membrane loading. The membrane was first blocked in 5% milk in 1x tris-buffered saline (TBS), and then washed three times during 5 min with 1% TBS plus 0.1% Polysorbate 20 (Tween 20). The incubation with the primary antibody has been performed overnight at 4°C, and the previous sequential wash procedures were also made. The secondary antibody was incubated during at least 2h at room temperature. Finally, membranes were processed for protein detection using Immobilon (Merk Millipore Corp.) at ChemiDoc™ MP (Bio-Rad).

The same membranes could be used for several proteins detections. In order to re-use the same membrane, after each protein detection, membranes were incubated during 10 min with each stripping solution: stripping solution I (0.2 M Glycine and 0.5M NaCl, pH 2.8 adjusted with glacial acetic acid) and stripping solution II (0.5 M Glacial acetic acid and 0.5 M NaCl, pH 2.5)

### III.8.3.1. Antibodies for immunoblotting

Primary rabbit monoclonal antibodies: Pax6 (901301, Covance); MnSOD (sc-30080; Santa Cruz Biotechnology, Inc.); Oct4 (2750, Cell Signalling Technology® Inc.). Primary mouse monoclonal antibodies: Sox2 (MAB2018, R&D Systems® Inc.); βIII-tubulin (Tuj1, MMS-435P, Covance); Mnf2 (ab50838, Abcam); Drp1 (Sc-32898, Santa Cruz Biotechnology, Inc.); PGC-1α (ST-1202, Millipore). As loading controls: β-actin (A5441; Sigma-Aldrich Corp.). Secondary antibodies conjugated with anti-mouse and anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad Laboratories).
III.8.3.2. Densitometry

In order to measure the relative intensities of protein bands obtained, the Image Lab\textsuperscript{TM} Version 5.2.1 densitometric analysis program (Bio-Rad Laboratories) has been used.

III.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. When appropriate, statistical analysis was performed using two-tailed nonparametric tests, as Mann-Whitney test for independent samples. The software ranks the values from low to high and then the P-value was computed, which depends on the discrepancy between the mean ranks of two data groups. Error bars represent the standard error of the mean (SEM). When statistical analysis was applied, at least two independent samples were evaluated. A p-value less than 0.05 was considered statistically significant.
IV. Results and Discussion

IV.1. Impact of TUDCA towards expansion of hiPSC

IV.1.1. TUDCA increases hiPSCs during expansion

It has been previously described that pluripotent stem cells rely on anaerobic glycolysis for having substrates for biosynthetic demands and cell proliferation support (Prigione et al. 2010). This metabolic process produces energy with lower ROS generation, demonstrating that mitochondria oxidative rate has to remain depressed during cell proliferation (Elguero et al. 2012). Considering the role of TUDCA on NSC proliferation, tested by Xavier and colleagues (Xavier et al. 2014), in the present work it was decided to test the effect of this bile acid towards hiPSC expansion. The previously mentioned research group has also demonstrated that this specific bile acid is an inhibitor of apoptosis-mediated mitochondrial perturbations, modulating cell fate, increasing self-renewal and cell cycle progression of NSCs (Xavier et al. 2014). In addition, it was also revealed that TUDCA acts as a chaperone, supporting in vitro growth of hematopoetic stem cells (HSCs), by inhibiting protein unfolding and aggregation, which by accumulation of mis-folded proteins causes endoplasmatic reticulum ER stress and promotes cell death (Gani et al. 2015), (Sigurdsson et al. 2016).

To further explore the effect of TUDCA on hiPSC expansion it was used the xeno-free, chemically-defined E8 medium, being TUDCA added daily during medium exchange. Two optimal concentrations of TUDCA were tested, 5 \( \mu \text{M} \) and 10 \( \mu \text{M} \), based on previous obtained results concerning the optimization of TUDCA concentration (master thesis from Pinho 2015). At each cell passaging, after counting, cells were re-plated at an initial seeding density of 50,000 cells/cm\(^2\). The previous value was used to determine the fold increase of cell proliferation (FI), which gives a better insight of proliferation tendency under different TUDCA concentrations. The results show an increase in the final cell fold increase upon addition of 5 \( \mu \text{M} \) TUDCA (Fig. IV.1 A). Moreover, the overall cumulative fold increase in total cell number (CFI) was also higher in cultures treated with 5 \( \mu \text{M} \) of TUDCA (CFI=11.22±1.47 for negative control, CFI=89.91±21.02 for 5 \( \mu \text{M} \) TUDCA and CFI=48.54±42.83 for 10 \( \mu \text{M} \) TUDCA) (Fig. IV.1 B). For the best condition tested in terms of CFI (5 \( \mu \text{M} \) of TUDCA), the specific growth rate (\( \mu \)) (Fig. IV.1 C) is almost three times higher than control condition (\( \mu=0.172±0.004 \text{ day}^{-1} \) for control and \( \mu=0.359±0.018 \text{ day}^{-1} \) for 5 \( \mu \text{M} \) TUDCA). On the other hand, the average doubling time (Td) for 5 \( \mu \text{M} \) TUDCA condition is approximately half in comparison to the obtained for the culture control (Td = 96.87±2.254h for control and Td = 46.36±2.225h for 5 \( \mu \text{M} \) TUDCA). This data suggests a significant role of 5\( \mu \text{M} \) of the bile acid TUDCA during expansion of hiPSC cultures.
The effect of 5 µM TUDCA on hiPSC proliferation was further studied by cell division analysis using the PHK67 fluorescent dye (Fernandes et al. 2010). For this condition, and taking into account hiPSCs’ peak of proliferation in culture, day 8 (Fig. IV.1 A), PKH67 cell staining was performed at day 5 after cell counting. Afterwards cells were analyzed at day 7 and day 8 by using flow cytometry. In contrast with previous obtained results, no relevant differences were observed between control and TUDCA-treated cells. In fact, it is possible to observe that from day 7 (Fig. IV.2 A-I) to day 8 (Fig. IV.2 A-II) most of the cells left parental generation and were already at generation 3 and 4, which in indicative of at least two cell divisions performed, starting from a PKH67 labeled cell. However, the percentage of cells for each condition at each generation was equivalent, showing no apparent effect of TUDCA addition on hiPSC proliferation.

Figure IV. 1 - Addition of TUDCA during 13 days of hiPSCs expansion for four consecutive passages. Results were obtained by cell counting procedure. (A) Effect of TUDCA (5µM and 10µM) in total cell number. Results express the fold increase at each day of cell passaging. Results are expressed as the mean of three independent experiments. (B) Cumulative fold increase in total cell number of the previously described 13 days of hiPSC expansion. (C) Specific growth rates determined for control and for 5µM TUDCA. Results are expressed as the mean of three independent experiments. All error bars represent the standard error of mean (SEM).
Despite rapid progress in developing new efficient GMP protocols and culture conditions, there are still many problems associated with hiPSC expansion. During passaging, cells are often individualized, however hiPSC survive poorly after individualization as myosin-actin-dependent contraction leads to cell death, and cell-cell adhesions promote survival by contraction inhibiting (Chen et al. 2010). Since that finding, EDTA treatment started to be used as it partially dissociates cells and generates small aggregates strong enough to survive. However, this protocol still induces cellular stress, associated with excessive apoptotic events and spontaneous differentiation (Chen et al. 2014). The discordance between kinetic growth and PKH-67 fluorescence results could also be explained by the possible effect of TUDCA in reducing cell apoptosis, without influencing cell proliferation. This hypothesis was not experimentally tested, but the well-establish antiapoptotic effect of TUDCA could support a future investigation of this hypothesis in hiPSCs expansion protocol. Moreover, in these studies the proliferation assays using hiPSC treated with TUDCA must be repeated. Indeed, the lack of replicates in TUDCA-treated samples led to a lack of statistical significance in these conditions. In addition, the PKH-67 essay should be repeated, for longer times, allowing cells to achieve latter generations for more accurate results.

Figure IV. 2 - Cell division analyses using the PKH67 fluorescent dye during hiPSC expansion. Analyzes performed by flow cytometry. The percentage of cells at each generation is shown, since parental to G4, for control culture and for 5μM TUDCA addition. Results are presented as a mean of two independent experiments, n=2. (A-I) Cell percentage at day 7 of expansion and 5μM TUDCA addition. (A-II) Cell percentage at day 8 of expansion and 5μM TUDCA addition.
IV.2. Effect of TUDCA during neural commitment of hiPSCs

IV.2.1. TUDCA increases the number of neuroepithelial rosettes

Neural rosettes structures *in vitro* resemble a sagittal view of the neural tube, from which the first neurons are generated *in vivo* (Chambers et al. 2009). Following the dual-SMAD inhibition protocol, hiPSCs-derived NPs were replated at day 12 and further expanded in the presence of bFGF, thus allowing the formation of neuroepithelial rosettes that have been possible to obtain *in vitro*.

TUDCA has been previously demonstrated to have a neuroprotective effect by preventing apoptosis-associated events (Rodrigues et al. 2003). In addition, and based on the previous report showing TUDCA effect in increasing NSCs proliferation, by preventing cell cycle arrest modulating mtROS and ATP levels, (Xavier (a) et al. 2014), as well as on the previous positive impact of TUDCA in increasing the number of hiPSCs during the expansion protocol, the effect of this bile acid was also tested during neural induction of hiPSCs.

To assess the influence of TUDCA upon neural rosette formation, hiPSCs were exposed to 5 µM and 10 µM of TUDCA during 12 days of neural commitment, being the cells previously expanded in E8 medium without bile acid addition. Briefly, NPs were replated into laminin-coated plates at day 12, and until day 16 no more TUDCA was added, being cells only maturated with N2B27 medium and bFGF (since day 14). At day 16, the number and morphology of the obtained neural rosettes were analyzed. By staining the culture with anti Zo-1, the tight junction protein in the apical center of the rosettes, and with the NPs marker Sox2, the quantification of the rosette number was assessed through immunocytochemistry by using fluorescent microscopy (Fig. IV.3 A).

The number of rosette structures counted was then normalized with the mean value of the control culture. No significant differences were found between the normalized number of rosettes per cm$^2$ obtained in the presence of 5 µM of TUDCA and control. However, differentiating cultures exposed to 10 µM of TUDCA showed an increase of 76.6% (Fig. IV.3 B), of rosette structures number, when compared to control ($p<0.05$).
The previous results demonstrate a significant improvement by TUDCA towards the process of neural induction from hiPSC, reaching a higher number of NP cells at the end of day 16 of neural commitment protocol.

Once the number of neural rosettes increased in cultures treated with TUDCA, it was thought that by using confocal microscopy it would be possible to observe morphological differences when compared to control conditions. The neural rosettes are radially-elongated columnar cells with a center apical zone and could be continuously expanded and differentiated into neurons and astrocytes in vitro as well as in vivo (Zhang et al. 2001) (Elkabetz et al. 2008). Again, the staining with Zo-1 and Sox2 reveals higher number of neural rosette structures on treated culture (Fig. IV.4 A). Additionally, cells were stained with Pax6 and Nestin, that is a sort of intermediate filament protein which is transiently expressed in adult NSCs and immature neural progenitor cells (Lendahi et al. 1990). Indeed, this imaging tool allows the observation of differences in morphological organization and

Figure IV. 3 – TUDCA influence on hiPSCs neural commitment, at day 16, during neural rosettes structure formation. The cell culture analyzes were performed by immunocytochemistry as described in Materials and Methods. (A) Immunofluorescence staining with Zo-1, used to see polarization within rosettes and with Sox2, a NPs marker. Nuclei (blue) are stained by DAPI. The staining observation was made under a fluorescence optical microscope (Leica Microsystems CMS GmbH, model DMI3000 B). Scale bar: 50 µm (B) Number of counted rosettes per cm² after replating NPs into laminin-coated plates. The counted rosettes for TUDCA 5 and 10 µM were normalized with the mean of control’s rosette numbers. The results are presented as the mean of three independent experiments, each performed in duplicate. The error bars represent the standard error of mean (SEM). * denote statistical significance (P<0.05).
maturation of rosettes, being the NPs more structured and partially well-defined (Fig. IV.4 B). In TUDCA culture, a higher number of Pax6⁺ cells are observed in the organized rosette structure. It is rather observed, for TUDCA culture, that rosettes structures are organized side-by-side, being interconnected with each other, while in control neural rosettes are more dispersed along the culture.

**Figure IV. 4 - Impact of TUDCA in neural rosette formation at day 16 of neural induction of hiPSCs.** Cells were staining by immunocytochemistry procedures. (A) Immunocytochemistry staining with Zo-1 and Sox2 (B) Immunocytochemistry staining with Pax6 and Nestin. Images taken at confocal microscopy (Leica TCS SP5 laser scanning microscope), at 630x magnification. Scale bar: 75 µm.

**IV.2.2. TUDCA effect on cell proliferation during neural induction of hiPSC**

In order to clarify whether 10 µM TUDCA, the best concentration of the bile acid for increasing rosette number, would also have a positive influence on NPs proliferation during neural commitment process, flow cytometry analysis was performed at 0, 1, 3, 9, 12 and 16 days of neural commitment using the intracellular marker Ki-67. Human Ki-67 protein is strictly associated with cell proliferation, being detected within the nucleus during interphase. During the so-called active phases of cell cycle, G1, S, G2 and mitotic phase, this protein is present, being absent from resting cells phase, G0. The growth fraction of a determined cell population could thus be measured (Fig. IV.5). The results obtained revealed that Ki-67 positive cells, which are expected to proliferate, are clearly separated from the Ki-67 negative cells, measured at the same fluorescence intensity, only treated with secondary antibody. Cells that are Ki-67 negative have exit the cell cycle and remain quiescent.
Two distinct proliferative cell populations are observed, before 16 days of neural commitment. At day 0 and day 1, it is possible to observe an expected gated pluripotent population (later confirmed by pluripotency markers). At day 1 the proliferation seems to be higher on TUDCA conditions, but not significantly different (Fig. IV.5 A-I). The previous proliferative population is still present at day 3, although a new proliferative population was gated (Fig. IV.5 B). This expected new self-renewal and multipotent population must be NPs, which will be next confirmed by staining neuroectodermal markers. Indeed, this new gated population tends gradually to become the only proliferative population over the neural commitment time points. No differences are observed regarding the percentage of positive NPs population between control and TUDCA-treated cells, except for day 12, where NPs appears to be more proliferative in control conditions (Fig. IV.5 A-II). At day 16, both conditions showed a global increase in the percentage of dividing population. This increase is potentially due to the re-plating stimulus on day 12 and the addition of bFGF until day 16, which in turn is a growth factor with the ability of promoting proliferation.
Overall, the results obtained from these experiments are not completely in agreement with the previous outcome of TUDCA-induced increased in rosette structure number. In the future, it would be important to repeat these experiments, as it was only performed once, in order to have a better understanding of the TUDCA effect in hiPSC proliferation under neural commitment conditions.

**IV.2.3. Effect of TUDCA on gene and protein expression during neural commitment of hiPSCs**

After applying the neural commitment protocol, the supplementation of a defined culture medium with inhibitors of BMP and TGFβ is sufficient to generate NPs expressing Pax6, Sox1 and Nestin. To further explore the potential role of TUDCA in the process of neurogenesis, pluripotent and neural markers were analyzed by qRT-PCR and immunoblot throughout neural commitment process. The results obtained by qRT-PCR and Western blot are represented on Figure IV.6.
The profile of the pluripotency marker expression shows a significant decrease in both Nanog and Oct4 expression patterns throughout neural commitment process of hiPSCs, suggesting that pluripotency is almost totally lost since day 3 (Fig. IV.6 A-I, II). The expression of Oct4 was also corroborated by immunoblotting (Fig. IV.6 B-I). Nevertheless, no differences are observed between control and TUDCA condition for these pluripotency markers. Moreover, for both control and TUDCA-treated cells, the neuroectoderm fate determinant Pax6 rapidly increases its expression after day 1, reaching maximum levels at day 9 (Fig. IV.6 A-IV). These results were also confirmed by Western blot (Fig. IV.6 B-II). After peaking at initial stages of neural commitment, Pax6 expression declines at day
In turn, the expression of Sox1, other neural marker which functions primarily in neurogenesis, reached its peak at day 9, with a slight increase in control when compared with TUDCA conditions (Fig. IV.6 A-III). The decrease in Sox1 and Pax6 levels possibly indicates further differentiation into more mature neural cells. To sum up, no significant changes were observed in terms of protein quantification or relative gene expression of pluripotent and neuroectodermal markers on hiPSCs neural inducted after treatment with TUDCA.

Sox2, a transcription factor required in pluripotency maintenance, expressed in embryonic stem cells, is also expressed by developing cells from neural tube and neural progenitors in CNS (Favaro et al. 2009). By evaluating the expression of Sox2 by immunoblotting, a distinct behavior between conditions is observable (Fig. IV.6 B-III). It was expected to have high levels of Sox2 at day 0, when most of the culture is composed by pluripotent stem cells. Although, Sox2 expression is not null at day 0, it is rather reduced in view of the expressed levels during neural commitment time points. Moreover, at day 12, TUDCA treatment induces an increase in Sox2 expression when compared with the control. This evidence suggests that bile acid could potentiate NPs self-renewal at later stages of neural commitment.

The already reported experiments by Xavier and others showed the same effect for this bile acid, in maintaining NSCs self-renewing during differentiation (Xavier (a) et al. 2014). On the other hand, previous results in section IV.2.2 from KI-67 flow cytometry seems to contradict the last evidence of TUDCA effect in NPs self-renewal. Considering the in vivo system, neural stem cells (NSCs) are capable of proliferating and are multipotent in terms of differentiation capacity. During embryogenesis, NSCs are located in the ventricular zone of the neural tube, giving rise to the cell types that compose the CNS. Furthermore, it has been shown that neurogenesis is still occurring during adulthood, being NSCs responsible for this process. To support the existence of self-renewal and multipotent NSCs in vivo, Suh and colleagues have used a transgenic mouse and GFP reporter under the control of Sox2 promoter (Suh et al. 2007). The results demonstrated that Sox2+ NSCs enhances neurogenesis, by asymmetrical cell divisions, leading to the generation of neuronal precursors as well as Sox2+ NSC population. This two generated daughter cells are different, one reenters the cell cycle (G1 to S) and the other differentiates into young neurons (G0). It is also known that deficiency in Sox2-positive cells caused a decrease in NSC proliferation and reduced neuronal numbers at SVZ (Favaro et al. 2009).

However, Xavier and others verified that increased of NSCs self-renewal in vitro by TUDCA is more related with symmetrical divisions, generating daughter cells that are destined to acquire the equal fate (Xavier (a) et al. 2014). To further confirm the NPs division behavior the Sox2 cell pair assay should be performed. In addition, Feng and colleagues have identified that the overexpression of Sox2 elevates the expression of survivin, which is an inhibitor of caspase activation, and it also inhibits mitochondria-dependent apoptotic pathways (Feng et al. 2013). Regarding that, another hypothesis to explain the obtained results could be related with less triggered apoptotic events on day 12 in TUDCA condition, where Sox2 levels appear higher than control.

The TUDCA effect on proliferation and self-renewal of NPs was not conclusive. In this regard, addition experiments for proliferation must be repeated for more consistent results.
IV.2.4. TUDCA cumulative effect on neuronal differentiation

Neural rosette structures are capable of differentiating into various region-specific neuronal and glial cell types. In response to appropriate developmental cues, morphogens and mitogens confer different positional identities to NPCs (Mertens et al. 2016). In vitro, the differential competency is maintained allowing NPs to differentiate into more mature cells. By studying neuronal differentiation of NP’s cultures previously treated with TUDCA it was possible to validate the functionality of the NPs previous generated in the presence of this molecule in comparison to the control. The immunoblotting and immunocytochemistry results obtained for neuronal differentiation analysis are represented on Figure IV.7.

![Figure IV.7](image)

Figure IV. 7 – Neuronal differentiation and maturation of hiPSCs-derived NP cells under defined conditions exposed to TUDCA treatment during neural commitment. Results were obtained by Western blot and immunocytochemistry analyses. (A) Quantification of immunoblots during day 0, 3, 9, 12, 16 and 29 of neural differentiation by β-Tubulin (III) Protein levels were quantified by Western blot. Results were normalized to endogenous β-actin protein levels. (A-II) Representative immunoblot of Tuj1 and internal control β-actin in total extracts. (B) Immunofluorescent staining of Map2, NeuN and Tuj1 expression levels at day 70 for evaluation of neuronal maturation. Nuclei (blue) are stained by DAPI. Scale bar: 50 μm. The staining observation was made under fluorescence optical microscope (Leica Microsystems CMS GmbH, model DMI3000 B).

As observed in Figure IV.7, the expression of typical early neural markers Pax6 and Sox1 reached a peak at day 9 of neural commitment protocol, as previous reported. This was followed by up-regulation of the neuronal marker β-Tubulin (III) (Fig. IV.8 A-I). It is evident the increase in the expression of β-Tubulin (III) until day 29 of differentiation, and no significant differences are noticed between control and TUDCA addition. From around day 16 of differentiation, Tuj1-positive immature neurons outgrew from neural rosettes and differentiation into more mature neurons is achieved. By immunocytochemistry analyses neural markers Map2, NeuN and Tuj1 expression levels were evaluated at day 70 of neuronal differentiation. NeuN protein is located in nuclei and perinuclear cytoplasm of most of the neurons in the CNS of mammals (Gusel & Korzhevskiy 2015), while Map2 proteins are thought to be involved in microtubule assembly, an essential step in neurogenesis. NPs
derived from hiPSCs neural commitment in the presence of TUDCA treatment were replated at day 16 in a split ratio of 1:3 and again, at day 29 at a density of 100,000 cells/cm² into laminin-coated plates. The control and TUDCA treated culture were only supplemented with the previous described differentiation medium, N2B27, since day 16 until day 70. At this stage, both cultures were expressing the same markers, indicating an efficient neuronal maturation (Fig.IV.7 B). It was thus demonstrated that TUDCA exposure does not impair NPs differentiation.

Xavier and others have reported that TUDCA-influenced NSC lineage determination is a consequence of its effect on mitochondria and cell cycle progression (Xavier (a) et al. 2014). In fact, increased levels of mitochondrial damage are associated with elevated astrogliogenesis when compared to neurogenesis. Accordingly, Xavier et al., revealed that TUDCA mediates neuronal rather than astroglial conversion (Xavier (a) et al. 2014). In order to evaluate whether this phenomena also occurred during differentiation of hiPSC-derived NP’s, the previous neural differentiating cultures at day 70 were also stained for the astrocyte marker GFAP. However, at this stage of differentiation, no fluorescent staining was detected in either control or TUDCA-treated cultures, not allowing to determine if the potential effect of TUDCA, previous described for NSCs, is also verified for hiPSCs-derived NP’s.
IV.2.5. TUDCA effect on mitochondrial mass and metabolism during neural commitment of hiPSC

Upon cell differentiation, large amounts of energy are required to sustain specialized functions, and so a mitochondrial maturation and a metabolic change take part. A transition from predominant glycolysis-based metabolism in hiPSC to an activation of mitochondrial aerobic metabolism occurs. Glycolysis is less energetically efficient than ATP production through OXPHOS. Mitochondria biogenesis also increases mitochondrial number and content, respiratory chain complex density as well as ATP and ROS levels (Cho et al. 2006) (Xu et al. 2013).

The characterization of hiPSCs neural commitment protocol at an energetic level and the consequent effect of TUDCA in improving mitochondrial mass, viability and function have been evaluated by measuring mtDNA copy number by qRT-PCR, staining mitochondrial organelles, and ultimately by assessing ATP levels throughout this process – Figure IV.8.

![Figure IV.8](Image)

**Figure IV. 8 – TUDCA modulation of mitochondrial alteration on hiPSCs under neural commitment.** Results were obtained by qRT-PCR and by luminescence measurements. (A) qRT-PCR analysis of relative mtDNA copy number at day 0, 1, 3, 9, and 12 of dual SMAD inhibition protocol. The results are presented as the mean of three independent experiments n=3, each performed in duplicate (B) Fold increase data of ATP content in cells at day 0 and at day 12 of neural commitment. The results are presented as the mean of three independent experiments n=3, each were performed in triplicate. The error bars represent the standard error of mean (SEM). * denote statistical significance (P<0.05). (C) TUDCA effect on mitochondria at day 12 of neural commitment. Images obtained by immunocytochemistry. Staining of live cultures with MitoTracker_ Red CMXRos. The Nuclei (blue) are stained by Hoechst. Images taken at confocal microscopy (Leica TCS SP5 laser scanning microscope), at 630x magnification. Scale bar: 25 µm.
As shown in Figure IV.8, during 12 days of the dual-SMAD inhibition protocol, the mtDNA copy number data contradicts the general tendency of increase during the differentiation process (Fig. IV.8 A). Although TUDCA appears to rescue the decrease in mtDNA copy number at all analyzed time-points, being graphically more noticed at day 12, no statistical significance was achieved.

Beside demonstrating an increase of NSCs proliferation by TUDCA, Xavier and colleagues, have also shown TUDCA regulatory effect on NSC proliferation is a mtROS and ATP-dependent mechanism, consequently retarding cell cycle arrest (Xavier (a) et al. 2014). Although the results previously presented in section IV.2.2 show inconclusive findings regarding the proliferative effects of TUDCA, it is still possible that TUDCA might regulate NPs self-renewal and neural differentiation by a similar mechanism. In conclusion, at day 12, the self-renewal potential and mtDNA were both increased, while proliferation results display a decrease in TUDCA condition.

Being aware of the mtDNA results at day 12, the total ATP levels generated by either mitochondrial (OXPHOS) or non-mitochondrial (glycolysis) were measured by a generation of a luminescent signal proportional to the amount of ATP present. The data obtained indicate significant different results, with TUDCA improving the ATP levels at day 12 of differentiation induction (Fig. IV.8 B).

Taking into account the previous results in mtDNA increase for the bile acid condition, cell culture at day 12 were incubated with MitoTracker_ Red CMXRos probes in order to stain mitochondria structures and observe this organelles by confocal microscopy. No significant differences were observed for the quantification by using MitoTracker_ Red CMXRos probes as unspecific staining occurred (Fig.IV.8 C). The staining protocol, as incubation time and concentration, should be optimized.

Mitochondrial apoptosis-associated events were shown by Xavier and others to be critical during the early stages of NSC differentiation, partly caused by the higher rates of mitochondrial oxidative stress (Xavier, et al. 2014). The same behavior of high levels of initial apoptotic events may be observed in other cell types, mainly during hiPSCs neural differentiation. Many possibly consequences may be the increase in ROS production through (OXPHOS) stress induced by high levels of cell confluence and also related with medium defined composition exchange and small molecules addition. TUDCA has been shown to be an apoptosis-modulator, being described as an inhibitor of Bax translocation from cytosol to the mitochondria, release of cytochrome c and consequent activation of downstream caspases (Rodrigues et al. 1999), (Xavier (b) et al. 2014). In both cell types, hepatocytes and neurons, TUDCA treatment reduces mitochondrial membrane perturbation by the previous described activity (Rodrigues et al. 2000). TUDCA has also demonstrated a crucial role in mitigating mitochondrial apoptosis activity, being neuroprotective in a transgenic mouse model of Huntington’s disease (Rodrigues et al. 2000) (Keene et al. 2002). Regarding all these evidences, TUDCA role in increasing mtDNA content could rely, in part, on its effect in preventing mtDNA stress-damage and consequently mitochondrial damage and degradation, acting as a potential-mitochondria protector. As mitochondria are known to be a vital component in many cell processes, including differentiation and apoptosis, analysis of structural and dynamic-related mitochondrial factors may corroborate this hypothesis. As verified by Xavier et all, TUDCA improves the mtDNA content and ATP levels at early
stages of NSCs differentiation (Xavier (a) et al. 2014), being the same tendency observed at day 12 during hiPSCs neural commitment protocol.

Evidence by the work of Xavier et al. showed relieve of mitochondrial-stress by this bile acid allowing NSCs to re-enter in cell cycle and enhancing proliferation. On the other hand, mitochondrial dysfunctions activates two retrograde signals to modulate cell cycle, such as mtROS increase and decrease in ATP levels, which in turn could induce cell cycle arrest (Xavier (a) et al. 2014). The increase in ATP levels with TUDCA treatment are consequently related with enhanced mitochondrial respiratory function and energy metabolism during the course of maturation and also could be an indication of cell cycle progression or NP cell proliferation. ROS levels modulation could emphasize the previous hypothesis, as an increase in ROS could potentiate cell cycle arrest, and consequently impair proliferative potential.

In order to understand the impact of TUDCA in regulating mitochondrial oxidative stress in hiPSCs neural derivatives, the quantification of MnSOD mitochondrial levels, the major scavenger of mtROS, was performed by immunoblots – Figure IV.9.

![Figure IV. 9 – TUDCA influence on MnSOD expression during neural commitment.](image)

Since ROS is a by-product of OXPHOS, the incomplete reduction of O₂ to water results in the formation of superoxide anions (O₂⁻), which is one of the main consequences of DNA damage during early stages of differentiation and also triggers mitochondria membrane depolarization. MnSOD, an anti-oxidant enzyme, converted superoxide anions into hydrogen peroxide, which is not so reactive (Candas & Li 2014). As expected, MnSOD levels increased during hiPSCs neural commitment, possibly as consequence of mtROS increase during differentiation days of commitment (Fig. IV.9 A-I). Otherwise, the TUDCA treated culture displays higher levels of MnSOD, which could be associated with bile acid effectiveness in the role of reducing mitochondrial-stress and consequently cells’ apoptosis, which for the control culture remains fairly stable.
It has been shown that apoptotic-related events occur at either early stages of NSC differentiation or hiPSCs neural commitment, as cells are subject to a similar set of stressed morphological, energetically and metabolic alterations (Investigacion et al. 2010) (Elguero et al. 2012) (Solá et al. 2012). Therefore, it is expected an increase in ROS levels during differentiation, as OXPHOS is more expected to occur. Again, two distinct analyses might be speculated. First, TUDCA displays higher levels of the antioxidant enzyme, which could indicate less oxidative stress in culture, consequently preventing mitochondrial membrane depolarization, mtDNA damage and mitochondrial dysfunction. On the other hand, the possibly decrease in ROS levels by the bile acid action, could influence cell cycle modulation, inhibiting NPs of arresting cell cycle. This last data corroborates with ATP increase by TUDCA, which may be indicative of TUDCA effect on proliferation. Again, proliferative tests may be repeated, otherwise if it does not corroborate proliferation induction on NPs, the effect of mtROS and ATP modulation in mitochondria functionality still be a viable consequence of TUDCA in enhancing neural rosettes numbers.
IV.2.6. TUDCA effect on mitochondrial dynamics and biogenesis

NSCs and hiPSCs neural differentiation and survival relies on dynamic behavior of mitochondria, allowing cell response to the change of physiological conditions. The quality of mitochondria is maintained through mitophagy, or a balance control between fusion and division, that are controlled by a range of dynamic proteins (Westermann 2010). Considering that the differentiation process of both cell types aforementioned requires an increase in mitochondria activity, the role of TUDCA in modulating the expression of mitochondrial dynamic proteins and other regulators was explored in this work. The total levels of PGC-1α, a master regulator of mitochondrial biogenesis and mitochondrial dynamic-associated proteins, such as Mfn2 and Drp1 were assessed during hiPSC neural commitment by immunoblot analysis (Fig.IV.10).

![Graphs](image-url)

Figure IV. 10 - TUDCA effect on the expression of mitochondrial biogenesis- and dynamic-related proteins during hiPSCs neural commitment protocol. The total levels of PGC-1α, Drp1 and Mfn2 were evaluated by Western blot. Quantification of immunoblots during day 0, 3, 9 and 12 of neural commitment protocol. (A-I/II/III) Quantification of PGC-1α, Drp1 and Mfn2 protein levels. Results were normalized with Ponceau protein levels. (A-IV) Representative immunoblot of PGC-1α, Drp1 and Mfn2 and internal control Ponceau in total extracts.
PGC-1α is a transcription coactivator that promotes mitochondrial biogenesis and it is also a powerful regulator of ROS removal by increasing the expression of ROS-detoxifying enzymes (Austin & St-pierre 2012) (Liang et al. 2016). As it can be observed in Figure IV.10 A-I, at the first days of differentiation induction, the levels of PGC-1α increase, being further upregulated with TUDCA. Regarding the role of PGC-1α in mitochondrial biogenesis, the results seem to be in almost all the time points coherent with mtDNA content, as it decreases along commitment time, with an improvement in the presence of the bile acid. In spite of decreasing levels, it is slightly improved by TUDCA at day 12, when a higher mtDNA and ATP production was also observed. ST-pierre and others investigated the role of PGC-1α in increasing the expression of MnSOD, which in turn removes superoxide anions. And it also found that the absence of PGC-1α, renders the brain more sensitive to neurodegeneration, with increased oxidative damage and apoptotic cell death (St-pierre et al. 2006). However, the results, even for control, do not reveal a coherent relation between PGC-1α levels and MnSOD.

Mitochondrial fusion and fission events may be mediated by several GTPases in the outer mitochondrial membrane. Drp1 is a mediator of mitochondria fission, which mainly occurs with mitochondrial division, and could be associated with cells that undergo apoptosis under critical stress conditions. Mitochondrial oxidative stress is thought to trigger mitophagy, being higher the occurrence of fission process (Xavier et al. 2015). In fact, until day 12, the levels of Drp1 generally increase, although it is observable a positive influence of TUDCA correlated with lower levels of Drp1 (Fig.IV.10 A-II). As hypothesis, increasing in fission activity can facilitate damaged mitochondria mitophagy, so TUDCA treatment decreases Drp1 recruitment which could be indicative of less apoptotic events and less mitochondrial damage when compared with control condition.

On the other hand, the expression of Mfn2 regulates mitochondrial fusion, which ensures the unifying and mixing of mitochondrial components (Westermann 2010). Tondera and colleagues have discovered that mitochondria hyperfusion requires metabolic active mitochondria, with an increase of ATP, conferring an adaptive response against stress conditions (Tondera et al. 2009). Fusion processes afford protection against mitophagy and promote cell survival. The levels of Mfn2 were found to vary along the neural commitment period, starting to decrease since day 3 and being less recruited when TUDCA treatment occurs (Fig.IV.10 A-III). However, while Mfn2 levels at control are still decreasing, from day 9 to 12, the presence of TUDCA seems to trigger the Mfn2 levels. This result is in concordance with the increase in mtDNA content and with the increase in ATP levels. Indeed, Fang and colleagues demonstrate that Mfn2 overexpression could be related with more mature neuronal phenotypes, displaying higher content of mitochondria and more developed ones, also associated with higher mitochondrial membrane potential (Fang et al. 2016). Increased Mfn2 expression found in TUDCA conditions might be indicative of enhanced mitochondria bioenergetics functions, associated with a higher maturation of NPs obtained and analyzed at day 16, when rosettes structures were counted.
V. Conclusions

Neuronal differentiated hiPSC-derived NSCs have been a powerful tool regarding their potential application in disease modeling, being extremely useful in patient specific therapies (Fernandes et al. 2013). In fact, hiPSCs neural inducted cells provide an unrestricted access to CNS, by the in vitro generation of neural cells, being considered as a source for research and a model for neurodegenerative diseases. In spite of many protocols that have already been used, a lot of improvements must be investigated to close research to tangible applications. In this regard, new culture strategies should be developed, whereas it is crucial to understand the molecular mechanisms that regulate cell proliferation and differentiation potential, as well as survive functions.

In this study, an endogenous bile acid, TUDCA was tested by adding 5 µM and 10 µM to a defined medium and culture conditions, during hiPSCs expansion protocol and neural commitment induction. This aimed was initially defined based on the knowledge that TUDCA is a potential inhibitor of apoptosis-mediated mitochondrial perturbations in neurons, also acting as a mediator of NSC proliferation (Xavier et al. 2014). During hiPSCs expansion, a culture defined protocol was followed and TUDCA was added every day. The analysis from the growing kinetics and CFI demonstrated a higher number of hiPSCs treated with 5 µM TUDCA, being the specific growing rate three times higher and the doubling time half when compared with control condition. Nevertheless, no significant differences were observed in terms of cell proliferation as determined by the PKH67 assay. Of note, the already described TUDCA properties, mainly its effect on inhibiting mitochondrial-apoptotic events, could justify the increase in the hiPSCs pool, in place of proliferation induction.

Since, there are no reports in the literature describing a possible effect of TUDCA in hiPSCs neural commitment induction into NPs, we decided to address this specific issue in this thesis. For that, the number of hiPSC-derived neural rosettes was counted after 12 days in dual-SMAD inhibition protocol and after replating the immature NPs into laminin-coated plates. For 10 µM of bile acid treatment, a significant difference in rosettes number was attained, with an increase of 76.6% when compared with control. Taking into account this finding, it was decided to perform a more detail analysis in order to better understand the mechanisms behind this positive effect of TUDCA. For that, three different possibilities were studied with the best concentration obtained (10 µM): the influence of TUDCA on cell proliferation, as determined by the Ki67 assay, the TUDCA effect on the expression of markers of neural commitment, and, at last, the effect of this bile acid in modulating mitochondria during neural induction of hiPSCs. Firstly, we began to clarify whether the increase in NPs pool could be correlated with the already reported proliferative effect of TUDCA in NSCs (Xavier (a) et al. 2014). However, the intercellular staining with Ki-67, measured by flow cytometry, showed no relevant differences for almost all the time points studied, except at day 12, where the control showed higher proliferative rates than the treated culture. However, immunobloting of Sox2, revealed that at the same time point an increase in Sox2 levels observed after TUDCA treatment could be indicative of the presence of higher number of proliferative NPs. A precise conclusion about TUDCA effect of mediating NPs proliferation is unclear.
A neural commitment characterization of pluripotency and neural markers was performed to further understand the influence of bile acid in neural differentiation pathway. No significant differences were observed, as both TUDCA and control cultures showed an expected immature neural phenotype. In spite of bile acid inducing an increase in NPs pool, this does not guarantee that NPs obtained were functional. By obtaining more rosettes structures, it was not sure the capacity of those precursors in generating functional and differentiated mature neurons. In order to verify this, hiPSCs were differentiated for 70 days and cultures were then analyzed by immunocytochemistry with neural markers characteristic of more mature neurons. Nevertheless, analysis of NeuN, Map2 and Tuj1 expression levels indicate that TUDCA treatment does not delay neuronal differentiation per se. The inconclusive results obtained, guided the work for the study of other known valences of the bile acid. This new stage of the research began to explore mitochondrial alteration induced by TUDCA addition, taking into account the role of TUDCA in preventing mitochondrial membrane-damaging and regulating apoptosis and oxidative stress. By measuring the levels of mtDNA by qRT-PCR it was possible to notice that TUDCA rescues the decrease in mitochondrial mass during neural differentiation. Importantly, it was demonstrated a considerable improvement in mtDNA content, at day 12 of neural commitment. The ATP levels measured at the same time point corroborate the TUDCA impact during neural commitment, as it was found that this molecule mediates a significant increase in ATP levels. Due to the stress, morphological and bioenergetic alterations associated with early stages of hiPSCs neural differentiation, higher apoptotic levels are perceived. One of the main consequences for cell apoptosis is intrinsically related with ROS generation. ROS levels are associated with DNA damage, mitochondria-membrane depolarization and also with mitophagy (Candas & Li 2014). The ROS levels are increased by the anabolic metabolism, characteristic of cells following differentiation. To further explore whether the bile acid interfere with ROS levels elimination, the total MnSOD levels were analyzed. Western blots revealed that MnSOD levels increases in general during neural commitment, being further increased in TUDCA treated cells. This antioxidant enzyme is responsible for the reduction of ROS levels at the mitochondrial level, and consequently for apoptosis reduction, which for the control culture, the antioxidant system, remains fairly stable. Mitochondrial dysfunction activates the two retrograde signals, which results in cell cycle modulation. The effect of TUDCA in mediate mitochondrial integrity, decreasing ROS levels and enhancing ATP levels could also be indicative of inhibiting cell cycle arrest of NPs, corroborating the observed enhance in self-renewal potential of NPs presented at later stages of hiPSCs neural induction. The proliferative analysis of this cell model must be repeated, as Ki-67 staining revealed antagonist results for hiPSC proliferation, not corroborating the previous described hypothesis.

In addition, the study of mitochondrial biogenesis- and dynamic-related proteins expression were also help to better elucidate the role of TUDCA during neural commitment of hiPSCs. PGC-1α expression pattern indicated that mitochondrial biogenesis was higher in almost all analyzed time points after TUDCA exposure, generally decreasing during time of commitment. The levels of Drp-1, in turn, were lower for TUDCA condition, possibly being associated with less fission mechanisms, less occurrence of mitophagy and less apoptotic-associated events. In contrast, Mfn2 showed a variable behavior, almost opposite from those found for Drp1. While Mfn2 levels at control were still
decreasing, from the day 9 to 12, TUDCA seemed to induce the Mnf2 levels. This previous result is in agreement with the increase of mtDNA and improvement in ATP levels verified for the same time point, which is a possible indication of the effect of TUDCA on mitochondria respiratory and bioenergetics function. This previous results and hypothesis are indicative of mitochondrial dynamic alterations during hiPSCs neural commitment due to the TUDCA treatment, which might be responsible for promoting maturation of NPs. The bile acid treatment inhibited the events that usually triggers apoptosis while some dynamic mechanisms are recruited increasing the mitochondria energetic content and functionality. This balanced alteration in mitochondrial behavior during neural commitment induction of hiPSCs, induced by TUDCA, may interfere with NPs pool, which per se will certainly influence the number and consequently the morphology of neural rosettes.

In conclusion, bearing in mind that TUDCA effect is well established in NSCs in vitro (Xavier (a) et al. 2014), the precise role of this bile acid has never been explored on hiPSCs model. In fact, TUDCA increases both hiPSCs and hiPSCs neural differentiated pools. The strongest evidence of bile acid effect could be associated with its anti-apoptotic and antioxidant capacities, but also with its regulatory function on mitochondrial and metabolic cell behavior, which in turn easily may influence proliferation and self-renewal of NPs pool.

Altogether, these studies provide evidence of the influence of TUDCA in other cellular context, in both proliferation and differentiation conditions, which rely on modulation of mitochondrial dynamic activity, biogenesis and oxidative stage. The bile acid tested in hiPSCs model provides a new framework to further explore the treatment and associated mechanisms of neurological pathologies, as well as ageing features, in patient-specific derived cells, avoiding ethical issues related with other used cell models.
VI. Future perspectives

Despite the clear evidence of TUDCA effect on neural differentiated hiPSCs, a lot of improvements and repetitions must be performed, to acquire more reliable results. As a general future work, the results with no statistical significance must be repeated, at least three times, for more accurate discussable data. The repetition should be performed on the same lineage of cells, TCLab, but it should also be accomplished using other hiPSCs cell lines, to attest the same behavior independent of cell lineage.

Firstly, in hiPSCs expansion protocol, the maintenance of pluripotency characteristics during TUDCA treatment must be tested. In vitro expression assays with pluripotency markers Oct4, Sox2 or Nanog could be determined by flow cytometry or qRT-PCR. In addition, spontaneous differentiation of hiPSC, as embryoid bodies into cells of the three germ layers, would largely contribute to confidently verify the pluripotency maintenance.

As PKH67 dye did not reveal any significant different in hiPSCs proliferation, Ki-67 staining could be an alternative in order to verify the percentage of proliferative population. Despite that, a different alternative should be followed, as the study of cell death, apoptosis and viability, evaluating the proof of concept of TUDCA role in mediating apoptotic events.

Regarding the positive shown effect of the bile acid during hiPSCs neural commitment, complementary tests may be performed. The proliferative assays must be repeated, for an accurate conclusion about TUDCA effect on NPs proliferation positively obtained by Sox2 levels observation. Sox2 cell pair assay could determine the number of progenitor pair undergoing proliferative or differentiation-related cell divisions (Xavier et al. 2014). Cell cycle progression must be studied, for clarifying the possible role of TUDCA in cell cycle modulation, and consequently on cell proliferation.

It remains to be determined the influence of TUDCA in the mediating neuronal rather than glial conversion of hiPSCs. To attest the previous described effect, TUDCA treatment must be prolonged, as neuronal differentiation takes long time until obtaining mature neurons and culture staining positively for GFAP marker. Also p27 and p21 expression levels could be measured in NP’s cells, as it is related with cell cycle exit and glial terminal differentiation (Xavier et al. 2014).

The obtained results demonstrate an effect of TUDCA in modulating energetic mitochondrial-related parameters, and mitochondrial stress events. In order to strongly corroborate the increase in mtDNA content by other technique, the staining with MitoTracker_ Red CMXRos should be also optimized. The protocol improvement should be performed for both the incubation time and concentration of the dye. After that, the confocal images should be treated with thresholding method, which is a technique of image segmentation, using an appropriate software, and the mitochondrial-cell ratios must be determined by the number of mitochondrial pixels divided by the number of nuclei pixels in the complete image set (Birket et al. 2011).

The bile acid incubation promotes the mitochondrial integrity, predominantly during early stages of differentiation. The measure of mitochondrial p53 translocation, mitochondrial ROS detection and cytochrome c may complement the protein and molecular results indicatives of TUDCA incubation effect. Mitochondrial ROS levels may be determined, in order to confirm the influence of TUDCA in mediating oxidative levels, attesting the action of MnSOD activity. Since mitochondrial translocation of
p53 was shown to induce mitochondrial survival during early stages of differentiation, mitochondrial levels of p53 could be also measured in cell cultures treated with TUDCA (Xavier (b) et al. 2014).

Finally, cytochrome c release is associated with caspases activation and consequently with mitochondrial perturbations. The improvement in the collapse in membrane potential and the opening of mitochondrial permeability transition pore may be also studied regarding the establishment of chaperone activity of TUDCA. Regarding the extensive number of dynamic proteins and other mitochondrial regulators, a more complete study, as proteomic analysis, may be also useful to dissect the role of this bile acid on hiPSC neural fate. On this way, the TUDCA condition and the control may be compared for different expression proteins profiles.

Given the interest in exploring the role of TUDCA in regulating the expression of mitochondria regulators and dynamic proteins and regarding the energetic characterization and molecular study, in the future, a neurological disease modeling, in hiPSCs model, associated with NSCs lost and apoptosis could be used to extend our studies regarding TUDCA.
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