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INTEGRATION OF TRANSCRIPTIONAL PROGRAMMING AND MICRO-TECHNOLOGIES FOR IN VITRO MODELLING OF NEURAL DEVELOPMENT

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Coordinator: Ch.mo Prof. Carlo Giaquinto

Supervisor: Ch.mo Prof. Maurizio Muraca

Co-Supervisor: Ch.mo Prof. Nicola Elvassore

Ph.D. student: Anna Maria Tolomeo

…ovunque io sia…

…la vostra Forza!

AnnaMaria

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SOMMARIO

Fino ad oggi, la maggior parte degli studi riguardanti il cervello umano ed i disturbi neurologici sono stati effettuati su biopsie *post-mortem* o su tessuti allo stadio terminale della patologia. I modelli animali potrebbero rappresentare una possibilità per comprendere alcuni dei meccanismi neuronali, ma risultano limitati e spesso non riproducono fedelmente il fenotipo del paziente. Recentemente, lo sviluppo di modelli cellulari *in vitro* ha fornito una nuova strategia per studiare le patologie neurologiche umane, partendo dalle cellule del paziente stesso e studiando lo sviluppo della specifica patologia *ex vivo*. In aggiunta, con tali modelli è possibile caratterizzare i relativi difetti metabolici o condurre *screening* farmacologici mirati. Purtroppo, le colture cellulari primarie hanno un tempo di vita limitato ed è spesso difficile isolare la popolazione cellulare d'interesse, ad esempio nel caso del sistema nervoso centrale. La tecnologia delle cellule staminali pluripotenti indotte (iPSC, *induced pluripotent stem cells*) ha aperto la strada a nuove prospettive per la creazione di modelli cellulari di malattia *in vitro*, poiché offre l'opportunità di riprogrammare cellule somatiche (es. fibroblasti cutanei) ad uno stadio simil-embrionale che può essere in seguito differenziato nel tipo cellulare desiderato, dando vita a veri e propri "*disease in a dish*".

Parallelamente a queste tecnologie, lo sviluppo di piattaforme microfluidiche ha potenziato le capacità dei ricercatori di controllare e manipolare il microambiente cellulare e le condizioni sperimentali. Infatti, l'uso di sistemi microfluidici presenta diversi vantaggi fra cui bassi costi di produzione, ridotti volumi di reagenti e possibilità di effettuare analisi multiple su colture compartimentate ma funzionalmente collegate fra loro, per non parlare del fatto che ricapitolano *in vitro* un ambiente di coltura cellulare fisiologicamente molto più simile a quello presente *in vivo*, se confrontato con i sistemi di coltura convenzionali. Infatti, i fattori solubili sono molto più omogeneamente concentrati a livello delle cellule e queste sono sottoposte ad un sistema di perfusione che mima quanto succede *in vivo*.

In questo contesto, mentre la riprogrammazione verso la pluripotenza è ora ben codificata e viene realizzata con diverse metodiche, il differenziamento non sempre risulta riproducibile e richiede ulteriori ottimizzazioni, specialmente per le linee neuronali. Nello specifico, la derivazione *in vitro* di neuroni post-mitotici da iPSC e la conseguente maturazione funzionale richiede tempi sperimentali di coltura molto prolungati, della durata di 30 o più giorni. Per questo motivo, l'identificazione di nuove strategie per abbreviare il processo di differenziamento neuronale *in vitro* rappresenta una sfida cruciale per realizzare il pieno potenziale delle iPSC nell'ambito della ricerca di base, nello sviluppo di modelli di patologie umane e, di conseguenza, anche in generale nella medicina rigenerativa. Per risolvere questo problema è stata sviluppata una strategia di induzione neuronale innovativa, veloce ed efficiente, basata sull'espressione forzata di un singolo fattore di trascrizione esogeno, in grado di indurre rapidamente il differenziamento di determinate popolazioni di neuroni funzionali a partire da iPSC.

In quest'ottica, l'obiettivo di questa tesi è la messa a punto di protocolli di differenziamento neuronale *in vitro* a partire da iPSC, in piattaforme miniaturizzate, sfruttando la tecnologia microfluidica, per generare modelli neuronali *high-throughput in vitro*.

La prima parte del progetto riguarda la miniaturizzazione, in piattaforme microfluidiche, del processo di induzione neuronale basato su vettori lentivirali. In particolare, è stato sfruttato il sistema TetOn per indurre l'espressione di Ngn2 in iPSC mediante somministrazione di doxiciclina. Inoltre, questo approccio è stato usato con una prospettiva innovativa: regolando la durata e la frequenza dell'espressione di Ngn2, attraverso la somministrazione regolata di doxiciclina, è possibile imitare, *in vitro*, il profilo oscillatorio di Ngn2 osservato *in vivo*. *In vivo*, infatti, i progenitori neurali sono caratterizzati da un pattern di espressione di Ngn2 oscillatorio, mentre i neuroni mostrano un'espressione sostenuta e costante di Ngn2. La possibilità di generare sia cellule staminali neurali che neuroni utilizzando un singolo fattore (nello specifico Ngn2) semplicemente variando la frequenza di espressione di un fattore pro-neurale, come Ngn2, offre l'opportunità

senza precedenti di modificare *in vitro* il destino cellulare, a seconda del bisogno specifico di ottenere cellule neurali progenitrici o neuroni maturi. Inoltre, dal momento che il corretto rilascio di fattori esogeni, importanti per la generazione di neuroni, ad alta efficienza, deve essere finemente regolato, e questa regolazione è spesso difficile da eseguire manualmente da un operatore, abbiamo progettato una piattaforma microfluidica automatizzata in grado di rilasciare in modo controllato i fattori solubili necessari per la generazione *in vitro* di neuroni da iPSCs.

I vettori lentivirali, però hanno la potenzialità di generare modificazioni genomiche, per questo motivo il passo successivo è stato quello di indurre la formazione di neuroni utilizzando un *integration free-system*, ovvero somministrando sintetici mRNA modificati (mmRNA) codificanti per il fattore di trascrizione pro-neurale Neurogenin 2 (Ngn2), a colture cellulari di iPSC. Tale metodo è stato inoltre accoppiato alla tecnologia microfluidica. Gli mmRNA offrono l'opportunità di indurre l'espressione della proteina d'interesse *in vivo* o *in vitro* in maniera temporalmente definita. In condizioni fisiologiche, tali mmRNA, una volta introdotti nella cellula bersaglio, vengono tradotti in proteina dai complessi endogeni di traduzione proteica, della cellula stessa. Nell'ultimo decennio gli mmRNA non solo sono stati usati per indurre la produzione di proteine mancanti o alterate in pazienti affetti da patologie ereditarie o acquisite, caratterizzate, appunto, dalla sintesi disfunzionale di specifiche proteine, ma sono anche stati usati per riprogrammare cellule somatiche ad iPSC promuovendo l'espressione dei fattori di riprogrammazione. La capacità dell'mmRNA codificante per Ngn2 (Ngn2-mmRNA) di indurre programmazione trascrizionale non è mai stata testata prima. Sono disponibili solamente dati riguardanti la programmazione trascrizionale neuronale per generare, in soli 10 giorni, neuroni motori funzionali, mediante un cocktail di 5 mmRNA codificanti per fattori di trascrizione ((NEUROGENIN1 (NGN1), NEUROGENIN2 (NGN2), NEUROGENIN3 (NGN3), NEUROD1 (ND1), NEUROD2 (ND2)), accoppiati a Dual-SMAD inhibition in piattaforme convenzionali di coltura cellulare.

In questo lavoro di tesi è stata testata la capacità di Ngn2-mmRNA di promuovere programmazione trascrizionale neuronale sia in chip microfluidici che in convenzionali piastre multi-well. Abbiamo dimostrato come l'efficienza di differenziamento di iPSC in cellule neuronali, ottenuto mediante la sovra-espressione di un singolo fattore di trascrizione, sia maggiore in piattaforme microfluidiche (2.5%) che in supporti convenzionali (0.002%), suggerendo una maggior efficienza delle piattaforme microfluidiche nel veicolare fattori solubili alle cellule bersaglio.

Dopo aver definito le condizioni ottimali di coltura, lo studio si è focalizzato sull'aumentare l'efficienza di differenziamento, attraverso la modulazione di vie di trasduzione del segnale fondamentali per lo sviluppo *in vivo* del sistema nervoso centrale. In particolare, ci siamo concentrati sul ruolo di Fgf2, della *Dual-SMAD inhibition* e del metabolismo del glucosio. Inoltre, sono state testate cellule neuroepiteliali come cellule di partenza, è stato modulato il dosaggio di Ngn2-mmRNA ed è stato studiato l'utilizzo di ROCK inhibitor per migliorare la sopravvivenza cellulare.

Possiamo concludere che, nelle nostre mani, la miglior combinazione che fornisce la massima efficienza di induzione neuronale, tra quelle testate, è quella che consiste nell'uso di Fgf2 per 2 giorni all'inizio del protocollo di differenziamento, in combinazione con il Rock inhibitor e 3,1 mg/ml di glucosio. Inoltre, abbiamo osservato che il trattamento con Rock inhibitor da una parte aumenta l'efficienza dell'induzione neuronale, ma dall'altra promuove la differenziazione di progenitori neurali.

SUMMARY

To date, a large amount of neurological diseases and human brain studies have been performed on post-mortem biopsies or on tissues collected at late disease stages. The animal models could represent a possibility to understand some neurological mechanisms, but they are limited and sometimes do not fully recapitulate the patient phenotype or human-specific processes. Recently, the development of *in vitro* cellular models has provided a new way to investigate human neurological pathologies, starting from patients' own cells and studying the development of their pathology *ex vivo*. Indeed, it is possible to characterize the metabolic defects or perform a specific drug screening. However, primary cell cultures have a limited lifespan, and often it is difficult to take the cellular population of interest, as shown in the case of the central nervous system (CNS). Induced pluripotent stem cells (iPSC) technology has opened new perspectives for *in vitro* disease modelling, because it offers the opportunity to reprogram somatic cells (e.g. skin fibroblasts) to an embryonic-like stage that can be subsequently differentiated to the desired cell type providing the so-called "disease in a dish". In parallel to these technologies, the development of microfluidic platforms enhanced the capabilities of investigators to control and manipulate the cellular microenvironment and experimental conditions. In fact, the use of microfluidic systems has many advantages, including low production costs, reduction in drug sample volumes, and the ability to analyse multiple, environmentally isolated but functionally connected cultures, not to mention the fact that they recapitulate *in vitro* a more physiological environment for cell culture, compared to conventional culture systems. Indeed, the soluble factors are concentrated around the cells and the cells are subjected to a perfusion system that could resemble what happens *in vivo*.

In this scenario, while reprogramming for pluripotency is now well codified, and can be implemented using different methods, the differentiation is not always reproducible and is still under optimization, especially for the neuronal

lineage. In this specific case, the *in vitro* derivation of post-mitotic hiPSCs neurons, and the consequent functional maturation, require a very extensive cell culture period, lasting 30 days or more. Therefore, identifying innovative strategies to shorten the *in vitro* differentiation process of neurons, represents a crucial challenge for the realization of the full potential of hiPSCs, in basic biology and in the modelling of human diseases and consequently in regenerative medicine. To solve this issue a new fast and efficient strategy for neuronal induction has been developed, based on the forced expression of only one exogenous transcription factor (i.e. Ngn2) that rapidly induces the differentiation of a defined population of functional neurons from hiPSCs.

From this perspective, the focus of this thesis is the downscaling of *in vitro* neuronal differentiation protocols starting from hiPSCs, taking advantage of microfluidic technology and transcriptional programming, for the generation of a high-throughput neuronal *in vitro* model.

We performed a downscaling of neuronal induction based on lentiviral vectors in the microfluidic system. In particular we took advantage of a TetOn system to induce Ngn2 expression in hiPSCs regulated by doxycycline administration. However, we used this approach with an innovative prospective: regulating the timing and the pattern of Ngn2 expression through the regulated administration of doxycycline, it is possible to mimic, *in vitro*, the oscillatory profile of Ngn2 observed *in vivo*. *In vivo*, indeed, neural progenitors show an oscillatory Ngn2 pattern whereas neurons show a sustained and constant expression of Ngn2. The possibility of generating both neural stem cells and neurons using a single factor (i.e. Ngn2) and different expression frequencies offer the unprecedented opportunity to modify *in vitro* the cell fate based on the specific needs for progenitors or mature neurons. Furthermore, since the correct release of exogenous factors, important for the generation of neurons with high efficiency, need to be finely times regulated, and is difficult to regulate when performed manually, we designed an automated microfluidic platform able to release in a controlled way the soluble factors necessary for *in vitro* generation of neurons from iPSCs.

Due to a potential characteristic of lentiviral vectors, to generate genome modifications we decided to induce the generation of neurons by an integration free-system, introducing synthetic modified mRNA (mmRNA) encoding for Ngn2 (Ngn2-mmRNA) into hiPSCs and coupling to this method with the microfluidic technology. mmRNAs offer the opportunity to induce the expression of a protein of interest *in vivo* or *in vitro* in a time-defined fashion. The mmRNA, when introduced in the host cell, is translated by the cellular translation machinery, under physiological conditions. In the last decade, mmRNAs have been used to induce the production of missing or defective proteins in patients affected by hereditary or acquired genetic disorders characterized by dysfunctional synthesis of specific proteins, but also to reprogram somatic cells into pluripotent stem cells promoting the expression of the reprogramming factors. The transcriptional programming efficiency of Ngn2-mmRNA has never been tested before. The only report available showing neuronal transcriptional programming, to generate functional motor neurons, in only 10 days, used an mmRNA cocktail of 5 transcription factors (NEUROGENIN1 (NGN1), NEUROGENIN2 (NGN2), NEUROGENIN3 (NGN3), NEUROD1 (ND1), NEUROD2 (ND2)) and Dual-SMAD inhibition, in conventional devices.

In this thesis we tested Ngn2-mmRNA neuronal transcriptional programming both in microfluidic chips and in conventional multi-well plates. We showed that the differentiation efficiency of hiPSCs into neuron-like cells, obtained with the overexpression of only one transcription factor, Ngn2, is very high on chip (2,5%) compared to well plates (0.002%) suggesting that in the microfluidic platform the delivery of the soluble factors to the cells is more efficient.

We explored various cellular signalling playing important roles during the *in vivo* development of the central nervous system, with the aim of further increasing the differentiation efficiency. In particular we focused on the role of Fgf2, of glucose metabolism, of Dual-SMAD inhibition or the use of neuroepithelial cells as cell source, on the increase of the dose of Ngn2-mmRNA and finally on the use of Rock inhibitor (Ri). We can conclude that, in our hands, the best

combination that gives the highest neuronal induction efficiency, among the conditions that we tested, consist in the use of Fgf2 for 2 days at the beginning of the differentiation protocol, in combination with Ri and 3.1mg/mL of glucose. Moreover, we observed that the treatment with Ri on one hand increases the efficiency of neuronal induction but on the other hand promotes the differentiation of neural progenitor-like cells.

FOREWORD

The work presented in this thesis was performed at the Fondazione Istituto di Ricerca Pediatrica "Città della Speranza" of the Department of Women's and Children's Health, of the University of Padova, under the supervision of the Prof. Maurizio Muraca and at the Venetia Institute of Molecular Medicine (VIMM) in Padua, under the supervision of Prof. Nicola Elvassore, of the Department of Industrial Engineering, at the University of Padova.

During my time as a Ph.D student, I had the opportunity to work in a multidisciplinary environment and for that I want to thank Prof. Maurizio Muraca, because he gave me the opportunity to work on two topics seemingly very distant from each other but in fact interconnected. On one hand I worked on the evaluation of the therapeutic effect of extracellular vesicles (EVs) isolated from mesenchymal stem cells in an animal model of inflammatory bowel diseases, through the development of isolation and a quantification protocol of EVs and the evaluation of both the angiogenic and the immune modulatory activity of these nanoparticles in different, *in vitro-in vivo*, models. On the other hand, I had the opportunity to work under the supervision of Prof. Nicola Elvassore and Dr. Cecilia Laterza, who they taught me to enjoy the challenges of neurobiology/engineering cross-talk and expand my skill set beyond the traditional biological education by studying the neural development in a microfluidic context. Working on two different topics can initially be frightening, but *in vivo* every organ communicates with the rest of the body and in addition, it has been largely demonstrated that some neurological disease can generate chronic inflammation and inflammatory bowel disease is an example about that. I would like to thank my mentors for giving me the opportunity to move my first steps toward a future characterized by a 360°C study of the pathology, taking advantage of this multidisciplinary environment and this thesis represented the first step toward this future.

Mandatory thanks are due to the people who supported me scientifically and personally in this work, which include all the present and past Regenerative Medicine and BioERA group's members.

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During this 3 years of Ph.D studies the following publication has been produced:

- M. Muraca, M. Piccoli, C. Franzin, **A.M. Tolomeo**, M. Jurga, M. Pozzobon, G. Perilongo (2017) Diverging Concepts and Novel Perspectives in Regenerative Medicine.Int J Mol Sci. May 9;18(5). pii: E1021. doi: 10.3390/ijms18051021. Review.

and the following abstracts have been published:

- **A.M. Tolomeo**; M. Piccoli; M. Pozzobon; M. Grassi; C. Franzin; M. Jurga; A. Fierabracci; M. Scarpa; A. Porzionato; I. Castagliuolo; M. Muraca (2018) Annexin a5(An5)-bound extracellular vesicles (EVs) from mesenchymal stromal cells (MSCs) show enhanced and specific anti-inflammatory effects. (2018) ISEV2018 abstract book, Journal of Extracellular Vesicles, 7:sup1, 1461450, DOI: 10.1080/20013078.2018.1461450.
- M. Grassi; M. Piccoli; **A.M.Tolomeo**; C. Franzin; M. Jurga; B. Lukomska; C. Marchiori; A. Porzionato; I. Castagliuolo; M. Pozzobon; M. Muraca (2018) Extracellular vesicles (EVs) secreted by mesenchymal stem cells (MSCs) exert opposite effects with respect to their cells of origin in mice with DSS-induced colitis. (2018) ISEV2018 abstract book, Journal of Extracellular Vesicles, 7:sup1, 1461450, DOI: 10.1080/20013078.2018.1461450.
- A. Carraro, S. Negrisolo, G. Fregonese, E. Benetti, **A.M.Tolomeo**, L. Murer (2017) Urinary exosomes isolation: different methods to discover novel biomarkers of kidney rejection. 50Th Anniversary meeting of the European

Society of Pediatric Nephrology, Pediatr Nephrol 32:1643-1834. DOI 10.1007/s00467-017-3753-x (25/08/2017).

Lastly, the following laboratories have been visited:

- Prof. Giuseppe Testa, Laboratory of Stem Cell Epigenetics, Department of Oncology and Haemato-oncology, European Institute of Oncology and European School of Molecular Medicine – University of Milan, Italy – 1-month period for rapid neuronal induction via lentiviral transduction of transcription factor encoding for Neurogenin 2, proneural gene;
- Prof. Zaal Kokaia, Laboratory of Stem Cells and Restorative Neurology – Lund University Stem Cell Center, Sweden – 1-month period for generation of neural stem cells with different protocols;
- Prof. Antonella Viola, Laboratory of inflammation and immunity, Department of Biomedical Sciences, University of Padova, Italy – 1-year period for study the angiogenetic activity of extracellular vesicles isolated mesenchymal stem cells.

Chapter 1:

INTRODUCTION

In this section I will provide an introduction, describing the state of the art of all the technologies used in this thesis projects and the main topics treated. In particular I will introduce the concepts of the pluripotent stem cells, human CNS development with the description of the principal signalling pathways, the microfluidic platform and finally the different approaches used for the in vitro generation of neurons.

1.1. ADVANCED TECHNOLOGIES FOR *IN VITRO* MODEL OF HUMAN CENTRAL NERVOUS SYSTEM (CNS)

1.1.1. HUMAN PLURIPOTENT STEM CELLS (hESCs AND hiPSCs)

Pluripotency refers to the capacity of a cell to develop into cells from the three germ layers of the early embryo, in vitro and in vivo, and to self-renew by dividing generating all cells of the adult body except the extra-embryonic tissues such as the placenta (Abdulrazzak et al., 2010); thus, they have become an attractive source for in vitro studies concerning the early development and the modelling of human diseases. In addition, the pluripotent stem cells thank their characteristic of self-renewal and differentiation are consider an important promise as an inexhaustible resource for cell-based therapies in human degenerative diseases (Malgrange et al., 2011). To data, is possible generate three types of hPSCs (Fig. 1.1): human embryonic stem cells (hESCs) derived directly from human embryos through isolation of inner cell mass from blastocyst; induced pluripotent stem cells (iPSCs) obtained by reprogramming of somatic adult cells via exogenous expression of transcription factors; stem cells generated through somatic cell nuclear transfer (hPSCs-SCNT) (Yu and Cowan, 2016). In this thesis the first two cell typologies will be discussed.

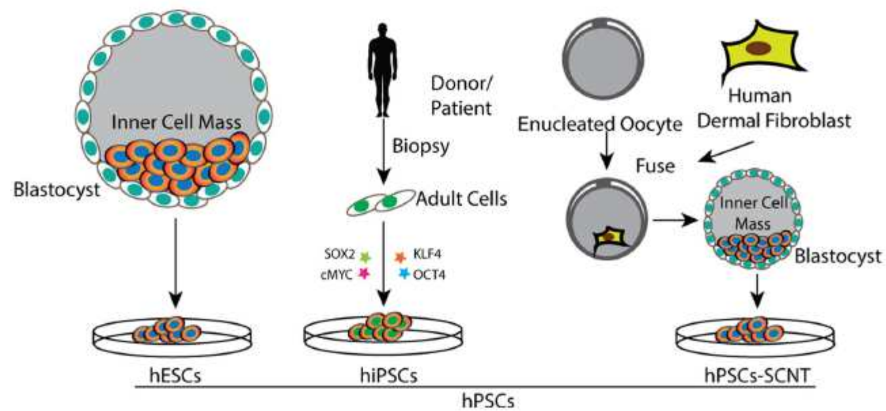


Figure 1.1: Overview of generation of three types of hPSCs. hESCs generated through isolation of inner cell mass from blastocyst; hiPSCs generated through reprogramming of adult cells by exogenous expression of transcription factors; hPSCs-SCNT generated through SCNT (Yu and Cowan, 2016).

1.1.1.1 Human embryonic stem cells

Embryonic stem cells are pluripotent cells lines derived generally from the compact inner cell mass (ICM) of a blastocyst, thus they are present transiently during embryogenesis (Rathjen, 2014). They have the potentiality to propagate indefinitely in an undifferentiated state, in culture. The first report about the in vitro growing cultures arrived only in the 1980s, in fact the researchers, during studies on cells isolated from teratocarcinoma obtained important information on the culture conditions suitable for growing cells with pluripotent characteristics (Barbaric and Harrison, 2012). In 1981, two independent labs reported the isolation and in vitro maintenance of pluripotent ESCs lines obtained from mouse embryos, but this condition was not easy to accomplish with primate-derived cells (Vazin and Freed, 2010). In the same years, Gail Martin showed that these particular cell lines to avoid in vivo alteration and that if cultured in a medium previously conditioned by teratocarcinoma stem-cell line stable, the cell survival increased. Only in 1998, a further breakthrough came out, when James Thomson et al., in Science, described the isolation of ESCs from human blastocysts and confirmed that these cells, after 4-5 months of in vitro culture, could still

differentiate into various cell types. Unfortunately, the discovery sparked ethical firestorms, because critics argued that human embryos constitute human beings. To circumvent this problem researches stabled alternative plans to derive ESCs from embryos through a process called somatic-cell nuclear transfer. A method characterized by the transfer of a nucleus of an adult donor cell into a human egg, in which its nucleus was previously removed– the same technique used to create Dolly the sheep. The goal of this method, called “therapeutic cloning”, was to offer to the researches the possibility to study the complex genetic diseases “in a dish” using dynamic cells with the donor-DNA. However, during the first 15 years, much ESCs research focused to understanding the pluripotency potentiality, the amazing ability, of these cells, to become any of body’s 200 plus cell types.

The pluripotent potency of ESCs may be verified with specific tests, all focused on the evaluation of the capacity of ESCs to differentiate in all the three germ layers:

- *In vivo* chimera assay: cells are injected into a carrier embryo supporting its growth and development and its descendants are found in all organs and tissues including the placenta;
- *in vivo* teratoma formation: undifferentiated ESCs are injected into immunocompromised mice to promote the development of specific tumours, called teratomas, which contain cells belonging to embryonic ectoderm, mesoderm and endoderm layers;
- *in vitro* embryoid bodies formation: ESCs in non-adherent cultures have the capacity to form 3D multicellular structures with the potentiality to produce embryonic ectoderm, mesoderm and endodermic;
- *in vitro* differentiation of specific embryonal lineage: embryonic ectoderm, mesoderm and endoderm.

To date, six clinical trials based on ESCs have been started worldwide and on March the 19th the ophthalmologist Pete Coffey described the implant of ESCs into damaged retinas of two patients that, after one year regained the ability to read.

1.1.1.2 Human induced pluripotent stem cells

Induced pluripotent stem cells are adult somatic cells-derived pluripotent stem cells with the typical molecular and functional properties of embryonic stem cells. The two principal characteristics that associate iPSCs to ESCs are the self-renewal and possibility to give rise cells from all three embryonic germ layers. This peculiar technology offers, to the regenerative medicine, the possibility to work with autologous cell sources in the replacement therapies field, and with patient-specific iPSCs for *in vitro* disease modelling and drug screening. Thank to this revolutionary discovery Shinya Yamanaka of Japan's Kyoto University was awarded with the 2012 Nobel Prize (Omole and Fakoya, 2018).

In 2006, Shinya Yamanaka, a stem-cell biologist of Japan's Kyoto University, focused his researches on how to return adult mouse somatic cells to an embryonic-like state using four specific pluripotent-associated genes (Takahashi and Yamanaka, 2006). In brief, twenty-four gene important for the pluripotency were screened from Yamanaka's group and introduced into mouse fibroblast cells by retroviral vectors and they verify that only four genes of the twenty-four were necessary to generate induced pluripotent stem cells. Oct4, Sox2, Klf4 and c-Myc, collectively named "OSKM factors", were the transcription factor genes used for the adult cells reprogramming. The mouse iPSCs obtained showed:

- Unlimited self-renewal;
- Functional pluripotency: *in vitro* embryoid body formation;
- *In vivo* teratoma and fetal chimera generation.

In 2007 Yamanaka et al, using the OSKM factors, reprogrammed, for the first time, human adult fibroblasts into embryonic-like cells via retroviral transduction and in the same year, Thomson et al obtained similar results using different factors (Oct3/4, Sox2, Nanog, Lin28) (Yu et al., 2007). These results have inspired a lot of studies (Fig. 1.2), in which human fibroblasts, first of all, have been successfully reprogrammed in iPSCs and, subsequently, used to generate of a wide variety of cell types, as neural stem cells, pancreatic B cells, melanocytes, stomach and liver

cells, mature B cells, adipose stem cells confirming the universal capacity, of this technology, to alter cellular identity.

In these years a variety of reprogramming methods to derive iPSCs have been developed (Table1). The reprogramming process produces compact colonies with a well-defined edge, where the cells have a large nucleus, large nucleoli and scant cytoplasm. Unfortunately, only a small percentage of these iPSCs colonies have comparable molecular and functional features with ESCs, although all the colonies derived from the reprogramming are characterized from morphology like ESCs; thus, it is important to distinguish between the full reprogrammed iPSC colonies from the partial reprogrammed, this through the evaluation of a series of molecular hallmarks.

	Reprogramming	Factors	Cell type	Efficiency %	References
Integrating methods	Retroviral transduction	OSKM	Mouse fibroblast	0.001–1	Takahashi and Yamanaka, 2006a
		OSK + VPA	Neonatal	1	Huangfu et al., 2008a
	Lentiviral	OSKM	Human fibroblast	0.1–1	Yu et al., 2007
		OK + parnate + CHIR99021	Neonatal	0.02	Li et al., 2009
	Inducible lentiviral	OSKM	Human fibroblast	0.1–2	Maherali et al., 2008
Non-integrating methods	Sendai virus	OSKM	Human fibroblast	~0.1	Fusaki et al., 2009
	Adeno viral transduction	OSKM	Mouse fibroblast	~0.001	Stadtfeld et al., 2008a
	Plasmid DNA transfer	OSK	Fibroblast	0.00	Okita et al., 2008
	lox p lentivirus	OSKM	Fibroblast	0.1–1	Somers et al., 2010
	PiggyBAC	OSKM	Fibroblast	0.01	Woltjen et al., 2009
	Polyarginine tagged polypeptide	OSKM	Neonatal fibroblast	0.00	Kim et al., 2009b
	RNA modified synthetic mRNA	OSKM	Human fibroblast	4.40	Warren et al., 2010

Table1: Methods for reprogramming somatic cells to iPSC cells (Singh et al., 2015).

The iPSCs fully reprogrammed express a network of characteristic typical of the pluripotency state, which permit to consider them as embryonic-like cells:

- Expression of Oct4, Sox2 and Nanog in levels comparable to ESCs, but only this information is insufficient because also intermediately reprogrammed cells express these markers;
- Reactivation of the expression of telomerase genes;

- Expression of embryonic antigens, such as SSEA3, TRA-1-60, TRA-1-81, DNA methyltransferase 3B and REX1 obtained from the activation of endogenous pluripotency;
- Degree of genome-wide epigenetic reprogramming evaluated on the promoter's methylation status of the genes responsible for the maintenance of pluripotency and the differentiation;
- Reactivation of silent X chromosome, which is normally silenced during the late embryogenesis, thus, it is consider a hallmark of ground-state of pluripotency.

Masayo Takahashi at the RIKEN Center for Developmental Biology led the first clinical trial with iPSCs to treat the macular degeneration. This trial unfortunately was halted in 2014, because researcher preferred to simplify the procedure using donor-derived cells, rather than patient-derived stem cells. Only in the 2017 the trial restarted, but in January 2018 it hit another roadblock, after the development of a membrane in the eye of the patient, surgically removed later. But despite this, iPSCs circumvent the ethical controversy characteristic of ESCs and gained popularity over human ESCs. In fact, a lot of laboratories, around the world, use this revolutionary technique.

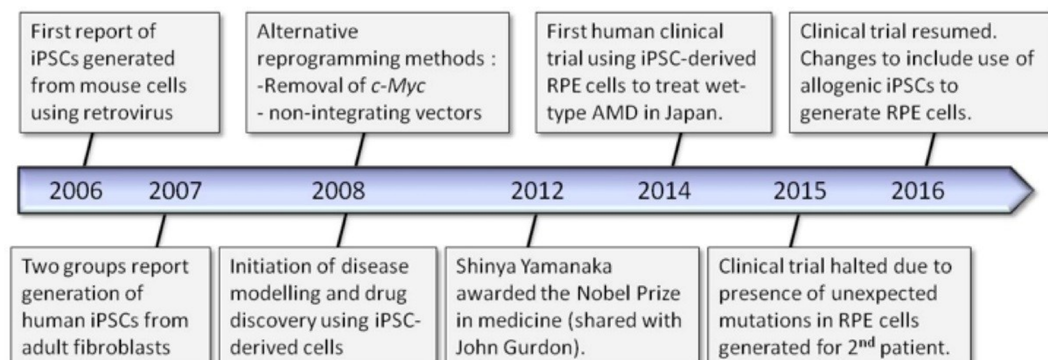


Figure 1.2: Major milestones timeline for iPSC technology starting from the first reports of iPSCs ending with in-progress human clinical trial (Sargent, 2016).

1.1.2. POTENTIAL APPLICATIONS OF hiPSCs-DERIVED MODELS

The iPSCs have the intrinsic potential to become a clinical tool to model diseases with the aim to understand the molecular mechanism at the base of the onset of the diseases, develop candidate drugs and finally deliver cell-replacement therapy in regenerative medicine contexts. In this perspective easily-accessible cell types (such as skin fibroblasts) could be obtain from patient biopsies and can be reprogrammed in order to recapitulate the patient's disease in a dish. These cells could be used for:

- Autologous cell replacement therapy. The cells, in fact, derived from the patient minimize the immune rejection of the differentiated derivates. This with the advantage of a reduction in the use of immunosuppressive drugs that accompany the transplanted cells;
- Generation of cell lines with genomes predisposed to disease, when genetically inherited diseases affect tissues that cannot be easily accessed;
- Production of cell types compromised or destroyed from the disease. For example, the iPSCs derived from ALS patients that are differentiates in motor neurons destroyed in the disease.
- Drug screening and discovery, in order to determine the effects of candidate drugs and new compounds and identify target pathways. These together with toxicity tests represent human preclinical "trials in a tube" that allow the introduction of "the patient" in early stages of the drug discovery process (Fig. 1.3).

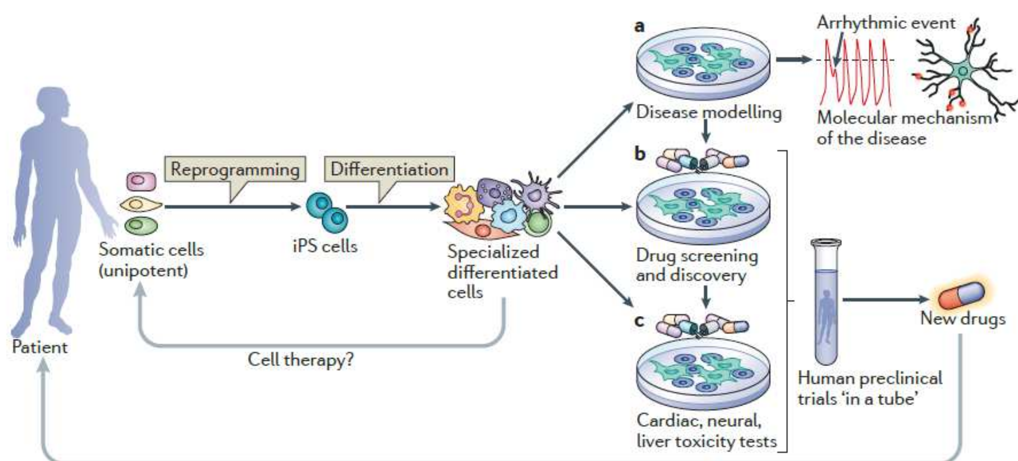


Figure 1.3: Human hiPSC derivation, differentiation and applications. Somatic cells (unipotent) from patient can be reprogrammed into hiPSCs. After the *in vitro* induction of differentiation, hiPSCs form specialized cells that have several applications. (a) hiPSCs can be used in disease modelling to understand the molecular mechanisms underlying disease phenotypes; (b) hiPSCs can be used for drug screening and discovery, in order to determine the effects of candidate drugs and new compounds and identify target pathways. (c) hiPSCs are also valuable, for example, in cardiac, neural and liver toxicity tests to assess cellular toxic responses. Drug screening and toxicity tests are considered human preclinical 'trials in a tube' that allow the introduction of 'the patient' in early stages of the drug discovery process (Bellin et al., 2012).

In this optic the researches are generating a lot of iPSCs specific for the different pathologies. For example, fibroblasts and bone marrow-derived mesenchymal cells have been used as cell source to generate iPSCs from patients suffering from various disease including ALS, adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophies, Parkinson's disease, Huntington's disease, type 1 diabetes mellitus, Down syndrome/trisomy 21, and spinal muscular atrophy.

Although much additional basic research is required before iPSCs can be applied in the clinic, the iPSCs provide a model for the development of normal and disease-specific pathologic tissues to better understand pathologies or an

unknown developmental process. It is expected that discoveries made using these cells will inform future drug development or other therapeutic interventions.

1.1.3. CURRENT LIMITATIONS AND FUTURE DIRECTIONS

The iPSC technology is considered a promise for regenerative medicine because it offers the unique possibility to obtain an autologous cell for replacement therapy and patient-specific iPSCs to model in vitro disease mechanisms and to perform drug tests. But this promise is obscured by recent findings of genetic and epigenetic variations:

- Genetic variation in iPSCs: the genome of iPSCs may be characterized from a wide range of different variations, such as aneuploidy, subchromosomal copy number variation and single nucleotide variations. All these variations can be introduced during the generation and the maintenance of iPSCs, from different sources (Fig. 1.4). First, the heterogeneous genetic composition of the cell source contributes to the generation of genetic variation. Although the variation occurs only at low frequencies at the source cell level, due to the low efficiency and clonal nature of the derivation of iPSCs, the single iPSCs are able to capture the genetic variation of the starting cells. Moreover, when certain genetic variations in the source cell facilitate the generation of iPSCs, these variations will be propagated in the iPSCs. Second, the reprogramming process may be mutagenic introducing de novo variations. Third, the maintenance in culture of the iPSCs could introduce or select specific genetic alteration promoting cell propagation.

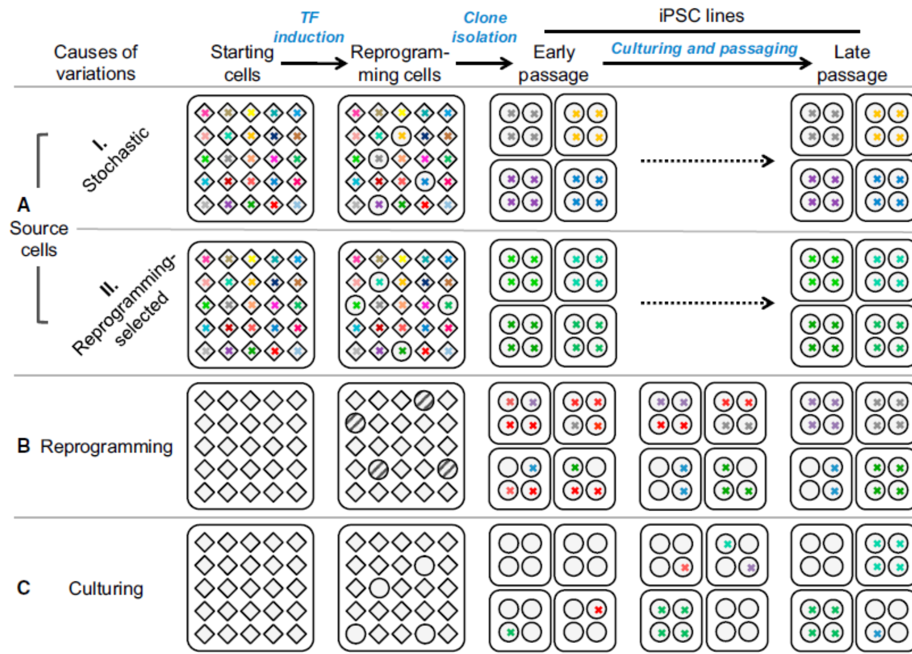


Figure 1.4: Sources of Genetic Variations in iPSC Lines. (A) the single cells source (diamond) in a culture (big square) have genetic variations (colored crosses) that can be capture from the iPSCs (circle of the early passage column). (AI) the iPSCs may have random pattern because in the starting cell population stochastic reprogramming occurs. (AII) when the reprogramming takes place in cells with genetic variations, these variations may promote a functional enrichment of the iPSCs (green crosses). (B) The cells that undergo the reprogramming process may have an increase of genomic instability, with the direct generation of de novo mutations in iPSCs, because the reprogramming per se introduces variations (striped circles). In the early stages the iPSCs are characterized from mosaicism of de novo mutations; the mutations that confers advantages in self-renewal and proliferation (green crosses) will be positively selected; the mutations deleterious for the survival of the cell will be negatively selected (red crosses) and the neutral mutations undergo genetic drift (crosses with remain colors). (C) The mutations that occur during a cell culture prolongated undergo selection like the one that occurs in (B) (Liang and Zhang, 2013b).

- Epigenetic Variations in iPSCs: the iPSCs generation promotes the resetting of epigenetic landscapes. In brief (Fig. 1.5), the reprogramming factors when introduce into a fibroblast, drive the cell to overcome the first barrier promoting the acquisition of epithelial properties acquisition through mesenchymal to epithelial transition (MET). When a cell has acquired

epithelial properties, a second barrier may be encountered for the pluripotency acquisition. This cell is considered an intermediate epithelial cell and may become a nascent iPSCs, in this stage it can self-renew independently of introduced transcription factors. After that, the nascent iPSCs must overcome additional barrier(s) to become an authentic pluripotent stem cell like ESCs. But, when a cell is not able to migrate to the next stage remains trapped in the intermediate stage named partial reprogramming. In this condition, epigenetic variations into the iPSC population may exist compared with ESCs or the completely reprogrammed iPSCs and the retention of the cell memory, the epigenetic signature of the source cell, is a clear example of that. However, this epigenetic variability can occur also during a prolongate culture. Variation in X chromosome inactivation, local epigenetic variations in different parts of the genome respect to X chromosome are considered epigenetic variations.

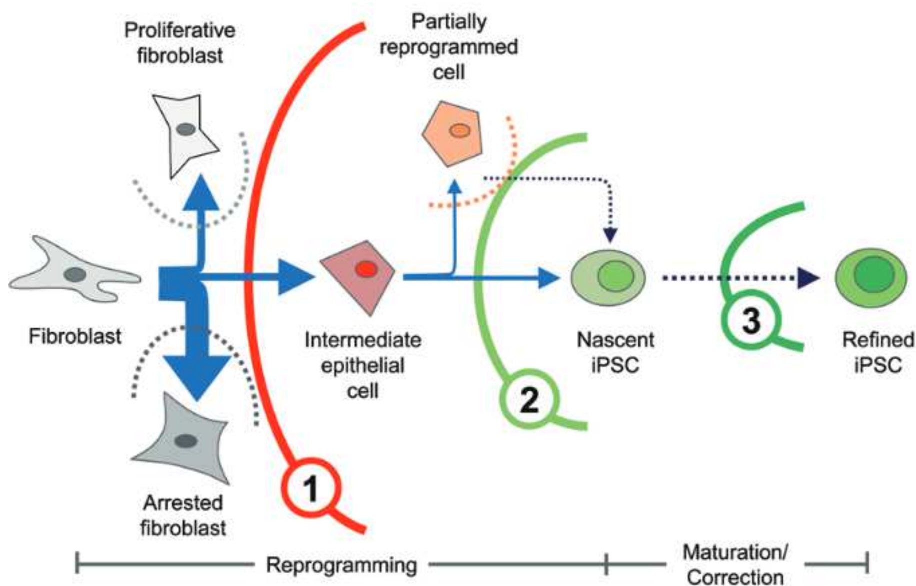


Figure 1.5: The putative epigenetic barriers during the path of a somatic cell to a refined iPSCs. Schematic illustration of the iPSCs reprogramming process, which the putative epigenetic barriers are numbered and shown in solid arcs. In dotted arcs are shown other potential barriers (Liang and Zhang, 2013a).

To reduce the occurrence of variations is necessary to optimize the reprogramming strategy and the culture conditions. To ensure the iPSCs quality used for the disease modelling or for the clinical trial is fundamental to have a complete and rigorous characterization of the genome and the integrity of the epigenome. The continuous request of iPSCs and the consequent generation of these specific cells will permit a better comprehension of the ontology of genetic and the epigenetic variations in order to provide solutions for overcoming the limitations caused from the variability.

1.1.4. MICROFLUIDIC LAB-ON-CHIP PLATFORMS

Lab-on-a-chip technology is characterized from the miniaturized devices, which offer the possibility to integrate one or several tests, usually done in a laboratory, into a single chip. Generally, the research on la-on-a-chip focuses on human diagnostic and DNA analysis, but in these last years also on the synthesis of chemicals. Cost efficiency, parallelization, sensitivity and diagnostic speed are the advantages of biochemical operations development in miniaturization contests. Lab-on-a-chip puts its foundation on two main pillars: the microfluidics and the molecular biology (Yılmaz and Yılmaz, 2018). Furthermore, the miniaturization characteristic of the microfluidic system can expand the capability of analysis techniques leaving unchanged the nature of molecular reactions, the molecular diffusion, laws of scale for surface per volume and finally the heat transport promotes an increase on throughput (Gagliano et al., 2016). The actual applications focus on:

- Molecular biology in microscale: lab-on-a-chip for the amplification and the detection of DNA/RNA offers advantages in terms speed for the detection maintaining the same sensitivity obtained with the traditional devices. The microscale offers the opportunity to perform high-speed thermal shift and this is considered very important for the DNA

amplification using PCR, based on thermal cycles (Chang et al., 2013) and data have demonstrated that with the microscale the PCR completes its processes in less 1 min (Jayamohan et al., 2017). Furthermore, the lab-on-a-chip technology promotes a reduction of the times of sequencing of DNA and RNA, because with an array of DNA probes is possible to sequence the genomes time faster compared with the first human genome sequencing. Lab-on-a-chip application in all the biological molecular applications evidence a great potential for the identification of disease biomarker. In this context also, the immunoassay can be done time faster if compare with the macroscopic technologies;

- Proteomics in microscale: lab-on-a-chip, in this field, provides the advantage to perform all the steps, of the protein analysis, in the same chip, from the cell extraction of the protein to the mass spectrometry analyses; the microscale offers the opportunity to do this in few minutes compare with hours characteristics of the traditional devices. Also, the protein crystallization takes advantage from the microscale, because all the different parameters enabling of the protein crystallization can be controlled simultaneously, this in order to speed up the crystallization conditions and then to study the protein structures;
- Cell biology in microscale: lab-on-a-chip provides the opportunity to control the cells at the single-cell level when the microchannels of the chip have the same size of the cells and can handle, in few seconds, many cells. Furthermore, with the microscale there is the possibility to control the flow promoting a precise regulation of the flow rate. furthermore, taking advantage of the use of fast optical detectors, in microscale a given cell made fluorescent with a specific antibody can be the identified and the isolated;
- Chemistry in microscale: microscale facilitates fast cooling and heating promoting higher efficiency of the chemical reactions. Also, in contexts in which is request the use of dangerous compounds the lab-on-a-chip

technology results more convenient than the macroscale, because the microscale contains the risks, since the volumes work are smaller.

Based on the application of the microfluidic devices, lab-on-a-chip uses different manufacturing technologies and various polymers, such as PDMS. PDMS (polydimethylsiloxane) is an elastomer flexible and transparent. It is cheap and very easy to fabricate by casting. This particular polymer permits the integration of microvalves for the control flow and pression of the medium used for the cell growth. Unfortunately, the PDMS has few limits related to the industrial production for problems concerning the aging and the absorbed hydrophobic molecules. To prevent these issues the researchers takes their steps to the research of innovative materials for the fabrication of the microscale devices, such as glass, silicon or paper.

Microfluidic lab-on-a-chip is characterized from different advantages, such as:

- fine control of flows: in microfluidic the behaviour of fluids is different than the macroscale because the laminar motion prevails on the turbulent on, permitting a fine control of molecular diffusion kinetics (Fig. 1.6). In this way the analyte administration to the cells is finely controlled because the mixing between different fluids takes place only for diffusion and not for turbulent motion, even if the two flows of liquid flow contiguous (Sackmann et al., 2014). In this way it is also possible to create precise gradients of concentration;

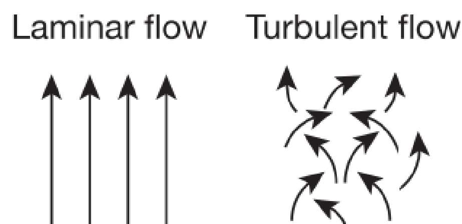
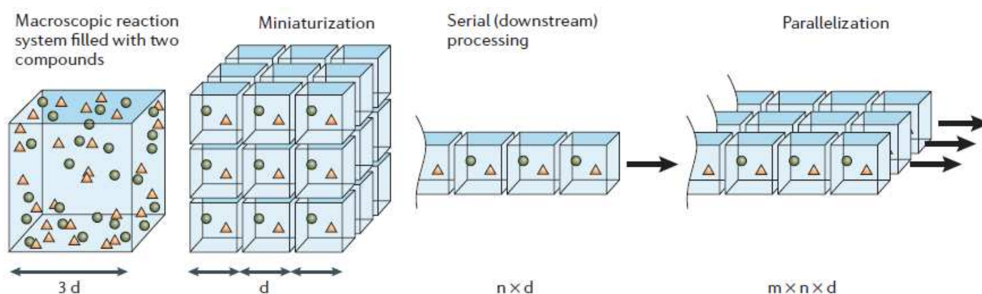


Figure 1.6: Laminar versus turbulent flow. The dimensionless quantity that describes the ratio of inertial to viscous forces in a fluid, is defined as the Reynolds number (Re). It is proportional to the length scale and the characteristic velocity of the fluid and inversely proportional to the fluid viscosity, of the system. Microfluidic

systems are characterized from a Re in a laminar flow regime, promoting a highly predictable fluid dynamics compared with the traditional systems in which the high-Re fluids have flow profiles that increasingly mix stochastically (turbulent flow). Furthermore, at this scale, also molecular transport changes dramatically because convective mixing does not occur, enabling predictable diffusion kinetics (Sackmann et al., 2014).

- control of the cellular microenvironment: *in vivo* some growth factors are immobilized near the cell thanks to direct ties with collagen or fibronectin of the extracellular matrix, or they are directly anchored to the cell membrane for increase local concentration and avoid the spread of these molecules. This condition can also be obtained *in vitro* creating an engineered cell adhesion surface with characteristics similar to the extracellular matrix present in the tissues *in vivo*. Other growth factors are found in the cellular environment in soluble form, they can be paracrine factors produced by cells stromal, exogenous or autocrine and contribute to maintaining homeostasis of cells of a fabric. For example, in the stem niches the factors secreted by already differentiated cells contribute to the homeostasis of the stem cells to keep the two cell types balanced. Many of these factors influence cells in a concentration-dependent manner and in certain temporal periods. It is therefore necessary to recreate *in vitro* the conditions of concentration and temporal distribution of the growth factors to which cells are exposed *in vivo*. Taking advantage of the high surface-to-volume ratio of microscale systems, it is possible to simulate, *in vitro*, the dynamics of the accumulation of soluble factors (autocrine, paracrine or exocrine) typical of *in vivo* cellular environments, but it is important use the correct temporal delivery strategy of medium. Indeed, the accumulation of toxic metabolites due to too sparse medium changes or the growth factor washout caused by medium changes too frequent, they can have cytotoxic effects on cell culture;

- low cost: reduction of the price of each individual analysis performing numerous tests on the same chip;
- high parallelization: preformation of simultaneously hundreds analysis on the same chip (Fig. 1.7). Doctors are able to target specific illnesses and consequently the best-suited therapy for the patient;
- low volume samples: reduction of reagents quantity, because lab-on-a-chip systems requires a small amount of samples reducing the expensive chemicals necessary for the tests. This technology will require a very small amount of patients' blood;
- compactness and ease to use: integration of a large operation numbers in a small volume and allowing analyses comparable to those conducted in traditional laboratories. Less handling and operations particular complexes will be necessary, then could be done directly on site by a nurse;
- reduction of human error: reduction of human handling for the presence of automatic diagnoses performed in a lab-on-a-chip context;
- faster time and diagnose responses: time reduction of the chemicals and heat diffusion. It is possible to change the parameters in milliseconds to enable faster reactions;
- sensitivity increasing: real time control of the environment of culture conditions, leading to more controlled results.



Parameter	Macroscopic example	Factor change	Microscopic example
Length of edge	1 mm	d	1 µm
Surface	1 mm^2	d^2	1 μm^2
Volume	1 µl	d^3	1 fl
Number of molecules	10^9	d^3	1
Diffusion time over d ($D = 10^{-6}\ cm^2 s^{-1}$)	15 min	d^2	1 ms
Example: in flowing systems			
Linear flow rate	1 µm/s	d	1 mm/s
Separation time	10^5 s (>1 day)*	d^2	100 ms
Example: in planar array			
Number of volumes per microwell plate	96	d^2	10^8

*Typically, for example, high-performance liquid chromatography in packed column.

Figure 1.7: miniaturization of reaction systems. Different opportunities emerge from reaction systems in microscale and this is described from the scaling laws (figure and table). The high surface to volume radio permits the reduction of transport time of heat and mass allowing a fine regulation of chemical processes (Dittrich and Manz, 2006).

In conclusion, lab-on-a-chip will change the actual way of practicing medicine, because it offers the unique opportunity to perform a complete patient diagnosis during the consultation time and the analysis will be done by nurses, thus enabling doctors to focus only on treatment. The survival chances will increase for emergency service patients and will permit to give the appropriate treatment to each patient. The antibio-resistance will be reduced thank to the complete diagnosis and the cost of the medicines will decrease changing the way we see the actual medicine allowing us to detect the pathologies in the early stages and consequently to treat them as soon as possible.

1.1.4.1 Neuron-on-chip models

A fundamental challenge of the neuroscience is the deciphering the mosaic of the signals that direct the neuronal maturation and the formation of the axons, dendrites, and the establishment of synapses between two or more neurons during the wiring of the brain. So far, the conventional methods of cells culture have impeded to understand how the local signals (chemicals, physicals and fluidics) shape the neurons during the brain development.

Within the cytoarchitecture of the brain, the mature neurons and their extensions are densely packed, filling all the space and this condition impedes the *in vivo* analysis of the subcellular domains of the neuronal membrane. The conventional devices promote the maturation of neurons with complex morphologies like the *in vivo* ones, but with a lower density and orientation growth. For these reasons neurites often are located in different physical, fluidic and chemical micro-environments. New approaches to resolve these issues are necessaries, in order to implement the local analyses at high-resolution of the interactions between the extracellular signals and intracellular answers responsible of the brain modelling during the development (Fantuzzo et al., 2017).

Currently, the microfluidic devices offer exactly the opportunity to extent the subcellular analyses understanding the nervous information flux. The interface of the biological methodologies with the engineering technologies permits the fabrication and the application of microfluidic-based systems important for the maintenance and consequently for the study of cerebral cells and their circuits and this not only in a neuronal network context but implementing even the investigation of the neuronal cell or the single neuronal process.

Here I will report only few of the new perspectives related to the neuronal development using the microfluidic devices:

- differentiation and polarity of neurons: the microfluidics allow a fine control on the neuronal development taking advantage of the micropatterning technologies that permit a chemical and topographic

disposition of the developmental signals. In this condition is possible to model different attractive and repulsive stimulus with controlled geometries, such as physical cues can be used to test the neurite navigation. In the conventional devices there are no instructive patterns, so the control on the in vitro neuronal development is lesser. Fabrication methods of the microfluidic platforms permit the modelling of specific substrate chemicals in various geometric forms in order to design the arrangement of the neurons and of their connections. This for the study of neuronal signalling pathways to understand the mechanism of neural repair and of the construction of the functional neuronal systems;

- differentiation and guidance of axon: during the neuronal development the axon, after the establishment of the polarity, begins the growth phase traversing different structures, fluid microdomains. Study with traditional devices have determined the axon preferences for specific substrate molecules. The microfluidic platforms allow a great flexibility in the design of the microenvironment in order to evaluate the axon reactions to different stimulus; administrating single or multiple exogenous factors is possible to study the behaviour of a single axon or a population (Pujic et al., 2016). In addition, the spatial control of the neuronal morphology through the isolation of a single axon permits the assess to its behaviour and to its molecular content;
- formation of the synapse and dendritic signalling: microscale platforms are characterized from the capacity to control the microenvironments of the single dendrites enabling the studies of formation mechanisms of the synapses and their regulation. The interconnected compartments of a microfluidic devices facilitate the control of the dendrites and their sub-regions through the administration of the chemoattractant ultra-thin fluids that regulate the neurotransmitter pulses. Into traditional devices is difficult to control the exogenous signals because of a static-fluid condition and because of the dynamic of plumes. On the contrary the microfluidics allows the control of the direction of neurites, of the connections axo-

dendritic and of the cortico-striatal synapses (Millet and Gillette, 2012). The confounds of traditional dishes are circumvented by the microfluidic devices due to the identification of specific typologies of neurons into the co-cultured cell populations and the identify of two neuronal populations without hinder the axo-dendritic interactions of neurons. Recent studies have demonstrated, for example, that the microfluidic platforms promote the generation of striatal neurons that means longer dendrites, higher density of spines, increased of phosphorylation of extracellular signals regulated by kinase activation and finally activation of cortical neurons through the promotion of Ca^{2+} synchronous oscillations.

- evaluation of the neuronal stimulus-response: microfluidics, compared with traditional devices, permits the delivery to cultured neurons of the neuromodulators, activators or inhibitors and reagents for imaging tests. These microdevices allow the manipulation of the axonal transport of single-molecule, the stimulation of dendrites, the detections of molecular changes and the study of the neurogenerative states. The microfluidic design is able to probe a large-order neuronal array to investigate the specific neuromodulator that influences a particular neuronal population. In this field, the spatial-fluidic control of the stimulus in the cell culture has permitted the investigation of different classes of combinatorial responses and this analyse should be translated to a neuronal network in order to better understand the components and circuits that are implicated into the myriad of neuronal stimuli. Furthermore, coupling the microfluidic analyses and the analytical chemistry is possible to identify the neurochemical identity of the chemical substances secreted by the neurons. Taking advantage of the important potentiality of the microfluidics, recently, a lot of groups are analysing neuropeptides, mRNA, etc.

1.1.4.2. Organ-on-chip models

To date, the development of a specific drug, with the traditional devices are used is characterized from the high rate of failure but taking advantage of the recent steps forward in tissue engineering and in the microdevices, in recent years researchers start to orient their interests on the development of organ-on-a-chip models that recapitulate the function of the human organs.

Tissue chips considered as microphysiological systems are devices designed to obtain and culture cells in three-dimensional structure, defined organoids, in order to mimic the typical functions of the organs, that constitute the human body. Furthermore, these chips allow to the organoids to react to the exposure to cell signalling molecules, drug, biochemical stressors and hormones (Low and Tagle, 2017). To date, there are various platforms with specific design offering different cell culture possibilities. Cells, for example, are directed to self-organize into organoid-type structures or some platforms are characterized from a highly prescriptive designs in which cells are placed in well-defined device positions or are compartmentalized in order to recapitulate specific functional units of organs (i.e the proximal tubule of the kidney or the sinusoid of the liver). This system can be particularly useful for modelling the cytoarchitecture of the brain.

Neurons are cells that build functional organized structures in different parts of the CNS. An important structural motif of CNS is represented from the axons fascicle, corticospinal tract, peripheral nerves and corpus callosum are perfect examples of these structures (Stewart, 2003). Literature data have demonstrated that during the formation of an axonal fascicle the native axons follow and gather close to a previous “pioneer axon” taking advantage of the *in vivo* axo-axonal interactions (Ahsan et al., 2007). In the neurodegenerative diseases, such as amyotrophic lateral sclerosis, axon fascicles are disrupted and, although the two-dimension cell cultures have produced a variety of insights into nervous system developmental, still very little is known about the

pathophysiological mechanisms. Neurons, derived from human pluripotent stem cells cultured with a technology that allows the generation of multi-cellular organizations or more specifically defined as three-dimensional brain “organoids” that are able to recreate the microenvironment of the internal human nervous system, *in vitro*, could provide an important platform for the CNS diseases modelling (Wevers et al., 2016). In particular the develop of methods for the generations of cell-intrinsic self-assembly mechanisms that conduce to the formation of organoids of specific regions of the nervous system represent an important step forward into the comprehension of the specific neuronal structures and circuitries that characterize diseases of the CNS (Wang et al., 2018). As showed in Figure 1.8 has demonstrated how providing a spatially confinement, such as the microchannel of a chip the axons, in a spontaneously way, have the tendency to form a unidirectional fascicle into the microchannel (Kawada et al., 2017). This final nerve organoid can be used to model the neuronal damage and degeneration for high-throughput drug screening of specific neurological diseases or more in general to model in vitro neuronal development to better understand the molecular mechanisms at the base of the generation of neurons in order to able to study the pathological onset condition step by step during the differentiation process.

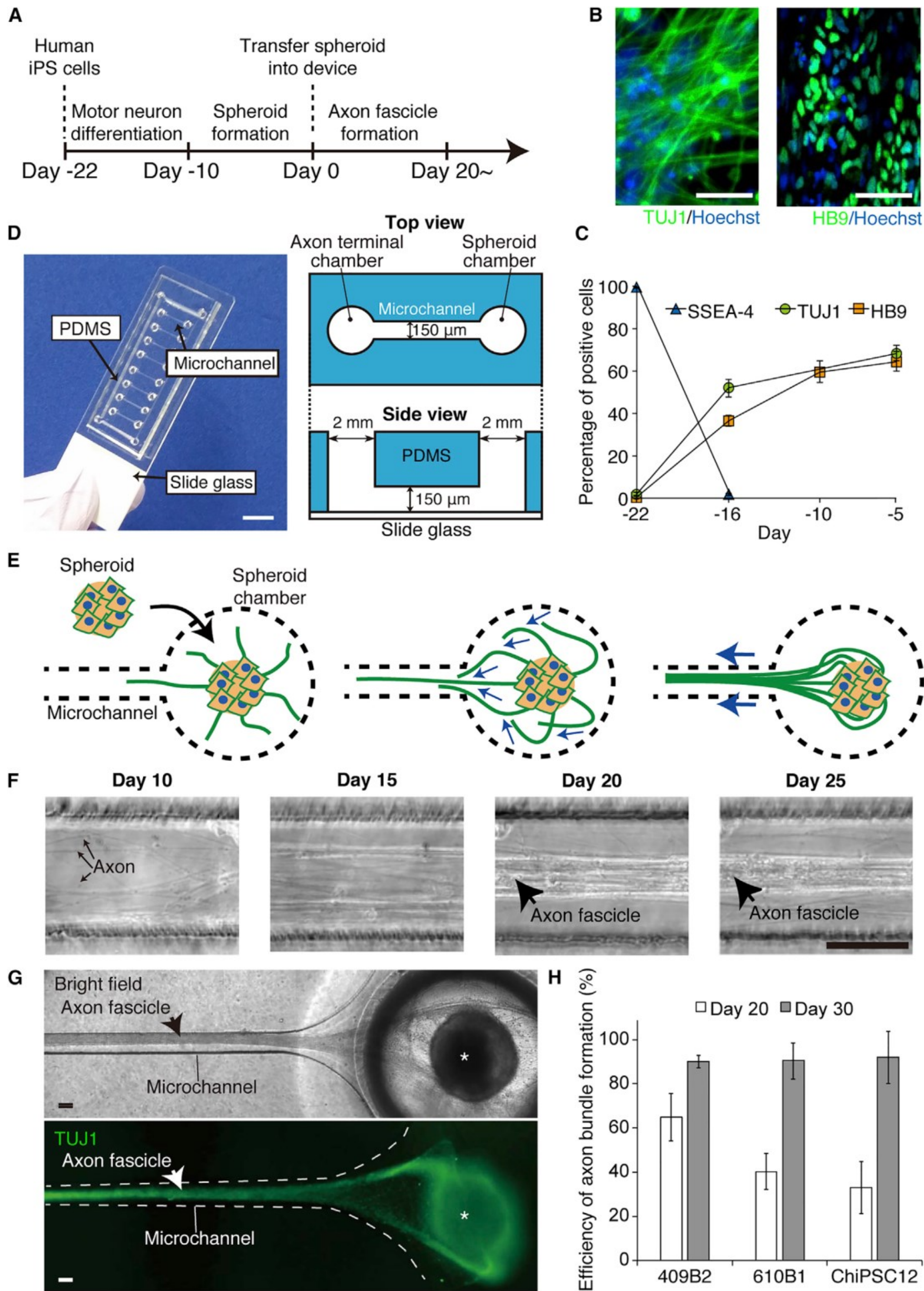


Figure 1.8: Formation of Nerve Organoid in Microscale

(A) Timeline of the generation of nerve organoid starting from hiPSCs. Motor neuron are differentiated in 12 days, and the neurons are subjected to the formation of spheroid for 10 days. At day 0, after these preliminary steps, the spheroids are transferred into chips. After a culture, in microdevices, of 20–30 days the axon fascicles are formed. (B) Immunofluorescent images of differentiated cells immunostained with TUJ1 antibody (left) and HB9 antibody (right) at day 5. (C)

Percentage of SSEA-4-, TUJ1-, and HB9-positive cells during the differentiation process. The error bar denotes the SE of means of values acquired from three independent experiments. (D) A photograph (left) and schematic drawings (right) of the microdevice. (E) Schematic drawings of spontaneous axon fascicle formation in the microdevice, illustrating the growth of axons from the spheroid within a chamber (left), axons following the preceding axon in the microchannel (middle), and axon fascicle formation and growth (right). (F) Representative time-lapse images of axons and an axon fascicle within a microchannel. (G) A bright-field representative image of a nerve organoid (top) and a fluorescent image of the same organoid immunolabeled with TUJ1 (bottom) within a microdevice. Asterisks indicate a spheroid comprising the nerve organoid. (H) Success rate of axon fascicle formation using three independent hiPSC lines. An assembled bundle of axons with a diameter of over 25 mm was counted as an axon fascicle. The error bar denotes the SE of means of values acquired from three independent experiments. A total of 90 samples were analysed. Scale bars, 10 mm (B), 100 mm (F and G), and 4 mm (D) (Kawada et al., 2017).

After the definition of organ-on-a-chip the multi-organ-on-a-chip concept is approaching (Fig. 1.9). Recently, in fact, a variety of design for the multi-organ platforms have been introduced and an example is represented from the interconnection of different human organ-specific tissues in microscale three-dimensional chambers (Rogal et al., 2017). These platforms offer the opportunity to work with organoids that mimic the essential functions of the organs in a simultaneous way reducing the needed of animal models for drug screening or more in general for basic research. In fact, to test the efficiency of a drug is important to simulate a drug's journey though the human body considering how the combination of organs responds to a specific drug.

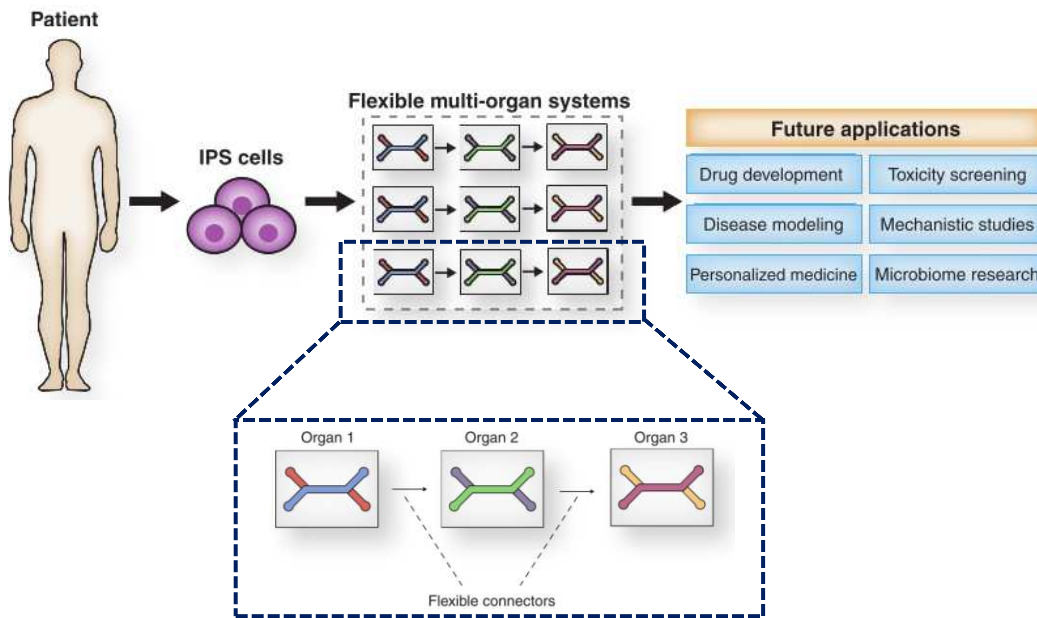


Figure 1.9: The future potential of the flexible 'mix-and-match' multiorgan toolbox. In combination with the technology of iPSCs, flexible multi-organ systems will significantly contribute to future advances in a variety of domains of research. The multifarious application areas of the multi-organ system will include drug development and toxicity screening, disease modelling and mechanistic studies, as well as personalized medicine and research on the human microbiome. iPSC: Induced pluripotent stem cell (Rogal et al., 2017).

1.2. THE CENTRAL NERVOUS SYSTEM

1.2.1. DEVELOPMENT OF CENTRAL NERVOUS SYSTEM

CNS development is an extremely complicated process, in fact is characterized by highly regulated mechanisms, that involve molecular as well as cellular signalling. Brain development starts in third gestational week and it is well-known that molecular cues emanating from a mesoderm-derived structure, the notochord, initiates the ensuing events of brain development (Smith and Schoenwolf, 1989). In a simplified overview of CNS development, the ectoderm under the control of specific morphogens gives rise to the neural plate (neuroepithelium), neural folds and epidermal ectoderm. The neural tube (Neurulation) and neural crest arise from the alteration of the neural plate, concurrently with the neural folds. The neural folds become the neural crest which later gives rise to a number of different cell lineages such as neurons and glia of the peripheral nervous system, melanocytes, smooth muscle cells as well as cartilage and bone of the cranium and face (Huang and Saint-Jeannet, 2004). By the time the neural tube takes origin all the different types of neural progenitor cells that differentiate into different neuronal cell populations of the CNS are formed. These various neural progenitor populations have different functionalities and based on which they give rise to the various parts of the human brain. In brain development the first well-defined structure is represented from the neural tube (Fig. 1.10).

The expression of morphogens by the notochord and other organizing centres (Sander and Faessler, 2001) controls the formation of the neural plate, neural crest and epidermis. The superfamily of transforming growth factor (TGFβ) ligands is a major component of stem cell niches and is implicated in many developmental processes. BMPs are morphogens of this superfamily and are secreted during gastrulation from the trophoblast. The expression of BMPs' antagonists, such as noggin, chordin, that mitigate the influence of BMP-4 on the midline of the ectoderm (i.e. to the cells in the immediate vicinity of the

notochord. Instead, the lateral areas of the ectoderm that are not under the influence of the BMPs' inhibitors promote the binding of BMP-4 and -7 to their receptors activating genes associated with the epidermal ectoderm fate. In brief, the neural plate arises by the cells of the ectoderm overlying the notochord. The neural crest arises by the edges of the neural plate and the rest of the ectoderm turns into the epidermal ectoderm.

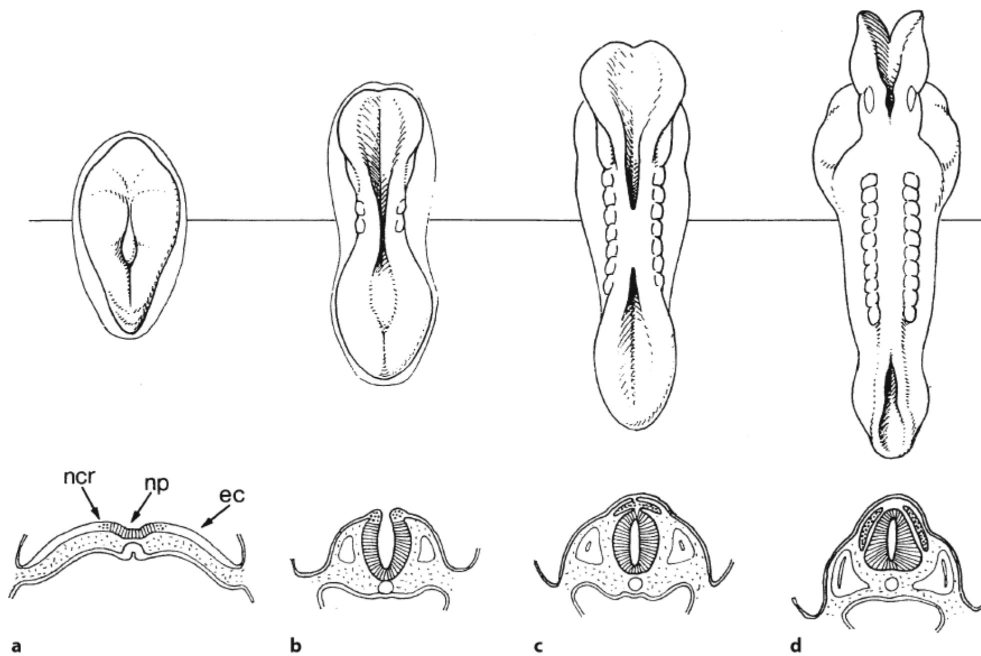


Figure 1.10: The neural tube and neural crest formation. Dorsal views and transverse sections are shown for human embryos at different stages: stage 8 (a), 9 (b), 10 (c, seven somites) and 10 (d, ten somites). ec ectoderm, ncr neural crest, np neural plate (Ten Donkelaar and van der Vliet, 2006).

1.2.1.1. Neural tube formation: the neurulation process

The neural plate, as previously mentioned, takes origin during the GW3, the ectoderm creates two folds called neural folds and the cells occupying this area, in-between the folds cells, start thickening. During the primary Neurulation the

median hinge point of the neural plate represents the midline (Fig. 1.11 left a). This part of the neural plate gets anchored to the notochord and gradually deepens forming the neural groove (Fig. 1.11 left b). During the secondary Neurulation the folds rise in tandem with the deepening of the median hinge point and eventually merge, so the structure now resembles a hollow tube (Fig. 1.11 left c). During GW3, the closing of the anterior neuropore and the posterior neuropore promote the closing of the neural tube in central regions. The anterior neuropore of the tube, prior to its closure, expands and forms the three primary vesicles; the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain). The prosencephalon gives rise to the telencephalon and diencephalon while the rhombencephalon gives rise to the metencephalon and myelencephalon (de Lahunta et al., 2016). The mesencephalon does not experience further division (Fig. 1.11 right). These vesicles are referred to as the secondary brain vesicles and are responsible for the establishment and further development of the central nervous system and are situated along the rostral-caudal axis of the developing embryo (Stiles and Jernigan, 2010).

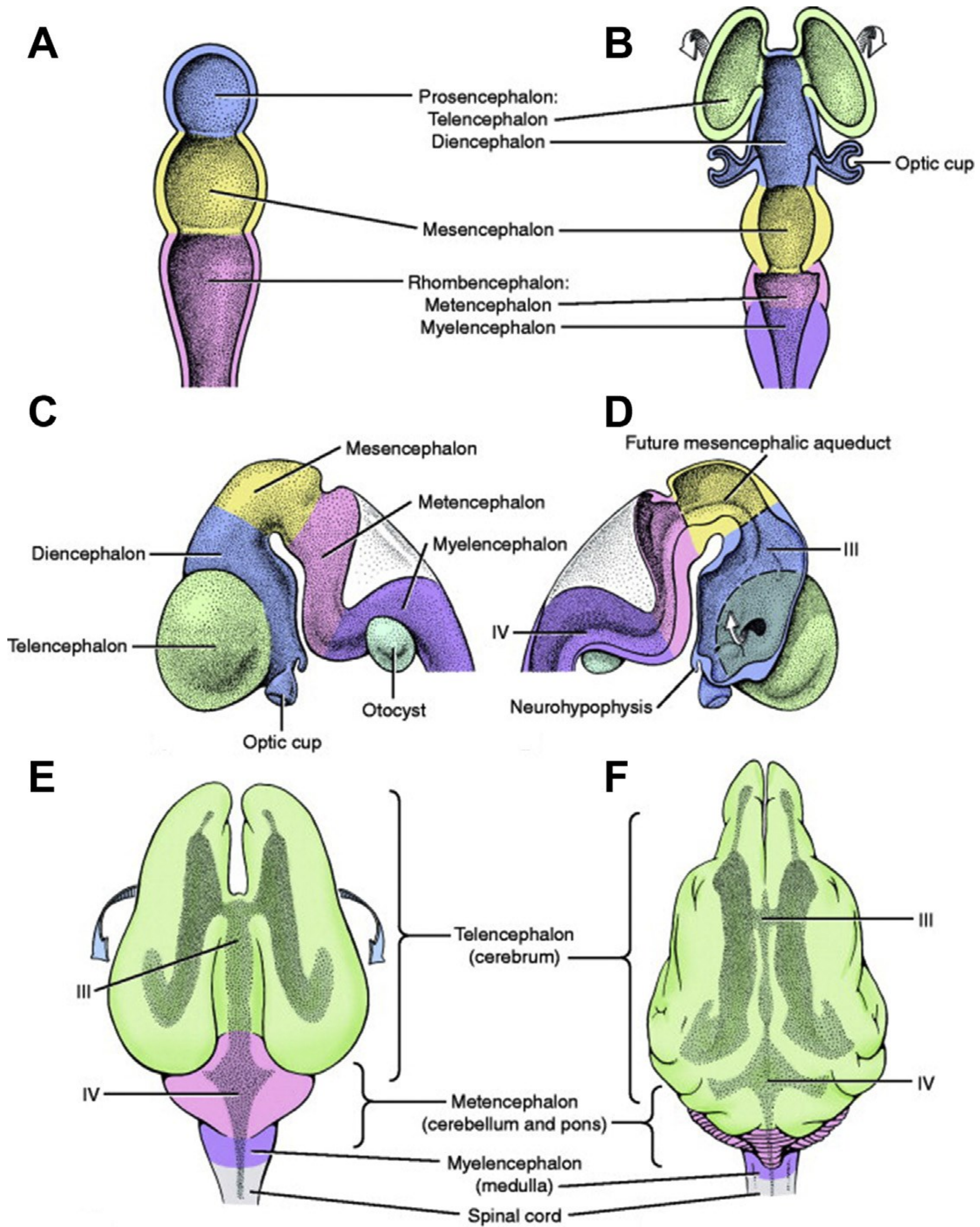


Figure 1.11: Development of brain vesicles. (A) Stage of three vesicles. (B–F) Stages of five vesicles; III, IV: ventricles (de Lahunta et al., 2016).

1.2.1.2. Dorsal-ventral polarization

The transformation of the neural plate into the neural tube is promoted by two fundamental signalling centres: the notochord by secreting the morphogen Sonic Hedgehog (SHH) (Jessell, 2000) and the epidermal ectoderm by secreting BMP4 and -7 (Chizhikov and Millen, 2005). The “French flag” model of Wolpert proposed in the 1969 describes the morphogens’ impact on cell fate which is concentration- and time-depended. Two opposing diffusion gradients of BMP4 and SHH are present in which high expression of the former or the latter or the same expression level of both influence cell fate. On the neuronal fate of cells occupying the neural tube, the synergistic effect of the concentration gradients, of such morphogens, has a pivotal impact. As the five secondary vesicles are generated from the neural tube, cells occupying the various brain vesicles are associated with the respective part of the brain they occupy. Hence, the neural stem cells are exposed to molecular signal that dictate the functionality and therefore regionality of the generated neuronal populations. The effect of morphogens is referred to as caudalization when the neuronal fate is shifted towards cells which accommodate functionalities associated with the myelencephalon while the opposite condition, the shift towards the telencephalon is considered rostralization. The dorsalizing effect of morphogens is referred to cells occupying brain regions situated towards the back of the embryo while the ventralization is referred to the opposite effect (Fig 1.12).

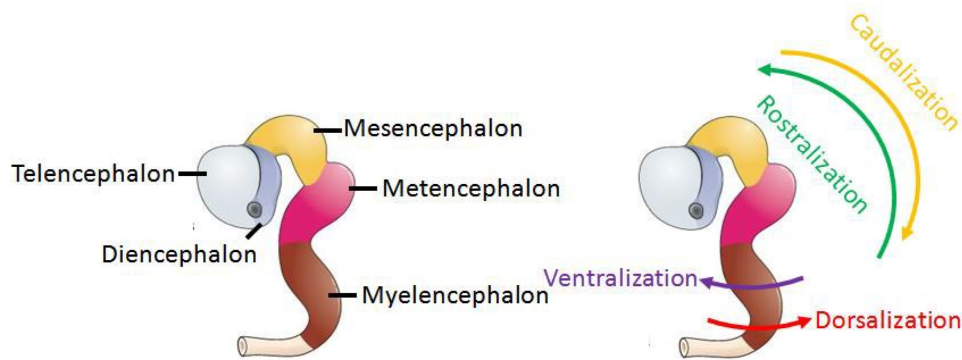


Figure 1.12: Lateral view of the embryo in GW5. The combined concentration gradients of different morphogens define the regional identities of the neuronal populations. The ability of morphogens to impart different cell fates promotes the formation of rostrocaudal and dorsoventral axis. During the rostralization the cells are shifted towards the telencephalon while during the caudalization the process shifting cell fates towards the myelencephalon. When the morphogens define cell fates towards the back of the embryo is considered dorsalization while the opposite effect as ventralization (Voulgaris, 2016).

The dorsal region of the neural tube is called the roof or alar plate, here BMP4 exerts its effect forming a gradient along the dorsal-ventral axis. Cells in the ventral region (floor plate) of the neural tube express SHH and form a concentration gradient along the ventral-dorsal axis in an opposing fashion of BMPs' (Fig.1.13). The dorsal-ventral regionality of the developing brain is generated by the concentration gradients of the two aforementioned morphogens that act in concert (Wilson et al., 1997, Patten and Placzek, 2000).

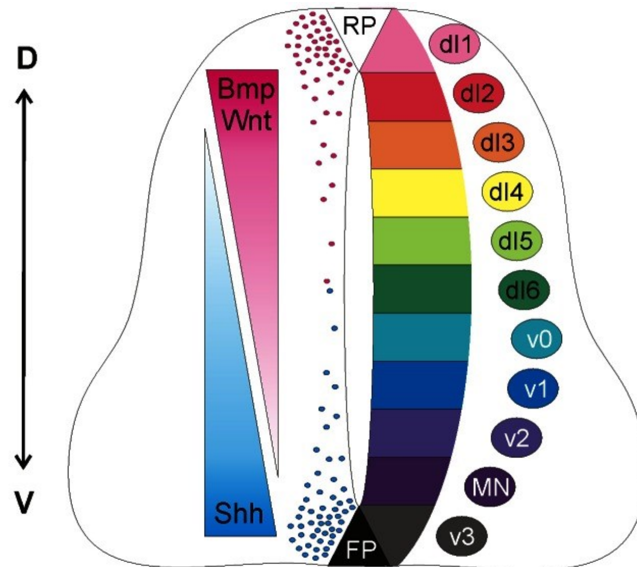


Figure 1.13: Antagonistic activities of Shh and BMP/Wnt pattern. The concentration-dependent differentiation of precursor cells along the dorso-ventral (D-V) axis is induced by the counteracting gradients of Shh, secreted from the floorplate (FP), and Wnt/BMP, derived from the roof plate (RP). Shh and Wnts have antagonistic functions, during morphogenesis. In turn, the specific combinations of transcription factors induced by Shh and Wnts generate a cell identity code that specifies the neural progenitor subtypes. As these cells exit the cell cycle, they distribute laterally in a specific order along the dorso-ventral axis (dI1-v3) (Aviles et al., 2013).

The generation (in order of increasing ventral identity) of V0, V1, V2 interneurons, motor neurons and V3 interneurons is influenced by Shh's effect on ventralizing the neural tube. Neuronal populations in the dorsal region are subdivided into 6 types (dl1-dl6) and BMPs influence only the most dorsal neuronal populations (dl1-dl3), in the alar plate (Lee et al., 2000), the rest (dl4-dl6) are generated regardless of the presence of BMPs (Müller et al., 2002). The region-specific gene products determine the inclination of neural stem cells to assume different regional identities and consequently different functionalities, such as the cells of neuroectoderm. Cells that characterize the neuroectoderm (i.e. the neural plate) express, among others, Pax6, Sox1 and Sox2 genes. Pax6 and Sox1 are the earliest genes to be expressed in the neuroectoderm denoting a neural commitment of the ESCs/iPSCs (Casarosa et al., 2013). Cells that characterize the neuroectoderm (i.e. the neural plate) express, among others, Pax6, Sox1 and Sox2 genes. Pax6 and

Sox1 are the earliest genes express in the neuroectoderm differentiated starting from ESCs/iPSCs (Casarosa et al., 2013) and p75 is a neural crest marker (Lee et al., 2007a).

1.2.2. SIGNALING PATHWAYS INVOLVED IN CNS DEVELOPMENT

1.2.2.1. TGF-β signalling pathway

The TGFβ pathway results in the differentiation of hESCs/hiPSCs to different germ layers and TGFβ ligands play a crucial role in this sense (Dupont et al., 2005). The TGFβ pathway revolves around the phosphorylation of a TGFβ type I receptor (a serine/thionine transmembrane receptor kinase) catalysed by a TGFβ type II receptor. The TGFβ pathway revolves around the phosphorylation of a TGFβ type I receptor (a serine/thionine transmembrane receptor kinase) catalysed by a TGFβ type II receptor. BMPs, Activin, Nodal are among others proteins/growth factors of the superfamily of TGFβ ligands. The intracellular SMAD proteins, namely receptor-regulated SMAD (R-SMAD) and common-mediator SMAD (co-SMAD), are activated and are able to modulate gene expression after the activation of the transmembrane TGFβ type I receptor (Fig. 1.14).

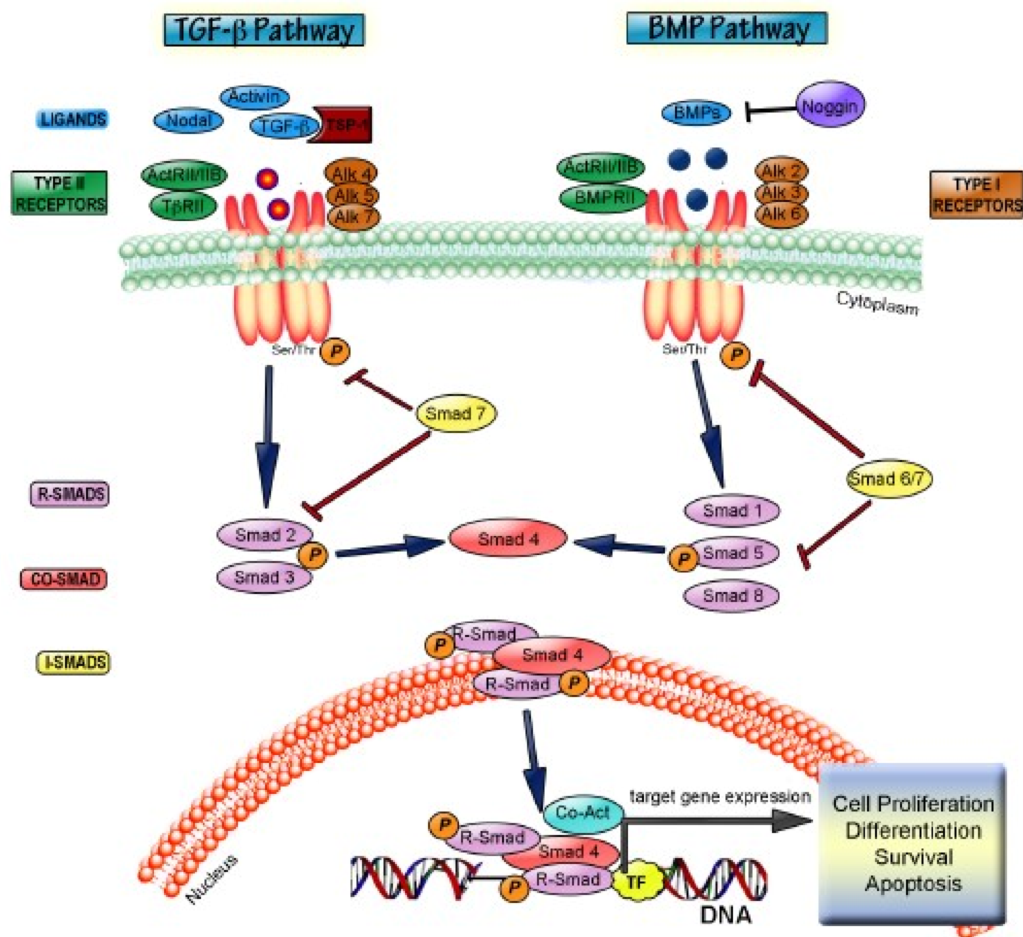


Figure 1.14: Inhibition of the TGFβ and BMP-4 pathway by small molecules/proteins such as SB431542 and noggin. The expression of genes is altered by failure of TGFβ ligands to bind their TGFβ type I receptors. Resultantly, the accessibility of the transmembrane TGFβ type I receptors regulate cell fate (Villapol et al., 2013).

The variables that greatly influence the induction of germ layers interplay are characterized from extra- and intracellular molecules (Xu, 2006, Ross and Hill, 2008) and from the impact of different growth factors on the TGFβ pathway (Massague and Xi, 2012). The proteins noggin, chordin and follistatin, along with others, are the molecules that have been documented to play a major role in the neuroectoderm induction. The inhibition of BMP-4 is mediated by Noggin, a protein which is a natural antagonist of BMP-4 allowing ESCs to differentiate into the mesoderm and has been used in many neuroectoderm induction protocols

(Lee et al., 2007b, Elkabetz et al., 2008). Moreover, SB431542 utilized in neuroectoderm induction protocols, is another candidate molecule that has been explored for its implication in the TGFβ pathway (Smith et al., 2008). SB431542 blocking the phosphorylation of TGFβ type I receptors, inhibits the Activin/Nodal pathway.

1.2.2.2. Wnt signaling pathway

The activation of the Wingless-related integration site (Wnt) pathway plays a crucial role in the neural commitment and in the self-renewal of neural stem cells. For this reason, it is widely used in the differentiation protocol aimed and deriving neuroectoderm from PSCs (Li et al., 2011, Lu et al., 2013). Wingless-related integration site pathway is considered as an evolutionary pathway, because it is involved in stem cell renewal (Nusse, 2008), regulation of calcium, cell polarity and cell mobility inside the cell. Wnt signalling, in fact, is implicated in the cell growth and differentiation control during the nervous system development. The proliferation of the early NPCs is promoted by the Wnt signaling, while it seems to play important roles during the neurogenic phase. This is probably due to the differences of epigenetic statuses in the early and late NPCs. Studies, indeed, demonstrated that this signalling induces the differentiation of neurons and astroglia but suppresses the oligodendroglial differentiation. Furthermore, during the embryonic development, it plays an important pivotal role in the formation of axis of human body (van Amerongen and Nusse, 2009). The Wnt pathway can be divided into Wnt/β-catenin depended pathway (canonical) and Wnt/β-catenin independent (non-canonical), that can be divided into the Wnt/Ca2+ pathways and the Planar Cell Polarity (Habas and Dawid, 2005). Here, we will describe only the canonical pathway, since this is the pathway involved in neural development. The activation of Wnt pathway mediates the gene expression though the control of the translocation and accumulation of the cytoplasmic protein β-catenin into

the nucleus (Fig. 1.15). The Wnt pathway is composed from extracellular glycoprotein, these molecules bind to the extracellular receptor complex consisting of the protein Frizzled (Fz) and the low-density-lipoprotein-related protein 5/6 (LRP 5/6).

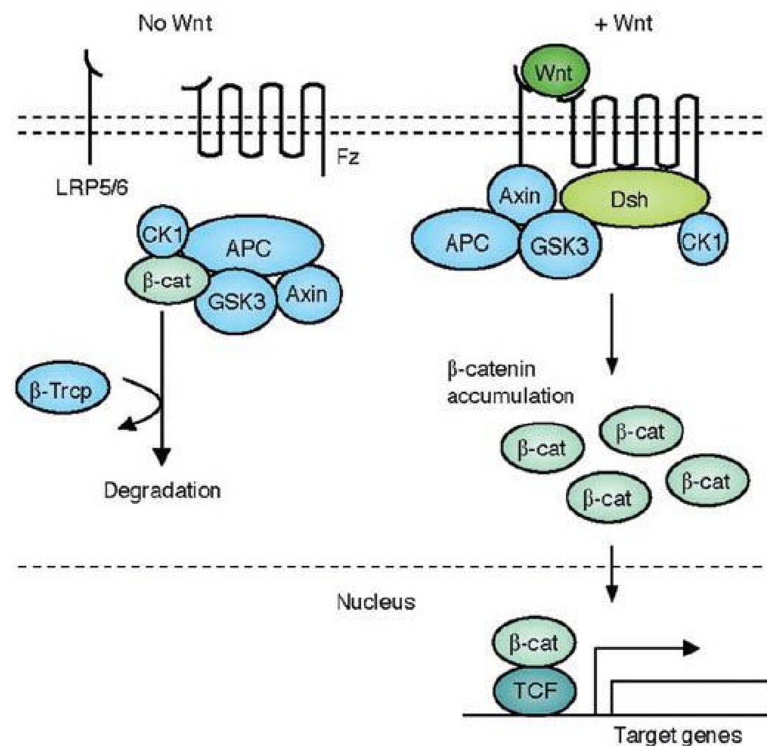


Figure 1.15: Schematic representation of the Wnt/β-catenin depended pathway. β-catenin is degraded by the proteosomal machinery (β-TrCP) when there is no activation of the Fz – LRP 5/6 complex, while if the glicoproteins promote the activation of the Fz - LRP 5/6, this condition imparts conformational changes to the destruction complex preventing the degradation of β-catenin. The consequently accumulation of the β-catenin act in a co-transcriptional capacity to transcriptional factors such as T-cell factor activating target genes (Komiya and Habas, 2008)

Wnt glycoproteins promote the recruitment of LRP 5/6 and concurrently the activation of the protein Fz, a transmembrane protein. The synergistic action of Fz and LRP 5/6 transduces the prosphoprotein Dishvelled signals rendering the β-

catenin destruction complex. This complex is composed by Axin, Adenomatosis Polyposis Coli, glycogen synthase kinase 3 and casein kinase 1a (Gordon and Nusse, 2006). The targeting and the degradation of β-catenin is promoted by its phosphorylation obtained through the destruction complex, which flags it for ubiquitination and the consequent degradation by the proteosomal machinery (β-TrCP). The Wnt pathway has been simulated *in vitro* with the use of pharmacological inhibitors (e.g. CHIR99021) of GSK3, which imitate the Dsh activation. This condition conduces to the accumulation of β-catenin and consequently of the Wnt pathway downstream genes. Lastly, canonical Wnt pathway promotes the increase of Ngn1 and Ngn2, proneural basic helix-loop-helix (bHLH) proteins, through direct activation of their promoters by the β-catenin/TCF complex. Conditional knockout of β-catenin gene in the cerebral cortex and neural crest stem cells shown the critical role of β-catenin in Ngn1 and Ngn2 expression. All these results demonstrate the important role of the Wnt pathway in neurogenesis. Indeed Wnt/ β-catenin pathway promotes proliferation via upregulation of cyclin D1, cyclin D2 and c-Myc expression in other systems, while, via upregulating Ngn1 and Ngn2 expression in the neocortex, it appears to promote neuronal differentiation.

1.2.2.3. Induction of neuroectoderm

Different studies are extensively conducted to determine the neural induction in vitro as well as the evaluation of various patterning methods to induce neuronal cells with distinct functionality, this through the simulation of the in vivo environment (stem cell niche) during development of nervous system, and more specifically of the autocrine and paracrine signalling that drive the induction of neuroectoderm and direct the region-specific identity of the cells. At the beginning, prior to 2009, neural inductions were carried out by the BMP-4 inhibitors introduction, essential for neural induction, such as noggin. Starting

from 2009, the combined activity of noggin and SB431542 (Chambers et al., 2009) a pharmacological inhibitor of the TGFβ pathway, greatly improved the differentiation yield to neural stem cells. The combination of the two molecules inhibited the two Smad-mediated pathways in the TGF-b signalling pathway. Data suggest that noggin and SB431542 work synergistically to efficiently direct both ESCs and iPSCs to a neuroectodermal lineage.

1.2.2.4. Small molecules pathway

The mode of action of the 3D structure of noggin, BMP-4 antagonist, lies in the binding of noggin to the active site of the BMP-4 protein (Groppe et al., 2002). However, Noggin is a recombinant protein with high production cost and batch-to-batch differences (Surmacz et al., 2012). The research of new candidate molecules has conducted to the use of small molecules relatively stable, cost-efficient and manufactured with high purity. The Small Molecules used to inhibit the BMP-4's action is Dorsomorphin, LDN193189 and DMH1.

These small molecules and noggin prevent the activation of BMP-4 pathway acting at different levels. The small molecules directly bind to the TGFβ type I receptors blocking its activation, while noggin, through the binding to the active site of the ligand responsible of the eventual activation of type I receptors (via type II receptors), intercepts the activation of TGFβ type I receptors and the activin receptor-like kinases (ALK) 2, ALK3 and ALK6 (Fig. 1.16).

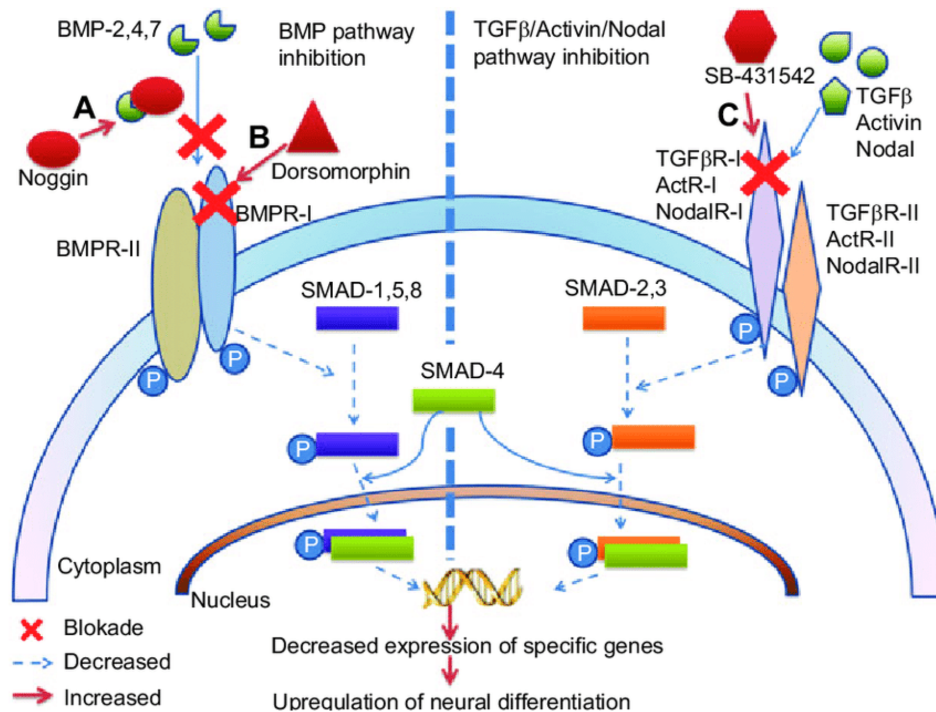


Figure 1.16: Mechanisms for SMAD inhibition pathway to increase the neural cells differentiation. (A) Noggin can prevent the ligation of bind bone morphogenetic protein (BMP)-2,4,7 by bind with high affinity BMP-2, BMP-4, and BMP-7 receptors. (B) Dorsomorphin inhibits selectively the BMP type I receptors (ALK2, ALK3, ALK6) and in addition can blocks BMP-mediated SMAD-1,5,8 phosphorylation. (C) SB-431542 blocks phosphorylation of SMAD-2 and SMAD-3 by binding transforming growth factor-beta (TGF β) type I receptor (ALK-5), activin type I receptor (ALK-4) and nodal type I receptor (ALK-7) (Chambers et al., 2009, Zhu et al., 2012).

Chambers et al. (2009) have used Dorsomorphin in neural induction protocols demonstrating the inhibition of both BMP-4 and TGFβ1. In this way they have shown the suppression of the differentiation, in hESCs, to the trophectoderm mesoderm and endoderm and the enhanced of the differentiation of neuroectoderm. However, the action mode of the dorsomorphin seems to be vary; Zhou and colleagues in 2010 have postulated that dorsomorphin alone inhibits type I receptors in both TGFβ1 and BMP-4 pathways, so the addition of SB431542 (TGFβ1) was consider insignificant. Instead, the group of Reinhardt, in 2013, used both molecules to differentiate neuroectoderm with high efficiency.

LDN193189, a chemically modified small molecule, resulted by a structure-activity relationship study aimed at increasing the Dorsomorphin potency (Cuny et al., 2008). LDN193189 has the same action pathway of Dorsomorphin, for this reason it is considered a chemical analogue. This type of BMP-4 inhibitor has been employed to inhibition of the both TGFβ1 and BMP-4 (Kriks et al., 2011, Chambers et al., 2012, Vazin et al., 2014).

Even though the small molecules are employed in many induction protocols of neurons, with success, using hiPSCs/ESCs as a starting material, but different hiPSCs lines exhibit different concentrations of endogenous proteins. The protocols, for this reason, should be re-evaluated every time and the small molecules concentrations should be optimized for each hiPSC line.

1.2.3. ROLE OF NEUROGENIN 2 (NGN2) DURING NEUROGENESIS AND NEURONAL SPECIFICATION

1.2.3.1 Notch signaling pathway: 'salt-and-pepper' pattern

Activation of Notch regulation promotes the manipulation of NSCs. In the developing forebrain of mouse embryos, ectopic expression of Hes1, target of Notch pathway, inhibits the differentiation of neurons and maintains HES1-positive cells at the embryonic ventricular zone. Notch maintains multipotent NSCs renewal by inhibition of their differentiation until the correct differentiation cue is available.

During the CNS development, it is important to maintain neurons in an appropriate proportion depending on the brain region and to initiate gliogenesis in time. Therefore, when the neurons have reached the correct mature phenotype and number at individual positions, the expression of proneural gene finishes. This control is named "lateral inhibition", a mechanism defined as the commitment to

a neural cell fate by one cell had the consequence of inhibiting its neighbours to follow the same fate. In the progenitor cells, Notch ligand activates the Notch signalling cascade in neighbouring cells, increasing Hes/Her/Esr genes expression, which, in turn, promote the direct downregulation of proneural gene expression (Kageyama et al., 2008). Through this action mechanism, the proneural genes expression is restricted only to the single cells that enter in a specific neural differentiation pathway. In particular, in vertebrate, during the neurogenesis, Mash1 and Ngn2, proneural genes, trigger the transcriptional activation of Notch receptor ligand Delta (Shimojo et al., 2011). Indeed, when the embryos lack the Notch function, they exhibit massive neuronal differentiation, because the interruption of the Notch-dependent cellular communications promotes, in the neighbouring cells, their consequent development into neuronal progenitors (Fig.1.17). In embryo characterized from mutant forms of Notch effectors, virtually all the neural stem cells differentiate prematurely into neurons, generating a severe disorganized neural tube and absence of normal brain structures, highlighting the importance of Notch signalling in controlling appropriate cellular composition of nervous system by preventing the formation of a neuron surplus and promoting glial progenitor-to-astrocyte transition.

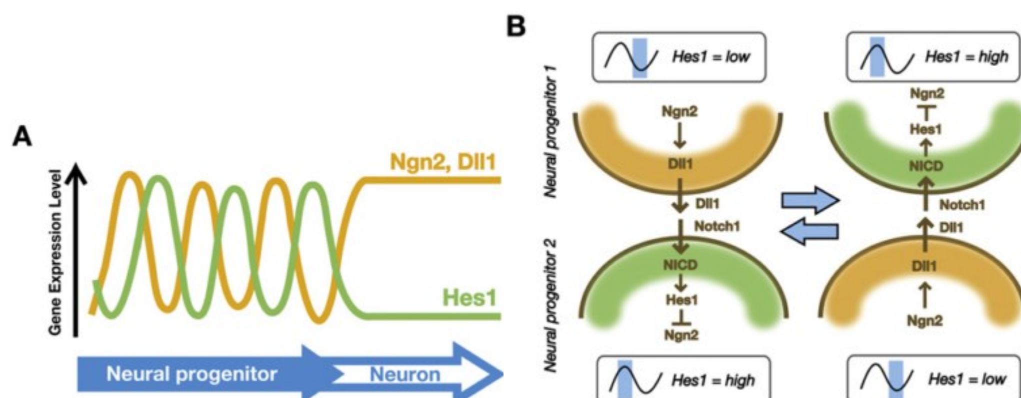


Figure 1.17: Model of oscillations in Notch signalling. (A) In neural progenitor cells, the expression of Hes1 oscillates with a period of ~2-3 hours. Also, the expression of Ngn2 and DII1 oscillates, in

these cells. Indeed, the expression of Hes1 protein exhibits an inverse correlation with the expression of Ngn2 protein and DII1 mRNA. In contrast, Ngn2 and DII1 expression is sustained in post-mitotic neurons characterized from the loss of Hes1 expression. (B) Mutual activation of Notch signalling for maintenance of neural stem/progenitor cells. When the expression of Hes1 is low in a cell, expression of Ngn2 and DII1 becomes high, promoting the activation of Notch signalling and the up-regulation of Hes1 in neighbouring cell. Hes1 has a short half-life, this means that its expression becomes low after ~2-3 hours, Ngn2 and DII1 expression becomes again high, leading Notch signalling activation in the former cells (Shimojo et al., 2008).

Furthermore, the increased of Hes1, as a result of activation of Notch signalling pathway, is also known to act at cell cycle level, by promoting G1 phase progression by the downregulation of cyclin-dependent kinase inhibitors. In brief, in cycling cells, on transition through late G1 and S phase into G2 and mitosis, cdk levels rise rapidly, promoting the increase of Ngn2 phosphorylation by cdk2 and cdk1 (Ali et al., 2011). This condition results in a reduction of promoter occupancy, which is insufficient to activate the Ngn2-responsive promoters of genes required for differentiation of neurons, such as NeuroD. On the contrary, cell cycle lengthening results in a longer time spent in G1 phase, so the ckd levels are low and the inhibitors of cdk accumulate. This favours, more stable unphosphorylated Ngn2, which binds E box DNA more tightly (Fig. 1.18). Furthermore, Ngn2 is characterized from many direct downstream targets for epigenetic modification, and consequently for the recruitment of chromatin modifiers, before the activation. For example, NeuroD gene promoter responds slowly at expression of Ngn2 and requires extensive chromatin modification for the activation. All together, these considerations explain a model in which is required a stable promoter association to drive the differentiation of neurons and these promoter’s activation are Hes1-dependent.

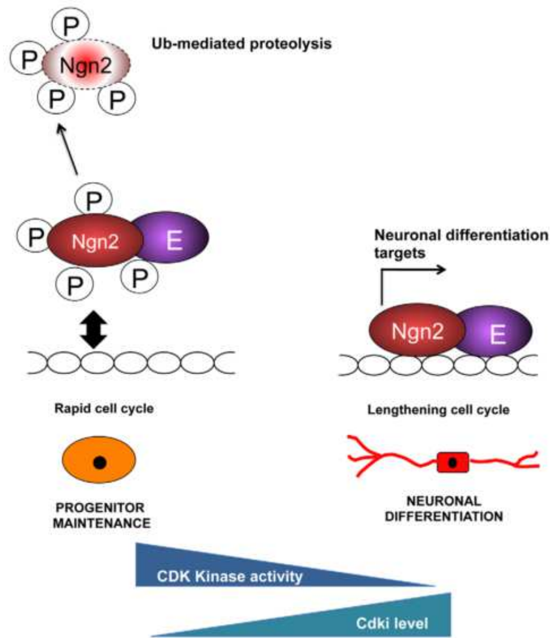


Figure 1.18: Control model of differentiation of neurons. Ngn2 protein phosphorylation characterizes the rapid progenitor cell cycles. Cell cycle lengthening promotes an accumulate of un(der)phosphorylated Ngn2, enhancing promoter binding and consequent activation of downstream target genes, which drive neuronal differentiation (Ali et al., 2011).

E, E protein; Ub, ubiquitin; P, phosphorylation.

1.3. DIFFERENTIATION APPROACHES IN NEUROSCIENCE

The inaccessibility of brain tissues or relevant cerebral cell types hinders the progresses of the study of the human nervous system. Likewise, the multifactorial and polygenic nature of particular neurological diseases has hindered the mouse model generation that recapitulate the diseases phenotype. The possibility to reprogram adult human somatic cells into hiPSCs for the neuronal generation and the direct conversion of human fibroblast directly into neurons offers the unique chance at the researchers to study fundamental aspects of the function and development, at a cellular level, of the CNS (Fig. 1.19).

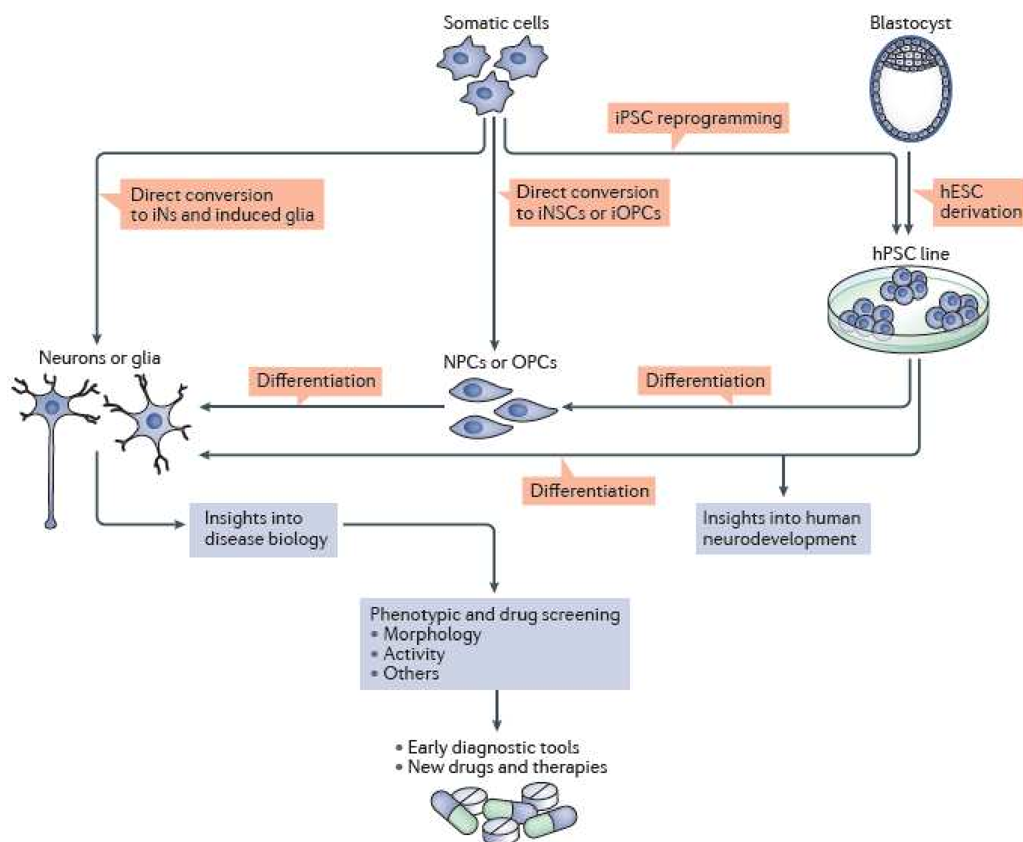


Figure 1.19: Neural cells can be generated from somatic cells through somatic tissue reprogramming, which produces induced pluripotent stem cells (iPSCs), or by direct conversion. Human pluripotent stem cells (hiPSCs or hESCs) can be differentiated into both neural progenitor cells (NPCs) and oligodendrocyte progenitor cells (OPCs), moreover, also fibroblasts can be converted into NPCs or OPCs by direct neural conversion (induced NPCs or iNPCs). Both

differentiation-derived NPCs and iNPCs can be further differentiated into mature neurons or glial cells. These models allow to study human neurodevelopment. Direct conversion of somatic cells or iPSCs into induced neurons allows the bypassing of the NPCs. Models based on cell cultures of neurons or glia are suitable for the study of neurological diseases pathobiology and to develop patient-specific assays where cells electrical activity, morphology and connectivity can be evaluated. Such models can be used for drug screenings, to screen molecular compounds that can improve the patient-specific phenotype. Diagnostic approaches, as well as new therapeutic drugs can be developed with these cell-based systems (Mertens et al., 2016).

To date, reprogrammed cells have allowed to investigate the various human-specific cellular adaptation through the survey of epigenetic, transcriptional and functional signatures. Further, this technology promotes the generation of patient-specific models of different genetic diseases facilitating the monitoring of the progression of neurogenerative diseases. In this field, the combination between the cellular reprogramming and the microfluidic technology allows the molecular characterization of a single cell offering a platform for drug screening. But clearly, in order to successfully perform neurodisease in a dish, it is necessary produce neurodevelopment models through the in vitro generation of specific human neurons subtypes.

Currently, there are two different approaches for the *in vitro* development neurons: the neuronal differentiation from hPSCs following all the developmental stages, and the transcriptional programming. The opportunity to use hiPSCs or ESCs for the differentiation of neurons offers the possibility to use different combinations of factors, such as morphogens or mitogens, to gradually define the identity from positional and temporal point of view, of the different neuronal populations characteristic of each stage of nervous system development; the direct conversion via transcriptional programming, on the other hand, takes advantage of the overexpression of transcription factors specific for a particular cellular lineage, and promotes a variation of the cellular identity toward the desiderate cell typology reducing the differentiation timing.

In this section the differences between the two approaches considering will be discussed their advantages and disadvantages.

1.3.1. NEURONAL DIFFERENTIATION FROM HUMAN PLURIPOTENT STEM CELLS (hPSCS)

During the *in vitro* generation of neurons, the human pluripotent stem cells (hiPSCs and ESCs) transit through specific stages, which they are like to the neuronal progenitor cell populations present in the typical phases of the *in vivo* neurogenesis. As showed in Figure 1.20 the hPSCs that thanks to their characteristics can be consider as the typical cells of the inner cell mass of the blastocyst, can generate *in vitro* neuroepithelial stem cells which can look like to the neuroepithelial stem cells that constitute the neural plate *in vivo*. *In vivo* during the neurulation, take place the close of the neural tube, the polarization of the axes and the generation of the first neurons. *In vitro*, this phase can be represented by the rosette-type NPCs developmental stage. Lastly, the radial glia-like NPCs that rise from the rosette-type NPCs, further differentiate into postmitotic neurons, as it happens during the *in vivo* neurogenesis (Fig. 1.20) (Mertens et al., 2016).

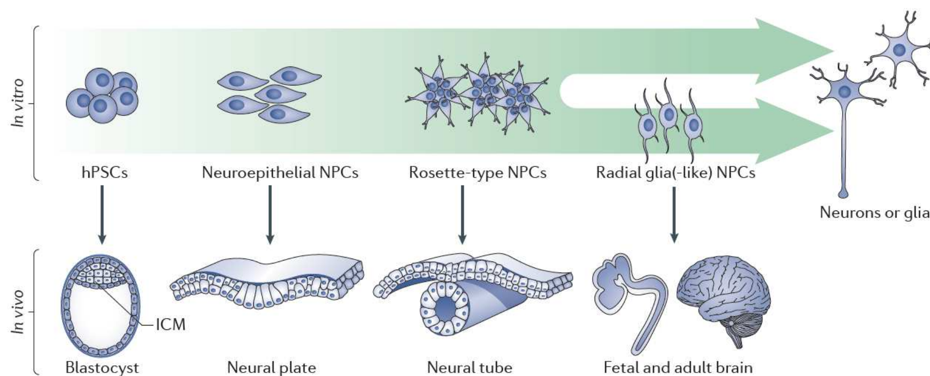


Figure 1.20: *in vitro* and *in vivo* neuronal differentiation stages. In vitro, when pluripotent stem cells differentiate into neurons (upper part), the in vivo neurogenesis stages (lower part) are

recapitulate, passing through the different neural progenitor cells (NPC) stages. For example, hPSCs correspond to the inner cell mass (ICM) of the pre-implantation blastocyst, then they are converted into neuroepithelial stem cells, which represent the cells forming the neural plate in vivo. Next, NPCs are converted into rosette-like structures, with an organizational pattern similar to the neuronal tube that is formed during in vivo neurulation. Finally, during neurogenesis, postmitotic neurons are derived from radial glia. Similarly, from NPSs rosette-like structures, radial glia-like neural progenitor cells can be differentiated (Mertens et al., 2016).

1.3.1.1. Signaling pathways involved in the neuronal differentiation

The pioneering work of many scientists on animal models of development of nervous system has permitted the derivation of method for the induction of NPCs from hPSCs identifying the key events of the neural commitment and the regionalization in the early mammalian. These researches have shown the neural rosettes represent the early neural tube and are generated by the intense proliferation of neuroectodermal NPCs. Radial glial cells derived from the neural rosettes, later proceeding with the development, produce most of neurons.

In the neural tube, the different identities of the NPCs are conferred by mitogens and morphogens. An example is represented from the SHH secreted from the ventral zone of the neural tube, the notochord and the floor plate, or from the BMP and WNT proteins secreted from the dorsal zones; this reflect the various morphogen gradients that specify the different NPCs subtypes along of both anterior-posterior and dorsal-ventral axis. In particular in the first case the specification of neural progenitor fates is promoted from the sequences of events relate to the timing development and the signalling cues, as those generated by fibroblast growth factors and retinoic acid. Generally, these factors fulfil mitogenic and morphogenic functions, at the same time, even though their effect are related to the development stage, localization, target neural progenitor cells and concentration. Some examples:

- Midbrain or spinal cord motor identity: adding a combination of posteriorizing factors (such as FGF2 or FGF8, SHH and RA), and promoting the activation of WNT signaling. Dopaminergic neurons resided in the substantia nigra pars compacta of the midbrain are an example of this category and are *in vitro* generated by administrating FGF2 or FGF8, to hPSCs-derived NPCs, to promote posteriorization and SHH, a ventralizing factor. Furthermore, the hPSCs to become dopaminergic neurons requires the activation of WNT signalling and the floor neural plate pattering in order to activate the transcription factors forkhead box protein A2 and LIM homeobox transcription factor 1α.
- Hippocampal dentate gyrus identity: adding the antagonists of SHH pathway and the anti-posteriorizing cocktail factors, such as Noggin that blocks BMP pathway, Dickkopf-related protein 1 (DKK1) that blocks the pathway of WNT, and small molecules, as SB431542 which blocks the transforming growth factor-β (TGFβ) pathway;
- Forebrain identity: inhibiting WNT and SMAD signalling pathways and activating the SHH signalling. The excitatory neurons rise in the dorsal area of this region and their genetic pathway are directed by FEZF2 (FEZ-family zinc finger 2) and CTIP2 (COUP-TF-interacting protein 2) that promote the specification of all populations of subcortical projection of neurons and the fasciculation of neuron axons, respectively. Instead, the ventral telencephalic fate is characterized from the specification of interneurons by inhibition of WNT and SMAD signalling responsible of the generation of medial ganglionic eminence-like progenitors starting from hPSCs;
- Hindbrain or spinal cord radial glia: immunoisolating a positive population for CD133 marker and neural regionalizing with traditional methods.

1.3.2. TRANSCRIPTIONAL PROGRAMMING OF HPSCS INTO NEURONS

The growing demand of active human neuronal cells has produced the generation of alternative technologies able to development specific neuron types. In the 1980s the first demonstrations that the cell identity can shift directly into another identity took place thanks to the transgenic expression of particular transcription factors.

Pathologies of nervous system are characterized by gradual loss of neurons and consequently of cognitive and physical functions. To date, there are no disease-modifying therapies able to slow or stop the neurodegeneration. Transcriptional programming provides an alternative strategy for disease modelling and for regenerative medicine. More in detail, obtaining a patient-specific subset of neuronal cells gives the opportunity to work with an autologous alternative, to produce personalized cell therapy or to identify new drug-target.

In this section I will provide a comprehensive overview of the recent technologies to generate neurons in a rapid manner.

1.3.2.1 Efficient induction of neurons from hPSCs with the expression of a single transcription factor

Recent data have demonstrated as under specific experimental conditions, somatic cells (adult differentiated cells) can become reprogrammed into a different cell type losing their germ layer or tissue specification.

In this thesis I will describe two rapid approaches to direct neuronal differentiation: induced neurons through the transcriptional programming of hiPSCs via lentiviral transduction of transcription factors and the development of induced neurons via synthetic modified mRNA encoding for specific proneural gene.

The first method has been developed by the group of Thomas C. Sudhof. The slow and variable conventional methods to differentiate ESCs or hiPSCs into neurons have pushed Sudhof and colleagues to determinate a rapid protocol, for the generation of neurons, characterized from a neuronal culture with the 100% yield and purity of conversion in less than only two weeks (Zhang et al., 2013). Previous data, in fact, have demonstrated that the overexpression of Sox2, together with additional transcription factors, is able to induce the conversion of mouse and human fibroblast into NPCs (Lujan et al., 2012). While exogenous Ngn2, a proneural gene, alone, induces the differentiation of neurons by activating a cascade of transcription factors comprehensive of the endogenous Ngn2. In this field, the forced overexpression of a single transcription factor, Ngn2 or NeuroD1, in ESCs and hiPSCs, is a sufficient condition to rapidly convert hPSCs into neurons (Fig. 1.21) and this induction is obtained using the Tet-On system. Two different lentiviral delivery, one for the constitutive expression of rtTA and one for the tetracycline-inducible overexpression of exogenous proteins driven by a tetO promoter (Ho et al., 2016).

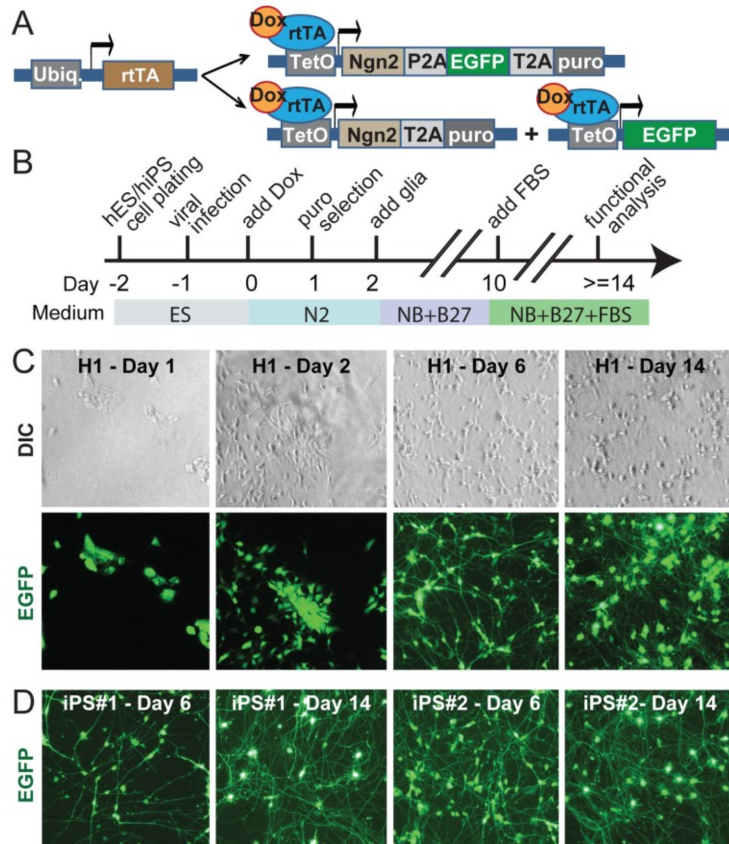


Figure 1.21: Rapid generation of human iN cells. (A) Lentiviral vectors for Ngn2-mediated conversion of hESCs and hiPSCs cells into iN cells. Cells are transduced with (1) a virus expressing rtTA and (2) either a single additional virus expressing a Ngn2/EGFP/puromycin resistance gene as a fusion protein linked by P2A and T2A sequences, or with two different viruses that separately express Ngn2/puromycin resistance gene and EGFP. (B) Flow diagram of iN cell generation. (C) Time course illustrated from representative images of the conversion of H1 ES cells into iN cells. Pictures of differential interference contrast (DIC) and GFP fluorescence are shown on top and bottom. (D) Representative images of converted iN cells from two different hiPSCs cells lines at day 6 and day 14. iN cells are identifiable on day 6 (Zhang et al., 2013).

Staining performed on iNs generated with rapid neuronal induced protocol has demonstrated the abolishment of stem-cell marker (Oct4 and Nanog) expression, confirming the conversion of hPSCs into neurons and a quantitative RT-PCR analyses showed high levels of neuronal markers expression, such as NeuN, MAP2 and in particular of endogenous Ngn2. Furthermore, a high expression of excitatory cortical markers, Brn2 and FoxG1 (Fig. 1.22) has been observed.

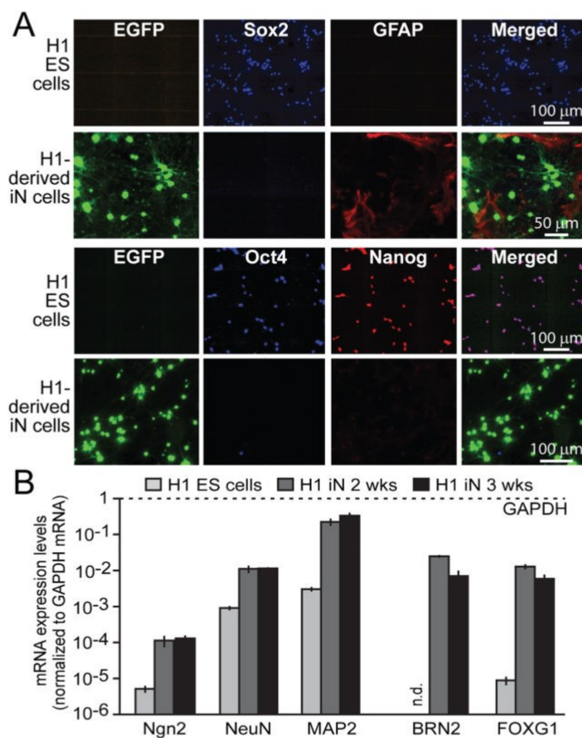


Figure 1.22: Characterization of the properties and yield of Ngn2-iN cells. (A) Immunofluorescence images of H1 ES cells and H1 ES cell-derived iN cells. H1 ES are positive for markers Nanog, Sox2 and Oct4 compared with H1-derived iN cells, while GFAP is only present in co-cultured astrocytes. (B) mRNA levels quantification in H1 ES cells and in H1 ESC-derived iN cells after 2 and 3 weeks of lentiviral infection. Levels are normalized for GAPDH mRNA levels as an internal control and are shown on a logarithmic scale. To notice that endogenous Ngn2 is expressed ~20-fold, and endogenous Brain-2 and FOXG1 expression is induced >1,000-fold. Data are means ± SEM (n=3 independent experiments)(Zhang et al., 2013).

Fluidigm-dependent mRNA quantitative assay conduced on 73 genes of more than 100 iN cells has demonstrated, first of all, that this method produces a reproducible generation of neurons with the same properties with both cell line used, ESCs or hiPSCs. Secondary, the neurons generated with this method express specific telencephalic markers, such as FoxG1, Brn2 and Cux1, typical markers of immature excitatory layer2/3 cortical neurons, of the dorsal forebrain, and the receptors GluA1, GluA2 and GluA4 AMPA-type glutamate, vGlut2 and GABAA-receptors are highly expressed. Lastly, all Ngn2-iN cells express pan-neural markers and have lack the expression of markers for stem cells or glia cell types (Fig. 1.23).

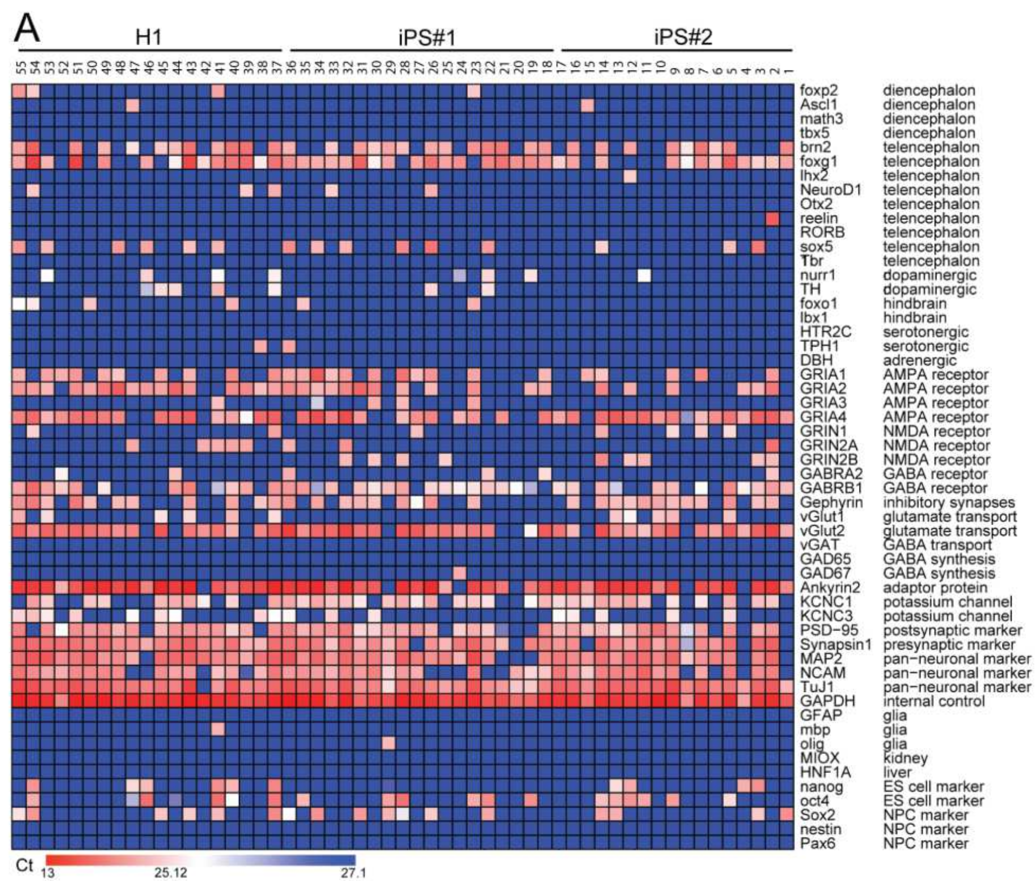


Figure 1.23: Generation of iN cell involves changes in gene expression. (A) Single-cell quantitative RT-PCR analysis (Fluidigm) of the levels expression of the genes (on the right) after 3 weeks of induction. On the bottom, the expression levels (expressed as Ct values) of the colour coded (Zhang et al., 2013).

The generation of neurons, with this method, proved to be reproducible between the different lines investigated. In fact, a striking concordance of the gene expression patterns has been revealed with the comparison between the iNs differentiated from ESC and hiPSC lines. As shown in Figure 1.24 there is no major differences between the cell lines tested in the expression profile of the gene investigated. This means that the forced Ngn2 overexpression override the epigenetic differences of the cell lines used for the neuronal generation (Zhang et al., 2013).

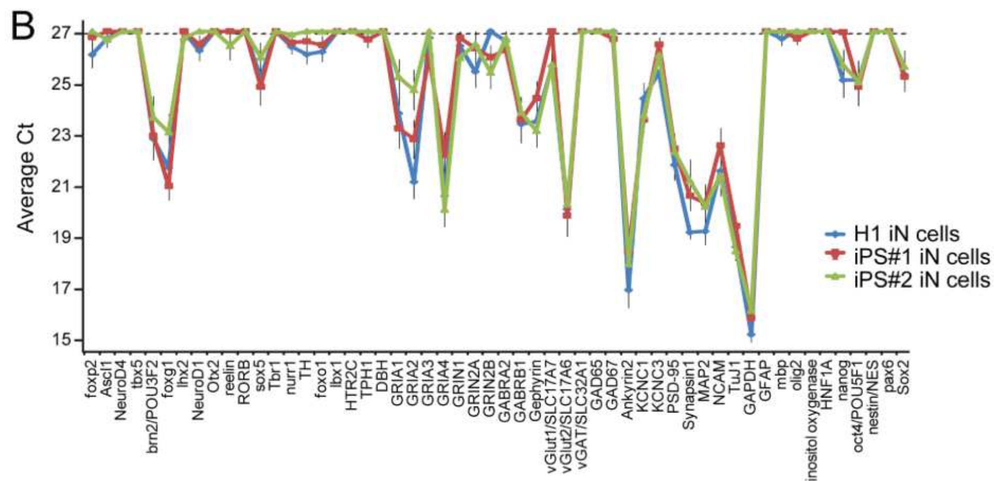


Figure 1.24: Comparison of gene expression profiles in iN cells. iN cells are generated from H1 ES and two different lines of hiPSCs cells. At the bottom, the plot depicts average Ct values of the genes, with a cut-off of 27 cycles (on top of the 18-cycle pre-amplification) (Zhang et al., 2013).

The co-culture of the iNs with mouse astrocytes promotes the formation of robust synapses, but so far there are no informations about the synaptogenic factors supplied by the glia. In fact, robust action potentials and voltage-gated Na^+/K^+ currents are observed in iNs and prove to be identical in the hPSCs lines used for the neuronal differentiation. Furthermore, Ngn2-iNs are characterized by massive synaptic activity and this data has been demonstrated with the administration of CNQX, an antagonist of AMPA-receptor, and consequently arrest of the synaptic activity. All together these data permit to maintain the rapid neuronal induction allows a rapid turnaround of the experiments and the resulting neurons form *in vitro* mature pre- and post-synaptic specializations characterized from voltage-gated currents or displayed excitatory postsynaptic currents, while *in vivo*, when transplanted in mouse brain are able to integrate into network of the existing neurons.

Finally, for what concerns the method so far discuss, with this protocol the synapses of the neurons generated with the rapid conversion of hPSCs, are characterized from full function, short-term plasticity ad direct modulation by factors as retinoic acid offering a valid platform for the evaluation, on the synaptic transmission, of the pharmacological or mutation agents.

The second method used for rapid differentiation of neurons is characterized from the use of an integration free system represented by synthetic modified mRNA encoding for specific transcription factor. Approaches based on synthetic modified mRNA have many important features for strategies of cell differentiation (Pang et al., 2011). Unlike lentivirus or plasmids, mmRNAs do not integrate in the genome of the host cell, obtaining footprint-free gene product delivery. Upon the entry into the cells, the mmRNA gets quickly translated, and multiple rounds of translation lead to high levels of expression in short time (Sridhar et al., 2016). Furthermore, compared to the dosage of viral transfection, in synthetic mmRNA the dosage is easily controlled. At the time of transfection, multiple transcripts of mmRNA can be transfected together as a cocktail. Finally, synthetic mmRNAs allow a robust expression, integration free and dose-dependent, making them perfect for cell differentiation use (Warren et al., 2010).

Goparajo and colleagues described a novel, rapid and highly efficient modality to generate functional neurons from hPSCs by the use of an mmRNA cocktail of five transcription factors (Goparaju et al., 2017).

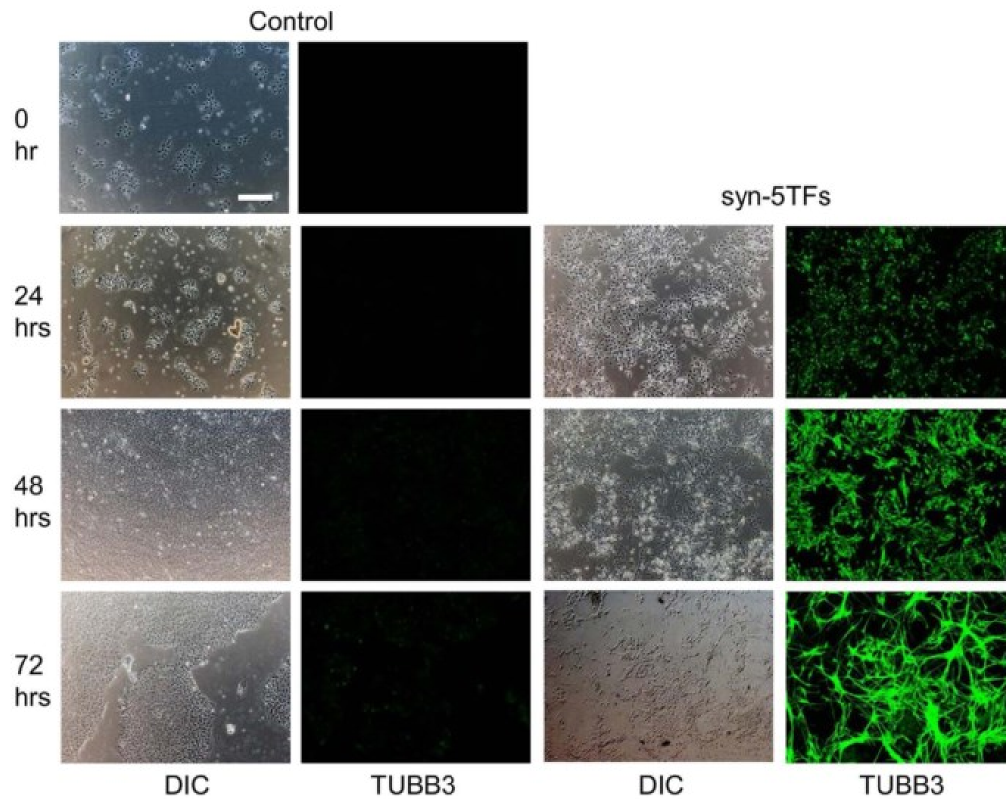


Figure 1.25: Morphological changes during the differentiation of neurons induced by the syn-5TFs cocktail. Kinetics of neuronal TUBB3 expression during syn-5TFs mRNA cocktail-induced compared with the control. Scale bar indicates 200 µm (Goparaju et al., 2017).

The *in vitro* transcription allows the production of synthetic mmRNAs and varying the dose of the mmRNA it is possible to obtain scalability in the production of neurons. Studies reveal that the motor neurons generated with this method express canonical markers such as ISL1, ChAT and HB9. The induction of neurons by synthetic mmRNA shows a much earlier βIIITubulin expression (Fig. 1.26) that the conventional neuronal differentiation protocols (Goparaju et al., 2017). Although NDs and Ngn, by themselves, are potent neuronal inducing factors, their combination makes possible to accomplish rapidity and high efficiency in motor neurons development.

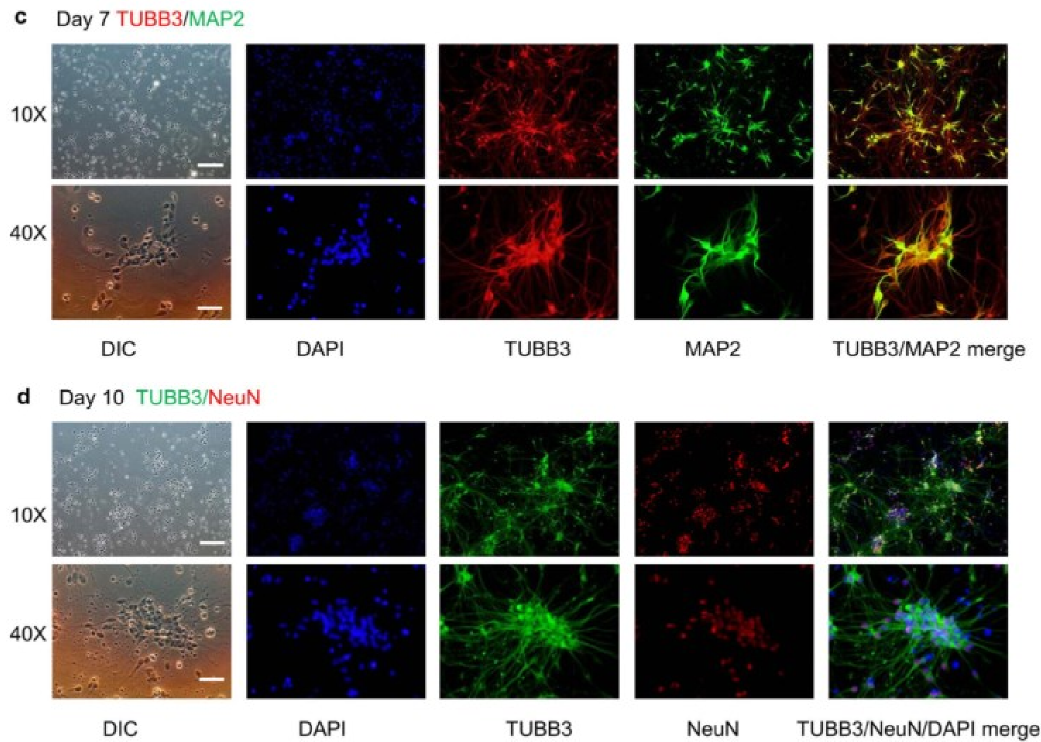


Figure 1.26: Neurogenesis induction in hiPSCs by syn-5TFs mRNA cocktail. (c) Highly efficient differentiation of neurons of hiPSCs by Syn-5TFs cocktail. TUBB3 (red) and MAP2 (green) neuronal markers, are expressed at Day 7. Upper row shows images of low magnification (10X). Scale bar 200 µm. Lower row shows images of high magnification (40X). Scale bar 50 µm. (d) Highly efficient differentiation of neurons of hiPSCs by Syn-5TFs cocktail. TUBB3 (red) and MAP2 (green) neuronal markers, are expressed at Day 10. Upper row shows images of low magnification (10X). Scale bar 200 µm. Lower row shows images of high magnification (40X) (Goparaju et al., 2017).

Furthermore, it has been demonstrated that the addition of well-known neural modulating small molecules, such as SMAD inhibitors (dorsomorphin and SB4315425, retinoic acid - a strong modulator of neurogenesis, and forskolin - a cyclic AMP enhancer), significantly improve the motor neuron derivation initiated by the syn-5TFs cocktail (Fig. 1.27). These results suggest that, both extracellular differentiation cues from small molecules and cell-intrinsic signals mediated by TFs are important for an efficient neuronal differentiation. Condition confirmed from the low efficient generation of neurons when small molecules are used without transcription factors.

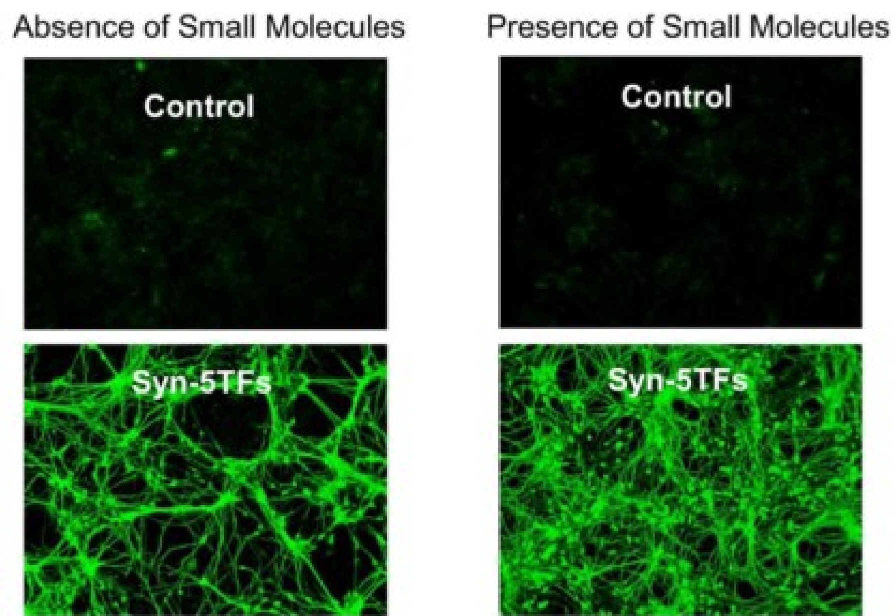


Figure 1.27: Small molecule modulators enhance the efficiency of neurons induction. Representative images of TUBB3 staining at Day 5. Scale bar indicates 200 µm (Goparaju et al., 2017).

Chapter 2:

AIM OF THESIS

The goal of this thesis is the downscaling of neuronal differentiation, starting from hiPSCs, using microfluidic and transcriptional programming technologies. The microfluidic lab-on-chip platform, used for the validation of neuronal induction protocols, offers the possibility to work in a context of tight control of culture conditions, high-throughput, efficient delivery of soluble factors. Instead, the induced pluripotent stem cells (iPSCs) technology allows to reprogram somatic cells, such as skin fibroblasts, to an embryonic-like phenotype and to obtain, in this way, a clonal expansion of undifferentiated cells that can then be differentiated in the desired phenotype (e.g. neuronal lineage). The combination between cell culture in microfluidics and iPSCs provides a very important contribution in the understanding of those unknown molecular mechanisms, responsible for specific pathologies, which could be useful for finding effective therapies.

This work finds its basis and strong motivation in a contest that, to date, does not provide exhaustive *in vitro* or *in vivo* models, because a large amount of neurological diseases and human brain studies are performed on post-mortem biopsies or on tissues collected at very late disease stages. The animal models, instead, could represent a possibility to understand some neurological mechanisms, but they are limited and sometimes do not fully recapitulate the patient phenotype.

In this scenario, we have focused our attention on the downscaling of *in vitro* neuronal differentiation protocols starting from hiPSCs, taking advantage first of all of lentiviral vectors for the overexpression of a proneural transcription factors, Ngn2, After the definition of the best culture condition, we focus our attention on the regulation of the expression pattern of Ngn2 in hiPSCs. Ngn2 is characterized by an oscillatory expression, in the early stages of the neurogenesis, and becomes constantly expressed on mature neurons. So, we evaluated weather we could reproduce *in vitro* this oscillatory expression pattern.

Furthermore, since the generation of neurons with high efficiency is influenced by the correct delivery of exogenous factors to the cells with a precise

timing, we developed a protocol of neuronal induction using an automated microfluidic platform.

Lastly, to avoid any genetic aberration caused by lentiviral vectors and to have a system that could be easily modulated in term of dose and frequency of administration, we induced the generation of neurons by introducing synthetic modified mRNA encoding for Ngn2 into hiPSCs and coupled this method with microfluidic technology. We focused on increasing the differentiation efficiency of neuronal differentiation, working on cellular signallings that play important roles during the *in vivo* development of the central nervous system.

Chapter 3:

MATERIAL AND METHODS

3.1. CELL CULTURES

In this section will be describe the origin and cell culture methods used in conventional and microfluidic devices, employed in this thesis.

3.1.1. HEK 293 CELLS

The HEK 293T are embryonic cells of human kidney with a starry morphology. Such cells they contain the coding gene sequence permanently integrated into the genome the antigen T of the Monkey Vacuatory Virus (SV40), necessary for an efficient replication of plasmid vectors bearing the origin of replication SV40. The cell line was grown in DMEM medium added with 10% (v / v) of inactivated FBS and 1% (v / v) of penicillin antibiotics (100 U/ml) and streptomycin (100 µg/ml). Cell cultures were maintained at a constant temperature of 37 °C in an incubator with a 5% (v/v) humidified atmosphere of CO2 and subjected to periodic checks to rule out any contamination.

3.1.2. HUMAN INDUCED PLURIPOTENT STEM CELLS

Coating chips. Prior to beginning with the differentiation protocol is necessary to coat the surface of the channels or the multi-well plates. Thaw a Matrigel aliquot (Corning Matrigel Matrix - Growth Factor Reduced, SACCO - Corning, #354230) and prepare a dilution at 1% of Matrigel with cold DMEM/F12 (Gibco by Life Technologies, #11320074), immediately aliquot 12µl of Matrigel solution into each channel (or 1 ml for each well of a 6-well plate). Place the devices into 37°C incubator for 30 minutes or leave they at room temperature from 1 hour. After

that aspirate the coating mixture and wash the channels directly with 12µl of hiPSCs medium (1 ml of Matrigel solution for each well of a 6-well plate). Seal with parafilm and store at 2-8°C overnight.

Defrosting. Cells are stored in cryovials at -80 ° C for a few months, or in liquid nitrogen vapours for longer times. Defrosting must be as quick as possible to minimize it cytotoxicity of the cryoprotection agent (10% dimethyl sulfoxide, Sigma-Aldrich). The criovial extracted from the freezer or nitrogen is heated quickly soaking it in water at 37 ° C, when the entire cell suspension is thawed, it is transferred into a 15 ml tube and diluted, at least 4 times, with prewarmed culture medium. The medium must be added drop by drop to limit osmotic stress. Cells are centrifuged 1100 rpm for 5 minutes and the supernatant can be removed before resuspending the cells in volume of desired medium.

Split and seeding hiPSCs. Prior to beginning pre-warm at room temperature, the proliferative medium, TeSR™-E8™ (TeSR™-E8™ Basal Medium – STEMCELL Technologies, #05941), TrypLE™ (TrypLE™ Select Enzyme (1X), no phenol red – Thermo Fisher Scientific, # 12563011), Trypsin Inhibitor (Soybean Trypsin Inhibitor, powder - Thermo Fisher Scientific, # 17075029) and remove the parafilm at the chips (or the multi-well plates) and place them into 37°C incubator for 30 minutes.

Split single cells. The hiPSCs culture are grown in 6-well plates; aspirate hiPSCs medium and incubate hiPSCs with 0.5 ml/well of TryPLE for 5–10 minutes at 37 °C incubator. Once detached, add 0.5 ml of Trypsin Inhibitor and 1 ml of TeSR™-E8™ per well, transfer the cells into a 15 ml tube containing 2.5 ml of TeSR™-E8™ and centrifuge 5 minutes at 300g. Following aspirate and resuspend, the cells, in TeSR™-E8™ medium plus Rock inhibitor (Y-27632 - CAS 146986-50-7 – Calbiochem, # 688000). Incubate cells overnight.

hiPSCs propagation. hiPSCs grow in defined and compact colonies, similar to the embryonic stem cells. In order to avoid differentiation phenomena, it is important to prevent the merging of the colonies with each other by subculture the well. To detach the colonies, it is necessary to first wash them in PBS and then

they can be treated with 0.5 mM EDTA for 2-3 minutes by monitoring under a microscope the action of the reagent. Dissociation should be stopped by aspirating the reagent and adding growth medium when holes in the colonies begin to form. The total detachment of the colonies occurs mechanically thanks to a cell scraper. The colonies are thus resuspended and can be seeded on the surface functionalized with 0.5% MRF.

3.2. PRODUCTION OF LENTIVIRAL VECTORS

Lentiviruses are viruses used, for their potentiality to infect cells, such as vectors in the genetic therapy. In this section will be describe the procedure which to package of competent virus from DNA vectors. The packaging system is compound of two different plasmids (Fig. 3.1):

- the packaging plasmids:
    - pMDLg/pRRE (Plasmid #12251), encoding for gal and pol, provides reverse transcriptase and structural proteins
    - pRSV-Rev (Plasmid #12253), encoding for Rev, promotes efficient RNA export from the nucleus by binding to the RRE
- the envelope plasmid:
    - pMD2.G (Plasmid #12259), encodes the VSV-G, vesicular stomatitis virus glycoprotein. This plasmid promotes the expansion of the vectors tropism and allows the final virus concentration by ultracentrifugation.

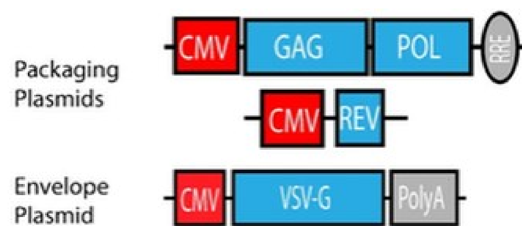


Figure 3.1: Third generation Lentivirus (http://www.addgene.org/).

3.2.1. GROWTH OF BACTERIA

Commercially available competent E coli DH5α cells (Promega, #L2015) have been used for plasmid transformation in accordance with the manufacturer's

instructions. Standard aseptic techniques have been used in bacteria handling. All cultures have been carried at 37°C for approximately 16 hours. A shaking incubator set at 180-200 rpm has been used for liquid cultures. Solid cultures on petri dishes and liquid cultures in universal tubes have been kept at 4°C for a short period of time (maximum two weeks). A 20%v/v glycerol stock of overnight cultures of clone colonies containing plasmids were stored at -80°C.

3.2.2. PLASMID AMPLIFICATION

Standard methods were used to prepare and store bacteria containing plasmids harboring cloned genes. Bacterial stocks were streaked onto agar plates with 100 µg/mL ampicillin and grown overnight at 37ºC. Single colonies were subsequently picked and used to inoculate liquid cultures of LB medium containing 100 µg/mL ampicillin (Ampicillin sodium salt powder – Sigma-Aldrich, #A9518). Small scale, mini-prep cultures were of 5ml and large-scale maxi-prep cultures were 200-500 mL in volume. Cultures were expanded at 37ºC in a shaking incubator overnight. Plasmid extraction from mini-prep and maxi-prep cultures was performed using the appropriate Qiagen kit and eluted in TE buffer as per the manufacturer's protocols. Concentrations of DNA were then determined using a NanoDrop 1000 Spectrophotometer and stored at -20ºC.

3.2.3. LENTIVIRAL PRODUCTION

Day 1: HEK cell seeding. It is recommended to start the procedure from 2 × 175 cm flasks of confluent 293T HEK cells grown in HEK medium - DMEM high glucose (ThermoFisher Scientific, # 10566-016) + 10% FBS (ThermoFisher Scientific, # 10270106) + NEAA (MEM Non-Essential Amino Acids Solution - ThermoFisher

Scientific, # 11140035) + Sodium pyruvate (Sigma-Aldrich, # S8636-100ML). Split 293T cells from two confluent 175-cm flasks to the 12 × 15 cm flasks (consider 2 flasks for each lentiviral production) pre-coated with Poly-L-ornithine (Sigma-Aldrich, # P3655-100MG), using 20 ml of HEK medium. Swirl the cells thoroughly during the seeding them in order to obtain even distribution across the surface of the dish. Incubate at 37 °C overnight. Cells should preferably be of low passage number and their use is not recommended after passage 20 or if growth is slow.

Day 2: Transfection with plasmid mix using CaPO4 precipitation. If cells are 80–90% confluent they are ready for transfection. In the morning refresh HEK medium (18 ml) and prepare the plasmid mix by aliquoting the four plasmids (transfer vector, pMDL (Gag/Pol), pVSVG (vesicular stomatitis virus glycoprotein) and REV). Left the plasmid mix at room temperature for 15 minutes. Add the transfection mixture (drop by drop) to each plate. Swirl the plates gently and incubate at 3% CO2, 37 °C overnight (16–20 h).

Day 3: Observe the cells and change the media. The cells should be reaching confluency. A visible marker (such as GFP) is normally present in the lentivector plasmid, for this reason the is possible verify the transfection efficiency, because it may be assessed visually. Ideally, transfection efficiency should be >90%. Remove media, add 19 ml of fresh HEK medium to each flask and transfer to 37 °C incubator overnight.

Day 4: Collect first harvest of supernatant. Collect and pool supernatant from flasks into a 50 ml tube. Important, supernatants and suspensions contain infectious lentiviral particles. Add 19 ml of fresh HEK medium to each flask. Incubate dishes at 37 °C overnight. The tube containing the lentiviral supernatant should be stored at 4 °C.

Day 5: Collect second harvest of supernatant. Collect supernatant from flasks. Pool supernatant from first and second harvests. Clear the supernatant of cell debris by filtering through a 0.45-µm filter. The cleared supernatant may be used to directly transduce cells, although viral titer will be relatively low (~106 viral particles/ml).

Day 6: Concentrate the viral preparation. Concentrate the viral particles by ultracentrifugation. Centrifuge the supernatant at 50,000g for 2 h at 20 °C with a Beckman SW28 rotor (capacity for six tubes). Fill the tubes with 30 ml of supernatant. Pour off the supernatant and allow the remaining liquid to drain by resting the inverted tubes on paper towels. Siphon off remaining droplets using an aspirator in order to remove all liquid from the pellet. The pellet should be barely visible as a small translucent spot. Resuspend the viral pellets in 100 µl of 1× PBS. Without pipetting. Make 5 µl aliquots of the supernatant. Store at –80 °C (for periods >1 month). Avoid repeated freeze-thaw cycles (ideally <3).

3.2.3.1. Lentiviral titration using flow cytometry

Make a tenfold serial dilution of the lentiviral preparation (from undiluted to a dilution of 106) of the viral preparation in 1× PBS. he actual range to test will depend on the concentration of the viral preparation and must be determined empirically. Seed 75000 293T HEK cells in each well of the 6-well cluster plate. Add 20 µl of each viral dilution to the cells, mix thoroughly but gently and incubate the cells at 37 °C. GFP fluorescence should become visible 24 h after infection. Grow the cells for 48 h. Gently aspirate medium and wash cells twice with 1× PBS to eliminate leftover virus in the medium. Resuspend the cells in 1 ml of PBS by vigorously pipetting up and down (trypsinization is unnecessary). Determine the percentage of labelled cells (if the marker is GFP, count GFP+ cells, ideally). Determine the percentage of labelled cells (if the marker is GFP, count GFP+ cells, ideally.

Calculate biological titer. Calculate the transduction units per ml (TU/ml) according to the following formula: TU/µl = (P × N / 100 × V) × 1/DF, where P = % GFP+ cells, N = number of cells at time of transduction, V = volume of dilution added to each well and DF = dilution factor.

3.3. MICROFLUIDIC TECHNOLOGY

In microfluidics the cell culture is analogous to the traditional devices (ex: multi-well) for what concern the type of mediums and solutions used, but volumes and procedures change. The main points of difference between the two culture systems are:

- any liquid can be injected or flowed into the channels. In particular, if the channel is empty, the liquid is injected through the output channels, if the channel is already full enough to place one drop of liquid on the outlets and, thanks to the principle of communicating vessels and at the pressure guaranteed by the liquid contained in the reserves, the drop it is sucked through the canal and the fresh medium flows on cells;
- the total volume of the medium into the channel is 5.4 µl but is fluxed a total volume of 12 µl to change extensively the medium within the channels;
- to seed the cells at the desired density, the following formula must be applied:

$$Vres = \frac{\text{N°cell} * 0.2\ \text{mm}}{\text{Density}\ (\frac{\text{cell}}{\text{mm2}})}$$

Vres = volume in which to resuspend total cells

0.2 mm = height of the microfluidic channel

- the chips must be placed in a plastic holder and kept in PBS isotonic bath to ensure correct chip humidification and avoid a significant evaporation of the culture medium due to the reduce volume used.

3.4. PROTOCOLS FOR GENERATION OF NEURONS IN WELL AND ON-CHIP

In this section will be describe the different protocol for the generation of neurons, in traditional and microfluidic devices, used in this thesis.

3.4.1. GENERATION OF NEURONS VIA LENTIVIRAL NGN2-OVEREXPRESSION

Day-3: Coating chips. Prior to beginning with the differentiation protocol is necessary to coat the surface of the channels or the multi-well plates. Thaw a Matrigel aliquot (Corning Matrigel Matrix - Growth Factor Reduced, SACCO - Corning, #354230) and prepare a dilution at 1% of Matrigel with cold DMEM/F12 (Gibco by Life Technologies, #11320074), immediately aliquot 12µl of Matrigel solution into each channel (or 1 ml for each well of a 6-well plate). Place the devices into 37°C incubator for 30 minutes or leave they at room temperature from 1 hour. After that aspirate the coating mixture and wash the channels directly with 12µl of hiPSCs medium (1 ml of Matrigel solution for each well of a 6-well plate). Seal with parafilm and store at 2-8°C overnight.

Day-2: Seeding hiPSCs. Prior to beginning pre-warm at room temperature, the proliferative medium, TeSR™-E8™ (TeSR™-E8™ Basal Medium – STEMCELL Technologies, #05941), TrypLE™ (TrypLE™ Select Enzyme (1X), no phenol red – Thermo Fisher Scientific, # 12563011), Trypsin Inhibitor (Soybean Trypsin Inhibitor, powder - Thermo Fisher Scientific, # 17075029) and remove the parafilm at the chips (or the multi-well plates) and place them into 37°C incubator for 30 minutes.

hiPSCs split: the hiPSCs culture are grown in 6-well plates; aspirate hiPSCs medium and incubate hiPSCs with 0.5 ml/well of TryPLE for 5–10 minutes at 37 °C

incubator. Once detached, add 0.5 ml of Trypsin Inhibitor and 1 ml of TeSR™-E8™ per well, transfer the cells into a 15 ml tube containing 2.5 ml of TeSR™-E8™ and centrifuge 5 minutes at 300g. Following aspirate and resuspend, the cells, in TeSR™-E8™ medium plus Rock inhibitor (Y-27632 - CAS 146986-50-7 – Calbiochem, # 688000). Typically, 330hiPSCs/mm2 are seeded and evenly distributed. Incubate cells overnight.

Day-1: Lentiviral Transduction. Note: Lentiviral aliquots of FUdeltaGW-rtTA (reverse tetracycline transactivator, Addgene ID: 19780), pTet-O-Ngn2-puro (Addgene ID: 52047) expresses Ngn2 and puromycin resistant gene under the control of TetON promoter and Tet-O-FUW-EGFP (Addgene ID: 30130) enhanced green fluorescent protein, are produced by transfection of HEK293T cells (3.2. Production of lentiviral vectors).

Prior to beginning, pre-warm hiPSCs medium in a 37 °C water bath. Thaw lentiviral aliquots on ice and add they to hiPSCs medium. Achieve lentiviral Ngn2 transduction with either FUdeltaGW-rtTA, pTet-O-Ngn2-puro transduction. As a control, use either FUdeltaGW-rtTA and Tet-O-FUW-EGFP, in order to evaluate the transfection efficiency.

To transduce hiPSCs, aspirate the media and replace with TeSR™-E8™ containing the lentiviruses. Then incubate the plate at 37 °C overnight.

Day 0: Doxycycline treatment and switch to N2B27 medium basal. Dilute Doxycycline (Sigma-Aldrich, #D9891) at 1 µg/ml concentration in N2B27 medium basal (Table 3.1) and aliquot 12µl of medium in each channel of the chip (or 2 ml/well for 6-well plate). 24 hours following doxycycline addition, GFP fluorescence can be observed in most hiPSCs.

Day 1-5: Puromycin selection. Replace media with fresh N2B27 medium basal containing 1 µg/ml puromycin (Puromycin Dihydrochloride - ThermoFisher Scientific, # A1113803) and 1 µg/ml doxycycline (12 µl per channel of a 5-channel chip or 2 ml per well of a 6-well plate).

Day 6–10: switch to iN Medium. Doxycycline need to be added every day with fresh iN medium (Table 3.1).

Day 10: Fix. Remove the medium and wash twice time with pre-warmed PBS, incubate with PFA 4% for 10 minutes. At the end of the PFA incubation aspirate and wash twice times with PBS 1X for 5 minutes. Seal with parafilm and store at 2-8°C overnight.

N2B27 medium basal		
DMEM/F-12, HEPES		ThermoFisher Scientific, # 11330-032
N-2 Supplement	100X	ThermoFisher Scientific, # 17502-001
B-27™ Supplement serum free	50X	ThermoFisher Scientific, # 17504-044
iN medium		
BrainPhys™ Neuronal Medium		StemCell Technologies, # 05790
MEM Non-Essential Amino Acids Solution	100X	ThermoFisher Scientific, # 11140-035
B-27™ Supplement serum free	50X	ThermoFisher Scientific, # 17504-044
N-2 Supplement	100X	ThermoFisher Scientific, # 17502-001
Recombinant Human/Murine/Rat BDNF	10ng/ul	Peprotech, #450-02
Recombinant Human NT-3	10ng/ul	Peprotech, # 450-03

Table 3.1: Components of neuronal differentiative media.

3.4.2. NEURONAL INDUCTION BY NGN2 ENCODING mmRNA

3.4.2.1. Cellular transfection with mmRNA

The cellular transfection is characterized from blends of cationic lipids which are able to complex the mmRNA molecules to promote an efficient delivery of the different messengers into the cells. In this work are used StemMACS transfection reagent (Miltenyi Biotec) and one specific synthetic modified mRNA encoding for Ngn2 (StemMACS Ngn2 Miltenyi Biotec).

In this work are used 2 different synthetic mmRNA encoding for nuclear GFP (StemMACS Nuclear eGFP, Miltenyi Biotec) and 1 mmRNA encoding for the transcription factor Ngn2.

The nucleic acids single strand when released into the cells promote the activation of immune system of the host cell and the consequent production of interferon type 1, promoting death cell. In order to reduce the inflammatory cell response, B18R 0.2 ng/ul has been added to the neuronal differentiate medium, 2 hours before the first transfection and for every medium changing. This kit consists of two different components: a transfection buffer and a transfection reagent stored at +4 ° C. To form the lipocomplexes of particles lipid and mRNA the two solutions must be balanced at temperature environment. The transfection mixture is prepared by mixing two solutions: the first is obtained by diluting 5X 100ng/µl of mmRNA in the transfection buffer and the second diluting 10X the reagent transfection in the transfection buffer. Then the two solutions are mixed together and incubated for 20 min before being added to the cells and left in contact with them for the desired time.

The transfection can be continued for 4 hours and after this period to remove the transfection reagent from the cells is necessary to change the medium and replace it with fresh medium added with protein B18R to extend the protective effect against the cellular immune response. The analysis of the

transfection can be carried out 24 hours after the beginning of the transfection, when there is the peak of expression of the transfected mmRNAs.

With conventional devices, the mix of transfection must be added drop by drop over the cells and distributed into uniform way, while with microfluidic platform the quantities, of the different components, they must be adapted to the volumes applied in microfluidics. Furthermore, into the different microfluidics channels cannot be injected transfection mix, alone, but it must be previously diluted in culture medium. The volumes used in microfluidics are very small (5.4 µl total over the culture), therefore is necessary to increase the ratio of transfection mix/cellular medium compared to conventional devices without fell into in a significant wastefulness of reagents and increasing noticeably the overall efficiency of the process. The scale down has been done maintaining the same proportions between mmRNA, transfection buffer and transfection reagent, but decreasing the amount of medium in which the transfection mix is dissolved. In this work, transfection mixes have been used with a medium volume equal to 75%, 50% and 25% compared to traditional devices. Keeping in mind volume of medium present above the cells, at such ratios correspond the following absolute quantities of mmRNA: 5.5 pg mmRNA/100cell, 9 pg mmRNA/100cell e 16 pg mmRNA/100cell.

The working concentration is influenced by the experiment and the cell type used: for example, if for the experiment is necessary perform repeated transfections over time, it is advisable to start with a more diluted transfection mix to preserve cell viability (transfection stresses cells and can give cellular mortality if carried out at too high concentrations), then the concentration can be raised to increase the efficiency. To carry out a single transfection, can be used directly a very concentrated mix to obtain a high expression of the transfected gene.

3.4.2.2. Neuronal differentiation protocol

<u>*Day-1: B18R treatment*</u>. Before to start with the differentiation protocol of neurons is important to treat the hiPSCs cultures with B18R to reduce the immunological activity of the cells. Pre-warm at room temperature, the proliferative medium, TeSR™-E8™ (TeSR™-E8™ Basal Medium – STEMCELL Technologies, #05941) containing 0.2ng/ul of B18R (Vaccinia Virus B18R Carrier-Free Recombinant Protein – eBioscience™, # 34-8185-81) and place them into 37°C incubator overnight

<u>*Day 0: Seeding hiPSCs*</u>. *Coating chips*. Prior to beginning with the differentiation protocol is necessary to coat the surface of the channels or the multi-well plates. Thaw a Matrigel aliquot (Corning Matrigel Matrix - Growth Factor Reduced, SACCO - Corning, #354230) and prepare a dilution at 1% of Matrigel with cold DMEM/F12 (Gibco by Life Technologies, #11320074), immediately aliquot 12µl of Matrigel solution into each channel (or 1 ml for each well of a 6-well plate). Place the devices into 37°C incubator for 30 minutes or leave they at room temperature from 1 hour. After that aspirate the coating mixture and wash the channels directly with 12µl of hiPSCs medium (0.2 ml of Matrigel solution for each well of a 96-well plate).

Mix transfection preparation. As explain before, in the section 3.4.2.1. Cellular transfection with mmRNA, two different mix must be prepared:

1) MIX1 characterized from transfection buffer + mmRNA
2) MIX2 characterized from transfection buffer + transfection reagent

at the end, gently put the MIX2 into the MIX1 and incubate for 20 minutes at room temperature.

In the meantime, pre-warm at room temperature, the proliferative medium, TeSR™-E8™ (TeSR™-E8™ Basal Medium – STEMCELL Technologies, #05941), TrypLE™ (TrypLE™ Select Enzyme (1X), no phenol red – Thermo Fisher Scientific, # 12563011), Trypsin Inhibitor (Soybean Trypsin Inhibitor, powder -

Thermo Fisher Scientific, # 17075029) and remove the parafilm at the chips (or the multi-well plates) and place them into 37°C incubator for 30 minutes.

hiPSCs split: the hiPSCs culture are grown in 6-well plates; aspirate hiPSCs medium and incubate hiPSCs with 0.5 ml/well of TryPLE for 5–10 minutes at 37 °C incubator. Once detached, add 0.5 ml of Trypsin Inhibitor and 1 ml of TeSR™-E8™ per well, transfer the cells into a 15 ml tube containing 2.5 ml of TeSR™-E8™ and centrifuge 5 minutes at 300g. Following aspirate and resuspend, the cells, in TeSR™-E8™ medium plus Rock inhibitor (Y-27632 - CAS 146986-50-7 – Calbiochem, # 688000). Typically, 330hiPSCs/mm2 are resuspended in TeSR™-E8™, comprehensive of the transfection mix, and seeded. Incubate cells overnight (12 µl per channel of a 5-channel chip).

Day 1-4: N2B27 medium. Switch to N2B27 medium (Table 3.2) containing B18R.

Day 5-9: Neuronal maturation medium. Replace media with fresh N2B27 medium containing 10 ng/ml of Brain-derived neurotrophic factor (BDNF), 10 ng/ml of Neurotrophin-3 (NT3) and B18R.

Day 10: Fix. Remove the medium and wash twice time with pre-warmed PBS, incubate with PFA 4% for 10 minutes. At the end of the PFA incubation aspirate and wash twice times with PBS 1X for 5 minutes. Seal with parafilm and store at 2-8°C overnight.

N2B27 medium		
DMEM/F-12, HEPES		ThermoFisher Scientific, # 11330-032
N-2 Supplement	100X	ThermoFisher Scientific, # 17502-001
Insulin	5ug/ml	
Neurobasal® Medium, minus phenol red and L-Glut		ThermoFisher Scientific, # 12348-017
2-Mercaptoethanol (50 mM)	500X	ThermoFisher Scientific, # 31350-010

glutamine		
MEM Non-Essential Amino Acids Solution	100X	ThermoFisher Scientific, # 11140-035
B-27™ Supplement serum free	50X	ThermoFisher Scientific, # 17504-044

Table 3.2: Components of neuronal medium

3.4.3. NEURONAL DIFFERENTIATION VIA DUAL-SMAD INHIBITIONS

The protocol described in this section is the same of the paragraph 3.4.2.2. Neuronal differentiation protocol with the unique difference that the cells receive the small molecules, such as 3uM dorsomorphin (Sigma-Aldrich, #P5499-5MG) and 10uM SB 431542 (Sigma-Aldrich, #S4317-5MG), in the Days 1-2 on one hand or Days 1-9 on the other hand

3.4.4. NEURAL STEM CELL GENERATION VIA DUAL-SMAD INHIBITION

Cells with a confluence about of 80% are splitted and seed as the protocols describe in the section 3.1.2. Human induced pluripotent stem cells. Incubate cells overnight.

Day 0-4: Dual-SMAD inhibition treatment. When cells have reached a 100% confluency remove the proliferative medium TeSR™-E8™ and switch to N2B27 medium (Table 3.2) containing 3uM dorsomorphin (Sigma-Aldrich, #P5499-5MG) and 10uM SB 431542 (Sigma-Aldrich, #S4317-5MG). After this period, the cells have been used for the differentiation protocol of neurons by synthetic mmRNA encoding for Ngn2.

3.5. CELL CULTURE ANALYSES

This section reports the procedure for statistical analyses and the protocol for immunodetection and histochemical assays performed on cell cultures.

3.5.1. STATISTICAL ANALYZES

Data are presented as means ± standard error of means (SEM). Data pairs were compared by non-directional Student's t-test, while group data by one-way ANOVA followed by Bonferroni's mean comparison. All data manipulation and computation has been performed with GraphPad Prism 7.00 software.

3.5.2. WESTERN BLOT IN MICROSCALE

Cell lysate is obtained by solubilizing the cells of each channel with 10 µl of lysis solution, composed of 5% deoxycholic acid (DOC, Sigma-Aldrich) and Complete protease inhibitor (Roche).

SDS-PAGE electrophoresis is performed with gradient gel polyacrylamide 4-12% NuPAGE (Life Technologies) and MOPS travel buffer (Life Technologies). Preparation of samples obtained from platforms microfluidics plans to add 5 µl of protein buffer to 13 µl of protein extract loading (Life Technologies) and 2 µl of reducing agent (Life Technologies). The electrophoresis is conducted at 150 V for 1 h and 45 min, and then the proteins separated are transferred to a polyvinylfluoride membrane (PVDF, Life Technologies) for 2 h at 45 V and 400 mA. The transfer is confirmed with the Ponceau colouring.

Once the membrane is prepared, the saturation of sites is performing by

binding with a 5% milk-based locking solution in TTBS (Tris-buffer saline and 0.1% Tween 20, Sigma-Aldrich) for 40 min at room temperature. After saturation the membrane is incubated overnight at +4 ° C with the primary antibody diluted in milk 5%. Followed by 3 5-minute washes with TTBS and during the third wash, the secondary antibody dilution is carried out 5% milk. Incubation with the secondary antibody occurs for 40 min a room temperature. Followed by a further 3 5-minute washes with TTBS.

The identification of the target protein occurs through chemioluminescence: the development solution (Novex ECL Chemiluminescent Substrate Reagent Kit, Life Technologies) is oxidized by HRP peroxidase conjugated to secondary antibody (mouse, Bio-Rad, rabbit, Life Technologies). The emitted luminescence, of the secondary antibody, is detected by a photographic plate (Carestream films, Kodak) and the signal obtained is analyzed with the software ImageJ (Nih).

Signal strength is proportional to the amount of target protein contained in the extract but must be normalized on the signal given by one housekeeper protein (GRP75 in our case) to correct any differences in protein quantity in different samples.

3.5.3. IMMUNOFLUORESCENT PROTOCOL

3.5.3.1. Immunostaining of neurons

For immunostaining, medium was removed per channel, to which 12 µl of 4% paraformaldehyde (PFA) was directly added. The PFA solution was incubated for 10 min at room temperature. Following aspiration of the pfa, 3 washes with permeabilization buffer (PBS containing ca2+ and mg2+ thermofisher scientific was performed. After that PBS supplemented with 0.1% triton-X-100 (Sigma-Aldrich, #93426) and 5% donkey serum (Sigma-Aldrich, # S30-100ML) was gently

added. Neurons were incubated with blocking/permeabilization buffer for 1 hour at room temperature.

During blocking and permeabilization, the primary antibody (Table 3.3) solution was prepared in ice-cold blocking/permeabilization buffer. Neurons were incubated with the primary antibody solution overnight at 4 °c.

Antibody	Species	Dilution ratio	Company (catalog #)
Nestin	Mouse	1:100	Millipore (MAB5326)
p75	Rabbit	1:2000	Promega (G323A)
Sox2	Rabbit	1:400	Millipore (AB5603)
Ngn2 (D2R3D)	Rabbit	1:100	Cell Signalling (13144S)
Map2	Mouse	1:1000	Sigma-Aldrich (M2320)
Sox1	Goat	1:50	Abcam (AF3369)
Pax6	Rabbit	1:300	BioLegend (901301)
Oct4-3/4 (C-10)	Mouse	1:200	Santa Cruz Biotechnology (sc-5279)
Tubb3 (clone Tuj1)	Mouse	1:5000	BioLegend (801202)
Alexa Fluor™ 594 donkey	Mouse	1:200	Jackson immuno. (715-165-151)
Alexa Fluor™ 594 donkey	Goat	1:200	Jackson immuno. (705-165-147)
Alexa Fluor™ 594 donkey	Rabbit	1:200	Jackson immuno. (711-585-152)
Alexa Fluor™ 488 donkey	Mouse	1:200	Jackson immuno. (715-545-151)
Alexa Fluor™ 488 donkey	Goat	1:200	Jackson immuno. (705-545-147)
Alexa Fluor™ 488 donkey	Rabbit	1:200	Jackson immuno. (711-545-152)
Alexa Fluor™ 647 donkey	Mouse	1:200	Jackson immuno. (715-605-151)
Alexa Fluor™ 647 donkey	Goat	1:200	Jackson immuno. (705-605-147)
Alexa Fluor™ 647 donkey	Rabbit	1:200	Jackson immuno. (711-605-152)

Table 3.3: List of antibodies.

Following incubation, the primary antibody solution was removed, and the chips were washed with ice-cold PBS with 0.1% triton-X-100 three times as described above.

Neurons were then incubated with secondary antibody (Table 3.3) prepared in ice-cold blocking/permeabilization buffer for one hour at room temperature, followed by two washes with PBS with 0.1% triton-X-100 and one with only PBS. Nuclei were counterstained with Hoechst (1:5000 - Life Technologies, #H3570) for 5 min and then neurons were washed with PBS three additional times.

3.5.3.2. Preparation of neurons for confocal microscopy

Fluoroshield™ with DAPI - histology mounting medium (Sigma-Aldrich, # F6057-20ML) has been equilibrated to room temperature. One drop (~12 µl) of mounting solution was placed onto each channel. Mounted chips have been air-dried in the dark overnight at room temperature. Pictures were taken by epifluorescence Leica DMI6000B microscope equipped with a mercury short-arc reflector lamp or Leica SP5 confocal microscope equipped with Ar laser.

Chapter 4:

RESULTS

4.1. DOWNSCALING OF NGN2-MEDIATED NEURONAL INDUCTION VIA LENTIVIRAL TRANSDUCTION ON CHIP

This section will describe in detail the protocol and the results related to the neuronal induction of the hiPSCs using a traditional cell culture device and the microfluidic system.

First of all, we defined the titre of the batch of virus that we produced, taking as reference a constitutive lentiviral vector encoding for GFP. This allows us to have an idea of the titre of all the different viruses produced in the same batch and it is important to fix the correct viral concentration that allows obtaining with high efficiency neuronal conversion.

Briefly, we seeded 75 cells/mm^2 and six different scalar virus dilutions (10^{-3}-10^{-8}) have been tested. After 3 days from the infection, cells have been collected and underwent cytofluorimetric analysis (Fig. 4.1).

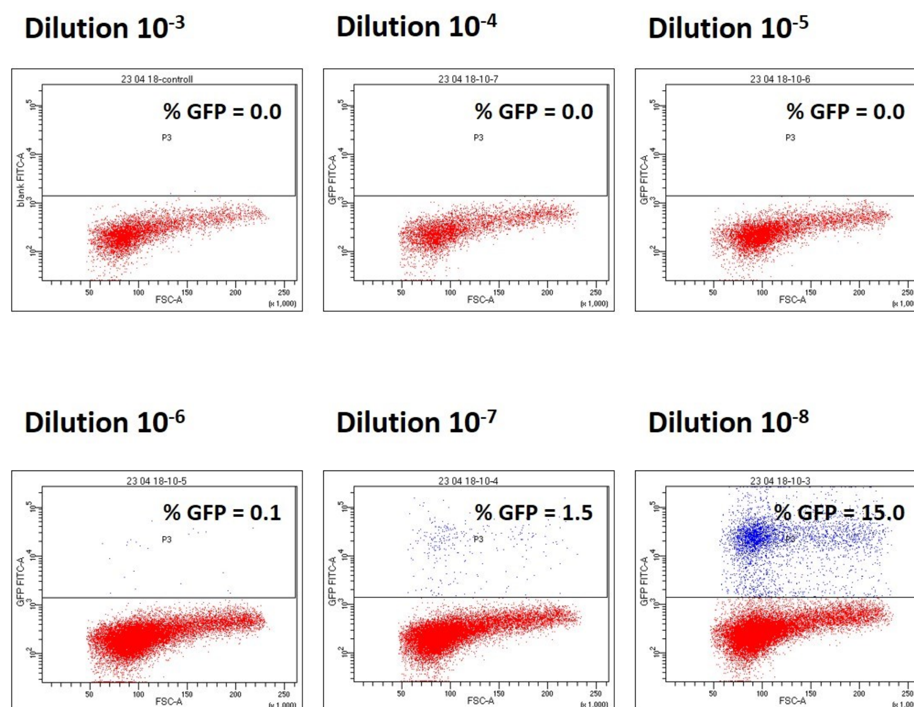


Figure 4.1: Flow cytometric analysis of expression of GFP protein. The dot plot diagrams represent the mean fluorescence intensity (MFI) of GFP. The upper quadrants show the percentage of GFP-positive cells related to the different concentration of virus used to infect the hiPSCs.

Taking advantage of the GFP expression level, we estimated the percentage of infected (GFP expressing) cells and we calculated the viral titre expressed as total transducing units per ml (TU/ml) corresponding to 1.13E+07 TU/ml. Since we produced in parallel different viral vectors encoding for different transgenes, we can assume that the different lentiviruses necessary for the protocol of neuronal induction would have a similar titre. This allows us to calculate the number of microliters of viruses that need to be used to obtain a defined number of integrations per cell (multiplicity of infection, MOI).

We are now performing a more detailed titration of the viruses using specific primers that recognize only the lentiviral sequence and taking advantage of two different cell lines containing a known number of lentiviral copies as reference.

To promote the conversion of hiPSCs into neuronal-like cells we took advantage of a lentiviral inducible system, named Tet-On system, based on the use of two different lentiviral constructs: one carrying a constitutive expression of tetracycline-controlled transactivator (rtTA), that binds to the DNA and activates gene expression only in the presence of Tetracycline (or its derivative Doxycycline (DOX)) and one carrying the tetracycline-inducible expression of Ngn2 together with a puromycin resistance to select only cells expressing the transgenes (Fig. 4.2).

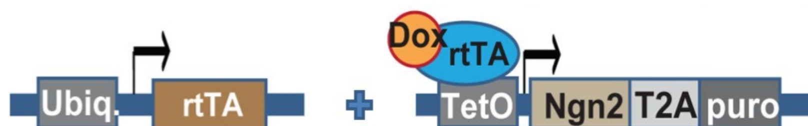


Figure 4.2: Lentiviral vectors design. Cells are transduced with a first virus expressing rtTA (a) and with a second combination of two different viruses one expressing Ngn2-P2A-puromycin and one expressing EGFP. Figure modified from (Zhang et al., 2013).

Before starting the neural induction experiments, we tested the transfection efficiency of the Tet-On system using a lentiviral construct expressing GFP protein under the control of Tet-O responsive region (Fig. 4.3-a) and counting the number of GFP-positive cells upon infection of 75000 cells with two different MOI, 0.012 and 0.023.

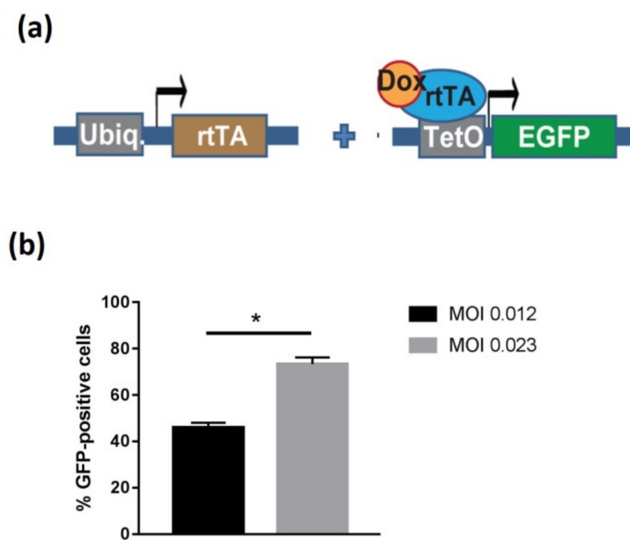


Figure 4.3: transfection efficiency. (a) hiPSCs are transduced with a virus expressing rtTA and a virus expressing Tet-O-EGFP. Figure modified from (Zhang et al., 2013). (b) Graph showing the percentage of neuronal-like cells induced with the two different MOI. n=3 random fields are analysed for each independent experiment; *p<0.05.

As we can see in Figure 4.3 (b) the MOI 0.012 promoted a lower transfection efficiency compared to the MOI 0.023, resulting in a 48.9% ± 2.14 [mean ± SEM] (n=3) and in a 73.4% ± 2.8 [mean ± SEM] (n=3) of GFP-positive cells respectively at day 5, counted on the total of cells/field.

To convert hiPSCs into neurons via Ngn2 overexpression (Fig. 4.4), hiPSCs were plated at day −2, infected with the two lentiviruses at day −1, and Ngn2 expression induced with doxycycline (2.5 ug/ml) at day 0. After 24 h from the induction (day 1), the puromycin selection period was started and maintained until day 5.

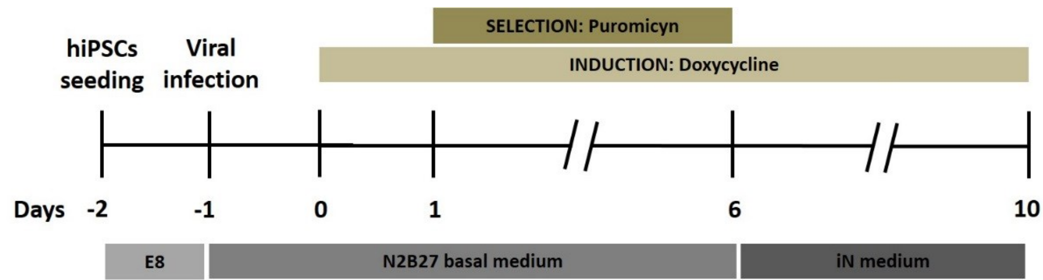


Figure 4.4: Time course for Ngn2 based neuronal conversion. Schematic representation of the timing used for the neurons induction starting from hiPSCs. Three different media were used: TeSR™-E8™ Medium (E8), N2B27 medium and iN medium, for the composition see section Material and Methods (3.4.1. Generation of neurons via lentiviral Ngn2-overexpression).

Based on the results obtained with Tet-O-eGFP, we tested two different MOI to try to improve neuronal conversion of iPSCs: 0.023 and 0.045. As we can see in Figure 4.5, the MOI 0.023 promoted a neuronal differentiation with lower efficiency compared with the MOI 0.045, resulting in a 50.5% ± 5.82 [mean ± SEM] (n=3) and in a 98.7% ± 2.3 [mean ± SEM] (n=3) of neuronal-like cells respectively at day 5, counted on the total of cells/field. So, we decided to use the MOI of 0.045 as condition for neuronal induction, and we characterized the whole process step by step and the neuronal-like cells obtained at the end of the process by means of immunofluorescence.

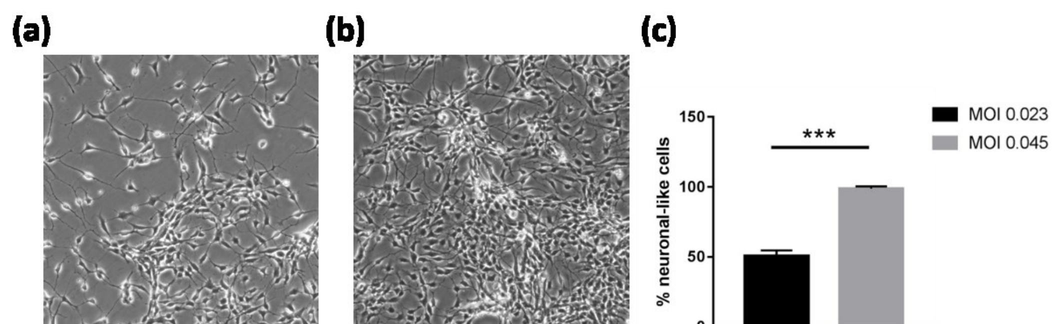


Figure 4.5: Efficiency of neuronal generation in conventional system. Neuronal-like cells obtained with (a) MOI 0.23 and (b) MOI 0.045; (c) graph showing the percentage of neuronal-like cells induced with the two different MOI; ***p<0.001.

As we can see in Figure 4.6 at day 1, only 24 hours after the induction with DOX, the cell morphology was still round with a big nucleus, characteristic of pluripotent stem cells, and cells were still organized in compact colonies. Proceeding with the protocol of neuronal differentiation, the cells started moving out from the compact iPSC colony with an epithelial to mesenchymal transition and acquired a more elongated morphology, assuming at the end of the process the typical neuronal conformation, where is possible distinguish a central body (soma) and the neurites that depart from the soma of neurons.

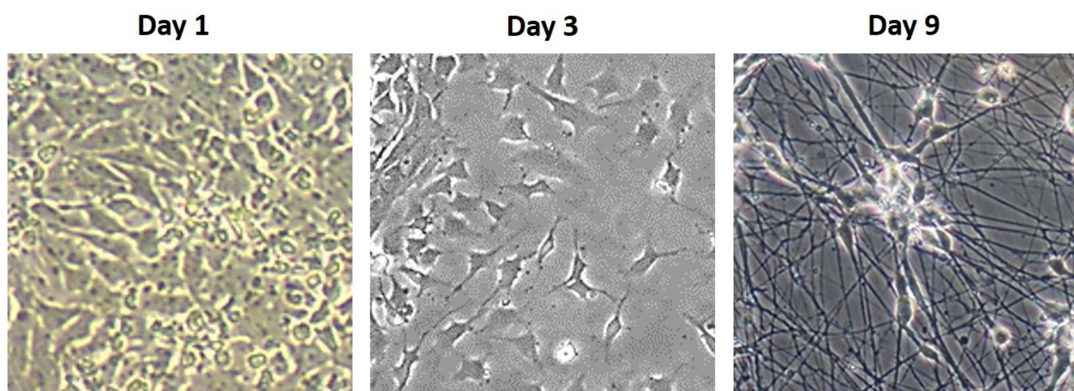


Figure 4.6: Induction of neurogenesis via lentiviral transduction of Ngn2. Time course of morphological changes during the conversion of hiPSCs into neuronal-like cells.

In Figure 4.7 we can appreciate that, after 10 days of differentiation, pluripotency markers (i.e. Oct4) are not expressed anymore, indicating a transition from a pluripotent to a differentiated state. To confirm the neuronal identity of the differentiated cells, we performed stainings for typical neuronal markers such as Nestin (neural progenitors), βIIITubulin (immature neurons), Map2 and NeuN (mature neurons) and to exclude the transdifferentiation in astrocytes (GFAP). As we can see in Figure 4.7, consistent with the morphological conversion of hiPSCs into neuronal-like cells, cells show a homogeneous expression of all these markers. To further evaluate the maturation level of the neuronal cells obtained, we

investigated the expression of Synapsin (pre-synaptic) and PSD95 (post-synaptic) markers. However, as we can see in Figure 4.7, a clear signal is not present yet.

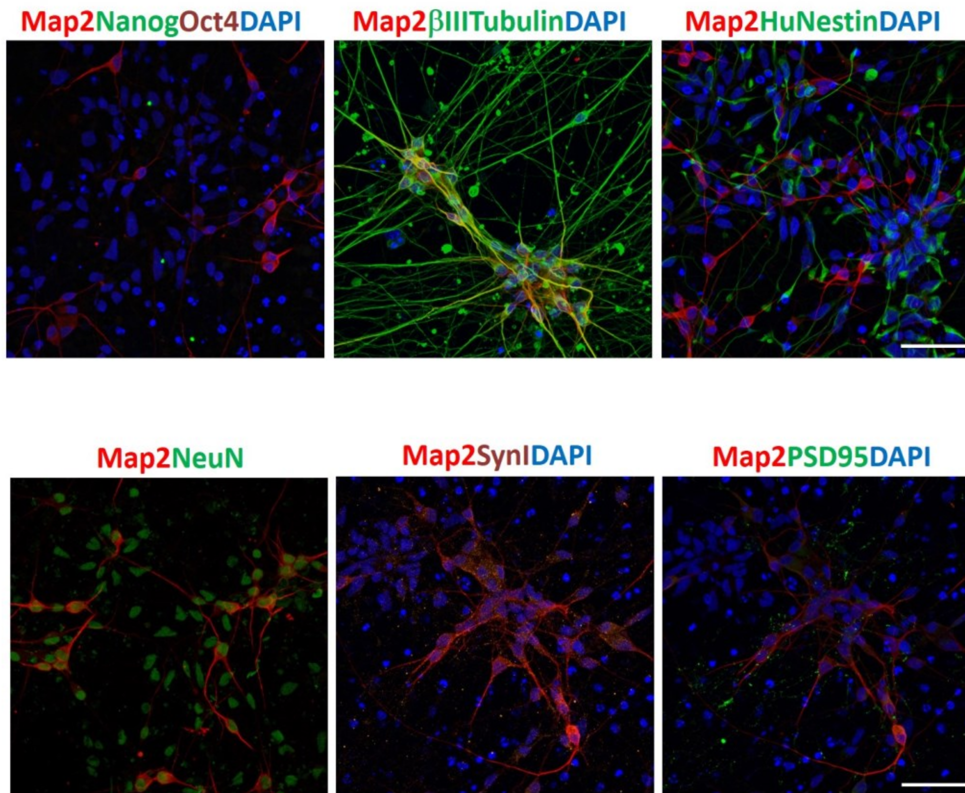


Figure 4.7: Induction of neurogenesis via lentiviral transduction of Ngn2 on conventional devices. Representative images of pluripotent markers (Nanog and Oct4), immature neurons markers (βIIITubulin and HuNestin), mature neurons markers (Map2 and NeuN), presynaptic marker (SynI), and postsynaptic marker (PSD95) staining at Day 10 of the differentiation protocol, in well, obtained with MOI 0.045. Scale bar 100um.

These data, all together demonstrated that the forced of the Ngn2 expression, alone, converts hiPSCs cells into neuron-like cells in less than two weeks (10 days).

After we tested the ability of the Tet-O-Ngn2 system to induce neuronal induction in traditional cell culture devices, we tested the same protocol in micro-scale taking advantage of the microfluidic platform. This system requires only few

microliters per day of differentiating medium compared with a six-well plate. Furthermore, this platform permits a homogenous delivery of soluble factors to the cells and allows to perform several experiments in parallel.

We tested, in parallel, the same MOI previous investigated in a conventional cell culture system, 0.023 and 0.045.

In Figure 4.8 is possible observed that we were able to generate neuronal-like cells in microfluidic platform and that the efficiency of neuronal induction, obtained from the two MOI of virus used were comparable.

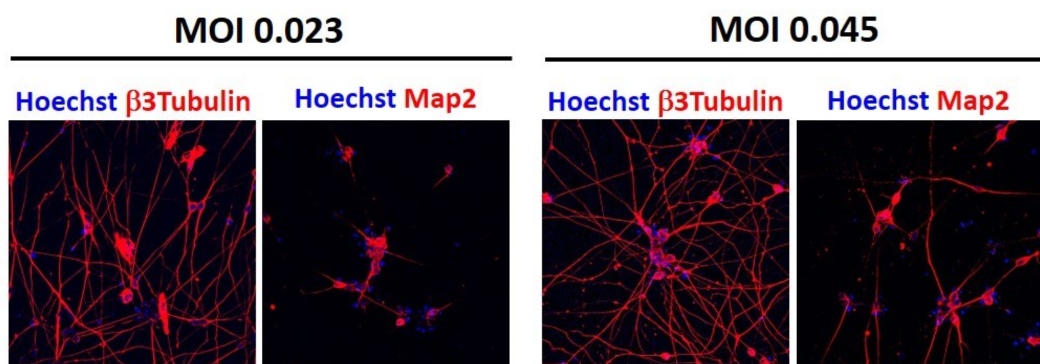


Figure 4.8: Induction of neurogenesis via lentiviral transduction of Ngn2 on chip. Representative images of βIIITubulin and Map2 staining at Day 10 of the differentiation protocol, on chip, obtained with both MOI, 0.023 and 0.045. Scale bar 50um.

As previously observed in conventional culture system, also in microfluidics cells don't express stem-cells markers anymore (data not shown), but neuronal markers are homogeneously present indicating a transition from a pluripotent to a differentiated state.

4.2. TIME-DEPENDENT NGN2 GENE EXPRESSION REGULATED VIA DOXYCYCLINE-INDUCIBLE LENTIVIRAL SYSTEM

As we mentioned in paragraph 1.1.2. (chapter 1), in the early neurogenesis, Ngn2 expression oscillates with a period of ~4 hours, maintaining a correct equilibrium between neural stem cells and mature neurons (Shimojo et al., 2008). This is an important condition for the correct brain development (Kageyama et al., 2008). So, we took advantage of the TetOn system, described in the first section of this chapter, to regulate the timing and the pattern of Ngn2 expression trying to mimic, in vitro, the oscillatory profile of Ngn2. In particular we induced Ngn2 expression in hiPSCs modulating DOX administration.

In brief, after 24 hours from the transfection with the lentivirus, iPSCs were induced to express Ngn2 via doxycycline administration. Since we wanted to induce oscillations, we combined treatments with DOX of different duration with different time points of protein extraction (Table 4.1).

	Time before extracting proteins							
Duration of DOX induction								
30 minutes	0 h	30 min	1 h	2 hrs	4 hrs	8 hrs	16 hrs	24 hrs
1 hour	-	30 min	1 h	2 hrs	4 hrs	8 hrs	16 hrs	24 hrs
2 hours	-	30 min	1 h	2 hrs	4 hrs	8 hrs	16 hrs	24 hrs
4 hours	-	30 min	1 h	2 hrs	4 hrs	8 hrs	16 hrs	24 hrs
24 hours	-	30 min	1 h	2 hrs	4 hrs	8 hrs	16 hrs	24 hrs

Table 4.1: Scheme of the time points used to recreate the Ngn2 oscillatory pattern. The incubation with DOX was performed for 30 min, 1 h, 2 h, 4 h and 24 h, and protein extraction was performed with different time points after the end of each incubations.

In detail, as describe in Table 4.1, we analysed 5 different treatment paradigms: administration of DOX for 30 min, 1h, 2h, 4h and 24h. After these pulses of

different length, cells have been lysated immediately (0 min), after 1 h, 2 h, 4 h, 16 h, 24h and the Ngn2 expression level analysed via western blot (Fig 4.9-4.13). In all these different experiments we observed that changing the duration of the DOX pulse we have been able to induce an oscillatory expression of Ngn2 with a different frequency. In general, the longer was the DOX treatment, the longer was the period between two Ngn2 peaks.

As shown in Figure 4.9 stimulating the cells with the DOX for 30 minutes was enough to induce an oscillation with a period of 16h. We observed a peak of Ngn2 expression after 1 hour from the end of the stimulus (time point 30 min + 1 h), dropping after 7h (time point 30 min + 8 h), rising again after 8h (time point 30 min +16 h) and finally dropping after additional 8h (time point 30 min + 24 h).

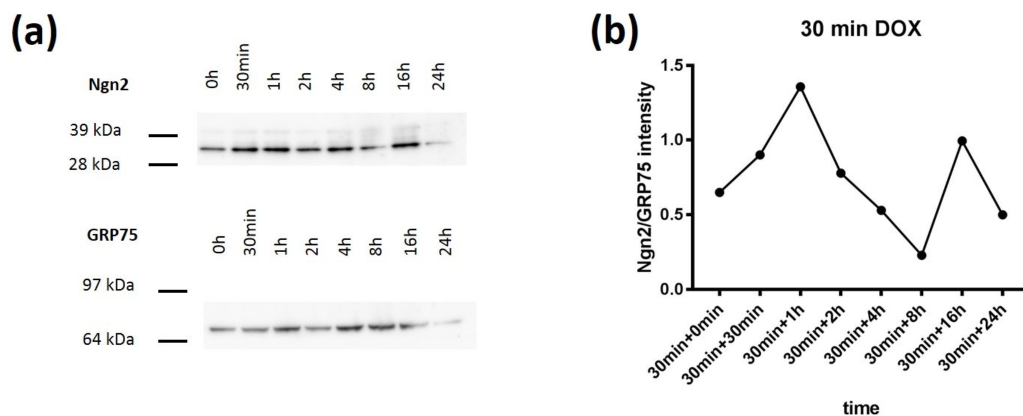


Figure 4.9: Time course of Ngn2 protein. (a) Expression of Ngn2 and GRP75 in lysates of hiPSCs transfected with lentivirus encoding for Ngn2. Representative images of the immunoblot for Ngn2 and GRP75. n=pull of 3 technical replicates (b) Graph of the oscillatory trend of the Ngn2 expression after 30 min of DOX stimulation. Shown is the ratio between the intensity of the band of the house keeping protein GRP75 and the intensity of Ngn2.

Then we gave a longer DOX stimulus and we treated the cells for 1 h (Fig. 4.10). However, in this case we observed a peak of Ngn2 protein level 4h after the end of the stimulus (time point 1 h + 4 h) followed by a drop 4h later (time point 1 h + 8 h), showing a delay in the Ngn2 increase as compared to the 30 min treatment.

Unfortunately, the data for the time points 1 h + 16 h and 1 h + 24 h are not clear enough to evaluate an oscillatory behaviour.

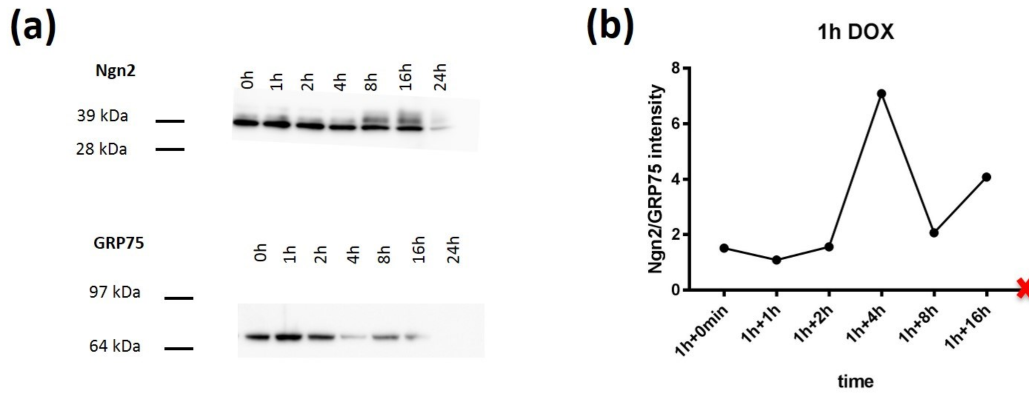


Figure 4.10: Time course of Ngn2 protein. (a) Expression of Ngn2 and GRP75 in lysates of hiPSCs transfected with lentivirus encoding for Ngn2. Representative images of the immunoblot for Ngn2 and GRP75. n=pull of 3 technical replicates (b) Graph of the oscillatory trend of the Ngn2 expression after 1 h of DOX stimulation. Shown is the ratio between the intensity of the band of the house keeping protein GRP75 and the intensity of Ngn2. Red cross for undetectable value.

Once we treated the cells for 2 h we observed a further delay in the increase of Ngn2 protein level (peak at time point 2 h + 8 h) followed by a decrease 8 h later (time point 2 h + 16 h). The blot for the time point 2 h + 24 h was good enough to be analysed (Fig. 4.11).

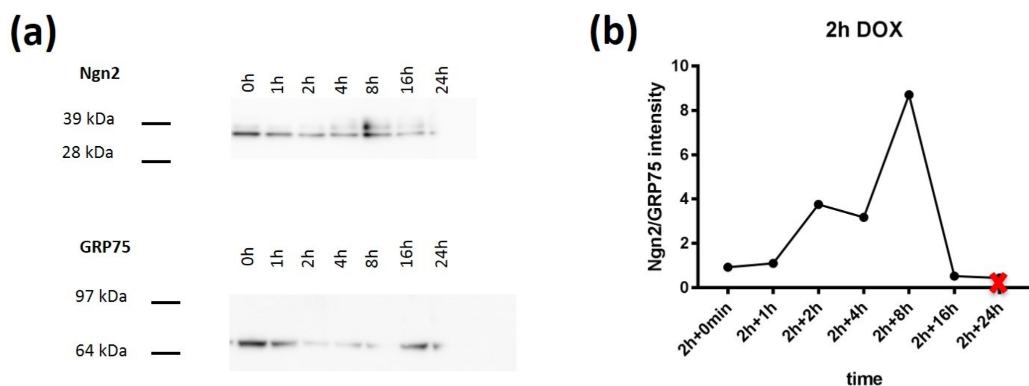


Figure 4.11: Time course of Ngn2 protein. (a) Expression of Ngn2 and GRP75 in lysates of hiPSCs transfected with lentivirus encoding for Ngn2. Representative images of the immunoblot for Ngn2 and GRP75. n=pull of 3 technical replicates (b) Graph of the oscillatory trend of the Ngn2 expression after 2 h of DOX stimulation. Shown is the ratio between the intensity of the band of the house keeping protein GRP75 and the intensity of Ngn2. Red cross for undetectable value.

In Figure 4.12 is illustrated the expression pattern of Ngn2 protein after 4 h of stimulation. In this case at the first time-point (4 h + 0 min) the intensity of the Ngn2 expression is high and gradually drops reaching the minimum level 8 h after the end of the stimulus (time point 4 h + 8 h) and increasing again in the following time points (4 h + 16 h and 4 h + 24 h). We can hypothesize that what we observe at the 4h + 0min time point is the beginning of the degradation phase of the protein, which takes about 8 hours, after which we can observe an increase in the intensity of Ngn2 expression that, however, does not reach the level protein level showed at the beginning. This suggest that, Ngn2 protein level would have further increased if we would have kept track of a longer time frame, and the oscillation period could be of around 24 h.

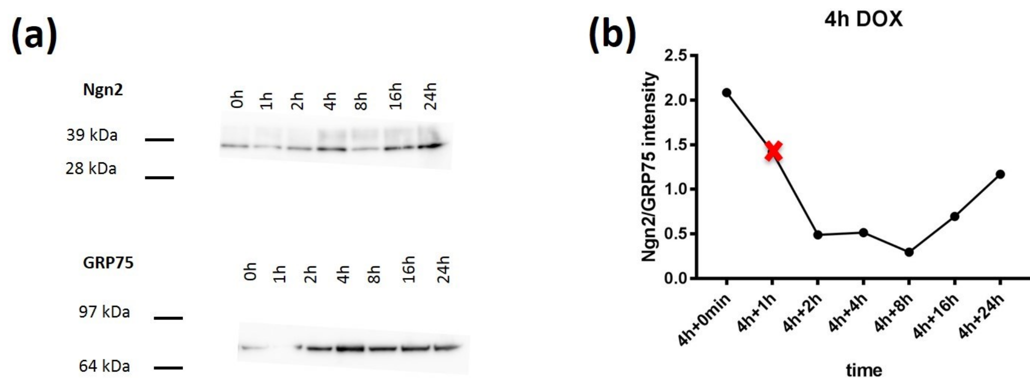


Figure 4.12: Time course of Ngn2 protein. (a) Expression of Ngn2 and GRP75 in lysates of hiPSCs transfected with lentivirus encoding for Ngn2. Representative images of the immublot for Ngn2 and GRP75. n=pull of 3 technical replicates (b) Graph of the oscillatory trend of the Ngn2 expression after 4 h of DOX stimulation. Shown is the ratio between the intensity of the band of the house keeping protein GRP75 and the intensity of Ngn2. Red cross for undetectable value.

Finally, we stimulated the cells for 24 h and we observed a gradual decrease in the amount of Ngn2 protein, reaching the lowest level after 16 h from the end of the stimulus and rising again at the following time point (24 h + 24 h) analysed (Fig. 4.13). This slow decay pattern of protein expression intensity that took about 16 h is indicative of the fact that the stimulation was carried out for 24 hours and the protein was produced continuously with a consequent accumulation, leading to a slower degradation. However, as it happened for the previous stimulation paradigm, the time points analysed were too short to allow the completion of this longer oscillation and to allow the visualization of a clear oscillatory pattern.

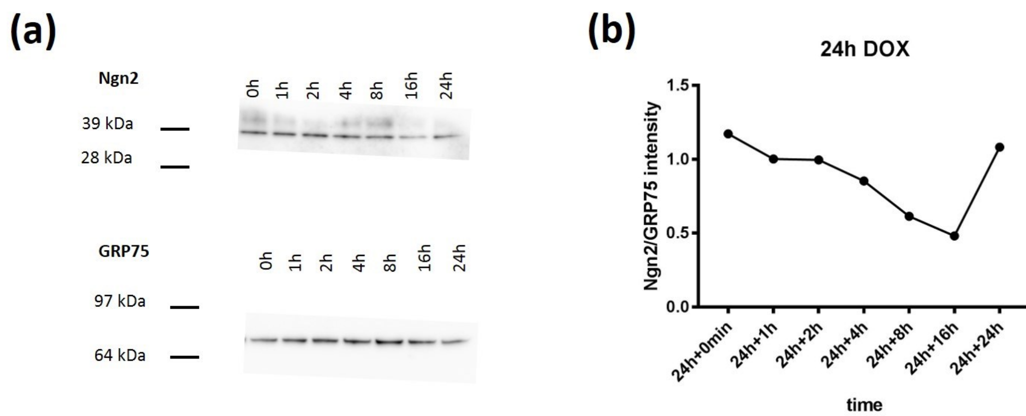


Figure 4.13: Time course of Ngn2 protein. (a) Expression of Ngn2 and GRP75 in lysates of hiPSCs transfected with lentivirus encoding for Ngn2. Representative images of the immublot for Ngn2 and GRP75. n=pull of 3 technical replicates (b) Graph of the oscillatory trend of the Ngn2 expression after 24 h of DOX stimulation. Shown is the ratio between the intensity of the band of the house keeping protein GRP75 and the intensity of Ngn2.

From these data we can conclude that the Tet-O system is suitable to reproduce in vitro an oscillatory pattern of Ngn2 gene expression. Moreover, modulating the stimulation length it is possible to impose a Ngn2 oscillatory expression with a defined period.

4.3. HIGH EFFICIENCY GENERATION OF NGN2-INDUCED NEURONS USING AN AUTOMATED MICROFLUIDIC DEVICE

Complex biological applications requiring precise timing of operations and many technical replicates are difficult to be manually performed by an operator and the experiment described in the previous section is a clear example.

In this last section of the results we took advantage of an automated micro-device to generate neuronal-like cells starting from an iPSC line stably infected with an inducible lentiviral vector expressing Ngn2.

The automated chip is a multilayer platform, formed by two principal layers: first layer composed by one outlet and two inlets connected with a particular structure of channels, in order to guarantee a correct control of the fluid inside the platform through the microvalves. This microfluidics design is due to the fact that while the first inlet allows the entry of fluid into the chambers, the second inlet functions as a kind of by-pass: keeping the valve closed, it is possible to discharge, by capillarity, the liquid contained inside the pressurized vial without having to change it. The second layer, the control layer (in yellow), is composed by two channels that intersect orthogonally the flow channels in order to ensure the correct functioning of the microvalves. As shown in Figure 4.14, this chip is characterized by 8 identical chambers of a rectangular form without angles, to avoid dead volume of fluid. The dimensions of the chambers are 26x2x0.25 mm with a surface of about 40 mm^2 and a volume of about 11 µl.

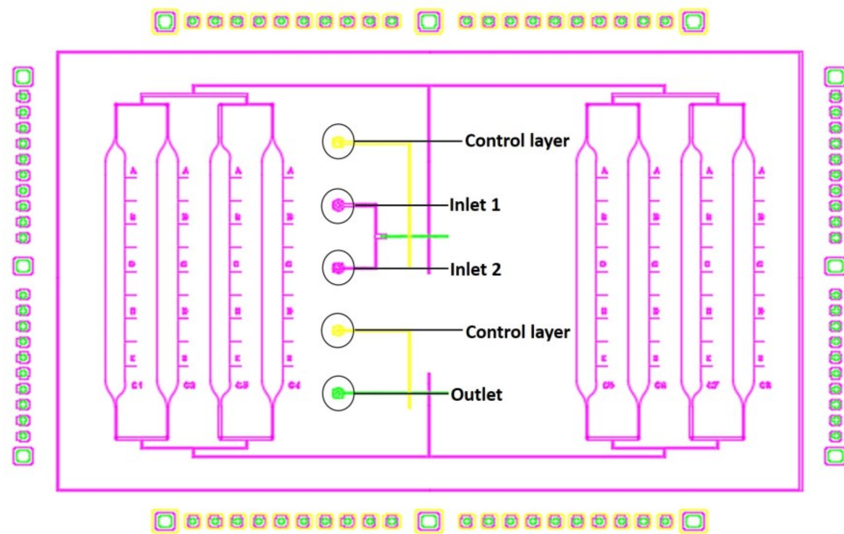


Figure 4.14: Design of the microfluidic platform. The flow layer characterized by two inlets are reported in violet, the flow layer characterized from the outlet is reported in green and the control layers are reported in yellow.

Previous experiments performed in our laboratory already validated this automated microfluidic device, demonstrating that cells (i.e. human fibroblasts) are able to attach and grow inside the platform for an extended period of time, and giving the proof that this platform is suitable for biological applications. So, taking advantage of this automated system, we investigated whether these automated devices were suitable for more complex experiments as the generation of hiPSC-derived neurons.

In brief, we took advantage of the hiPSC line used in the previous paragraph to measure the Ngn2 expression upon DOX treatment, which were stably infected with a lentiviral vector tested in the paragraph 4.1. Using the automated device, we seeded 500 cells/mm^2 and we started the neuronal induction via DOX treatment (Fig. 4.15). Since we used an automated device, we set the medium change to occur every 12 h, to reproduce the same medium change frequency that we usually adopt in the non-automated systems.

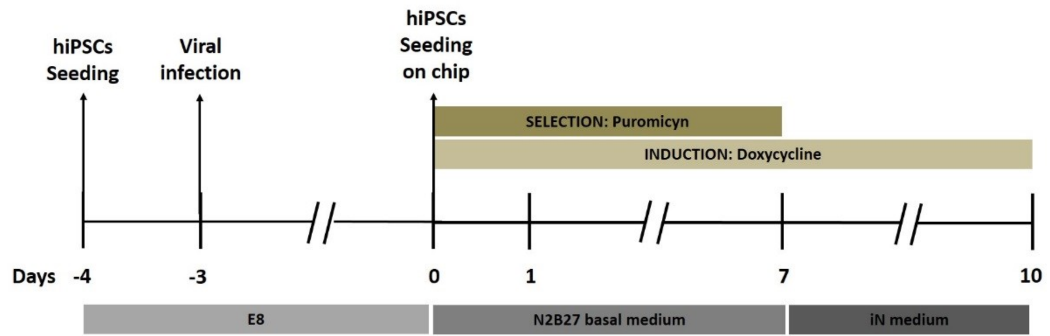


Figure 4.15: Time course for Ngn2 based neuronal conversion. Schematic representation of the timing used for the neurons induction starting from hiPSCs. Three different media were used: TeSR™-E8™ Medium (E8), N2B27 medium and iN medium, for the composition see section Material and Methods (3.4.1. Generation of neurons via lentiviral ngn2-overexpression). hiPSCs was infected, after 24 h the seeding, at day -3 and maintaining in culture for three days, until day 0, when cells were seeded on chip and neuronal induction protocol

As we can appreciate in Figure 4.16, after 10 days of differentiation, we obtained the neuronal conversion of hiPSCs with the automated microfluidic system, comparable to what we obtained using a non-automated chip. To confirm the neuronal identity of the differentiated cells, we performed stainings for typical neuronal markers such as βIIITubulin (immature neurons) and Map2 (mature neurons). As we can see in Figure 4.16, consistent with the neuronal-like morphology, cells show a homogeneous expression of all these markers.

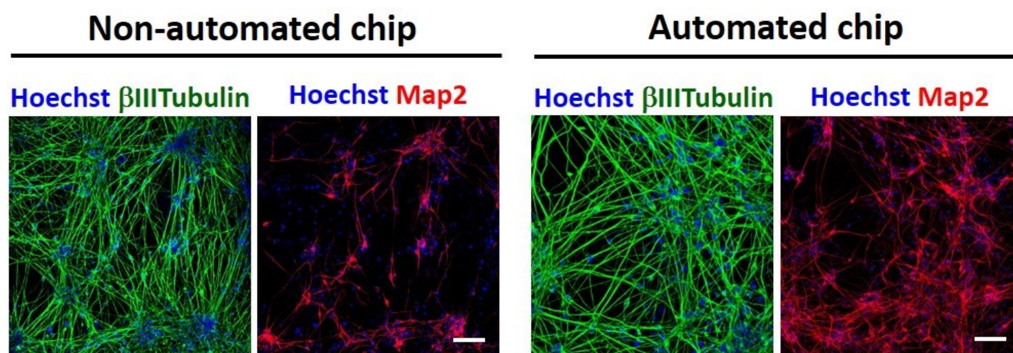


Figure 4.16: Induction of neurogenesis via lentiviral transduction of Ngn2. Representative of immunofluorescence images of neuronal-like cells generated in automated platforms at day 10. Scale bar 100um.

We can conclude that using an automated microfluidic platform it has been possible to generate hiPSC-derived neurons with comparable efficiency as using a non-automated device. In fact, immunofluorescence stainings of both microfluidic devices confirmed the efficient conversion of iPSCs into neurons in 10 days.

4.4. DOWNSCALING OF NGN2-MEDIATED NEURONAL INDUCTION VIA INTEGRATION-FREE SYSTEM ON-CHIP

In this section we will generate neuronal-like cells in micro-scale, using an integration free system (i.e. synthetic modified messenger RNA encoding for Ngn2), comparing the efficiency of the neuronal induction obtained in microfluidics with that obtained in a traditional cell culture platform.

4.4.1. OPTIMIZATION OF CELLULAR TRANSFECTION WITH mmRNA

The first parameters that need to be set when starting an experiment of transfection with mmRNA are the cell density and the mmRNA concentration. The fine-tuning of these parameters is required to obtain a correct equilibrium between transfection efficiency and cell death, caused by the toxicity of transfection reagents. This is particularly important when working with synthetic mmRNA and microfluidic system, characterized by an efficient delivery of exogenous factors to the cells.

We first performed a preliminary test with synthetic mmRNA encoding for GFP (GFP-mmRNA), to determine the best combination of cell density and the mmRNA concentration that maximizes the transfection efficiency, based on GFP expression. As described in Figure 4.17 the hiPSCs have been seeded at Day 0 in the presence of the transfection mix containing the GFP-mmRNA. In this way, cells started to be transfected immediately after seeding. The following transfections have been performed daily, until the end of the protocol.

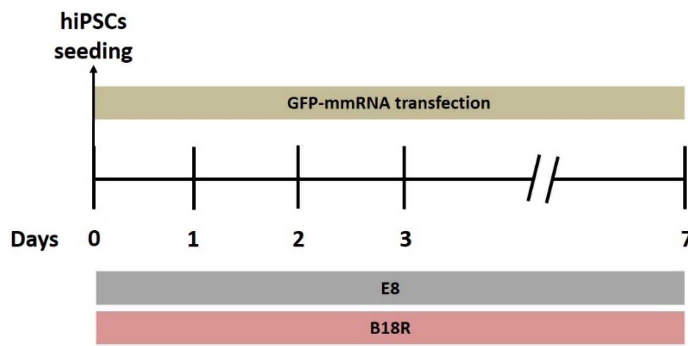


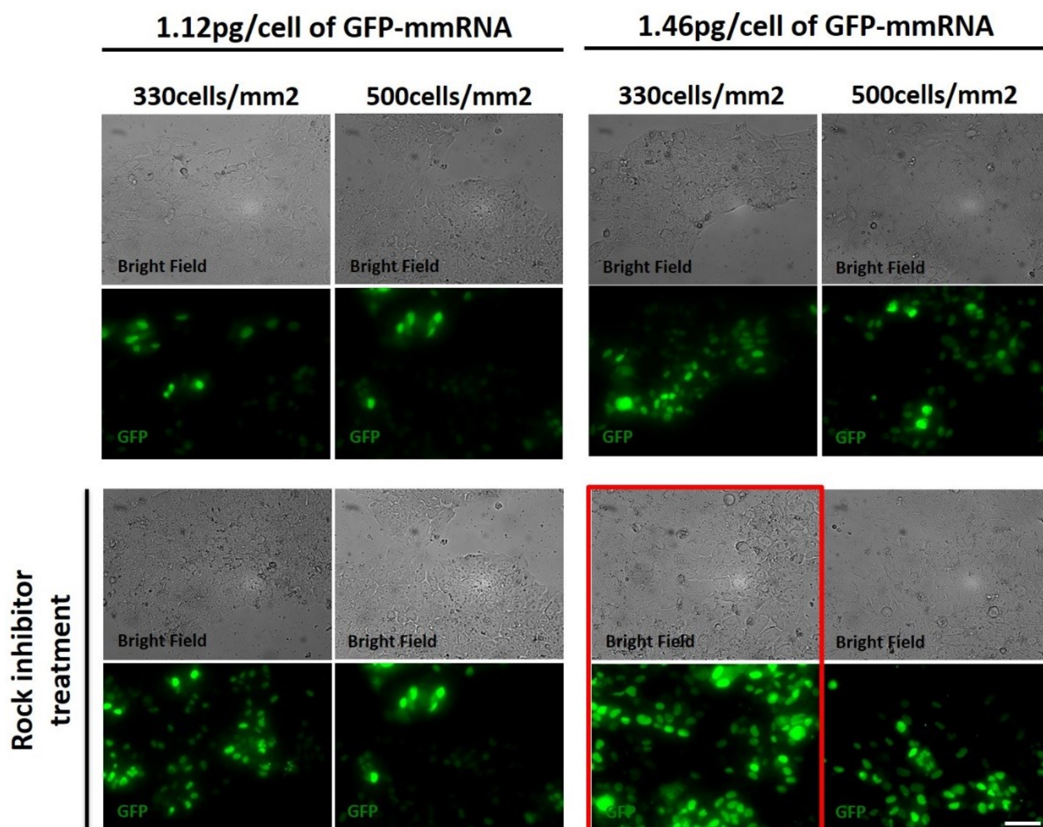
Figure 4.17: GFP-mmRNA transfection. Timing and medium used

As illustrated in Figure 4.18, we have tested two hiPSCs densities, 330cells/mm^2 and 500cells/mm^2, and two different GFP-mmRNA concentrations, 1.12pg/cell and 1.46pg/cell administrated every day. We decided to transfect once per day, based on other protocols of transcriptional programming and reprogramming using mmRNA developed in the lab and based on the peak of GFP-mmRNA expression after 24 h from the transfection. In this way we wanted to keep constant the dose exogenous Ngn2, as it happens during the neuronal induction via lentiviral transduction. The mmRNA concentrations have been defined by previous studies performed in our laboratory (data not showed in this thesis).

In addition, to improve the hiPSCs survival, contrasting the toxic effect of the transfection mix and the transfection efficiency, we tested the treatment with the Rock inhibitor (Ri) in conjunction with mmRNA administration. Ri is a kinase belonging to the PKA/ PKG/PKC family of serine-threonine kinases, it's involved mainly in regulating the shape and movement of cells by acting on the cytoskeleton (Zhang et al., 2011). In this specific case it acts on actin and keeps the cells separate and more isolated, counteracting the tendency of iPSC to reform compact colonies that are more difficult to transfect. To reduce the costs and perform multiple experiments in parallel, we performed these experiments directly in microfluidic devices. Moreover, this allowed us to set the perfect conditions that we then applied for the differentiation of neurons in micro-scale with high efficiency.

As expected, different doses of mmRNA leaded to different percentage of cells transfected, especially using a lower number of cells (i.e. 330 cell/mm^2) (Table

4.2). However, when the number of cells reached 500 cell/mm^2 the increase in the mmRNA dose was not paralleled by a substantial increase in the transfection efficiency. Moreover, the addition of Ri clearly enhanced the number of transfected cells in all the combinations tested (see table 4.2) but gave a statistically significant increase when we transfected 330cell/mm^2 with 1.46pg/cell of mmRNA giving 60.9% ± 2.9 vs. 48.9% ± 4.6 transfected cells with and without Ri respectively. This condition resulted the more efficient in terms of number of transfected cells and was the one we selected for the following experiments.



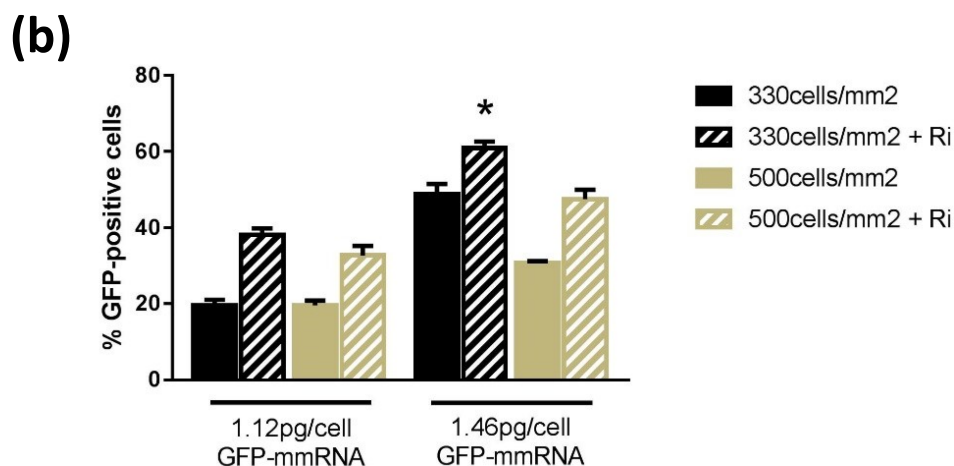


Figure 4.18: Transfection efficiency of hiPSCs with GFP-mmRNA. (a) hiPSCs were transfected with GFP-mmRNA for 7 days, once a day. Two mmRNA concentrations and two cell densities were tested. In addition, Ri treatment was evaluated. (b) graph of the transfection efficiency expressed as percentage of GFP positive cells on the total of cells. Cells have been analysed 7 days after transfection. n=2 biological replicates, n=6 total technical replicates. [mean±SEM], *p<0.05.

GFP-mmRNA concentration	Cell Density	Mean ± SEM
1.12pg/cell	330cells/mm^2	19.5% ± 2.6
	330cells/mm^2 + Ri	38.2% ± 2.8
	500cells/mm^2	19.6% ± 2.1
	500cells/mm^2 + Ri	32.7% ± 4.5
1.46pg/cell	330cells/mm^2	48.9% ± 4.6
	330cells/mm^2 + Ri	**60.9% ± 2.9**
	500cells/mm^2	30.7% ± 0.8
	500cells/mm^2 + Ri	47.6% ± 4.2

Table 4.2: Percentage of cells expressing GFP in the different conditions. The percentage of the GFP-positive cells has been estimated on the total number of cells. [mean±SEM]. In red the best combination between cell density, mmRNA concentration and treatment with Ri

After we set the best conditions to maximize the transfection of iPSCs, we moved to the use of Ngn2-mmRNA to demonstrate the efficient differentiation of neurons using mmRNA.

We first evaluated if there was any difference in the differentiation efficiency when using traditional cell culture supports vs. microfluidics. So, we tested the transcriptional programming efficiency of Ngn2-mmRNA on chip and in an 8-well Chamber Slides. In each device 330cell/mm^2, 1.46pg/cell of mmRNA and Ri have been used for the generation of neurons.

Both culture conditions have been treated with the same protocol schematized below (Fig. 4.19); in brief, hiPSCs have been transfected and diluted in the proliferative basal medium E8 plus Ri to help the cells survival. The day after, the medium has been changed with N2B27 differentiating medium and it has been maintained until day 4 of the protocol. Then, from day 4, neurotrophic factors BDNF and NT3 have been added to then medium until the end of the experiment (for detail about protocol, medium composition and factors concentration see section 3.4.2.2).

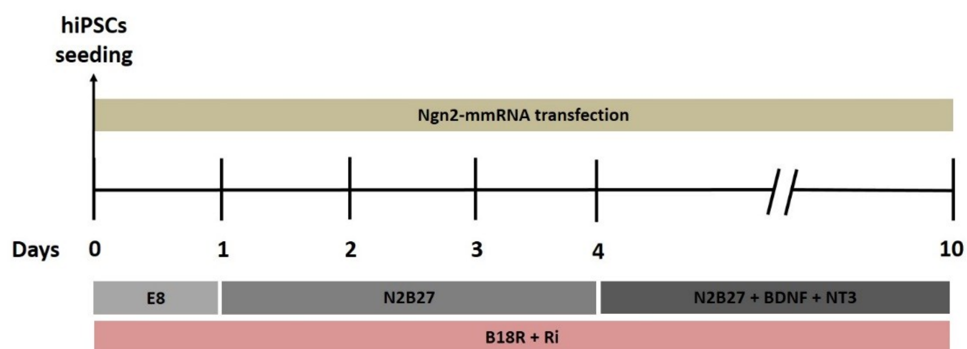
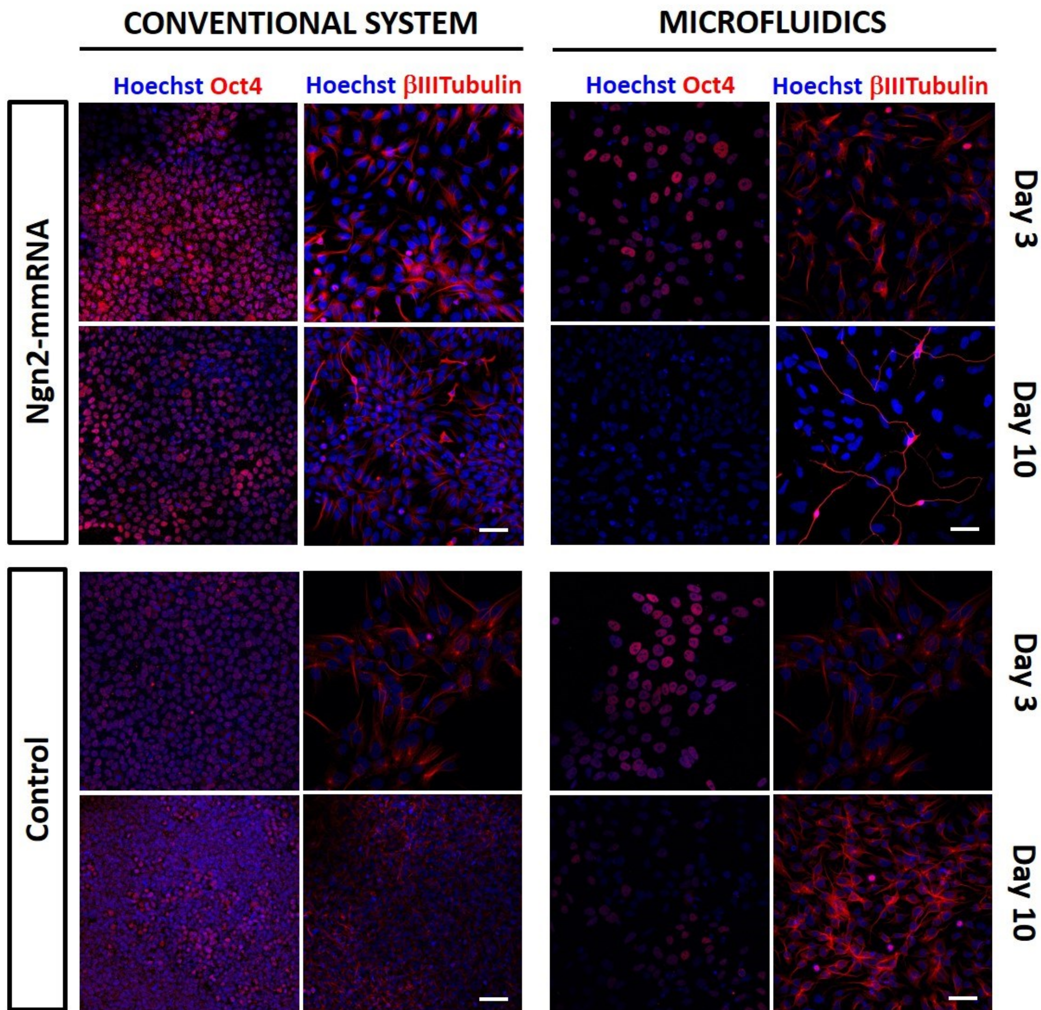


Figure 4.19: Ngn2-mmRNA transfection protocol. Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor).

To compare the two systems, we evaluated the number of neurons obtained (positive for the neuronal marker βIIITubulin) and the presence of residual cells expressing the pluripotency marker Oct4 at the end of the protocol (day 10). As shown in Figure 4.20 when using the conventional cell culture support, most of

the cells kept the expression of the pluripotency marker Oct4 until day 10, and the percentage of expressing βIIITubulin marker showed a very immature morphology, whereas cells with a clear neuronal-like morphology were very few (Fig. 4.20-a-b). On the other hand, in the microfluidic system the situation was different: at day 3 is still possible to observe some Oct4-positive cells in culture and there are only very immature neuronal-like cells; at day 10 we could not detect Oct4 positive cells and we observed the clear presence of βIIITubulin positive neuronal-like cells.



(b)

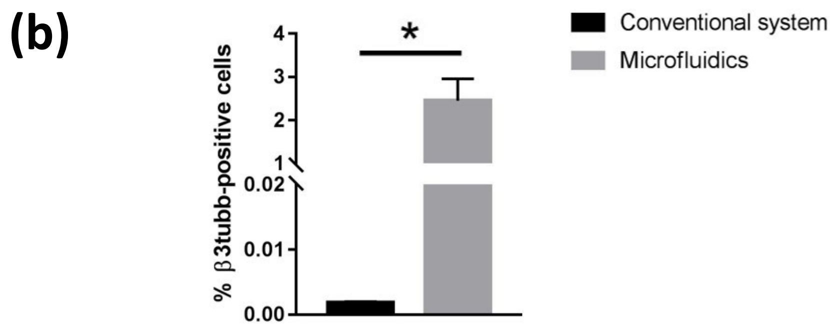


Figure 4.20: Induction of neurogenesis in human pluripotent stem cells by Ngn2-mmRNA. (a) Representative images of Oct4 and βIIITubulin staining at Day 3 and Day 10 of the differentiation protocol in both conventional system and microfluidics. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50um. (b) Percentage of βIIITubulin expressing cells cultured in the different devices (conventional device, 0.002% ± 0.0 [mean±SEM], microfluidics 2.5% ± 0.7 [mean±SEM], n=3; *p<0.05).

Our data demonstrated that, using the same parameters of cell number and mmRNA dose, the microfluidic platform is more efficient in promoting the mmRNA induced differentiation of hiPSCs into neuron-like cells than the conventional devices.

4.5. OPTIMIZATION OF NEURONAL INDUCTION BY NGN2-mmRNA

In this section of the results we will focus on increasing the differentiation efficiency, acting on cellular signalling playing important roles during the *in vivo* development of the central nervous system. In particular we will focus on the role of Fgf2, the combination of Ngn2 programming with Dual-SMAD inhibition, the role of glucose metabolism, the use of neuroepithelial cells as cell source and the increase of Ngn2-mmRNA dose. Finally, we will discuss the effect of the use of Rock inhibitor on the cell fate.

4.5.1. TREATMENT WITH FGF2 COUNTERBALANCES mmRNA TOXICITY AND PROMOTES NEURONAL DIFFERENTIATION

In this paragraph, we will discuss the role of Fgf2, to explore if acting on the cell survival we would increase the efficiency of neuronal differentiation.

To counterbalance the cell death caused by the transfection procedure and to increase the cell survival, we supplemented the media with Fgf2 for the first two days (Fig. 4.21) (Eiselleova et al., 2009). Fgf2 is a crucial growth factor both for human iPSCs and neural cells (Chen et al., 2007). The presence of Fgf2 increases the cell survival and proliferation and, possibly, reduces the oxidative stress caused by the absence of serum in the medium, which usually has anti-oxidative properties (Galderisi et al., 2013) decreasing DNA damage in the cells.

We first evaluated if the transcriptional programming efficiency of Ngn2-mmRNA on chip was affected by the addition of Fgf2 for the first two days, maintaining the number of cells (330cell/mm^2) the dose of mmRNA (1.46pg/cell) and the presence of Ri all along the protocol as we defined in the previous paragraph (Fig. 4.13) (for detail about protocol, medium composition and factors concentration see section 3.4.2.2).

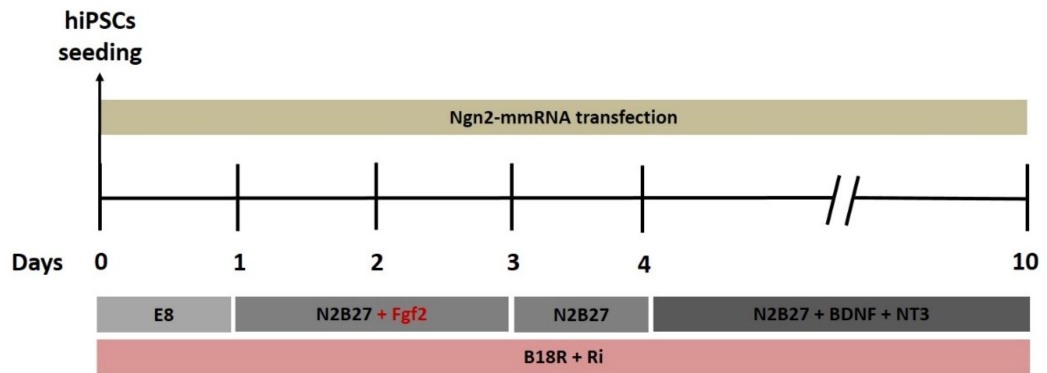
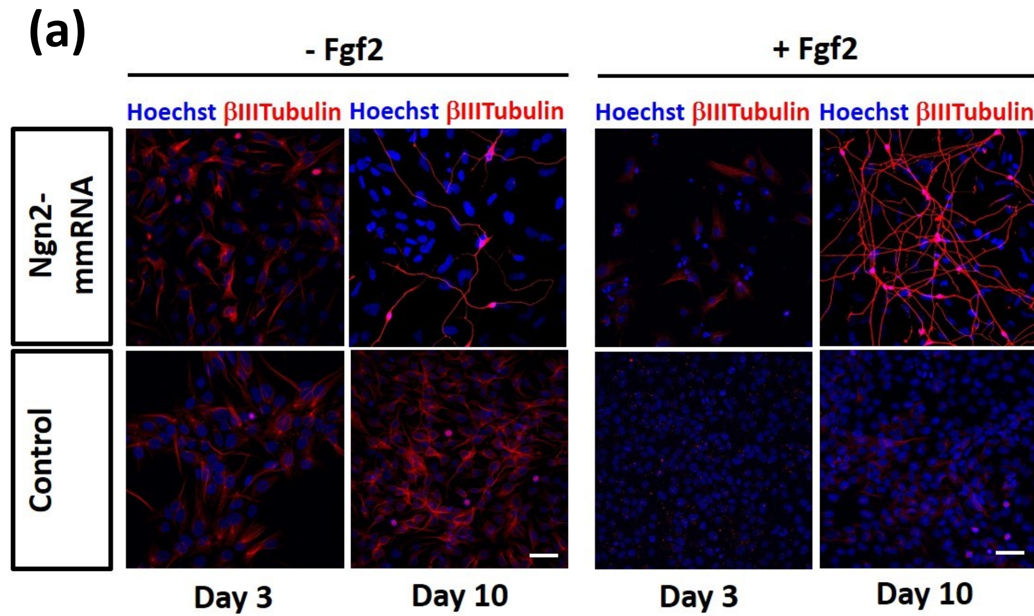


Figure 4.21: Ngn2-mmRNA transfection protocol. Timing and different media used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). Fgf2 was added only day 1 and day 2.

To compare the two protocols, we evaluated the percentage of neuronal-like cells positive for the neuronal marker βIIITubulin, obtained at the end of the protocol (day 10). As shown in Figure 4.22 we observed that adding Fgf2 to the medium for just 2 days was enough to promote a statistically significant increase of 4 times in the percentage of βIIITubulin expressing cells showing a clear neuronal-like morphology, passing from 2.5% ± 0.7 without Fgf2 to 10.7% ± 1.1 with the Fgf2 supplementation.



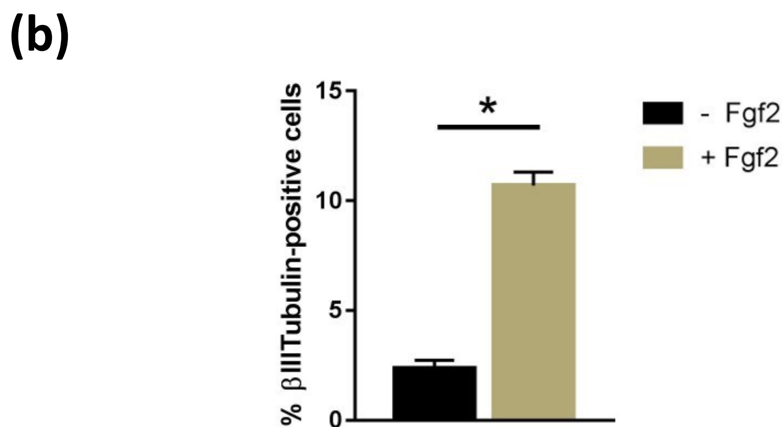
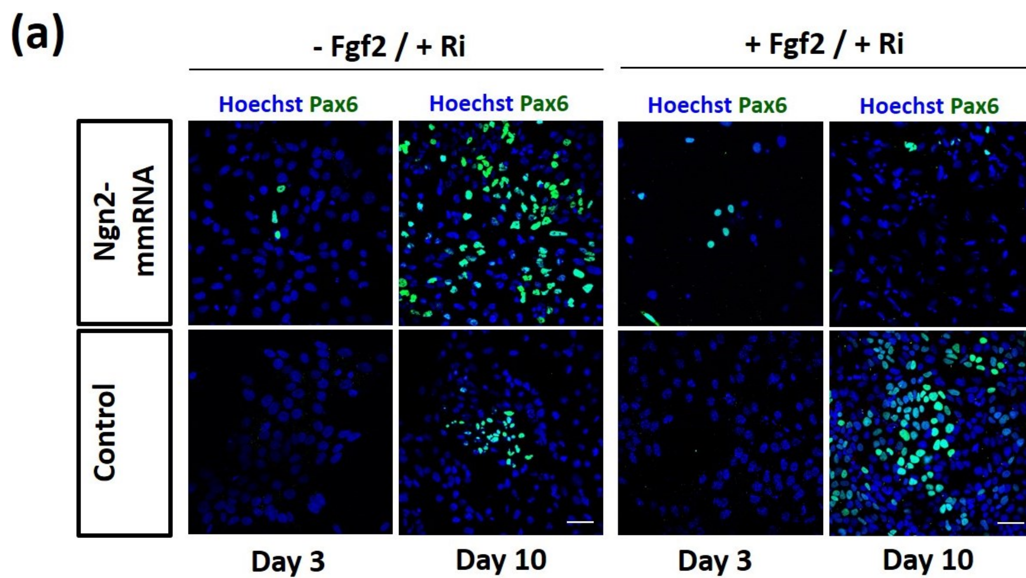


Figure 4.22: Induction of neurogenesis in hiPSCs by Ngn2-mmRNA with or without Fgf2 treatment. (a) Representative images of βIIITubulin staining at Day 3 and Day 10 of the two differentiation protocols, with or without Fgf2. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50um. (b) Percentage of βIIITubulin expressing cells cultured with the different protocols on the total cells measured at day 10 (– Fgf2, 2.5% ± 0.7 [mean±SEM], + Fgf2 10.7% ± 1.1 [mean±SEM]), n=3 independent replicates and n=3 technical replicates each independent replicate; *p<0.05.

Given this clear effect on neuronal differentiation that we observed adding Fgf2, we asked whether the treatment with this growth factor would have affected the composition of the culture. So we focused our attention on neural progenitors cells, since Fgf2 is known to have a mitogenic effect on those cells (Eiselleova et al., 2009). We observed that at day 3, soon after the treatment with Fgf2 (day1-2), the presence of Fgf2 significantly enhanced the percentage of neural progenitor cells positive for Pax6 (Fig. 4.23-a-b), as expected. However, at day 10 the situation was completely different and the condition without Fgf2 showed a significant higher percentage of neural progenitor cells compared with the condition treated with Fgf2 (Fig. 4.23-a-b). This correlates as well with the difference previously observed in the percentage of neuronal-like cells that was higher when cells were exposed to Fgf2 (Fig. 4.22-b).



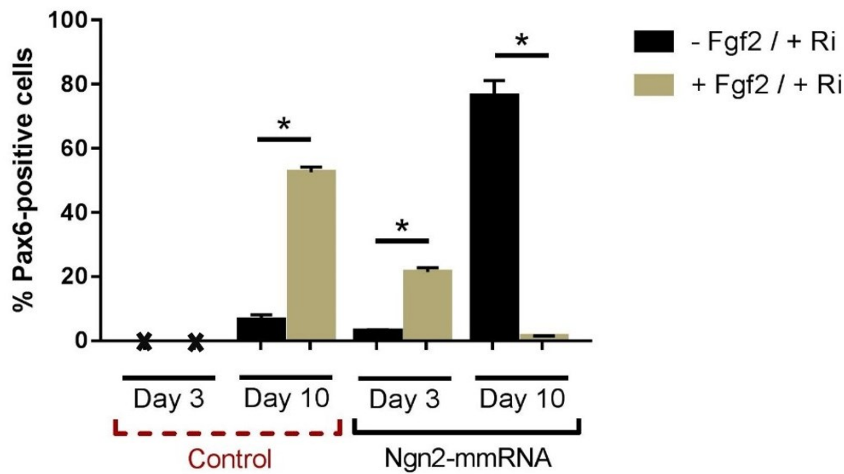


Figure 4.23: Induction of neural progenitor cells formation in hiPSCs by Ngn2-mmRNA. (a) Representative images of Pax6 staining at Day 3 and Day 10 of the two differentiation protocols, with or without Fgf2. (b) Percentage of Pax6 expressing cells cultured with the different protocols on the total cells measured at day 3 and day 10. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. n=3 independent replicates and n=3 technical replicates each independent replicate; *$p<0.05$. Black cross for undetectable value.

4.5.2. GLUCOSE TREATMENT DOES NOT INCREASE NEURONAL DIFFERENTIATION VIA NGN2-MMRNA

In order to counterbalance the glucose consumption during the neuronal differentiation, and possibly further promote neuronal differentiation, cells have been exposed to two different doses of glucose: 3.1mg/mL, concentration used in transcriptional programming protocols and 4.7mg/mL, concentration used to generate neurons from iPSCs using small molecules and growth factors. During the early stages of neuronal differentiation, cells relay on glycolytic metabolism (Agostini et al., 2016). Glucose is crucial for neuronal differentiation, neurite outgrowth, biosynthesis of neurotransmitters and synaptic formation. In addition,

once fully differentiated, neuronal cells require high energy levels to restore the neuronal membrane potential after depolarization.

We compared the protocol defined in the section 4.2.1, with Fgf2 and a glucose concentration of 3.1mg/mL with a protocol in which the concentration of glucose was increased to 4.7mg/mL (Fig. 4.24). The glucose was added at day 1 until day 10 (for detail about protocol, medium composition and factors concentration see section 3.4.2.2).

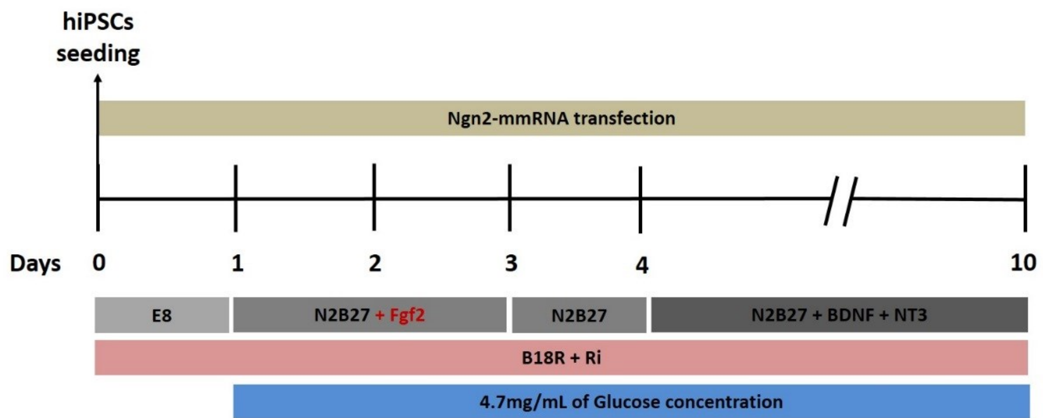


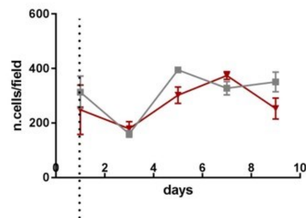
Figure 4.24: Ngn2-mmRNA transfection protocol. Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). 1.6mg/mL of glucose was added to the medium until the end of the experiment (day 1-10), for a total concentration of 4.1mg/mL.

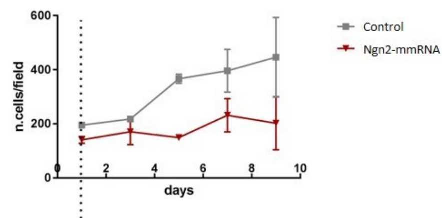
We first evaluated the proliferation of the cells in response to the different doses of Glucose and their glucose consumption during time, but we found a similar trend in both conditions. In Figure 4.25 is possible to observe how cell density and residual glucose found in the medium are inversely related: when the cell density increases, the consumption of the glucose increases as well, and the residual glucose measured in the medium decreases. Moreover, the representative bright field images at day 3 and at day 9, confirm the similar effect obtained with the two

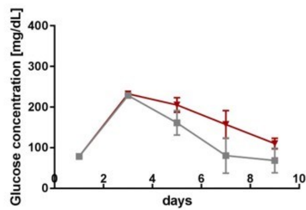
protocols tested. In particular it is possible to observe a proliferative effect of both glucose concentrations and cells show a similar change in their morphology, passing from single dispersed cells with a star-shaped morphology (day 3) to islands of more compact cells in which is hard to distinguish a clear morphology (day 9).

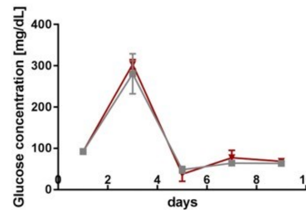
3.1mg/mL of Glucose conc.

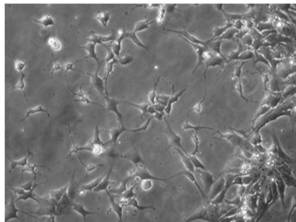
4.7mg/mL of Glucose conc.

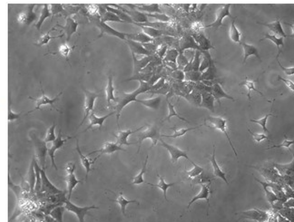


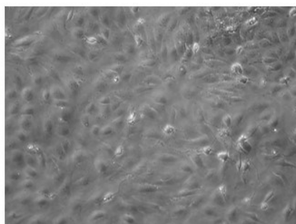












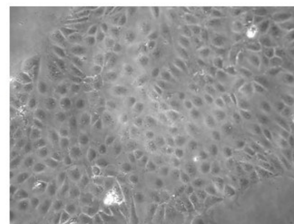


Figure 4.25: Induction of neurogenesis in hiPSCs by Ngn2-mmRNA. In the first graph, of both conditions, is shown the cell densities during time, while in second graph, of both treatments, is described the glucose concentration consumed by the cells during the neuronal development. In the last part of the figure are shown representative images of cell morphology. n=2 independent replicates and n=3 technical replicates each independent replicate.

Since we observed a similar effect in term of proliferation and cell morphology, we moved to analyse if the addition of Glucose could have had an impact on the differentiation process. We measured the percentage of βIIITubulin positive cells over the total number of cells and we observed that, instead of pushing further neuronal differentiation, higher levels of glucose had an opposite effect, reducing the percentage of neuronal-like cells measured at the end of the protocol (Fig. 4.26) from 10.7% ± 1.1 to 1.35% ± 0.7 (Fig. 4.27).

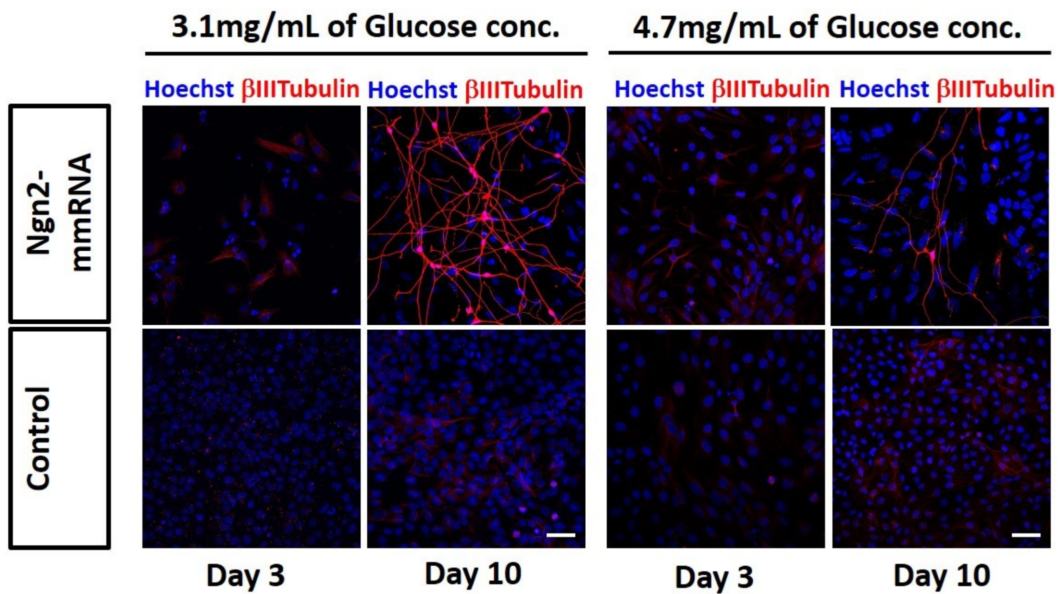


Figure 4.26: Cell density and glucose consumption during time. Representative images of cell morphology. (n=2 independent replicates and n=3 technical replicates each independent replicate)

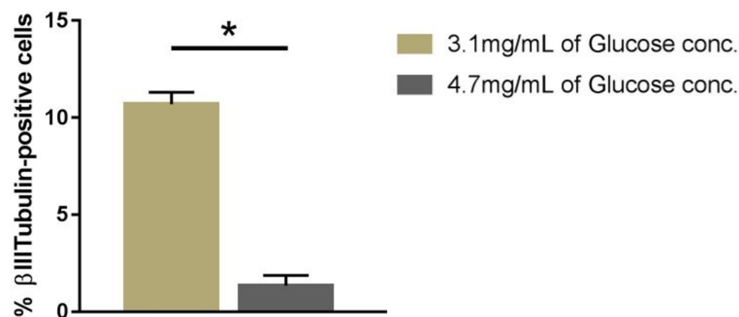


Figure 4.27: Induction of neurogenesis in hiPSCs by Ngn2-mmRNA. Percentage of βIIITubulin expressing cells cultured with the different protocols (3.1mg/mL of Glucose 10.7% ± 1.1

[mean±SEM], 4.7 mg/mL of glucose conc. 1.35% ± 0.7 [mean±SEM]. 3.1mg/mL of Glucose conc. is referred to the glucose concentration defined in the section 4.2.1, with Fgf2), n=2 independent replicates and n=3 technical replicates each independent replicate; *p<0.05.

These data confirmed that the use of Fgf2 for 2 days at the beginning of the differentiation protocol, in combination with Rock inhibitor and 3.1mg/mL of glucose is the combination that gives the highest neuronal induction efficiency, among the conditions that we tested.

4.5.3. TREATMENT WITH DUAL-SMAD INHIBITION DOES NOT INCREASE NEURONAL DIFFERENTIATION VIA NGN2-mmRNA

As we observed in paragraph 4.5.1., the population of cells obtained after 10 days of differentiation is not homogeneous but contains, among other cells, neuronal-like cells and neural progenitors that have the potential to give rise to neurons and represent an immature pool of cells that could be further pushed toward neuronal differentiation. In order to reduce the intrinsic cell culture variability, we thought to combine the transcriptional programming with dual-SMAD inhibition, to obtain a uniform cell population committed to neural-ectoderm.

In this section we will describe the combination of Ngn2 programming with dual-SMAD inhibition to homogenise the starting cell population and to further increase the conversion efficiency of iPSCs into neuronal-like cells. Dorsomorphin and SB431542 are two small molecules that inhibit type I TGFβ1 receptors and BMP-4 pathways respectively (Madhu et al., 2016) and their combination is named dual-SMAD inhibition (2i). Their use has been extensively applied to the neural induction of hESCs, since they efficiently suppress the trophectoderm, mesoderm

and endoderm differentiation of hESCs and enhance the neuroectoderm differentiation with high efficiency (Patani et al., 2009).

We used two different approaches for the dual-SMAD inhibition: the addition of the two inhibitors just for two days at the beginning of the differentiation protocol (Fig. 4.28-a) or the treatment with the inhibitors for all the duration of the differentiation protocol (Fig. 4.28-b).

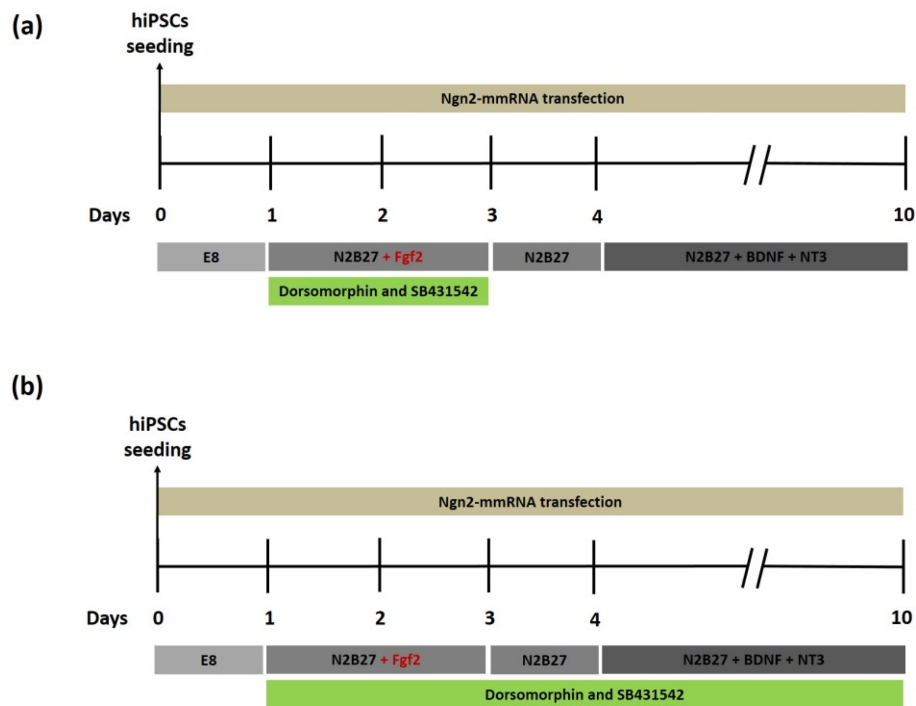


Figure 4.28: Ngn2-mmRNA transfection protocol. Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). (a) Treatment with Dual-SMAD inhibition was performed for two days (day1-2) (b) Treatment with Dual-SMAD inhibition was performed until the end of the experiment (day1-10)

However, as we can observe in Figure 4.29-a the use of small molecules, either for only two days or during all the protocol, does not increase the percentage of βIIITubulin expressing cells. On the contrary, the addition of the two inhibitors

significantly decreased the efficiency of neuronal-like cells induction, as illustrated in the graph below (Fig. 4.29-b):

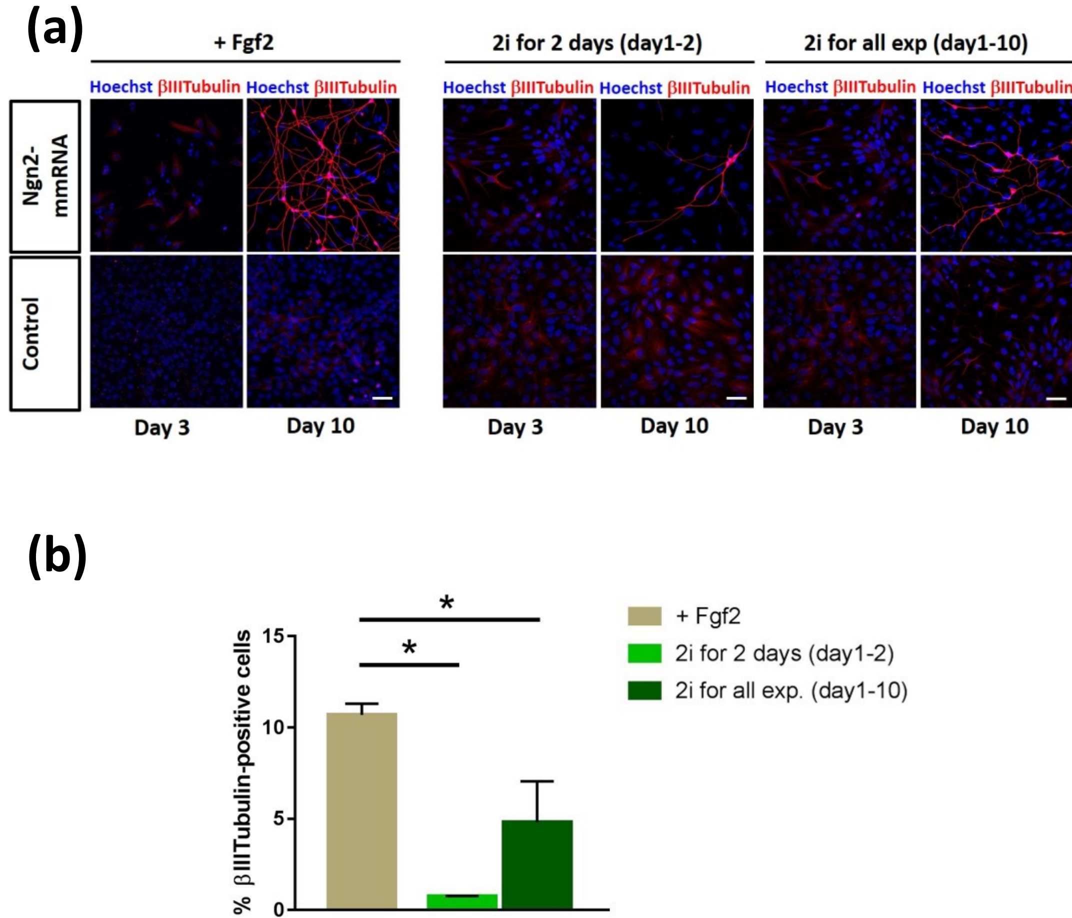


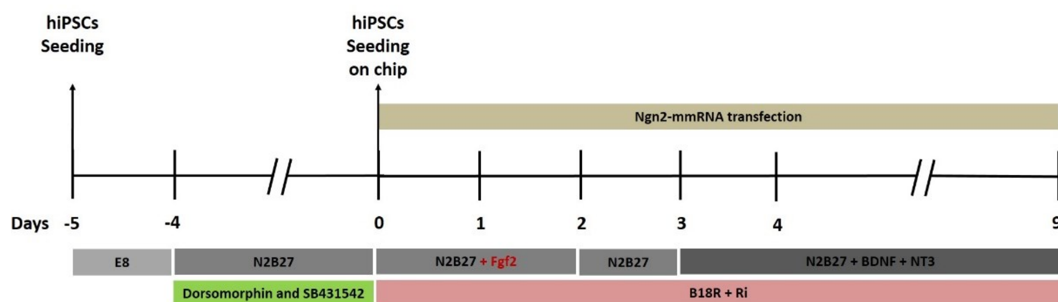
Figure 4.29: Induction of neurogenesis in hiPSCs by Ngn2-mmRNA via Dual-SMAD inhibition. (a) Representative images of βIIITubulin staining at Day 3 and Day 10 of the three differentiation protocols, + Fgf2, 2i for 2 days (day1-2) and 2i for all exp (day1-10). Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50 μm. (b) Percentage of βIIITubulin expressing cells cultured with the different protocols (Fgf2 10.7% ± 1.1 [mean±SEM], 2i for 2 days (day1-2) 0.76% ± 0.02 [mean±SEM] and 2i for all exp (day1-10) 4.81% ± 3.8 [mean±SEM]), n=3 independent replicates and n=3 technical replicates each independent replicate; *p<0.05.

4.5.4. NEUROEPITHELIAL CELLS AS CELL SOURCE DOES NOT IMPROVE NEURONAL INDUCTION VIA NGN2-mmRNA

Since we did not observe a benefit in terms of neuronal conversion when we combined Ngn2 transcriptional programming with dual-SMAD inhibition, we thought to change strategy and we first derived neural-ectoderm cells in conventional cell culture systems and then we subjected those cells to transcriptional programming with Ngn2-mmRNA.

hiPSCs have been treated with N2B27 medium supplemented with Dorsomorphin and SB431542, for four days, to generate a population of neural progenitors. Cells have been then detached and plated in microfluidics, where they underwent to the protocol of Ngn2-mmRNA based differentiation that we optimized in the previous paragraphs with Fgf2 for 2 days at the beginning of the differentiation protocol, in combination with Rock inhibitor and 3.1mg/mL of glucose (Fig. 4.30-a-b).

(a)



(b)

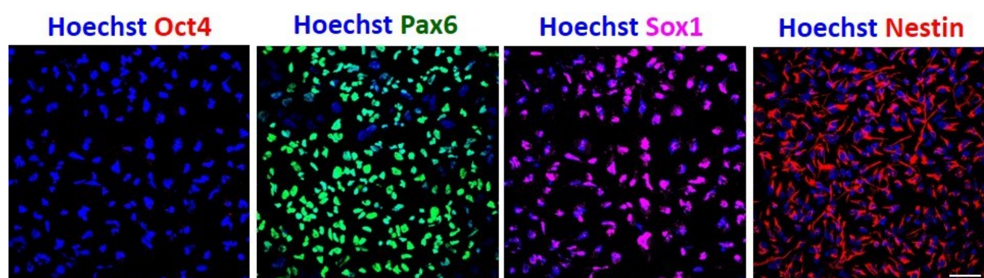
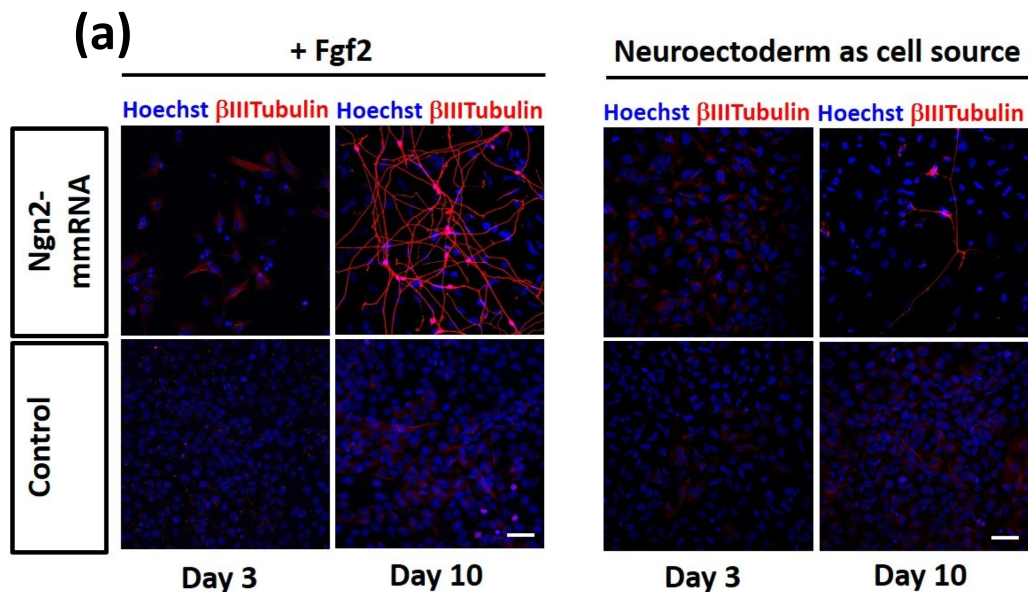


Figure 4.30: Ngn2-mmRNA transfection protocol. (a) Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). (b) Representative images of Oct4, Pax6, Sox1 and Nestin stainings after hiPSCs treatment with Dorsomorphin and SB431542, for four days. Scale bar 50 μm.

At the end of our experiment, the efficiency of neuronal differentiation was estimated by counting the percentage of cells expressing βIIITubulin. Surprisingly we observed that the generation of neuronal-like cells starting from neuroectoderm as cell source dramatically decreased as compared to the percentage of neuronal-like cells obtained starting from iPSCs, passing from 10.7% ± 1.1 to 0.4% ± 0.3 (Fig. 4.31). These data further confirm that the use of iPSCs as cell source represent the best condition to get the highest percentage of neuronal conversion.



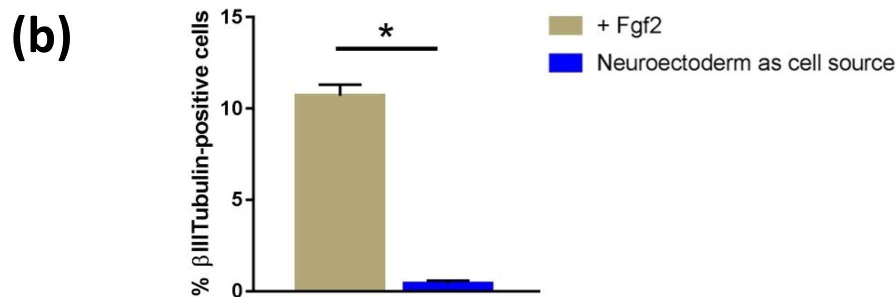


Figure 4.31: Induction of neurogenesis in neural progenitors' cells by Ngn2-mmRNA. (a) Representative images of βIIITubulin staining at Day 3 and Day 10 of the two differentiation protocols, + Fgf2 and Neuroectoderm as cell source protocol. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50um. (b) Percentage of βIIITubulin expressing cells obtained with the different protocols (Fgf2 10.7% ± 1.1 [mean±SEM], Neuroectoderm as cell source 0.4% ± 0.3 [mean±SEM]), n=2 independent replicates and n=3 technical replicates each independent replicate; *p<0.05.

4.5.5. INCREASING THE DOSE AND FREQUENCY OF NGN2-mmRNA DELIVERY SPEEDS UP NEURONAL CONVERSION BUT INCREASES CELL DEATH

Since we observed that the treatment with neural inducing stimuli did not promote a higher neuronal conversion mediated by Ngn2-mmRNA transfection, we tried to further push iPSCs toward neuronal fate increasing the Ngn2-mmRNA transfection frequency. Ngn2, indeed, is expressed in an oscillatory manner by neural progenitors, whereas becomes constantly expressed once neural progenitor undergoes neuronal differentiation (Shimojo et al., 2011). In the early stages of CNS development, the equilibrium between neuronal progenitors and

mature neurons is very important for the correct organ maturation and this is possible thanks to Ngn2 oscillatory pattern (Kageyama et al., 2008)

mmRNA treatment substantially differs from lentiviral mediated gene expression. In particular mmRNA are ready to use from the transfected cells, but once the cell has transcribed the entire dose that was transfected, the protein production rapidly stops. In case of lentiviral transduction, cells need to transcribe and translate the gene, but then the production of protein remains high and constant as long as the transgene expression is induced (in case of inducible systems).

We evaluated the mmRNA based Ngn2 expression pattern performing an experiment where we transfected the hiPSCs with Ngn2-mmRNA and GFP-mmRNA, in parallel, only one time/day and fixed cells after 0-2-4-6-8-16-24 hours after the transfection. Immunostaining analysis of Ngn2-transfected hiPSCs revealed expression of Ngn2 protein after 2 hours from the transfection and a rapid increase of cells expressing Ngn2 marker until 16 hours. At 24 hours the expression of Ngn2 decreases. Instead the expression of GFP protein is delayed over time, because we observed the GFP-positive cells starting from 8 hours after the transfection (Fig. 4.32).

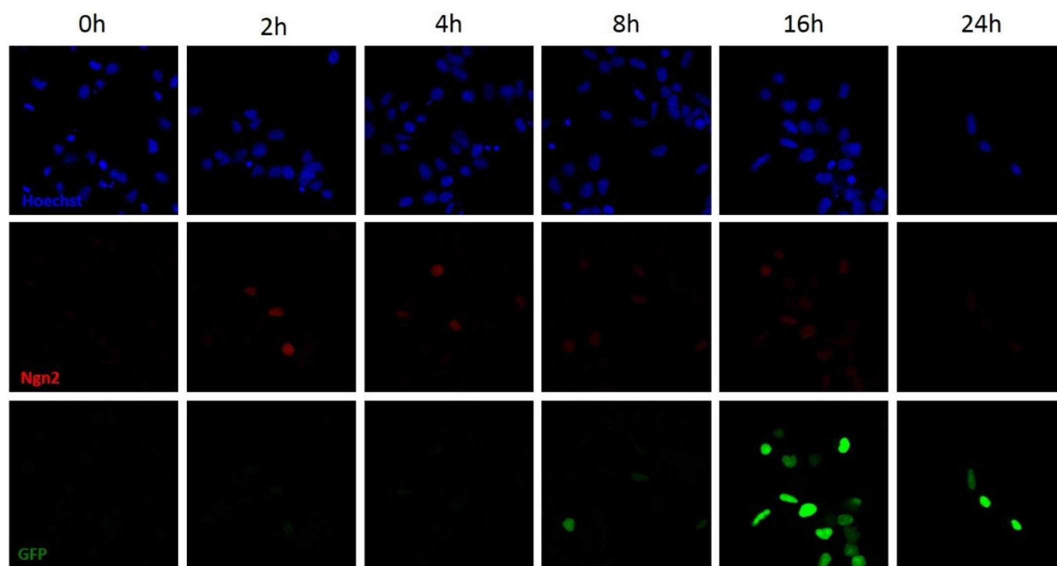


Figure 4.32: Time course of Ngn2 protein expression. (a) Representative images of Ngn2 and GFP expression at different time point. Scale bar 50um.

So, we tested in parallel two different Ngn2-mmRNA transfection frequencies from every 24 h (1.46pg/cell per day) to every 12h (2.92pg/cell per day) (Fig. 4.33).

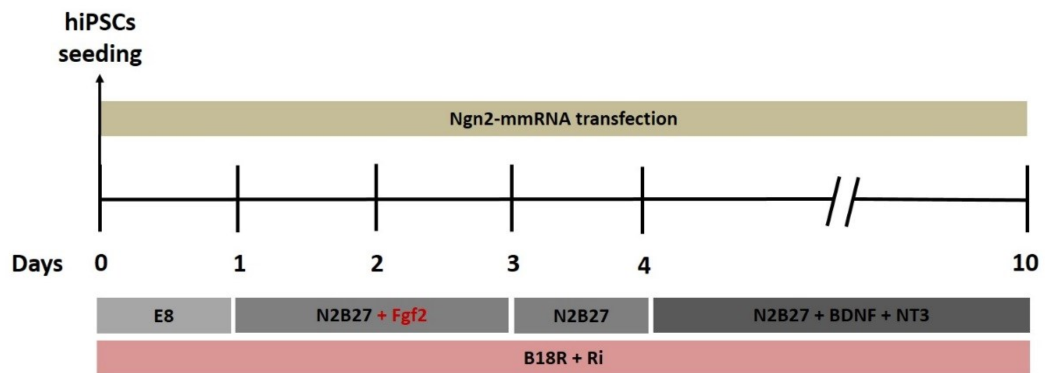
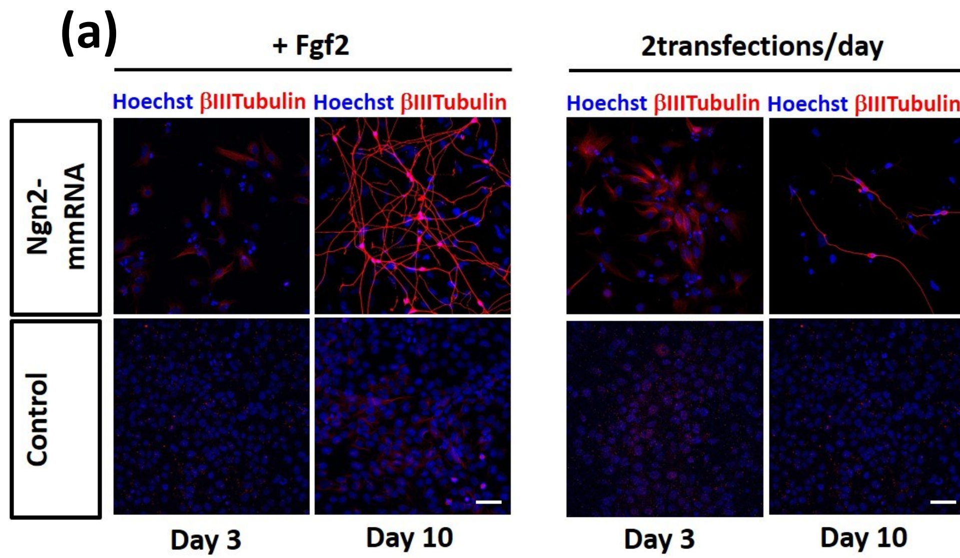


Figure 4.33: Ngn2-mmRNA transfection protocol. Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). In this experiment one group of hiPSCs received one transfection/day and one group two transfection/day.

To compare the two protocols based on the different Ngn2-mmRNA transfection frequency and dose, we evaluated the number of cells positive for the neuronal marker βIIITubulin. As shown in figure 4.34 transfecting iPSCs every 12h resulted in an increased cell mortality, which is reflected in a reduction in the overall cell number and in a low percentage of cells expressing βIIITubulin marker (Fig. 4.34). These data further confirm that the treatment of hiPSCs with Fgf2 for 2 days at the beginning of the differentiation protocol, in combination with Rock inhibitor and 3.1mg/mL of glucose and the transfection every 24h with 1.46pg/cell of Ngn2-mmRNA represent the most efficient strategy to obtain neuronal-like cells.



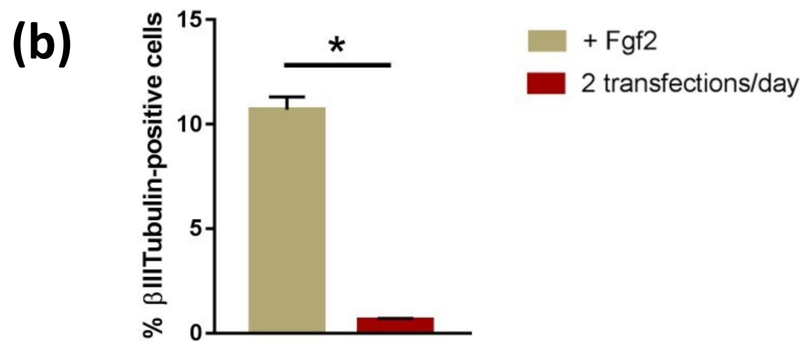


Figure 4.34: Induction of neurogenesis by Ngn2-mmRNA. (a) Representative images of βIIITubulin staining at Day 3 and Day 10 of the two differentiation protocols, + Fgf2 and 2transfection/day protocol. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50um. (b) Percentage of βIIITubulin expressing cells obtained with the different protocols (Fgf2 10.7% ± 1.1 [mean±SEM], 2transfection/day 0.7% ± 0.16 [mean±SEM]), n=2 independent replicates and n=3 technical replicates each independent replicate, *p<0.05.

4.5.6 ROCK INHIBITOR TREATMENT ASSOCIATED TO NGN2 TRANSFECTION AND FGF2 ACCELERATES THE NEURONAL-LIKE CELLS GENERATION BUT LEADS TO NEURAL CREST INDUCTION

As we mentioned in paragraph 4.4, the use of Rock inhibitor was instrumental to increase the transfection efficiency and counterbalance the detrimental effect of the single cell dissociation. Ri is a selective inhibitor of the p160-Rho-associated coiled kinase, used in iPSC research to avoid cell death and differentiation cause by single cell dissociation (Zhang et al., 2011). Moreover, it has been demonstrated that Rock inhibitor has a massive effect on cell shape and mechanotransduction, and in particular has been demonstrated that Rock inhibitor promote differentiation of human pluripotent stem cells into neural crest-like progenitors that are characterized by ability to differentiate into multiple cell types, including peripheral neurons (Kim et al., 2015).

Given these collateral effects of Ri we investigated whether the treatment with Ri just for the first day after seeding (Fig. 4.35) or for the entire experiment could alter the cell fate (Fig. 4.21).

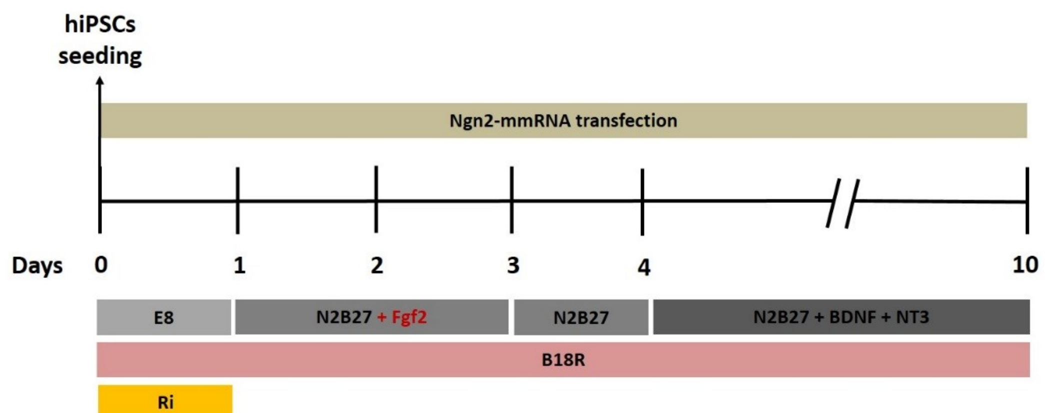


Figure 4.35: Ngn2-mmRNA transfection protocol. Timing and medium used during neuronal differentiation, E8 (TeSR™-E8™), N2B27 (N2B27 medium), Ri (rock inhibitor). Ri was added just for the first day after cell seeding.

To evaluate the effect of Ri treatment on the cell fate, and in particular on the generation of neural crest cells we evaluated the number of cells positive for the neural crest marker p75. As expected, hiPSCs differentiated into neural crest-like progenitors in response to prolonged Ri treatment. However, p75-positive cells are present only early during differentiation (day 3) but not at the end of the protocol (day 10). At day 10, indeed, both conditions show no p75 positive cells (Fig. 4.36-a-b).

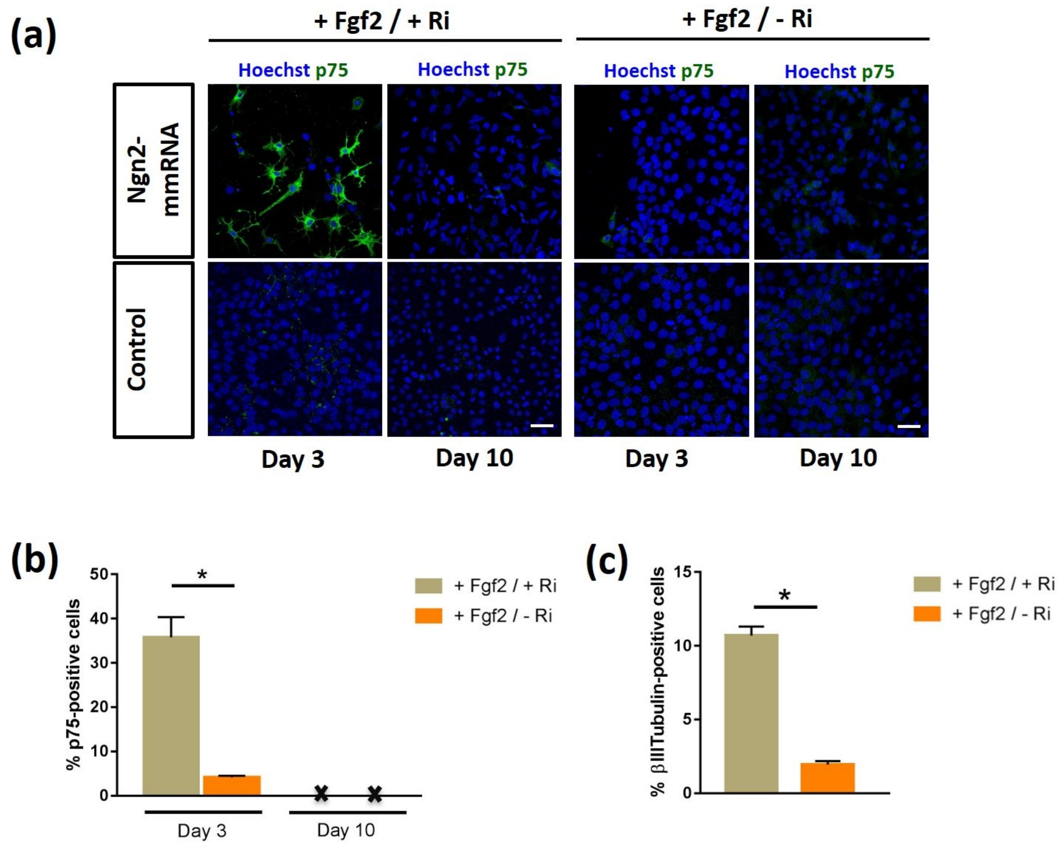


Figure 4.36: Induction of neural crest formation in hiPSCs by Ngn2-mmRNA. (a) Representative images of p75 staining at Day 3 and Day 10 of the three different protocols, +Fgf2/+Ri and +Fgf2/-Ri, respectively. Ngn2-mmRNA indicates cells that have been treated with synthetic mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection. Scale bar 50um. (b) Percentage of p75 expressing cells cultured with the different protocols (+Fgf2/+Ri 36.1% ± 10.1 [mean±SEM] and +Fgf2/-Ri 4.2% ± 0.8 [mean±SEM], all at day 3).The black cross means the absence of p75-positive cells for these

conditions. (c) Percentage of βIIITubulin expressing cells cultured with the different protocols, at day 10 (+Fgf2/+Ri 10.7% ± 1.04 [mean±SEM] and +Fgf2/-Ri 1.94% ± 0.4 [mean±SEM]), n=2 independent replicates and n=3 technical replicates each independent replicate; *p<0.05.

However, the absence of p75 positive cells in both conditions does not mean comparable differentiation efficiency. Indeed, as shown in Figure 4.37, restricting the Ri only to 24h after plating, most of the cells keep the expression of the pluripotency marker Oct4 until day 10, and the percentage of neuronal-like cells expressing βIIITubulin marker is significantly lower when compared to the condition with Ri treatment all along the differentiation protocol (Fig. 4.21).

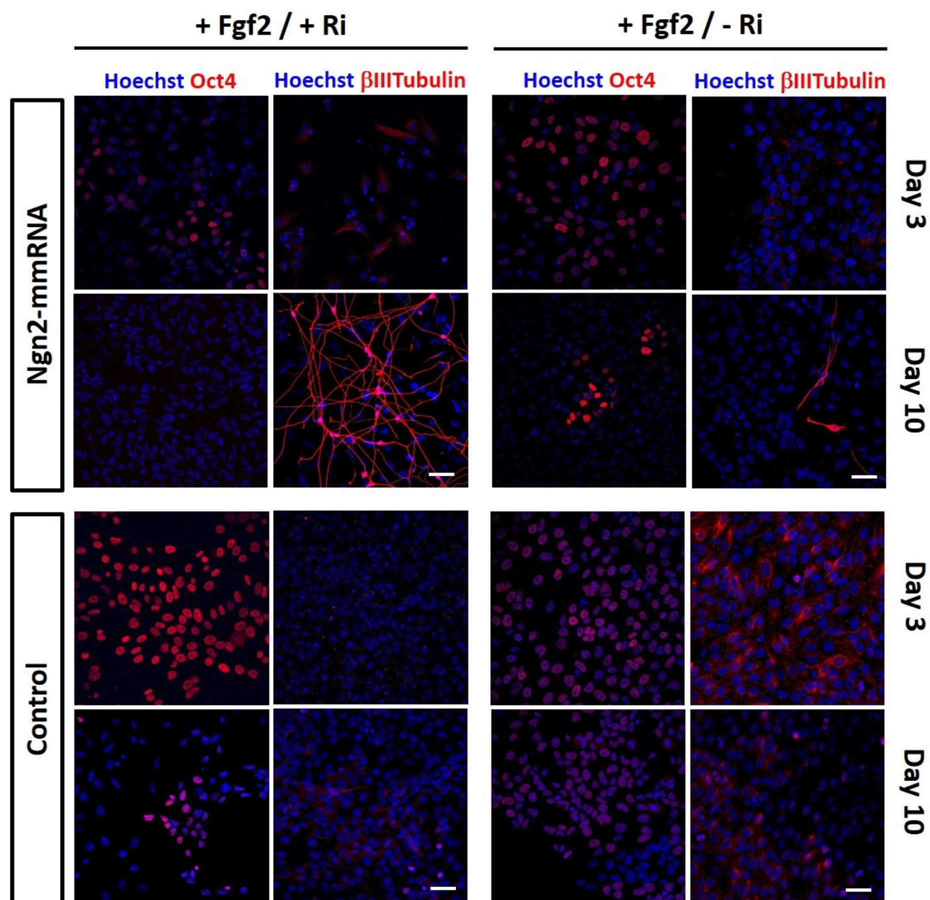
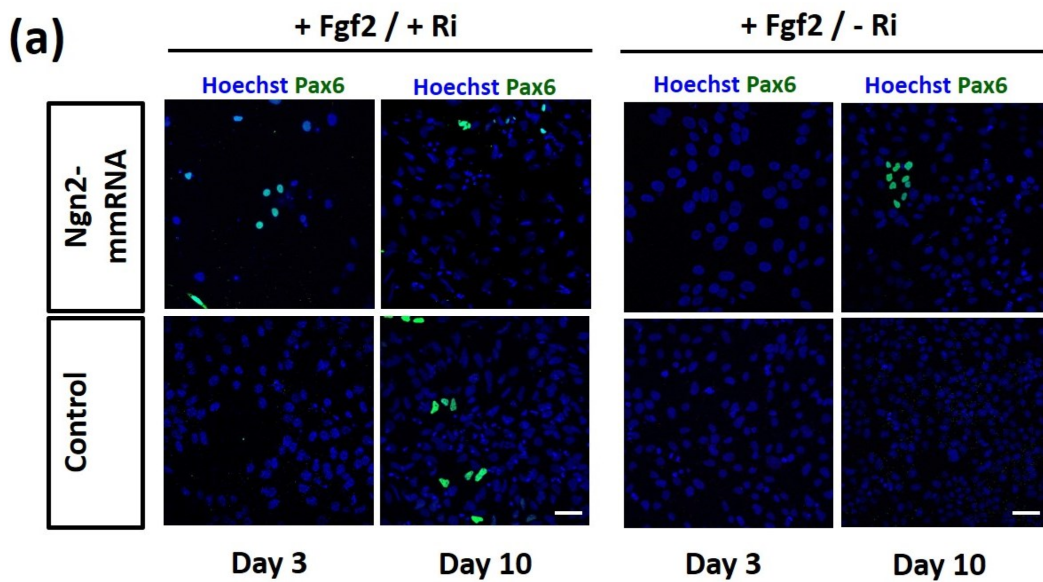


Figure 4.37: Induction of neurogenesis formation in hiPSCs by Ngn2-mmRNA. Representative images of βIIITubulin staining at Day 3 and Day 10 of the two different protocols, +Fgf2/+Ri and +Fgf2/-Ri, respectively. Ngn2-mmRNA indicates cells that have been treated with synthetic

mmRNA encoding for Ngn2. Control indicates cells that have been treated only the neuronal medium without the Ngn2 transfection, n=3. Scale bar 50um.

Moreover, the higher percentage of βIIITubulin-positive cells observed upon continuous Ri treatment (+Ri) (Fig 4.36-c) was coupled with a lower percentage of Pax6-positive at day 10 when compared to –Ri condition (Ri only for 24h) (Fig. 4.38). On the contrary, at day 3, the percentage of Pax6-positive cells in the +Ri condition results higher, also in the control group, when compared with the –Ri condition, in which Pax6 positive cells where undetectable at day 3, but present at day 10 (Fig. 4.38).



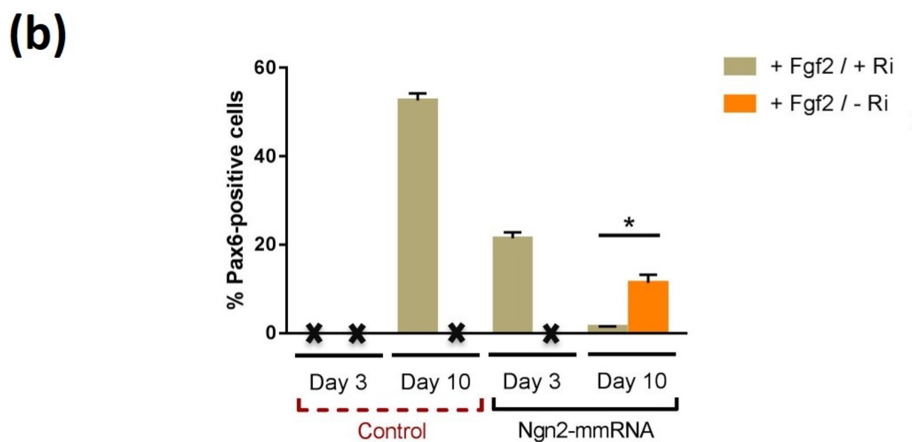


Figure 4.38: Induction of neural progenitor cells formation in hiPSCs by Ngn2-mmRNA. (a) Representative images of Pax6 staining at Day 3 and Day 10 of the three different protocols, +Fgf2/+Ri and +Fgf2/-Ri, respectively; Percentage of Pax6 expressing cells cultured with the different protocols [mean±SEM], *p<0.05. Black cross for undetectable value.

Chapter 5:

DISCUSSION

In this thesis work we optimized a system to generate neurons in microscale in less than 2 weeks, starting from hiPSCs as cell source, using both lentiviral mediated strategy and a non-integrating mmRNA-based strategy.

We centred our differentiation system on a well-defined protocol, set up by the group of Thomas Sudhof (Zhang et al., 2013), based on the lentiviral mediated expression of the pro-neural gene Ngn2 in hiPSCs, which leads to the conversion of pluripotent cells into neurons with high efficiency. We used in parallel conventional devices and microfluidic platforms and we observed that, in both systems, we were able to induce a complete conversion of hiPSCs from a pluripotent to a differentiated state, as indicated by the absence, of pluripotency markers (i.e. Oct4). The neuronal identity confirmed by presence of cells positive for neuronal markers (i.e. IIITubulin, Nestin or Map2), demonstrates how is possible to generate neurons in only 10 days also in microfluidics. This first result represents an important goal in the neurological research, because using the microfluidics to differentiate neurons, is possible to perform in parallel different experiments, using a little amount of sample, very convenient in case of patient-derived cells or drug screenings.

To date, there is a very limited amount of papers exploring the derivation of neurons in microfluidic devices, possibly because of the multiple competences required to develop such systems. In these few reports the authors demonstrated how is possible to recreate, in vitro, human brain circuitry. In particular, they developed a compartmentalized microfluidic device, for the spatial separation of the cell bodies of different human-derived neuronal subtypes (dopaminergic, excitatory and inhibitory), but with the capacity to spread the projecting processes. The induced neurons (iNs) cultivated in the device expressed the classic pan-neuronal markers and markers specific of the neuronal subtype. Moreover, if the neurons of one chamber were subjected to excitatory stimuli, it was possible to register evoked post-synaptic currents in the neurons of the adjacent chambers, showing how this system was able to create a functional neuronal network (Fantuzzo et al., 2017). Another example is represented by the differentiation of neurons by the use of a growth factor gradient generated by a microfluidic device.

With this system, the authors have attempted to recreate in vitro, the in vivo microenvironments, paying more attention to physiological processes, in order to determine the interactions between phenotypes and biological factors. In this study, neurons remained healthy, in the microfluidic device, during the entire period of cell culture, in response to the concentration gradient of growth factors. The authors demonstrated that overexpression of ASCL1 in NCPs increased neuronal differentiation depending on the concentration gradient of growth factors generated in the microfluidic gradient chip. This microfluidic system allowed to study concentration-dependent effects of factors within a single device, on the contrary a traditional system requires multiple independent cultures and a fix concentration of factors, suggesting that the microfluidic gradient-generating chip is a powerful tool for determining the optimal culture conditions (Kim et al., 2018).

Once we set-up the neuronal induction in microfluidics, we managed to regulate the timing and the pattern of Ngn2 expression in hiPSCs modulating doxycycline administration. Combining different stimulation length, we managed to impose and Ngn2 oscillatory expression with a defined period. This finding is extremely important and represents, to our knowledge, the first report of a chemically induced oscillation of Ngn2 using DOX stimulation.

The possibility to impose a defined Ngn2 oscillation pattern has a great biological meaning. Ngn2, indeed, is a crucial pro-neural transcription factor, able to instruct stem cells to maintain the pull of proliferative neural stem cells, when its expression is oscillatory, or to differentiate in mature neurons, when its expression is sustained. The possibility to chemically perturb this system using the TetOn strategy allows deriving, with one single transcription factor, cells at different maturation level, only by modulating its expression pattern. This idea paves the way to a new approach of transcriptional programming based, not only on the identity of the transcription factor required to induce a specific phenotype, but also on the timing required to induce one or multiple phenotypes.

To date, the transcriptional programming applied to the derivation of stem/progenitor cells is still at its very beginning. Indeed, protocols to derive mature cell types are much more developed and advanced (Ho et al., 2016, Frega et al., 2017). In the neuroscience field this is particularly true, since there are many papers reporting the derivation of various types of neurons (i.e. dopaminergic neurons (Yang et al., 2017), motor neurons (Busskamp et al., 2014), glutamatergic neurons (Peron et al., 2017), but protocol to efficiently derive neural stem/progenitor cells are still very few (Azmitia and Capetian, 2018). Using one single transcription factor in a frequency-encoded manner could represent a great advancement in the field of transcriptional programming.

This strategy can be further implemented using an automated micro-device. Indeed, complex biological applications that require precise timing of operations and many technical replicates are difficult to be manually performed and the reproduction of the oscillatory profile of Ngn2 is one of these examples. We showed that the automated device that was designed in our lab was suitable for prolonged cell culture and allowed the generation of Ngn2 induced neurons from iPSCs. In this preliminary experiment we imposed a medium change frequency of 12 h, to mimic what we usually perform manually. However, the system could be set to change the medium with different frequencies or to give pulses of drug-containing media, as for example containing DOX, further increasing the possibilities of large-scale production of neurons starting from hiPSCs in microfluidics.

However, the use of lentivirus to promote the overexpression of specific genes, is associated with the potential problem of host genome modifications due to integration of foreign DNA into the genome. For this reason, we focused our attention on the downscaling of in vitro neuronal differentiation protocols starting from hiPSCs but taking advantage of the use of non-integrative system, for the overexpression of a neuronal transcription factor, such as synthetic mmRNA encoding for Ngn2. mmRNAs offer the opportunity to induce the expression of a protein of interest in vivo or in vitro in a time-defined fashion and compared to lentiviral vectors, they do not integrate into the genome of the host cell,

preventing the risk of genomic mutation and oncogenesis. The mmRNA, when introduced in the host cell, is translated by the cellular translation machinery, under physiological conditions. The transcriptional programming efficiency of Ngn2-mmRNA has never been tested before. The only report available showing an mmRNA-based neuronal transcriptional programming generated, in only 10 days, functional motor neurons, using an mmRNA cocktail of 5 transcription factors (NEUROGENIN1 (NGN1), NEUROGENIN2 (NGN2), NEUROGENIN3 (NGN3), NEUROD1 (ND1), NEUROD2 (ND2)) and Dual-SMAD inhibition in conventional devices (Goparaju et al., 2017).

We tested the ability to induce transcriptional programming of Ngn2-mmRNA alone both in conventional and microfluidic devices. From our data we can clearly observe that, the differentiation efficiency of hiPSCs into neuron-like cells is higher in the microfluidic platform compared to the conventional cell culture systems. In the traditional system, in fact, at the end of the protocol (day 10) it is possible to observe the presence of residual Oct4-positive cells and the cells expressing βIIITubulin marker with a clear neuronal-like morphology are barely detectable. Instead, in the microfluidic platform, at day 10 we observed the clear presence of βIIITubulin positive neuronal-like cells. This difference can be attributed to the fact that the delivery of the soluble factors to the cells is more efficient in microfluidics and this increase the differentiation efficiency (Luni et al., 2016). The microfluidics, indeed, is characterized by a very high ratio media volume/surface, and this feature allows a fine and homogeneous delivery of the factors to the cells (Luni et al., 2016). This peculiar feature of the microfluidic systems allowed to reprogram with a high efficiency patient-derived somatic cell with non-integrating transcription factors, which are usually very inefficient (Luni et al., 2016).

Then, we focused on increasing the neuronal differentiation efficiency, acting on cellular signalling playing important roles during the in vivo development of the central nervous system. In particular we focused on:

- Fgf2 treatment: to counterbalance the cell death caused by the transfection procedure and to increase the survival of the cells, we supplemented the media with Fgf2 for the first two days of the protocol (Eiselleova et al., 2009). Fgf2 is a crucial growth factor both for human iPSCs and neural cells (Chen et al., 2007). The presence of Fgf2 increases the cell survival and proliferation and, possibly, reduces the oxidative stress caused by the absence of serum (as in neuronal medium), which usually has anti-oxidative properties. Fgf2 has the potentiality to reduce the oxidative stress with a consequent decrease of cellular DNA damage (Galderisi et al., 2013). Indeed, the administration of Fgf2 during the first steps of differentiation leds to an increase of about 4-fold in the number of neuronal-like cells generated from iPSCs without Fgf2 treatment. Our data shown that at day3 of the differentiation, soon after the treatment with Fgf2 (day1-2) is possible to observe the presence of neural progenitors (positive for Pax6), but their percentage decreases at day 10 leaving the place to βIIITubulin-positive cells. On the contrary, without the Fgf2 pulse we observed a significant higher percentage of neural progenitor cells at day 10 as compared with the condition treated with Fgf2. This data can indicate that, the administration of Ngn2-mmRNA can stretch the differentiation process that was usually observed using the lentiviral-based system, showing the appearance of a neural stem cell population not detected with the conventional lentiviral-based differentiation system. In fact, human pluripotent stem cells and fibroblast can be converted into functional neuronal-like cells with nearly 100% yield and purity in less than two weeks by forced expression of a single transcription factor (Zhang et al., 2013, Ho et al., 2016). However, it is important to specify that these protocols include a selection step, with puromycin, that selectively kills all the cells that have not integrated the lentiviral vector. So, the percentage to which the authors refer is relative to the number of cells that survived the puromycin selection. In our case, we do not perform any selection, so the cells that do not receive the Ngn2-mmRNA are only exposed to the

stimuli contained in the medium used. Moreover, the treatment with Fgf2 seems not only to increase the efficiency of neuronal-like cells differentiation, but also to speed up the process.

- Glucose treatment: During the early stages of neuronal differentiation, cells relay on glycolytic metabolism. Glucose is crucial for neuronal differentiation, neurite outgrowth, biosynthesis of neurotransmitters and synaptic formation. Once fully differentiated, neuronal cells require high energy levels to restore the neuronal membrane potential after depolarization (Knobloch and Jessberger, 2017). In addition, it has been showed that brain-derived neurotrophic factor (BDNF) has an effect in increasing glucose consumption by neuronal cells promoting the maturation of axons by activating the ATP-dependent Na/K pump, increasing the ATP request and consequently the use of glucose. In our experimental setup, we use BDNF in the last part of the neuronal differentiation protocol to support neuronal maturation. So, we tried to understand if, the percentage of neuronal-like cells obtained with Ngn2-mmRNA could have been limited by the availability of glucose in the media. However, we observed that supplementation of glucose not only did not increase the generation of neurons, but it reduced the percentage of neuronal-like cells measured at the end of the protocol. During neurogenesis, indeed, there is a different glucose demand from the cells at different stages of neuronal differentiation. While with the traditional neuronal differentiation protocols is possible to follow the different steps of the development of neurons and so to distinguish the different phases of the differentiation (i.e. hiPSCs, embryoid bodies, neural rosettes, neuronal progenitors and neurons) characterized by specific glucose demand, with the rapid generation of neurons is difficult to distinguish between the different phases and consequently to change the glucose concentration, according to the stage in which the cell is located. Furthermore, high glucose concentration suppresses embryonic stem cell capacity to differentiate into neural lineage cells. In particular Yang's group

demonstrated that high glucose concentration induces high levels of endoplasmic reticulum stress marker, CHOP and suppresses the expression of Sox1 and Nestin, βIIITubulin and GFAP. In addition, they showed that increasing the glucose concentration is possible to observe a decrease of neural crest differentiation, data supported by the low percentage of cells expressing Pax3 and Pax7 (Yang et al., 2016).

- Dual-SMAD inhibition: to further increase the conversion efficiency of iPSCs into neuronal-like cells we used two small molecules known to efficiently promote neuralization of hiPSCs, Dorsomorphin and SB431542. Several reports took advantage of this strategy to force the transcriptional programming system. Eggan's group reported that combining Ngn2 programming with SMAD and WNT inhibition allow to generate human induced excitatory neurons. In particular, they induced Ngn2 expression in TetO-NGN2-T2A-PURO/TetO-GFP lentivirally infected hPSCs by exposure to DOX 1 day after plating. Moreover, they inhibited TGF-β1 and BMP signaling, using SB431542 and LDN193189, and induced dorsalization by inhibiting Wnt signaling, through treatment with XAV939, a tankyrase inhibitor (Nehme et al., 2018). Furthermore, the Kokaia's group demonstrated that human fibroblasts can be directly converted into functional excitatory cortical neurons, through a combination of three transcription factors, BRN2, MYT1L, and FEZF2, small molecules (i.e. CHIR99021, SB431542, Noggin and LDN-193189) and microRNAs (i.e. miR-9/9* and miR-124) (Miskinyte et al., 2017). Another example is represented from the research of Ko's group, which promoted the rapid conversion of hiPSCs into mature neurons using a cocktail of synthetic modified mRNA encoding for five different transcription factors (NGN1, NGN2, NGN3, ND1, and ND2) in combination with a small molecule cocktail consisting of forskolin, SB431542, dorsomorphin and retinoic acid (Goparaju et al., 2017). In all this work the authors acted at different levels of the neurogenesis, forcing as much as possible the internal machinery responsible of the generation of neurons. However, in our hands, the use

of these small molecules, both for two days or until the end of the protocol, did not increase the efficiency of neuronal induction, if compared with the protocol in which cells were treated only with the Fgf2. This probably because combining Ngn2 programming with SMAD inhibition generates cells along a developmental continuum, ranging from poorly differentiated neuronal progenitors to neurons. So, what we observe, it is not an increase in efficiency in neuronal differentiation, interpreted as a percentage of βIIITubulin, but a different state of maturation or a greater uniformity in the population of neuronal progenitors obtained. In fact, it has been demonstrated that the combination between Ngn2 programming and SMAD - WNT inhibition generates human patterned iNs (hpiNs). Single-cell analyses showed that hpiNs cultures contained cells along a developmental continuum, ranging from poorly differentiated neuronal progenitors to well-differentiated, excitatory glutamatergic neurons (Nehme et al., 2018).

- Neuroepithelial cells as cell source: to reduce the intrinsic cell culture variability, we applied the transcriptional programming with Ngn2-mmRNA to a uniform population of cells committed to neuralectoderm, obtained through the treatment of hiPSCs with small molecules (Dorsomorphin and SB431542). At the end of our experiments we observed that the generation of neuronal-like cells with the use of neuralectoderm as cell source is extremely low (0.4%). We can explain this low efficiency of neuronal differentiation because the induction of neuroectoderm requires a state of confluence from the cells that make up the cell culture and the subsequently creation of a compact monolayer of neuroepithelial cells. By creating neuroectoderm in well, detaching them to single cells and transfecting them with mmRNA, we may have induced a series of stresses that negatively impacted the survival and differentiation induced by Ngn2. These results confirm that, in our hands, the best strategy for the rapid conversion of neurons is the direct treatment of hiPSCs with Ngn2-mmRNA without passing through the neuronal

progenitor stage reducing the time and the cost for the generation and maintenance of the neuroectoderm in culture.

- Increasing the dose of Ngn2-mmRNA: to further push iPSCs toward neuronal fate, we tested different transfection frequency of Ngn2-mmRNA, since we observed that the treatment with neural inducing stimuli did not promote a higher neuronal conversion mediated by Ngn2-mmRNA transfection. As explain before, Ngn2 is expressed in an oscillatory manner by neural progenitors, whereas becomes constantly expressed once neural progenitor undergo neuronal differentiation (Kageyama et al., 2008). Furthermore, in case of inducible systems as the lentiviral transduction, cells need to transcribe and translate the gene, but then the production of protein remains high and constant as long as the transgene expression is induced. On the contrary, mmRNA treatment is characterized by a context in which the mmRNA are ready to use, but once the cell has transcribed the entire dose that was transfected, the protein production rapidly stops. We confirmed the expression pattern of Ngn2 giving a pulse of Ngn2-mmRNA and testing the expression of Ngn2 at different time points. In our hand, at 2 h the Ngn2 protein is already detectable, remaining visible until 16 h from the transfection, when we observed the maximum percentage of Ngn2-positive cells, and then decreased at 24 h. Analysis of the induction kinetics of the Ngn2 protein in hiPSCs, analysed by Ko's group revealed a very rapid induction as early as 30 minutes after transfection, which continued up to 8 hours. Around 80% of the cells stained positive for Ngn2 protein expression by 90 minutes after transfection. However, it is important to underline that the synthetic mRNA used by this group were different form the one used in this thesis, and that the neuronal differentiation that has been induced by this group required synthetic mRNAs encoding for different neurogenins and NeuroD (Goparaju et al., 2017), while in our experiments we used only one transcription factor. These differences can explain the delay in the Ngn2 expression that we observed in our experimental setting. In our

experiments, the increase of Ngn2-mmRNA transfection frequency from once/24h to once/12h accelerated the neuronal-like cell formation as demonstrated by the increased number of βIIITubulin-positive cells with an immature morphology observed at day 3. However, transfecting iPSCs every 12 h resulted in an increase cell mortality, which is reflected in a reduction in the overall number of cells. This cell mortality could be explained thinking about to the nature of the transfection procedure. Transfection mix is composed by blends of cationic lipids, which are able to complex the mmRNA molecules, that could be toxic for the cell culture. To reduce this toxic effect but to increase the differentiation efficiency we could think to administer Ngn2 every 12 h, but using half of the dose in each transfection, thus leaving the total amount of RNA unchanged.

- Use of Rock inhibitor: Rock inhibitor Y27632 is a selective inhibitor of the p160-Rho-associated coiled kinase, used in iPSC research to avoid cell death and differentiation caused by single cell dissociation (Zhang et al., 2011). Moreover, it has been demonstrated that Rock inhibitor has a massive effect on cell shape and mechanotransduction. Indeed, the regulation of proliferation by cell shape and forces is particularly intriguing because there are many events during embryogenesis that involve dramatic changes in cell shape, structure, and mechanics and Rho Kinase (ROCK) is a potent regulator of contractility (Wozniak and Chen, 2009). Furthermore, it has been demonstrated that ROCK inhibitors promote the conversion of hESCs into neural crest-like progenitors that are characterized by ability to differentiate into multiple cell types, including peripheral neurons (Kim et al., 2015). In this project, to counterbalance the detrimental effect of the single cell dissociation, and to increase the transfection efficiency acting on cell shape, we supplemented the culture media with rock inhibitor Y27632 either for the entire experiment or just for the first day after seeding. We observed that, as expected, the treatment with Rock inhibitor efficiently induced an increase in the number of transfected cells. However, this treatment, substantially

modified cell fate instructing iPSCs to differentiate into p75-positive cells neural crest-like progenitors, at day 3. Neural crest progenitors have the potentiality to differentiate not only into mesenchymal cell types, but also in cell types belonging to the peripheral nervous system, including neurons. This could explain the increase of neuronal-like cells, after prolonged treatment with Ri. However, to confirm the neural crest derivation of those neuronal-like cells further analysis is required, such as stainings for specific markers like peripherin. These results open up new exiting prospective to the application of the Ngn2-mediated transcriptional programming. Indeed, to date, the derivation of neural crest from hiPSCs is mainly based on differentiation protocols that consider the rosette formation and a combination of factors, such as SOX10 and dbcAMP (Lee et al., 2007a). Currently, there is a unique work in which the authors generate a neural crest population, in 14 days, by forced the expression of a single transcription factor, SOX10, in combination with environmental cues including WNT activation (Kim et al., 2014).

In our experimental setting, we managed to obtain a very high percentage of neural crest cells (around 30%) after only 3 days of differentiation playing with mechanotransduction in combination with neural inducing stimuli (Ngn2), normally used to instruct iPSCs to acquire central nervous system identity. This finding opens up a completely new prospective for the transcriptional programming, showing how, using a single transcription factor but changing the physical properties con the cells, iPSCs can be instructed to acquire central or peripheral nervous system identity.

Finally, we observed that, the continuous treatment with Ri, not only was able to instruct hiPSCs to rapidly acquire a neural crest identity, but also affected the appearance of Pax6 positive cells, which are usually undetectable in lentiviral mediated neural induction. Neural progenitor cells have been detected only when lentiviral Ngn2 induction was coupled with dual SMAD and WNT inhibition (Nehme et al., 2018). This can be observed monitoring the percentage of Pax6 positive cells during time. At day 3, when the neural crest represents more than 30% of the total

cell population, Pax6 positive neural stem cells are representing around 20% of the total population, rapidly decreasing at day 10. On the other hand, in the condition without Ri, at 3 days Pax6 positive cells are undetectable, appearing only in a small percentage at 10 days of differentiation. The presence of Pax6 positive cells also in the control group treated with the neutralizing medium and Ri, but not transfected with Ngn2 could suggest that the combination of this signals is enough to induce a partial neutralization of hiPSCs, which is accelerated with the treatment with Ngn2, and the presence of Ri can further speed up the process.

CHAPTER 6:

CONCLUSIONS &

FUTURE PERSPECTIVES

In this thesis we showed how the phenotype displayed in vitro by human neurons derived by differentiation of pluripotent stem cells can be modified and driven. Promoting the maturation of human neurons, in a microfluidic context, is a desirable process for the generation of a robust and representative model for the study of the nervous system physiology and physiopathology. The applications of such cellular models span across all the field of in vitro studies, from disease modelling to compound screening and drug testing, down to basic understanding of the nervous system biology.

With this in mind, with this thesis we demonstrated how is possible to obtain human neurons generated, in only 10 days, in a high through-put context, represented by the microfluidic platform. We induced the differentiation of neurons, through the overexpression of a unique transcription factors encoding for Ngn2, proneural gene, by conveying synthetic modified mRNA into the host cells. Furthermore, we have demonstrated, how in only 3 days, is possible to differentiate neural progenitor cells or neural crest cells, starting from hiPSCs, such as hiPSCs-patient specific.

Furthermore, we demonstrated that taking advantage of neuronal differentiation via lentiviral transduction of Ngn2 is possible to regulate the timing and the pattern of Ngn2 expression mimicking, in vitro, the oscillatory profile of Ngn2. Indeed, different Ngn2 expression patterns can result in distinct cell populations: neural stem cells or mature neurons.

On the basis of our first results, the following steps will be realized: a coculture with astrocytes to improve the efficiency of differentiation of neurons and to carry out activity assays; a characterization of cells resulting from the induction of the Ngn2 oscillatory pattern, in order to confirm the identity of neural progenitor; a characterization of the most in-depth neural crest and of the neurons differentiated at day 10; a further mechanotransduction interference, mediated for example by latrunculin, instead of Ri, to confirm that the mechanism of induction of neural crest is really mediated by mechanotransduction.

The possibility to generate both neural stem cells and neurons using a single factor (i.e. Ngn2) and different expression frequencies offers the unprecedented opportunity to modify in vitro and in vivo cell fate based on the specific needs of progenitors or mature neurons. Indeed, in less than a week it is possible to obtain on one hand neural progenitors or neural crest cells, to be used for cell therapy, starting directly from the patient's cells or, on the other side, homogeneous populations of specific neuronal types for drug screening.

Lastly, the microfluidic technologies, through the automatization of the devices, could give an important contribute in this field, because offer the possibility of a periodic and finely regulated release of specific drugs or molecules. In case of Ngn2-mmRNA, for example, could permit the control of Ngn2 oscillatory pattern and consequently the generation of neural progenitors rather than mature neurons and vice versa or in case of soluble factor as the glucose, the media concentration could be modified according to the needs of the developing neuronal cell increasing the differentiation efficiency.

CHAPTER 7:

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…success is not final; failure is not fatal.

It is courage to continue that counts!

(cit. Winston Churchill)