"Mesmo quando tudo parece desabar,
cabe a mim decidir entre rir ou chorar,
ir ou ficar, desistir ou lutar.
Porque descobri, no caminho incerto da vida,
que o mais importante é o decidir"
Cora Coralina
(1889-1985)

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Abbreviations

5HT – Serotonin

AA – Ascorbic Acid

AADC – aromatic L- aminoacid decarboxylase

ABM – Ascl1, Brn2 and Myt1L

AD – Alzheimer's disease

ALDH – aldehyde dehydrogenase

ALS – amyotrophic lateral sclerosis

AN – Ascl1 and Neurogenin2

ASCs – adult stem cells

BDNF – brain-derived neurotrophic factors

BrdU – bromodeoxyuridine

cAMP – cyclic adenosine monophosphate

CB-SCs – cord blood-derived stem cells

CNS – central nervous system

cPPT - central polypurine tract

d-cAMP – dibutyryl cAMP

DA - dopamine

DAT – dopamine transporter

DCX – doublecortin

DNA – deoxyribonucleic acid

ESCs – embryonic stem cells

GABA – gamma-aminobutyric acid

GDNF – Glial cell line-derived neurotrophic factor

GSK-3β - glycogen-synthase kinase 3β

HD – Huntington's disease

hEF – human embryonic fibroblast

hESCs – human embryonic stem cells

hiN – human induced neuronal

hPGK – human phosphoglycerate kinase

iDA – induced dopaminergic

iN – induced neuronal

iPSCs – induced pluripotent stem cells

LTR – long terminal repeat

LVs – lentiviruses

mDA – mesencephalic dopaminergic

MEFs – mouse embryonic fibroblasts

miR – micro-RNA

MOI – multiplicity of infection

mRNA – messenger RNA

NCAM – neural cell adhesion molecule

Ngn – neurogenin

NMDA – N-methyl-D-aspartic acid

NMDAR – NMDA receptor

NPCs – neural progenitor cells

NSCs – neural stem cells

NT3 – neurotrophin 3

ORF – open reading frame

PCR – polymerase chain reaction

PD – Parkinson's disease

PPT – polypurine tract

PSA-NCAM – Polysialylated neural cell adhesion molecule

RA – Retinoic acid

RNA – ribonucleic acid

SGZ – subgranular zone

SM – small molecules

SVZ – subventricular zone

TF – transcription factor

TH – tyrosine hydroxylase

TRE – tetracycline response element

vGAT – vesicular GABA transporter

vGLUT – vesicular glutamate transporters

vMAT – vesicular monoamine transporter

WPRE - woodchuck hepatitis posttranscriptional regulatory element

Abstract

New findings in stem cell research show that it is possible to reprogram a somatic cell into a pluripotent stem cell, by delivering specific genes. The induction of pluripotency arouse questions related with the possibility of directly reprogram a somatic cell into another somatic cell type, also by delivering specific combinations of genes, without going through a pluripotency state.

Several reports have shown that this direct conversion is possible and in 2010, the first description of the conversion of mouse fibroblasts into induced neuronal (iN) cells was published. Since then, it has been established that it is possible to obtain subtype specific iN cells from human fibroblasts.

This thesis describes a project with the aim of directly convert human fibroblasts into striatal GABAergic projection neurons, by using a combination of defined transcription factors with important biological functions in neuronal development and differentiation of striatal neurons. Since these neurons are specially affected in cases of Huntington's disease and stroke, their generation through the direct conversion process would possibly be a valuable approach to obtain cells that could be used in cellular therapy, being transplanted into patients' brains.

In order to maximize the efficiency of neural conversion, a comparative study including several protocols for the generation of iN cells was also performed.

Resumo

Novas descobertas na área de investigação em células estaminais demonstram ser possível reprogramar uma célula somática numa célula pluripotente, através da entrega de genes específicos. Esta indução de pluripotência levantou algumas questões relacionadas com a possibilidade de converter directamente um determinado tipo célula somática noutro, também através da entrega de combinações específicas de genes, sem que esta célula passe por um estado de pluripotência.

Várias publicações demonstraram que esta conversão directa é possível e em 2010, a primeira descrição da conversão de fibroblastos de rato em células neuronais induzidas foi publicada. Desde então, foi estabelecido também que é possível obter células neuronais induzidas, de um determinado sub-tipo, a partir de fibroblastos humanos.

Esta tese descreve um projecto que teve como objectivo converter directamente fibroblastos humanos em neurónios GABAérgicos estriatais, utilizando uma combinação de factores de transcrição, com funções biológicas relacionadas sobretudo com o desenvolvimento neuronal e a diferenciação de neurónios estriatais. Pelo facto de estes neurónios serem especialmente afectados em casos de doença de Huntington e acidente vascular cerebral, a sua obtenção através de um processo de conversão directa constituiria um método de grande valor para a obtenção de células que poderiam ser utilizadas em terapia celular, servindo como material de transplante em cérebros de pacientes.

De modo a maximizar a eficiência da conversão neuronal, um estudo comparativo envolvendo diferentes protocolos para a obtenção de células neuronais induzidas foi também realizado.

Chapter I – Introduction

1.1 Stem cells and regenerative medicine in the central nervous system

Ever since Santiago Ramon y Cajal, the famous anatomist, wrote in the early 20th century that the central nervous system (CNS) does not regenerate once it is injured (Ramon y Cajal and May, 1928), this theory has been in fact accepted. Actually, there is a lack of regenerative properties from the CNS that may be attributable to several factors. It can be observed, for example, during a spinal cord injury, in which the inhibitory character of the CNS myelin and injury-induced glial scars, the apparent inability of endogenous adult neural stem cells (NSCs) in the spinal cord to induce *de novo* neurogenesis upon injury (Johansson et al., 1999), and the lack of sufficient trophic support (Widenfalk et al., 2001), can difficult regeneration.

The use of transplantation as a therapeutic approach to repair the brain and spinal cord has the specific goals of providing new neurons that either functionally reconstruct neural circuits, produce neurochemically active substances (neurotransmitters, growth factors, antibodies, or growth substrates), or remyelinate axons (Okano, 2002).

Decades later, some studies reported a promising and successful transplantation of peripheral nerves (Richardson et al., 1980) and fetal spinal cord (Bregman, 1987), that indicated that by introducing an appropriate environment into the injured site could cause injured axons to regenerate. Spinal cord regeneration is also described by other reports, including the promotion of the regeneration of injured axons by neurotrophic factors (Cai et al., 1999), and the identification of axonal growth inhibitors (Chen et al., 2000). During the late 1980's, studies involving the transplantation of human fetal mesencephalic tissue, obtained from aborted fetuses, to the striatum of Parkinson's disease (PD) patients were started. These grafts were able to survive and to induce symptomatic relief in some patients, reinnervating the striatum, releasing neurotransmitters and becoming integrated in the patient's brains (reviewed in (Lindvall and Bjorklund, 2004)). These findings opened a new era for regenerative medicine in the brain. However, although the feasibility of this technique has been demonstrated, its practical clinical application has found some technical barriers due to the small number of donor cells, the large variation in outcome

and adverse effects such as graft-induced dyskinesias in some patients (Bjorklund et al., 2003; Freed et al., 2001; Olanow et al., 2003; Winkler et al., 2005).

Stem cells are an unique type of cell that can be distinguished from the other body cells because of three major characteristics: a) they are undifferentiated and unspecialized; b) they have self-renewing and indefinitely division capacities; and c) they are able to become specialized into all cell types of the human body, from the three different germ layers (endoderm, mesoderm and ectoderm) (Verfaillie et al., 2002).

Stem cell self-renewal is the consequence of a particular cell division that takes place within a special microenvironment where stem cells reside, the niche. In the niche, the stem cells division can result in a progenitor daughter and in a daughter that remains a stem cell (asymmetric division) or in two stem cell daughters (symmetric division). Asymmetric stem cell division contributes to the correct replacement of daughter cells inside and outside the niche. Then, when subjected to specific molecular signals, progenitor cells generate a differentiated progeny (Cheng et al., 2008; Cowan and Hyman, 2004; Gaziova and Bhat, 2007; Rusan and Peifer, 2007; Segalen and Bellaiche, 2009; Tulina and Matunis, 2001; Yamashita, 2009; Yamashita and Fuller, 2008; Yamashita et al., 2005; Yamashita et al., 2007).

Stem cell-based replacement therapies, in which these cells are used as transplant material, are considered as an emerging powerful tool for regenerative medicine. Due to their regeneration properties, stem cells evoke a solid enthusiasm in the medical investigation field and allow predicting several therapeutic applications, including for neurodegenerative diseases (Orlacchio et al., 2010).

The term *neurodegenerative disease* is used for a wide range of either acute or chronic conditions, in which neurons and glial cells in the brain and spinal cord are lost. In the case of acute conditions, as a response to an ischemic stroke or a spinal cord injury, different types of neurons and glial cells die within a restricted area of the brain over a short period time. In chronic conditions, there is either a selective loss of a specific cell population, such as dopamine (DA) neurons in PD and motor neurons in amyotrophic lateral sclerosis (ALS), or a widespread degeneration of many types of neurons, such as the

one occurring in Alzheimer's disease (AD), over a period of several years (Lindvall and Kokaia, 2010).

According to its origin and differentiation capacity, several types of stem cells are considered to be interesting in the field of regenerative medicine. Each stem cell type possesses certain qualities and advantages, and the rationale for utilizing each depends on the desired applications and outcomes. Similarly, each set of stem cells is associated with its own set of problems as well.

1.1.1 Embryonic stem cells

At the 4th day after fertilization, the cells resulting from the divisions of the recently formed egg (totipotent cells) start to become specialized forming the blastocyst, from which it is possible to remove the cells present in its internal layer. Those cells are capable to origin any kind of tissue in the organism, of any of the three primary germ layers as well as the primordial germ cells, except the extra-embryonic structures (placenta and supporting tissues of the uterus) (Fischbach and Fischbach, 2004; Wobus and Boheler, 2005). These pluripotent cells are known as embryonic stem cells (ESCs).

ESCs have the ability to perpetually self-renew in culture and maintain undifferentiated phenotype, as well as a normal karyotype (Gage et al., 1995). The establishment of human ESCs (hESCs) from *in vitro* fertilized embryos (Thomson et al., 1998) and the demonstration of their developmental potential in vitro (Schuldiner et al., 2001; Thomson et al., 1998) have evoked widespread discussions concerning future applications of hESCs in regenerative medicine. Therefore, they were seen as excellent candidates for using as a source of neurons during the treatment of neurological disorders (Thomson et al., 1998).

However, the use of these cells has been associated with their own set of problems. A major hurdle is related to problems in generating large and defined populations of the desired types of neurons from hESCs (Li et al., 2008). Also, a good cell survival of the graft is essential for the effectiveness of the transplant during the treatment of those neurological disorders (Hagell and Brundin, 2001). A poor survival of the grafted cells was observed in some studies, which was explained by the possibility of those cells being relatively mature when harvested from culture (Freeman and Brundin, 2006), suggesting

that their maturation has passed a narrow optimal time window at the time of harvesting. Thus, a balance in the percentage of differentiation of the transplanted cells is needed.

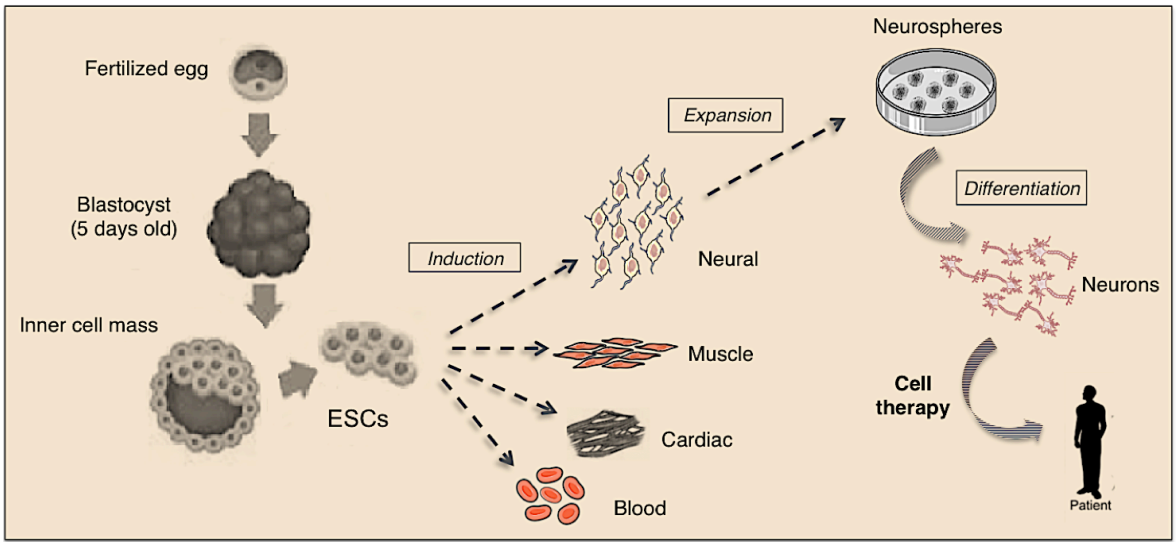


Figure 1 – Stem cell therapy for CNS disorders using hESCs.
Five days after fertilization, it is possible to remove the cells present in the internal layer of the recently formed blastocyst. From these cells, ESCs, neural precursors (neural stem cells) can be obtained (among others, as cardiac, muscle and blood precursors), expanded through neurosphere formation and differentiated into neurons that could be used for cell therapy purposes in neurodegenerative diseases' treatment.

While the excessive maturation of the cells is not advantageous, the excess of undifferentiated cells also brings up the risk of tumor formation after transplantation (Li et al., 2008). The delineation of the optimal developmental stage of transplanted cells remains a key issue. Candidate cells should be committed to their target specialization but must retain the plasticity of their precursors that is necessary for effective integration in the CNS (Einstein and Ben-Hur, 2008).

Even if the cell survival of the graft is optimal, another issue that can affect ESC-derived cell transplantation is the immune rejection of the grafts. Although the brain is considered to be an immune-privileged transplantation site (Li et al., 2008), strong immune responses occur in the brain in neurodegenerative disorders (Nguyen et al., 2002) or after intra-cerebral neural transplantation (Barker and Widner, 2004; Krystkowiak et al., 2007). Consequently, allogeneic cells and animal-derived products used during *in vitro* differentiation of the cells might trigger immune reactions and lead to graft rejection (Martin et al., 2005). Immunosuppressive treatments are used, but they do not fully

prevent chronic rejection and the risk of opportunistic responses increase (Brimble et al., 2004; Lopez et al., 2006). Another strategy is the generation of stem cells that are perfectly genetically matched to the host and can be generated by somatic nuclear transfer, also named therapeutic cloning (Barberi et al., 2003; Hipp and Atala, 2004). These cells would be identical to the recipient except for the proteins encoded by the mitochondrial genome. More recently, induced pluripotent stem cells (iPSCs), discussed in section 1.1.3, have become a possibility to generate genetically matched cells for transplantation.

The use of ESCs has also been strongly controversial due to the political, religious and ethical implications about the use of human embryos for their obtention.

Despite their use in transplantation therapy, ESCs remain as valuable research tools, namely for understanding the gene function in mammals, unlocking the function of many genes in normal development, normal physiology, and disease pathogenesis. The ability to create specific disease models was also considered as a possibility while using these cells, enabling drug screening on proximate models of human disease rather on animal surrogates (Verfaillie et al., 2002).

1.1.2 Neural stem cells

As the pluripotent cells become more specialized, they form specific tissues, and their differentiation potential becomes more restrict, so they can be called multipotent (Santner-Nanan et al., 2005; Verfaillie et al., 2002). In the adult organism it is possible to isolate multipotent stem cells from several tissues, such as: bone marrow, blood, adipose tissue, dental bulb and also from the skin, liver, pancreas and other organs. Those non-embryonic cells are somatic or adult stem cells (ASCs) and they are likely to replenish cells that are lost by physiological turnover, as well as pathological conditions including injury and degenerative diseases (Okano, 2002).

It has long been believed that stem cells are not present in the adult mammalian CNS, but many lines of recent evidence have shown that stem cells do exist in the adult brain and that they are self-replicative, as well as able to give rise to differentiated progeny (neurons, astrocytes and oligodendrocytes) *in vitro* and *in vivo* (Doetsch et al., 1999; Johansson et al., 1999; Kempermann and Gage, 1999; Morshead et al., 1994; Reynolds and

Weiss, 1992; Temple and Alvarez-Buylla, 1999). Those stem cells are known as neural stem cells (NSCs) and their multilineage potential is at least partially mediated by the generation of cell lineage-restricted intermediate progenitor cells that produce only neurons (neuronal progenitor cells (NPCs)) and glial progenitor cells that produce only astroglial or oligodendroglial cells (Luskin et al., 1988; Qian et al., 2000).

Although these stem cells have the ability to give rise to the three types of cells composing the CNS, it seems that the endogenous brain environment that is responsible for such differentiation process is not adequate and gives rise to a limited capacity of repair in the adult CNS of patients suffering from injury or diseases (Bjorklund and Lindvall, 2000).

During the last years, continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes, and these immortalized lines have advantages for basic studies of neural development and cell replacement therapy or gene therapy studies (Flax et al., 1998; Hoshimaru et al., 1996; Kim, 2004; Kim et al., 2008; Lee et al., 2007; Renfranz et al., 1991; Snyder et al., 1992). Cell replacement and gene transfer to the diseased or injured CNS with NSCs have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological disorders including PD, AD, ALS, stroke, spinal cord injury and brain tumors (Brustle and McKay, 1996; Flax et al., 1998; Gage, 2000; Goldman, 2005; Gottlieb, 2002; Kim, 2004; Lindvall et al., 2004; McKay, 1997; Temple, 2001). It has also become clear that the characteristics of the pathological environment, such as the magnitude of inflammation, play a crucial role in the survival, differentiation, and function of both grafted and endogenous cells (Becker et al., 2007; Biscaro et al., 2009; Di Giorgio et al., 2007; Hoehn et al., 2005; Lepore et al., 2008; Liu et al., 2007; Marchetto et al., 2008; Thored et al., 2009).

One of the major breakthroughs of the CNS stem cell biology was the establishment of clonogenic expansion of NSCs by neurosphere formation (Reynolds and Weiss, 1992), which enabled the definition of NSCs experimentally and the quantification of the multilineage potency and self-renewing ability of these cells. Using this culture

method, NSCs can proliferate in an undifferentiated state *in vitro*, allowing them to be expanded mitotically and harvested in bulk.

By taking advantage of the ease of NSCs harvesting after expansion *in vitro* by neurosphere formation (or similar methods), there have been numerous attempts to transplant NSCs into animals to treat damaged brains and spinal cords (Ogawa et al., 2002; Studer et al., 1998; Svendsen et al., 1996).

The mechanisms of action of stem cells and their progeny underlying behavioral recovery in animal models are better understood than they were just a few years ago (Lindvall and Kokaia, 2010). Besides cell replacement, stem cells are known to lead to improvements that could also be of clinical value through immunomodulation, trophic actions, neuroprotection, and stimulation of angiogenesis (Corti et al., 2007; Hwang et al., 2009; Kerr et al., 2003; Klein et al., 2005; Pluchino et al., 2005; Suzuki et al., 2007; Xu et al., 2009; Xu et al., 2006).

1.1.3 Induced pluripotent stem cells

Among some of the most relevant findings in the field of stem cell biology research, the possibility of reprogramming somatic cells to a pluripotent ESC-like state, by forcing the cells to express genes and factors important for maintaining pluripotency and to differentiate into cells of all three germ layers was absolutely stunning.

By using only four transcription factors (TFs) (Oct3/4, Sox2, Klf4 and c-myc) Takahashi and colleagues have demonstrated that somatic mouse cells (embryonic and adult) may be reprogrammed into induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Those factors function in the maintenance of pluripotency in both early embryos and ESCs (Oct3/4 and Sox2) (Avilion et al., 2003; Nichols et al., 1998; Niwa et al., 2000) and are frequently upregulated in tumors, contributing to the long-term maintenance of the ESCs phenotype and their rapid proliferation in culture (c-myc and Klf4) (Cartwright et al., 2005; Li et al., 2005). The resulting iPSCs had many of the characteristics of mouse ESCs, but failed to contribute to chimeras at term, which is considered the gold standard criterion for a pluripotent stem cell population, and showed differences in gene expression and chromatin organization when compared with ESCs

(Thompson and Yin, 2010). One year later, the same research group was able to generate iPSCs that contributed to chimeras (Okita et al., 2007) and moreover, they translated those remarkable findings from mouse to human (Takahashi et al., 2007), transducing cultures of adult human fibroblast populations from different donors with retroviral vectors carrying transgenes for the human versions of Oct4, Sox2, Klf4 and c-myc (Figure 2), and after thirty days under human ESC culture conditions, the culture plates were covered with iPSCs (Takahashi et al., 2007).

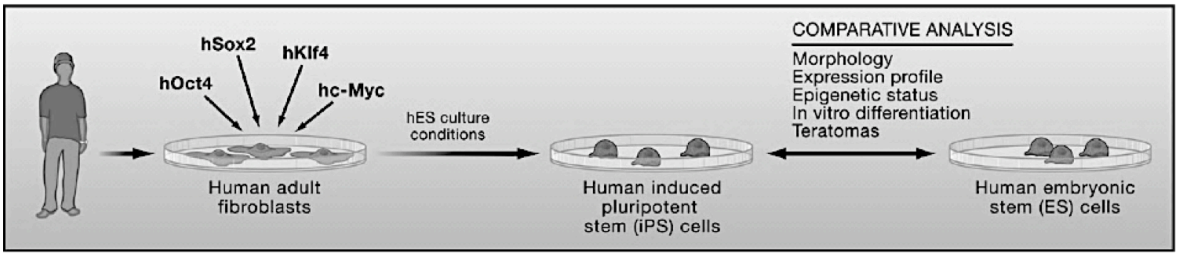


Figure 2 – Transcriptor factor-induced pluripotency.
Adult fibroblasts obtained from human donors were exposed to retroviral vectors expressing a cocktail of four transgenes encoding the human factors hOct4, hSox2, hKlf4 and hc-myc. Thirty days after transduction and further cultivation under hESCs growth conditions, human iPSC colonies that could be propagated and further expanded were isolated. Comparative analysis of human iPSCs and hESCs using assays for morphology, surface-marker expression, gene expression profiling, epigenetic status and *in vitro* and *in vivo* differentiation potential revealed a remarkable degree of similarity between these two pluripotent stem cell types (image adapted from (Zaehres and Scholer, 2007).

Yu et al., provided additional insight as to the mechanisms that induce pluripotency in human fibroblasts, in 2007, and also succeeded in reprogramming human somatic cells, but with a different cocktail of factors (Yu et al., 2007). Since the publication of comparable findings about human iPSCs underscores the validity of these cells and removes any doubt that their isolation might be irreproducible.

The obtained iPSCs may be used for many applications, circumventing the problems that are associated with ethical issues regarding the use of human embryos for the obtention of ESCs, as well as the problem of tissue rejection after transplantation into patients, since they could be derived directly from the patient's own somatic cells. Also due to its pluripotent characteristics, those cells could be used to generate other cell types, being a helpful tool in regenerative medicine (Takahashi and Yamanaka, 2006). In humans, many different cell types have been used for reprogramming into iPSCs, such as

keratinocytes, CD34+ hematopoietic stem cells, cord blood-derived endothelial cells, NSCs, amniotic fluid-derived cells, CD34+ peripheral blood cells, adult human adipose stem cells derived from lipoaspirate, among many others (reviewed in (Masip et al., 2010).

Furthermore, in the near future, iPSCs can also be used as disease models, helping to elucidate disease mechanisms, study metabolic pathways and/or screen new drugs, as well as models for normal development, oncology and differentiation processes in humans (Masip et al., 2010).

During development, the diverse cell types are defined by lineage-specific TFs that guide and reinforce cell type-specific gene expression patterns. Epigenetic modifications will further stabilize cellular phenotypes, allowing faithful transmission of cell-specific gene expression patterns over the lifetime of an organism (Bernstein et al., 2007; Jenuwein and Allis, 2001). Thus, the remarkable transformation of cells into a pluripotent state has been interpreted as a reversion of mature into more primitive developmental states, with a concomitant erasure of the developmentally relevant epigenetic information (Silva and Smith, 2008).

Although iPSCs and ESCs are very similar, they are not identical, as previously shown by Takahashi et al. (Takahashi et al., 2006). They show differences in gene expression signatures, as also shown by Chin et al. (Chin et al., 2009), as well as differences in DNA methylation patterns (Deng et al., 2009), and the efficiency to differentiate to specific lineages has been reported to be superior in ESCs compared with iPSCs (Feng et al., 2010).

Extensive tests will be required to confirm that the resulting cell lines are equivalent to those derived from embryos. Some authors also refer that would be a tremendous mistake to consider hESCs obsolete, since there are still many hurdles to overcome before we fully understand pluripotency and before obtaining human iPSCs that are suitable for therapeutic application. It has been demonstrated that mouse iPSC-derived chimeras frequently develop tumors, resulting from the activation of the oncogenes c-myc and Klf4 (Kim et al., 2009; Markoulaki et al., 2009; Okita et al., 2007). The use of genome integrative methods, such as retroviral/lentiviral vectors, may also cause, by itself, tumor formation. Although the expression of encoded genes is silenced in

fully reprogrammed iPS cell lines with retroviral integrative methods (Hotta and Ellis, 2008), and nearly complete silencing of lentiviral transgenes has been observed in the context of induced pluripotency (Ebert et al., 2009; Yu et al., 2007), the integrated foreign DNA remains in the genome and could disrupt/alter the host genome expression, causing tumor formation (Hochedlinger et al., 2005). Additionally, it has been proposed that residual transgene expression may explain some of the observed differences between ESCs and iPSCs, such as the altered differentiation into functional cell types (Soldner et al., 2009; Yu et al., 2007).

Despite all the questions that remain about iPSCs, reprogrammed cells have certainly a tremendous potential for new discoveries, which may help establish therapies for current and new diseases (Masip et al., 2010).

1.2 Direct reprogramming, iN cells and regenerative medicine in the central nervous system

1.2.1 Direct conversion, an old and new concept.

Reprogramming of somatic cells by using viral vectors, has raised the question of whether reprogramming could be successfully achieved by directly converting one differentiated cell type into another. This so called direct conversion process or lineage reprogramming has received significant attention due to the possible applications for cellular therapy (Masip et al., 2010).

In fact, several previous studies have shown the induction of direct conversion, generally within the same lineage, due to the ectopic overexpression of isolated factors. In 1987, Davis et al., were able to convert fibroblast-like cells into stable myoblasts by transfecting a single myoblast-specific cDNA (myoD) (Davis et al., 1987), but the same was not possible when transfected cells were hepatocytes (Schafer et al., 1990). Since then, other remarkable examples of cell-fate reprogramming by defined factors have been reported: the overexpression of IL-2 receptor and granulocyte-macrophage colony-stimulating factor receptor induced a myeloid conversion of committed lymphoid progenitor cells (Kondo et al., 2000); the expression of C/EBP alpha and beta in B cells

(Xie et al., 2004) as well as the overexpression of PU.I (also called Sfpi I) and transcription factors C/EBP alpha and beta in fibroblasts (Bussmann et al., 2009; Feng et al., 2008; Graf and Enver, 2009) induced macrophage differentiation. Moreover, the deletion of Pax5 can induce B cells to de-differentiate toward a common lymphoid progenitor (Cobaleda et al., 2007).

Significant evidence suggests that a specific combination of multiple factors, rather than a single one, may be the most effective tool to reprogram adult cells. Zhou et al. described the lineage reprogramming process in adult mouse pancreas from exocrine pancreatic cells to β-cells (Zhou et al., 2008) by adenoviral infection of three TFs (Ngn3, PdxI and Mafa) that are important in the embryonic development of the pancreas and β-cells, which were narrowed down from nine genes exhibiting β-cell developmental phenotypes when mutated (Jensen, 2004; Murtaugh and Melton, 2003). In 2009, Takeushi and Bruneau described the transdifferentiation of mouse mesoderm to heart tissue by transient transfection of Gata4, Tbx5 and Baf60c in cultured mouse embryos (Takeuchi and Bruneau, 2009).

It didn't take long until several reports showed to be possible to generate several different cell types from fibroblasts, an easily obtained cell source. Cardiomyocytes, blood progenitor cells, hepatocytes, epiblast stem cells and neural progenitors (Efe et al., 2011; Han et al., 2011; Han et al., 2012; Huang et al., 2011; Ieda et al., 2010; Sekiya and Suzuki, 2011; Szabo et al., 2010; Thier et al., 2012) were generated from mouse and human fibroblasts.

Fibroblasts exist in the interstices of various organs as a component of connective tissue and are one of several types of somatic cells that have been well established in culture. Human fibroblasts were shown to be able to directly differentiate into all three germ layer derivatives, and there are studies that indicate that human dermal fibroblasts have more plasticity than has been generally thought and that they have potential utility as a source for cell therapy (Osonoi et al., 2011). Therefore, the use of this cell source has become very common for cell reprogramming.

Some authors refer that it is not a surprise to verify that TFs are predominant in reprogramming experiments since they are the primary effectors of lineage decisions during normal development (Vierbuchen and Wernig, 2011). The use of other classes of factors, combined with TFs, is emerging though, as it will be further discussed.

A TF can be defined as a protein that has the capacity to bind to specific DNA sequences, controlling the transcription of genetic information contained in the DNA to messenger RNA (mRNA) (Karin, 1990; Latchman, 1997). According to the kind of TF, the transcription of the adjacent gene can be either up- or down regulated, i.e., they can act by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific genes (Lee and Young, 2000; Nikolov and Burley, 1997; Roeder, 1996). They perform this function alone or with other proteins in a complex and they use a variety of mechanisms for the regulation of gene expression (Gill, 2001) such as: stabilization or blockage of the binding of the RNA polymerase to DNA; catalyzation of the acetylation or deacetylation of histone proteins (involved in up- and down regulation of transcription, respectively); and recruitment of co-activator or co-repressor proteins to the TF DNA complex (Xu et al., 1999).

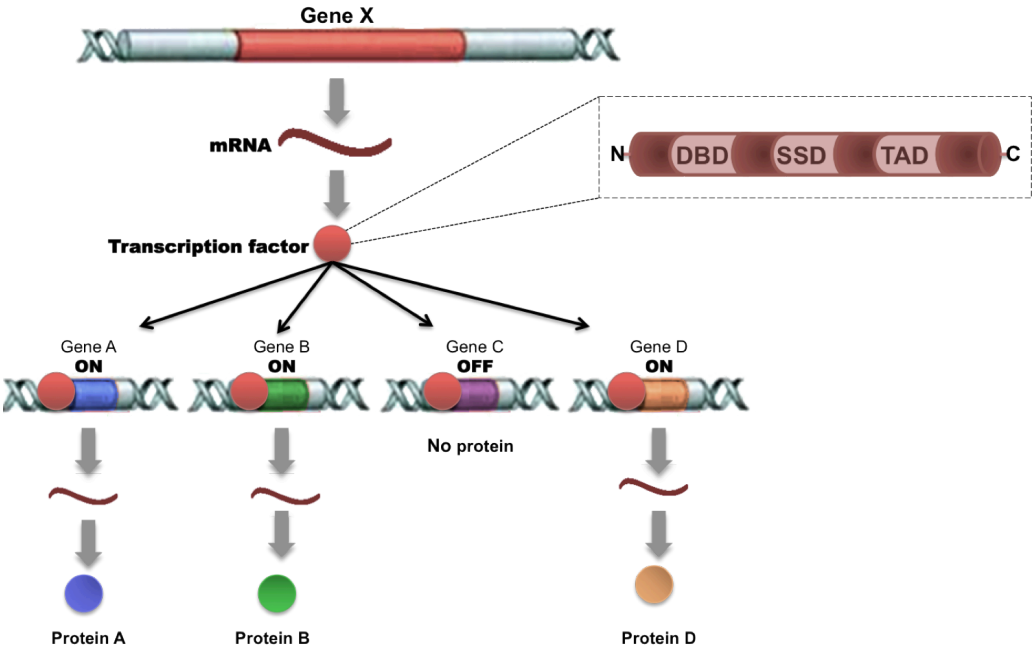


Figure 3 – Schematic representation of the activity of a transcription factor and its domains.

A gene (gene X) is transcribed to form a strand of mRNA, which is then translated by the cellular machinery into a protein, the transcription factor (TF). The function of a TF, though, is to migrate back to the nucleus, and bind to specific regions of DNA, affecting the transcription of other genes (it will up- or down-regulate them). In this example, the TF X turns on genes A, B and D, and turns off gene C. The proteins A, B and D can also act like transcription factors and they can feedback on each other and on other transcription factors that are not directly regulated by gene X. In the dashed box, a schematic diagram of the aminoacid sequence of a prototypical transcription factor is shown. It contains: a DNA-binding domain (DBD); a signal-sensing domain (SSD) (which senses external signals and, in response, transmits these signals to the rest of the transcription complex, resulting in up- or down-regulation of gene expression); and a transactivation domain (TAD) (that contains binding sites for other proteins such as transcription coregulators) (adapted from http://scienceblogs.com/pharyngula/2006/07/09/transcription-factors-and-morp/, as retrieved in 17 of June of 2012).

1.2.2 Direct conversion and iN cells

In 2010, the direct reprogramming of somatic cells into nervous system cells (neurons) was described by Wernig and co-workers. Initially, 19 genes specifically expressed in neural tissues or implicated in neural development were screened. After the screening process it was established that only three factors (Ascl1, Brn2 and Myt1L, (ABM)) sufficed to convert mouse embryonic fibroblasts (MEFs), carrying a green fluorescent Tau protein (Tau-GFP) reporter, into functional induced neuronal (iN) cells. In order to evaluate whether iN cells could also be derived from postnatal cells, similar assays were performed with tail-tip fibroblasts, and the results were identical to those that were obtained with MEFs. Although the single factor Ascl1 was sufficient to induce immature neuronal features, the additional expression of Brn2 and Myt1L generated mature iN cells with efficiencies of up to 19.5%. The generated cells displayed functional neuronal properties such as the generation of trains of action potentials and synapse formation, and they were on its majority excitatory cells expressing markers of cortical identity. A low proportion of iN cells expressed markers of GABAergic neurons, but no other neurotransmitter phenotypes were detected (Vierbuchen et al., 2010). Later, the same group proved that by combining the ABM factors with NeuroD1, they could convert fetal and postnatal human fibroblasts into iN cells showing typical neuronal morphologies and expressing multiple neuronal markers (Pang et al., 2011).

The combination of factors ABM by itself, also proved to be efficient in converting human embryonic and postnatal fibroblasts into mature human iN (hiN) cells (Pfisterer et

al., 2011a) as well as in converting adult human fibroblasts isolated from adult individuals, into functional hiN cells (Pfisterer et al., 2011b). In addition to these findings, Pfisterer and colleagues proved that by adding other TFs to the ABM cocktail they could generate a different neuronal type, as the dopaminergic (induced dopaminergic (iDA) neurons) (Pfisterer et al., 2011a). In order to prove this, they selected ten genes involved in midbrain patterning and specification of dopamine (DA) neurons, cloned them into lentiviral vectors and by infecting the fibroblasts with these lentiviruses, using different TF combinations, they found out that the highest numbers of tyrosine-hydroxylase (TH)-positive cells could be detected when using the factors ABM supplemented with the factors Lmx1a and FoxA1. The iDA were able to generate action potential and to give rise to currents, showing to be functional hiN cells. These subtype-specific hiN cells could be a helpful tool for the study of a treatment of neurological diseases such as PD and may serve as an interesting alternative to iPSCs for generating patient- and/or disease-specific neurons.

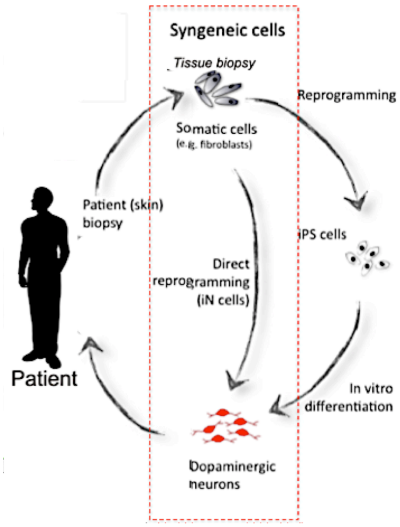


Figure 4 – Direct reprogramming of somatic cells into subtype-specific neurons.
The patient's cells are collected and used for reprogramming, creating patient-specific neurons, without going through a pluripotent state. Thus, the generated cells are considered as syngeneic, which means that they are genetically identical and immunologically compatible to allow for transplantation.

Several groups have now shown different cocktails of transcription factors and other molecules as being able to directly convert mouse and human fibroblasts into iN cells, also including dopaminergic and motor neurons, and even showing it to be possible to convert other terminally differentiated cell types into neurons. Table I shows some of

the most recent and relevant reports, describing the generation of these iN cells. Other classes of factors, such as components of chromatin modifying complexes, small molecules, microRNAs (miR) and other RNA species could also be able to induce lineage conversion, as described in some of the studies presented below.

Table I – Recent findings in iN cells' generation.

Factors used	Reprogrammed from:	Identity of the generated cells confirmed by:
***"Direct conversion of fibroblasts to functional neurons by defined factors"*, (Vierbuchen et al., 2010)**		
ABM	Mouse embryonic and postnatal fibroblasts Mouse postnatal fibroblasts	**Immunocytochemistry** **Electrophisiology**
"Direct conversion of human fibroblasts to dopaminergic neurons", (Pfisterer et al., 2011a)		
ABM ABM + Lmx1a and FoxA2	Human embryonic fibroblasts Human postnatal fibroblasts	**Immunocytochemistry** **Electrophysiology**
***"Direct generation of functional dopaminergic neurons from mouse and human fibroblasts"*, (Caiazzo et al., 2011)**		
Ascl1, Nurr1 and Lmx1a	Mouse embryonic fibroblasts Human fetal fibroblasts Human adult fibroblasts (from healthy and PD patients)	**Immunocytochemistry** **qPCR Global expression analysis** **High-performance liquid chromatography** **Electrophysiology** **Amperometry** ***In vivo* differentiation assays** **In vivo electrophysiology**
***"MicroRNA-mediated conversion of human fibroblasts to neurons"*, (Yoo et al., 2011)**		
miR-9/9*, miR-124, NeuroD2, Asc1 and Myt1L	Human neonatal fibroblasts Human adult fibroblasts	**Immunocytochemistry** **Electrophysiology** **Single-cell qPCR**
***"Induction of human neuronal cells by defined transcription factors"*, (Pang et al., 2011)**		
ABM and NeuroD1	hESCs Human fetal fibroblasts	**Immunocytochemistry** **Single cell RT-PCR** **Electrophysiology**
"Conversion of mouse and human fibroblasts into functional spinal motor neurons", (Son et al., 2011)		
ABM + Lhx3, Hb9, Isl1, Ngn2 and NeuroD1	Mouse embryonic fibroblasts Human embryonic fibroblasts	**Immunocytochemistry** **FACS** and **qRT-PCR** **Electrophysiology** ***In vivo* transplantation**
"Direct lineage conversion of terminally differentiated hepatocytes to functional neurons", (Marro et al., 2011)		
ABM	Mouse embryonic fibroblasts Postnatal hepatocytes	**Immunocytochemistry** **qRT-PCR** and **FACS** **Electrophisiology**

"Small molecules enable highly efficient neuronal conversion of human fibroblasts", (Ladewig et al., 2012)		
Ascl1 and Ngn2 (AN) + SB-431542 Noggin CHIR99021	Human postnatal fibroblasts Cord blood-derived stem cells (CB-SCs) Adult human fibroblasts (healthy donor)	**Immunocytochemistry** **Gene expression array** **Electrophysiology**

Other reports have also shown to be possible to generate iDA cells by using different combinations of TFs (Kim et al., 2011; Liu et al., 2012). A report describing the direct reprogramming of AD patient cells into functional neurons (Qiang et al., 2011) was also published. And additionally, another report showed to be possible to reprogram postnatal astroglia of mouse neocortex into functional, synapse-forming neurons (Heinrich et al., 2012).

The use of small molecules (SM) to enhance conversion of the cells into the desired phenotype had gained some attention when Ladewig et al. (Table I) described the highly efficient neuronal conversion of human fibroblasts into neurons. Initially, they used human postnatal fibroblasts, and infected these cells with Ascl1 and Neurogenin2 (Ngn2) (AN), which was the combination of factors that gave rise to the highest portion of βIII-tubulin. A synergistic SMAD pathway inhibition (dual SMAD inhibition) was used in their protocol, by applying the activin-like kinase 5 inhibitor SB-431542, together with Noggin. Additionally, inhibition of the glycogen-synthase kinase-3β (GSK-3β) was also used, through the application of the CHIR99021 inhibitor (Ladewig et al., 2012). The inhibition of SMAD signaling and of the GSK-3β have been used for highly efficient neural differentiation of hESCs and iPSCs ((Chambers et al., 2009; Li et al., 2010)). By doing so, Ladewig and colleagues were able to generate neurons with different neurotransmitter phenotypes, in very high yields and with high purities (Ladewig et al., 2012).

The fact that different combinations of TFs can drive the same cell fate conversion process suggests that many (if not all) of the key upstream TFs regulate one another (Vierbuchen and Wernig, 2011). Such variability in factors' combination and applications shows that neuronal conversion is feasible and reproducible under different conditions. However, the exact differences between the generated cells, obtained from different

combinations of factors, or between the generated cells and their target cells counterparts are still not completely addressed. Some evidence also suggests that the generated iN cells show differences in gene-expression, when compared with primary cells (Caiazzo et al., 2011).

It also remains unclear whether the direct lineage conversions fully reprogram the cells, or if any epigenetic memory of the previous cell fate remains. It is still not known if the intrinsic gene expression network is similar in reprogrammed cells and in differentiated cell types or if these networks are triggered during reprogramming. To answer this question, another one arises, concerning the fact of whether all the characteristics of the reprogrammed cells are maintained upon withdrawal of the inducing factors or not (Chambers and Studer, 2011). Experiments involving DNA excision or nonintegrating gene delivery have demonstrated that, for many passages, iPSCs maintain expression of pluripotent markers, differentiation capacity and epigenetic state in the absence of reprogramming factors (Hanna et al., 2010). This stability was associated with the induction of the intrinsic feedforward network of factors controlling pluripotency (Boyer et al., 2005). Furthermore, the way similarities and differences between converted cells and primary neurons, in terms of gene expression and chromatin structure, correlates with their functionality (neurotransmitter production, firing of action potentials and functional integration into neural networks) of the reprogrammed neurons, is a question that should also be addressed.

Since miRs and TFs affect the expression of other proteins involved in cell-fate switching and neuron differentiation, further work should access how the various chromatin-remodeling factors can affect gene expression, contribute to cell conversion and how can they be controlled (Sendtner, 2011).

The impact that the donor cell types have on the reprogramming efficiency and fidelity are also still unclear (reviewed in (Vierbuchen and Wernig, 2011)), and associated with iN cells' generation is the question of whether direct reprogramming will or not reset the developmental timing, or whether the age of the original cell used for reprogramming will impact the age of the obtained target cells (Chambers and Studer, 2011).

Finally, understanding how well the reprogrammed cells survive, integrate and respond to physiological cues *in vivo* has a very important role for translational applications (Chambers and Studer, 2011). It is important to know how gene expression and chromatin structure are shaped by intrinsic mechanisms and by the environment in which the cells are, after transplantation. Understanding how a diseased and/or aged brain's environment influences the functionality and gene expression profile of the transplanted iN cells, is of extreme relevance for the development of cell-based therapies for neurodegenerative disorders.

Within this scenario, future studies will be necessary to determine whether iN cells could represent an alternative method to generate patient-specific neurons. The differences between the converted cells and their corresponding primary neurons must be characterized, and whether they give rise to unwanted side effects should be explored (Sendtner, 2011).

The generation of iN cells is fast and devoid of tumorigenic pluripotent cells, a key complication of iPSC approaches in regenerative medicine. On top of that, iN cells are generated with a higher efficiency, show a relatively rapid conversion and also open the possibility for in situ reprogramming (Chambers and Studer, 2011). Therefore, iN cells could provide a novel and powerful system for studying cellular identity and plasticity, neurological disease modeling, drug discovery and regenerative medicine.

1.3 Lentivirus-mediated gene transfer in the CNS

Most studies on cell fate conversion use lentiviral vectors to deliver reprogramming genes. The advantages of using lentiviral vectors as tools in approaches involving gene transfer in cultured cells and in the CNS, are well known. LVs are good for these types of studies, since they have a great ability to efficiently transduce slowly or non-dividing cells (Zufferey et al., 1997) and a large cloning capacity, providing the possibility of carrying complex expression cassettes (Naldini et al., 1996a; Wiznerowicz and Trono, 2005). They also make it possible to broaden the spectrum of susceptible cells and alter the transduction efficiency. This occurs through the pseudotyping of LVs with a variety of envelope proteins from different viruses (Watson et al., 2002). For this purpose, the

vesicular stomatitis virus (VSV-G) envelope is widely used. Furthermore, LVs integrate genes in the chromosome of the target cells, leading to stable long-term expression (Azzouz et al., 2002; Blomer et al., 1997; Deglon et al., 2000; Naldini et al., 1996a) and elicit minimal inflammatory response that could compromise the viability of the transduced cells (Azzouz et al., 2002; Bensadoun et al., 2000; Deglon et al., 2000; Kordower et al., 2000; Mazarakis et al., 2001).

Lentiviral vectors can be split into two different groups, the primate, such as the ones based on immunodeficiency virus (HIV) (Naldini et al., 1996b) and simian immunodeficiency virus (SIV) (Negre et al., 2002), and non primate, such as those derived from equine infectious anaemia virus (EIAV) (Mitrophanous et al., 1999)(ref 18_Azzouz) and feline immunodeficiency virus (FIV) (Poeschla et al., 1998).

In order to make use of a LV, sequences that act in *cis* and *trans* within the genome have to be separated. The *cis* sequences contain signals required for packaging and integration of the viral genome as well as the polypurine tract (PPT) and the long terminal repeat sequences (LTR). In *trans,* sequence information coding is stored for proteins that compose the virus. Also, *trans* sequences are provided on more than one vector thus minimizing the risk of spontaneous recombination that could lead to replication-competent vectors. Additionally, a transfer vector containing the transgene of interest exists, which represents the lentiviral genome that will be integrated into the host genome (Vigna and Naldini, 2000).

Nonetheless the use of LVs in gene therapy approaches for example, HIV-based lentivirus-mediated overexpression of TFs for the direct conversion of both human embryonic and postnatal fibroblasts into neurons has proved to be successful (Pfisterer et al., 2011a). Therefore, a similar approach was used during the project described in the present thesis. For the delivery of the TFs into the target cells, the fibroblasts, two different types of lentiviral vector constructs (transfer vectors) were used. The first contained an internal human phosphoglycerate kinase (hPGK) promoter that drives the transgenes expression after integration. Additionally to the promoter sequence, the transfer vector contains two *cis*-acting elements, the central polypurine tract (cPPT) as well as the woodchuck hepatitis posttranscriptional regulatory element (WPRE). The latter enhances

the transgene expression levels and upgrades the transduction efficiency. The previously referred construct was used for carrying ten of the thirteen TFs used in the project (described in Figure 5). The second transfer vector, used for the three remaining TF's transgenes, contains a tetracycline-regulated promoter sequence, that drives the transcription of the factors upon doxycycline deliver to the cells, the so-called Tet-On system. To perform this regulation, an additional vector, encoding for a regulator protein (FuW), is necessary. In the presence of doxycycline, that protein binds to a region in the inducible promoter, the tetracycline response element (TRE), activating the transcription of the transgenes (Graham and Self, 2010).

Alongside the transfer vectors #1000-hPGK and Tet-O carrying the gene of interest, the envelope vector pMD2.G, containing the glycoprotein of vesicular stomatitis virus (VSV-G) as well as the packaging vectors pMDL/RRE and pRSV-REV, containing the sequences rev, pol, gag and tat were also used for LV production. Rev and tat sequences encode for proteins regulating viral transcription, while pol encodes proteins necessary for the viral life cycle (reverse transcriptase and integrase), and gag encodes structural proteins that form the viral capsid (Bour and Strebel, 2000).

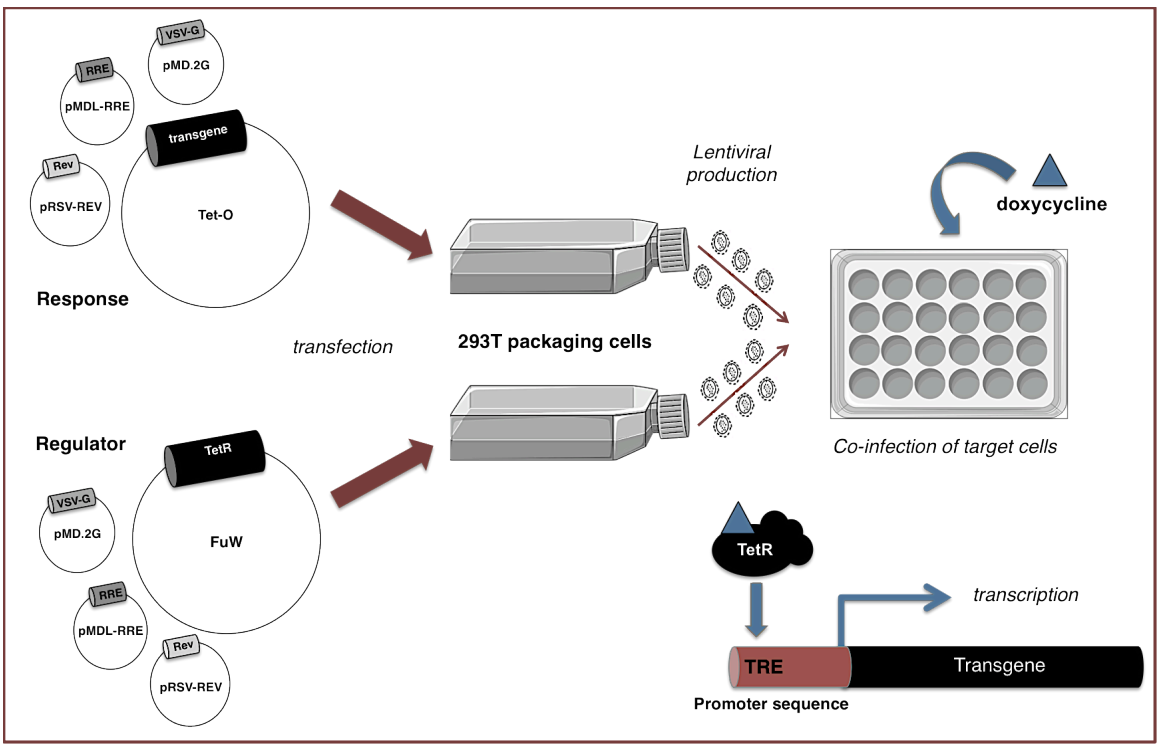


Figure 5 – Schematic representation of the doxycycline-regulated transcription system (Tet-On).

The upper represented 293T cells are transfected with the transfer vector (Tet-O), pseudotyping (pMD.2G) and packaging plasmids (pRSV-REV and pMDL-RRE), while the bottom cells are transfected also with pseudotyping and packaging plasmids, but with a different transfer vector (FuW), encoding for a regulator protein (TetR). The lentiviruses that are produced by these cells are used to co-infect the target cells (fibroblasts). When doxycycline is added to the media in the cells, this antibiotic binds to the TetR protein, giving rise to a complex that is able to bind to a specific site in the promoter (tetracycline response element (TRE)), allowing transcriptional activation of the transgene.

1.4 Hypothesis and Significance of the project

1.4.1 Hypothesis

The existing information about the ability of the TFs ABM to directly convert fibroblasts into functional neurons, and the fact that the addition of fate-specifying factors to this cocktail can lead to the generation of a different neuronal subtype (Pfisterer et al., 2011a), led us to formulate a hypothesis. The combination of those factors with an additional cocktail of TFs expressed during striatal development (Dlx2, Dlx5, Isl1, Nolz1, Gsx2, Gsx1, Gli1, Otx2, FoxG1 and CTIP2), could lead to the obtention of hiN cells with a striatal GABAergic phenotype. The selected TFs have been shown to have a relevant significance in brain processes related with striatal and more specifically, striatal GABAergic projection neurons' development (For more detailed information, see Table A1). These neurons are by far the most numerous neuronal type in the striatum (80-95%), being also known as medium spiny neurons (msn), and are involved in basal ganglia pathways, related with the control of movment (Squire, 2008). Since those neurons are particularly affected in cases of Huntington's disease (HD) and stroke, their generation through a transdifferentiation process would possibly be a valuable approach to obtain cells that could be used in cellular therapy for those diseases.

1.4.2 Significance of the project

In the striatum, there are four main types of neurons described according to the size of their cell body and the presence or absence of dendritic spines. These types of neurons are: medium spiny neurons (GABAergic), large aspiny neurons (Acetylcholinergic), medium spiny cells (Somatostatinergic) and small aspiny cells (GABAergic). By far, the most numerous neuron type in the striatum is the medium spiny neuron. With large and characteristic dendritic trees and expression of DARPP-32, a

dopamine and cAMP-regulated phosphoprotein of 32 kDa (Ouimet et al., 1984), these GABAergic projection neurons are neurochemically heterogeneous due to the presence of peptide neurotransmitters that are co-localized with GABA. Based on both the type of neurotransmitter and the type of dopamine receptor they contain, medium spiny neurons can be divided into two populations. One population contains GABA, dynorphin and substance P, primarily expressing D1 dopamine receptors, while the other population contains GABA and enkephalin and primarily expresses D2 dopamine receptors (Squire, 2008). The neurons that contain GABA, Dynorphyn and substance P as neurotransmitter project from the striatum to the internal segment of globus pallidus and substantia nigra, being involved in the direct pathway, while the neurons that contain GABA and encephalin project from the striatum to the external segment of globus pallidus and are involved in the direct pathway.

These medium spiny neurons receive the bulk of inputs to the striatum, receiving afferents from the cortex, thalamus and midbrain, as well as being the major output neuron of the striatum, projecting to various regions of the CNS, including the globus pallidus and the substantia nigra. As such, they form complex neuronal loops within the basal ganglia that are important for the control of movement, cognition and emotion (Nakano et al., 2000) (Figure 6).

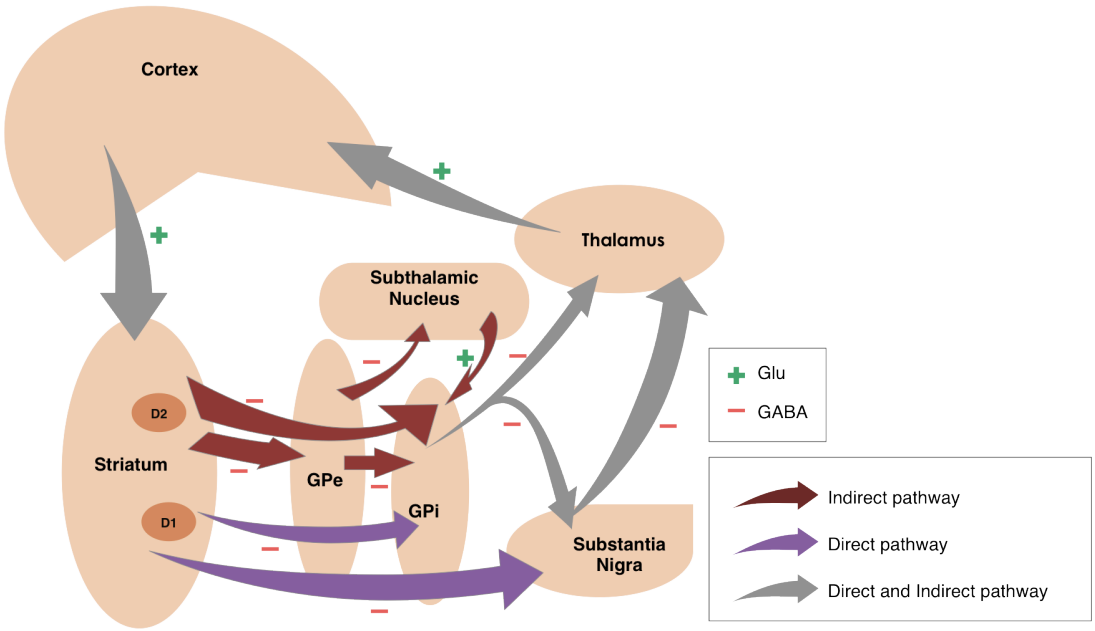


Figure 6 – Direct and indirect pathways in the basal ganglia circuitry for the control of movement.

The direct pathway contains two inhibitory GABAergic synapses (between the striatum and the internal globus pallidus or substantia nigra, and between the internal globus pallidus or the substantia nigra and the thalamus). Activation of this pathway produces desinhibition of the excitatory glutamatergic thalamic input to the sensory, motor and associated areas of the cortex. In the indirect pathway, there are one excitatory glutamatergic (between the subthalamic nucleus and the internal globus pallidus) and three inhibitory GABAergic synapses (between the striatum and external globus pallidus, between external globus pallidus and subthalamic nucleus, and between the internal globus pallidus and thalamus). In contrast to the direct pathway, the three inhibitory synapses result in net inhibition (inhibition of disinhibition) of the thalamic-cortical projections when the direct circuit is activated (Siegel et al., 1999).

Huntington's disease is an autosomal dominant neurodegenerative disorder, characterized by an expansion of the CAG polyglutamine repeat of the huntingtin protein. It involves the death of projection neurons (medium spiny neurons) in the striatum, enlargement of the ventricles and a corresponding shrinkage of the overlying cortex. These events of the disease lead to involuntary choreiformic movements, cognitive impairment and emotional disturbances (Kelly et al., 2009). Despite identification of the HD gene associated proteins, the mechanisms involved pathogenesis of HD remains largely unknown, hampering effective therapeutic interventions, and unlike other neurodegenerative disorders, like Parkinson's disease, where several options are available, albeit mainly for the early-to-moderate-stage patient, there is little in the way of disease-modifying treatment available for patients with HD (Kelly et al., 2009). The mechanisms of cell dysfunction and death in HD have been the subject of a number of studies, which led to therapeutic strategies largely based on the amelioration of mutant huntingtin-related metabolic impairment and cellular toxicity. Yet, in later stages of the disease, after cell death has become prominent, cell replacement therapy (either by direct cell transplantation or by mobilization of endogenous progenitors) may comprise a stronger potential avenue for therapy (Benraiss and Goldman, 2011).

Given the predominance of medium spiny neuronal loss in HD, a number of investigators have proposed that allogeneic striatal cell grafts, intended to replace lost projection neurons, might ameliorate disease progression as well as reducing the neuronal damage in HD brain. In fact, an improvement in motor and cognition performance in HD patients following fetal cell transplantation was documented (Bachoud-Levi et al., 2000). However, as previously referred, the difficulty in supplying sufficient amounts of

embryonic striatal tissue and the concomitant ethical issues associated with the use of human embryonic tissue made the use of these cells for transplantation more difficult.

Several donor tissues alternatives are now being actively explored. First, there is the potential to take into culture the primary tissue and NSC, either with a view to expanding the number of progenitors with the potential to differentiate into medium spiny neurons, thus avoiding the need for multiple fetal donors per patient, or by using them as a source for transplantation, after which they could develop and differentiate into regionally appropriate cell types in response to environmental factors (Flax et al., 1998; Gage, 2000; Gottlieb, 2002; Kelly et al., 2009; Kim, 2004; Lindvall and Kokaia, 2006; Temple, 2001). Other cell types were also used in animal models, such as ESCs, MSCs, primary human NPCs, and resulted in varying degree of clinical improvement (Armstrong et al., 2000; Kordower et al., 1997; Lee et al., 2005; Lee et al., 2006; McBride et al., 2004; Ryu et al., 2004; Visnyei et al., 2006).

A variety of disease-specific iPSCs, including those for HD, have been generated and have already found uses in drug screening and in the investigation of disease-specific molecular pathways (Beyene and Boockvar, 2008; Ebert and Svendsen, 2010; Park et al., 2008; Zhang et al., 2010). These cells also comprise an important source of cells for phenotype-specific cell replacement and tissue repair. However, since the risks of transplanting iPSC-derived cells are still under evaluation, an alternative source for generation of neurons would be necessary.

Since HD is a genetic disorder, and given the underlying CAG repeat expansions of the donor cells, when using patient-specific cells, further genetic modification in the donor cells would be needed before transplant. Several techniques have already been developed to achieve this end, all directed to the inactivation or blockade of mutant huntingtin gene expression (Benraiss and Goldman, 2011). Future studies involving iN cells for cell replacement therapies in HD would have to address this issue, by removing or inactivating the offending segment of poly-CAG expansion.

The stroke, medically known as cerebrovascular accident (CVA), is caused by blockage of a cerebral artery, leading to focal ischemia, loss of neurons (the most sensitive, like medium spiny neurons) and glial cells, and motor, sensory and cognitive

impairments. No effective treatment to promote recovery exists, so a therapy that produced even minor improvement would be valuable (Lindvall and Kokaia, 2006). Thus, the role of cellular therapy as an approach to repair has been explored.

The concept of restoring function after stroke by transplanting human neuronal cells into the brain was conceived in the mid-1990's (Bonn, 1998), and a variety of cell types have been tried for restoration of brain function after stroke, mostly in rodent models: fetal tissue, rat striatum, LBS neurons, NT2-teratocarcinoma-derived cells, ESC-derived neurons and marrow stromal cells (Borlongan et al., 1995; Borlongan et al., 1997; Borlongan et al., 1998a; Borlongan et al., 1998b; Chen et al., 2001a; Chen et al., 2001b; Hayashi et al., 2006; Ikeda et al., 2005; Li et al., 2002; Li et al., 2001; Li and Chopp, 2009; Li et al., 2000; Nishino et al., 1993; Saporta et al., 1999). These attempts to promote recovery of the affected subjects were in some cases successful, leading to restoration of cognitive and motor functions. A clinical trial was reported, in which stroke patients, with affected basal ganglia, received implants of neurons generated from the human NT-2 teratocarcinoma cell line into the infarcted area (Kondziolka et al., 2000; Meltzer et al., 2001). Some improvement was observed in some patients, but those were correlated with increased metabolic activity at the graft site (Meltzer et al., 2001).

The generation of neurons from a somatic source, like fibroblasts for example, could help find a solution for using in cell-based therapies for HD and stroke. Therefore, the use of iN cells with characteristics that could resemble the striatal GABAergic projection neurons' phenotype, expressing the same markers and with the same functional properties seems very appellative.

Chapter II - Materials and Methods

2.1 Cell cultures maintenance, expansion and sub-culture

2.1.1 293T cells

293T cells were grown in DMEM+GlutaMAX™-I (Dulbecco's Modified Eagle Medium with 4,5 g/L of Glucose; Gibco, Invitrogen), supplemented with 10% of standard Fetal Bovine Serum (Saveen & Werner) and 1% of Penicillin/Streptomycin (Gibco, Invitrogen), and maintained at 37°C in humidified atmosphere with 5% CO_2 in air (Forma Scientific incubator).

2.1.2 Human embryonic fibroblasts

Cultures of human embryonic fibroblasts (hEFs) were derived from legally aborted fetuses aged 5.5-7 weeks postconception with approval of the Swedish National Board of Health and Welfare and the Lund/Malmö Ethics committee. After neural tissue removal as well as removal of all red organ, hEFs were expanded in culture. Direct conversion experiments were performed on hEFs either in passage 3 or 4. Before plating the cells, all flasks and plates were coated with 0,1% Gelatin (Sigma Aldrich) (45 minutes, 37°C). The hEFs were grown in MEF medium, composed by: DMEM+ GlutaMAX™-I supplemented with 10% FBS (Gibco, Invitrogen), 1% 10 000 μg/mL Pen/Strep (Gibco, Invitrogen) and 1% 200mM Glutamine (Sigma Aldrich). All components were filtered (sterile filter; 0,2μm pores; Nunc, Thermo Scientific). The cells were kept at 37°C in humidified atmosphere with 5% CO_2 in air (Thermo Forma Steri-cycle CO_2 incubator).

2.1.3 NSE14 cells

In 2005, Conti et al. demonstrated that the combination of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) is sufficient for derivation and continuous expansion of pure monolayer cultures of mouse embryonic stem cell-derived neural stem cells (ES cell derived NS cells)(Conti et al., 2005). Conti and colleagues established the ES cell-derived NS cell line NS. Before plating the cells, all the flasks and plates were coated with 0,1% Gelatin (45 minutes, 37°C). The NSE14 cells were kept in culture and grown in Euromed-N medium (Optimized medium for neuronal precursor cells, EuroClone) supplemented with 1% 200mM L-Glutamine (Sigma Aldrich), 1% 10 000 μg/mL Pen Strep (Gibco, Life Technologies™) and 10x Hormone mix (prepared with 1 mg/mL Apo-transferrin, 250μg/mL Insulin, 96,6 μg/mL Putrescine, 51,8 ng/mL Sodium

Selenite and 62,9 ng/mL Progesterone (Sigma Aldrich) in Euromed-N medium). The medium was prepared by filtering all the components (sterile filter; 0,2μm pores; Nunc, Thermo Scientific). Before adding the medium to the cells, it was also supplemented with Growth factors: 0,1% 20μg/mL bFGF (Gibco, Life Technologies™) and 0,02% 100μg/mL EGF (R&D systems). The cells were maintained at 37°C in humidified atmosphere with 5% CO_2 in air (Thermo Scientific incubator).

2.1.4 Cells expansion and subculture

The medium in all the cell lines was changed every 2-3 days. The cells were split depending on their cell density and special care was taken to avoid cell densities above 75%. For cell splitting, the cell monolayer was washed with sterile DPBS 1X (Dulbecco's Phosphate Buffered Saline; Gibco®, Invitrogen) and then dissociated using Trypsin 1X (Gibco®, Invitrogen) (3-5 minutes, 37°C) or Accutase solution 1x (in the case of NSE14 cells; PAA laboratories GmbH) (3 minutes, 37°C). After pelletizing the cells (5 minutes, 400 x g or 1200 rpm, 20°C) (Rotina 35R Centrifuge, Hettich; Biofuge primo R, Heraeus) and removal of the supernatant, the cell pellet was resuspended in culture medium. The cell number was determined manually by using a Bürker chamber (Marienfeld) by counting the cell number within a minimum of three fields and cells were plated at the desired cell numbers into new T25 or T75 flasks (Nunc, Thermo Scientific).

2.2 Lentiviral production

Production of lentiviruses was performed in the variant of human embryonic kidney (HEK) 293 cells containing the SV40 largeT-antigen (293T cells). For viral production, 3rd generation lentiviral packaging and envelope plasmids were used. One day prior to transfection, 293T cells were plated in T175 flasks at a density of $10x10^6$ cells per flask. For the production of one virus batch, the supernatant of 2 flasks was pooled.

One day after plating and 2-3 hours prior to transfection, the medium in the cells was changed to IMDM+GlutaMAX™-I (Iscove's Modified Dulbecco Medium, 25 mM HEPES, Gibco, Invitrogen) supplemented with 10% of FBS and 1% of Pen/Strep.

Table II – Plasmids used for transfection and their correspondent necessary mass for the production of each batch of lentiviral particles.

Plasmids used for transfection		Mass/batch (μg)
Envelope plasmid	pMD2.G	21,1 μg
Packaging plasmids	pMDL/RRE	29,3 μg
	pRSV-REV	14,6μg
Transfer vector (*1)	Ascl1, Myt1L, Brn2, Dlx2, Dlx5, Isl1, Nolz1, Gsx1, Gsx2, Gli1, Otx2, FoxG1, CTIP2 and FuW (*2)	75μg

(*1) Each one of the vectors containing the different transcription factor's sequences is transfected in 293T cells independently.

(*2) FuW is the transactivator that allows the transcription of Ascl1, Myt1L and Brn2, whose expression is driven by a Tetracycline-regulated promoter. The remaining factors are transcribed under an ubiquitous and constitutively active promoter (hPGK).

The packaging, envelope and transfer vectors were added to 2500μL of TE-buffer 0,1% (TE-buffer 1X diluted 1:10 with distilled water; TE-buffer 1X: 10mL of 1M Tris-HCl pH 7.5, 2 mL of 0,5M EDTA pH 8.0 and 988 mL ddH_2O) at the desired concentrations listed above (Table II) Subsequently, $CaCl_2$ was added to the solution (292,5 μL) and left for 5 minutes at room temperature. After incubation, 2800μL of HeBS 2X (Hepes-buffered solution; 8,0 g NaCl, 6,5 g HEPES in 10 ml NA_2HPO_4 (5,25 g sodium phosphate dibasic in 500 mL distilled water), filled up to 500 mL with distilled water; pH 7,0) was added dropwise to the DNA/$CaCl_2$ solution, under vortex mixing (Vortex Genie, Scientific Industries, Labora). The final solution was then pipetted on cells carefully (2800 μL/T175 flask). The cells were maintained in the incubator at 37°C in a 5% CO_2 in air atmosphere.

All the procedures involving viruses handling were performed by trained personnel and according to *p2 laboratory safety guidelines*. Before discarding pipet tips, they were incubated in Virkon®, a strong antiviral agent and after usage, the bench, all the material and equipment used during the procedures were exposed to UV light for 20 minutes.

The culture medium was changed 12-14 hours after transfection. Approximately 30 hours post medium change, the medium of each batch was collected and centrifuged (800 x g, 10 minutes, 20° C). After filtering (0,45μm pore filter, Nunc), the supernatant was subsequently subjected to ultracentrifugation (Beckman Optima L-60 ultracentrifuge) (19500 rpm, 2 hours, 4°C). The supernatant was discarded diligently to avoid any contamination by remainder of medium. The pellet was then resuspended with 90μL of PBS (2 hours at 4°C) and aliquots of the viruses were stored at -80°C.

2.3 RNA studies

2.3.1 RNA extraction

Cells were plated in 6-well plates (2,0 x 10^5 cells/well) and infected with the different lentiviruses at desired multiplicity of infection (MOI = number of viral particles/ number of target cells). At various timepoints, cells were directly harvested in 350μL RLT lysis buffer (QIAgen) and RNA Extraction was performed using the RNeasy® Microkit (QIAgen) following main manufacturer's instructions (RNeasy Micro Handbook, 12/2007, pages 16-22).

The RNA concentration and grade of purity (Ratio $OD_{260nm/280nm}$ range 1.8-2.2) in the final samples was determined by using the Nanodrop 2000 (Thermo Scientific), and represented in ng/μL.

2.3.2 Reverse transcription and cDNA synthesis

Equal amounts of extracted RNA (range: 300-500 ng) were incubated at 65°C for 5 minutes in S1000 ™ Thermal Cycler (Bio-Rad) after being mixed with dNTP (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and random-primers mix (Fermentas), according to suplier's recommendations, added to a final volume of 13 μL with distilled water. After the incubation, 0,1 M DTT, First-Strand Buffer (5x) and Super Script® III Reverse Transcriptase (Invitrogen) were added to the samples. For each sample, a negative control was prepared, by adding the previously described reaction mix without the enzyme (referred to as –RT control). In both conditions, the final volume per sample was 20μL. Before performing the reaction, the samples were incubated in ice for 5 minutes. Reverse transcription was performed at 50°C for 1 hour for primer annealing and cDNA synthesis,

followed by 70°C for 15 minutes, in order to denature the reverse transcriptase. The sample was cooled to 4°C, diluted 1:5 in distilled water and stored at -20°C until further use.

2.3.3 Primers design and preparation

The primers designed for real time quantitative PCR (qRT-PCR) were used for detection of the target DNA of the ten transcription factors after lentiviral overexpression as well as for detection of the reference genes β-Actin and GAPDH.

All the primers used for qPCR analysis were designed using the open source software tool *Primer3* (v0.4.0). Primer pairs were designed complementary to the cDNA sequence of the individual detection targets. The primers were selected according to the following criteria: a) GC content about 50%; b) Melting temperature (Tm) of approximately 60°C; c) Primer length of 18-23 bp; d) Final product length of 70-150 bp; and e) Secondary structures and primer-dimers were avoided.

A first pair of primers was designed for each transgene in order to recognize a nucleotide sequence containing part of both the WPRE sequence (lentiviral) and transgene sequence (primers type A, figure 7), allowing the discrimination between the endogenous and virally mediated gene expression.

A second pair of primers was designed for Dlx2, Dlx5 as well as CTIP2, in order to recognize each individual transgene's open reading frame (ORF) (primers type B, figure 7). The primer pairs designed in the second set do not distinguish endogenous from exogenous gene expression, but enable for detection of an increase in gene expression in a dose-dependent fashion.

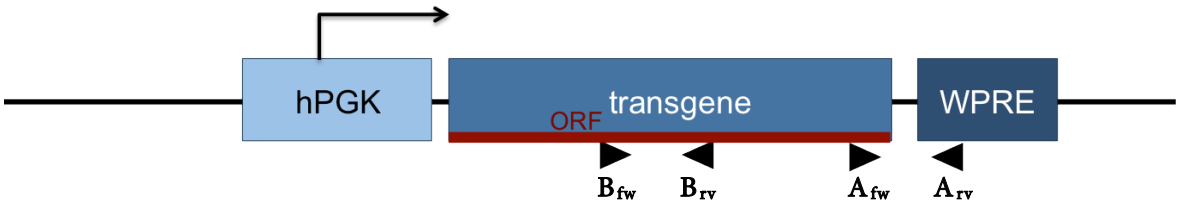


Figure 7 - Schematic representation of the sites of hybridization of the primers designed in both sets (A and B).
The expression of the transgenes is driven by the human phosphoglycerate kinase (hPGK) promoter. Primers type A (fw and rv) are amplifying a region spanned between the transgene sequence and WPRE sequence. Primers type B (fw and rv) recognize the ORF of each transgene.

The primers were ordered (Sigma-Aldrich) desalted, at standard concentrations, diluted (0,95μM) and stored at -20°C until further use.

In table III and IV, a list of the primers and the respective sequences are presented.

Table III – Forward and reverse sequences of the pairs of primers designed for qPCR analysis.

The primers shown here recognize both the WPRE sequence (rev primer) and part of the transgene sequence (fw primer).

Transgene	Forward Primer Sequence	Reverse Primer Sequence
Otx2 (mouse)	5'-GATCAGACGTCCTCATGGAAA-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
Gli1 (mouse)	5'-GCCTGGAGAGACACAATTCC-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
CTIP2 (human)	5'-AGGCCGAGAGGAGCTAAGTC-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
Dlx2 (human)	5'-GCGGGGACGATTTTCTAAGT-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
Dlx5 (human)	5'-GCCTCCGGGACACTCTATTA-3'	5'-AGCGTAAAAGGAGCAACATAGT-3'
FoxG1 (human)	5'-GGGTCTTCTTCCAACCCTTT-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
Gsx1 (human)	5'-AAGGACGACCGGGATCTTAC-3'	5'-GGCATTAAAGCAGCGTATCC-3'
Gsx2 (human)	5'-GCCAACGATGACAAGGAGAT-3'	5'-CCACATAGCGTAAAAGGAGCA-3'
Isl1 (human)	5'-AACAGCATGGTAGCCAGTCC-3'	5'-GGCATTAAAGCAGCGTATCC-3'
Nolz1 (mouse)	5'-CCTTATGCCCTCTACGGACA-3'	5'-CCACATAGCGTAAAAGGAGCA-3'

Table IV – Forward and reverse sequences of the pairs of primers designed for qPCR analysis (second set).
The primers presented here are primers that are able to hybridize centrally with the DNA sequence of the open reading frame (ORF) of the transgenes.

Transgene	Forward Primer Sequence	Reverse Primer Sequence
CTIP2 (human)	5'-CCATCCTCGAAGAAGACGAG-3'	5'-ATTTGACACTGGCCACAGGT-3'
	5'-TCCAGAGCAATCTCATCGTG-3'	5'-GTGCATGTGCGTCTTCATGT-3'
	5'-GGCAAGACCTTCAAGTTCCA-3'	5'-GTGCATGTGCGTCTTCATGT-3'
	5'-TCCAGAGCAATCTCATCGTG-3'	5'-TGCATGTGCGTCTTCATGT-3'
Dlx2 (human)	5'-AGCAGCTATGACCTGGGCTA-3'	5'-TCCTTCTCAGGCTCGTTGTT-3'
Dlx5 (human)	5'-TGAGAATGGTGAATGGCAAA-3'	5'-GCAAGGCGAGGTACTGAGTC-3'

2.3.4 Real time RT-PCR (qRT-PCR)

For gene expression analysis, qRT-PCR was carried out using the non-specific dye SYBR Green. The reaction was performed using a SYBR Light Cycler® 480 (Roche) according to standard procedures. The samples (including negative controls), SYBR Green mastermix (Roche) and individual primers (0,95μM) were pipetted in triplicates by using the VarispanArm (Perkin Elmer) robot, into 384-well plates. Data analysis was made according to the second derivative method (Abs Quant/2nd Derivative Max).

2.4 Lentiviruses titration and Validation of the constructs

2.4.1 Lentiviruses titration

For lentiviruses titration, and after the procedures described in 2.2, the viruses were used to infect 293T cells, which were plated on 6-well plates in a density of 1,0 x 10^5 cells per well, as described in 2.1. The 293T cells were infected with three different concentrations (3, 1 and 0,3 μL) of each virus, as well as a virus containing a reference gene, individually. The titer of the reference gene has been determined beforehand via FACS analysis for GFP. After DNA extraction (DNeasy® Blood and Tissue Kit, Qiagen),

performed three days after infection and according to the main manufacturer's instructions (Blood and Tissue Handbook, 07/2006, pages 25-27), real time PCR (qPCR) was carried out, as described in 2.3.4, using Taqman® primer probes specific for WPRE and Albumin genes, in order to determine the lentiviruses titers (U/mL).

2.4.2 Validation of the constructs

In order to validate the constructs containing the transcription factors' sequences, after the procedures described in 2.2, 293T and NSE14 cells were plated in 6-well plates as described in 2.1, in a density of 2,0 x 10^5 cells per well, and infected with three different increasing concentrations of each virus (1, 3 and 5µL). Three days after transduction, total RNA was extracted and cDNA synthesized (according to 2.3.1 and 2.3.2, respectively). qRT-PCR was performed (see 2.3.4) in order to detect an increase in transgene expression in a dose-dependent fashion, according to the different concentrations of viruses used for transduction.

2.5 *In vitro* differentiation studies

2.5.1 Screening approach

In 2011, Kim, J. et al. utilized a screening approach involving direct conversion of mouse fibroblasts to induced neurons (iN), to determine a crucial set of transcription factors sufficient to induce a dopaminergic fate in iN cells. The eleven initial TFs were divided in three different groups depending on biological function and expression patterns. Subsequently, the ability of each group of TFs to generate dopaminergic iN cells was tested by using a subtractive approach (Kim et al., 2011).

To investigate the *in vitro* differentiation process of human fibroblasts into striatal GABAergic projection neurons and the contribution of the TFs selected to perform the screening, thirteen TFs were organized in four different groups according to their biological functions and patterns of expression (see table A1, Appendix, detailed classification in categories of all individual TFs).

In **group 1** were the **conversion factors**, Ascl1, Myt1L and Brn2, known by their capacity to convert fibroblasts into neurons (Vierbuchen et al., 2010). In **Group 2**, the TFs CTIP2, Isl1 and Nolz1 were grouped, which are involved in the **striatal medium spiny neurons' development** specifically, while in **Group 3**, Dlx2, Dlx5 and Gsx2,

TFs that seemed to be related to the **striatal development.** Finally, in **group 4**, the TFs Gli1, Otx2, FoxG1 and Gsx1 that were grouped together since they are considered as **specific and related to early CNS development**.

One day prior to transduction, hEF cells at passage numbers of either 2 or 3 were plated in 24-well plates, at a density of 2,0 x 10^4 cells per well, according to the procedures described in 2.1.

2.5.2 Transcription factors' combination and infection

Solutions containing the individual viruses were prepared in MEF medium at a multiplicity of infection (MOI=number of lentiviral particles/number of target cells) of 4 for all the striatal and conversion factors, and an MOI of 8 for the transactivator lentivirus (FuW) (exceed of transactivator ensuring dox-mediated transgene expression). In the course of transduction, the entire medium per well was replaced by the previously described viral solution.

We designed a strategy in order to address the importance of the groups for the conversion of the hEFs. First, each one of the groups of factors, as well as a combination of all the groups of factors together were used to infect the hEFs. On top of that, all possible combinations of three groups per condition were tested. For each one of the conditions, three technical replicates (three wells per condition) were made, and 15 days after transgene activation, converted cells were quantified by using Immunocytochemistry.

For more detailed information about the grouping of the different TFs into the four groups, and the functions and patterns of expression of each TF individually, see table A1, Appendix.

2.5.3 Differentiation protocol

Post infection, the cells were cultured in MEF medium for 6 days and the medium was changed every 2-3 days, according to standard cell culture procedures (described in 2.1). Doxycycline (2 μg/mL) was added to the cells six days after transduction, and two days after this, the medium was changed to N2B27 (Neural differentiation medium, Stem Cells) supplemented with 1% PenStrep, and also supplemented with doxycycline (2 μg/mL) and neurotrophic factors (Ascorbic Acid (AA) 0,2 mM, GDNF 0,01 μg/mL, BDNF 0,02 μg/mL and Retinoic Acid (RA) 100 nM). Every 2nd to 3rd day, ¾ of the medium in the wells was changed until termination of the experiment. The protocol of differentiation lasted 15

days (after doxycycline was added) and the cells were then fixed with PFA 4% (Sigma Aldrich). The following figure shows a timeline for the *in vitro* differentiation studies.

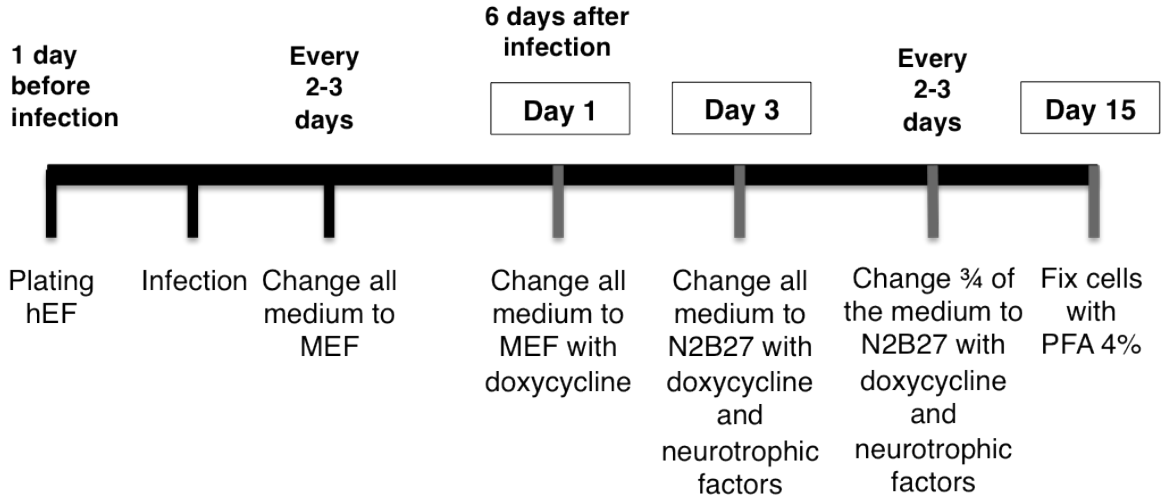


Figure 8 - Timeline for the differentiation protocol.
One day prior to infection, hEFs were plated at a density of 2,0 x 104. The infection was then made in the next day and the cells were kept in culture for 6 days, during which the medium was changed every 2-3 days. Doxycycline was added (Day 1) and 2 days after, all medium was changed to N2B27, supplemented with Doxycycline and neurotrophic factors (ascorbic acid, GDNF, BDNF and retinoic acid). ¾ of the medium were changed every 2-3 days until the end of the differentiation protocol (Day 15), when the cells were fixed with PFA 4%.

2.5.4 Comparison of differentiation protocols

The abovementioned differentiation protocol was selected based on previous experiments made in the lab that showed that delaying the doxycycline addition to the cells for six days yielded higher number of converted cells (unpublished data) than adding doxycycline one day after transduction (Pang et al., 2011; Pfisterer et al., 2011a; Vierbuchen et al., 2010).

In April 2012, Ladewig and colleagues showed that by using a combination of SM involved in the inhibition of the SMAD pathway and of the GSK-3β (SB-431542 together with noggin and CHIR99021), with a minimum number of TFs (Ascl1 and Neurogenin 2 (Ngn2) (AN)) it is possible to achieve a highly efficient neuronal conversion of human fibroblasts (Ladewig et al., 2012).

We set out to explore the potential of different differentiation protocols to generate hiN cells. In order to do so, we applied the conversion factors Ascl1, Brn2 and Myt1L (group 1) to hEF cells in five different settings of direct conversions as listed below:

Condition I – dual SMAD inhibition (according to (Ladewig et al., 2012));

Condition II – delayed doxycycline protocol (described above);

Condition III – dual SMAD inhibition with delayed doxycycline and using the neurotrophic factors suggested by Ladewig et al.;

Condition IV – No doxycycline delay (Pfisterer et al., 2011a);

Condition V - dual SMAD inhibition with delayed doxycycline and using the neurotrophic factors as described above (2.5.3).

In order to compare the new conversion protocols with the previously used and to evaluate maturation of generated hiN cells, two different timepoints were selected to fix the cells with 4%PFA (days 15 and 28). The combination of the conversion factors Ascl1 with Ngn2 was also tested in the same different conditions. Further analysis was performed by Immunocytochemistry.

SM were tested using the following final concentrations: 10 μM SB, 500 ng/mL Noggin (R&D systems) and 2 μM CHIR (Axon). For conditions I and III, a different cocktail of neurotrophic factors was used: BDNF (10 ng/mL), GDNF (2 ng/mL), Neurotrophin 3 (NT3; 10 ng/mL) (R&D systems) and dcAMP (0,5 mM) (Sigma Aldrich).

2.6 Characterization of the reprogrammed cells

2.6.1 Immunocytochemistry

Immunofluorescence stainings were performed on the fixed cells, starting with a 1 hour incubation with blocking solution, 5% normal donkey serum in 0,025% TKPBS (12,5 mL 10% Triton in 487,5 mL of KPBS; Sigma Aldrich), followed by an over-night incubation at 4°C with a solution containing the primary antibodies, which were diluted in blocking solution (5%) (Table V). Cells were then rinsed three times with TKPBS and after that, incubated again for 1 hour with blocking solution (5%). A dilution of secondary antibodies was then made in blocking solution (5%) (Table VI) and used in a 2 hours' incubation at room temperature, in the absence of light. Finally, the cells are rinsed three times in TKPBS and kept in this solution until they were used for fluorescence microscopy.

Table V – Primary Antibodies used in the immunocytochemistry assays.

Primary antibodies	Host	Dilution
βIII-tubuliin (Promega)	Mouse	1:1000
GABA (Sigma)	Rabbit	1:1000
DARPP-32 (Epitomics)	Rabbit	1:250
Isl1 (Hybridoma bank)	Mouse	1:100

Table VI – Secondary antibodies used in the immunocytochemistry assays.

Secondary antibodies	Company	Dilution
Cy2 donkey-α-mouse	Jackson ImmunoResearch laboratories, Inc.	1:200
Cy3 donkey-α-rabbit		
Cy5 donkey-α-rabbit		
DAPI		1:1000

2.7 Quantifications

In order to quantify the reprogrammed cells, 30-36 representative pictures were taken per well (condition) (Leica DFC360 FX + DMI 6000B). To calculate the average percentage of converted cells, $DAPI^+/\beta III\text{-}tubulin^+$ double positive cells were counted in 10 pictures from each condition, and the percentage of converted cells calculated for each picture, relating the number of $DAPI^+/\beta III\text{-}tubulin^+$ double positive cells to the total number of DAPI-positive cells. The average of the obtained percentages was then calculated, as well as the SD (standard deviation). In some conditions, with a very low number of converted cells, the number of taken pictures was lower, including only fields, which contained converted cells.

For the calculation of the number of neurons per mm^2, the total number of $DAPI^+/\beta III\text{-}tubulin^+$ double positive cells in 36 pictures was counted, and by relating to the surface area of each field, the total number of neurons per well was calculated, and thus

the number of neurons/mm^2 determined. In some cases, the exact number of βIII-tubulin$^+$ was determined, since there was a very low number of converted cells, and therefore there was no need to extrapolate a total value (the screening was performed through the analysis of the entire well)

The conversion efficiency was determined according to previously described analysis methods (Vierbuchen et al., 2010), in which the total number of converted cells id divided by the number of plated cells, and the result presented as a percentage.

Exact numbers of GABA-expressing cells were quantified by manually screening each individual well, with no use of any kind of extrapolation.

The percentage of GABAergic hiN cells was determined relatively to the total number of converted cells, per condition.

In the course of the last experiment (described in 2.5.4), the Cellomics array scan (Array Scan VTI, Thermo Fischer) was used. This array allows for unbiased high content analysis, quantifying the cells based on the presence of marker expression. As a first step, cells were identified as cell counts, based on the size of the nucleus and expression of the nuclear marker DAPI. Subsequently, intensity levels in different fluorescent channels representing different biological markers were determined based on a set of representative test fields, taken manually by the operator. Then, events were defined based on the combination of marker expression in the beforehand selected channels (DAPI$^+$/MAP2$^+$ or DAPI$^+$/MAP2$^+$/GABA$^+$, for example). The cellomics array scan screened the wells by taking 30 random pictures, in a spiral fashion, from center to outside. All images were acquired by using a 10x magnification objective. The obtained data is represented as total number of cell counts, positive for the defined events, thus allowing for representing the data as percentage of converted cells as well (MAP2$^+$/DAPI$^+$).

Chapter III – Results

3.1 Validation of the LentiStria vectors by qRT-PCR

3.1.1 Validation of seven of the LentiStria vectors by qRT-PCR, in 293T cells

This lab has successfully used lentivirus-mediated overexpression of TFs for the direct conversion of both human embryonic and postnatal fibroblasts into neurons (Pfisterer et al., 2011a). Lentiviruses are good tools for these types of studies, since they have a great ability to efficiently transduce slowly or non-dividing cells (Zufferey et al., 1997) and a large cloning capacity, providing the possibility of carrying complex expression cassettes (Naldini et al., 1996a; Wiznerowicz and Trono, 2005). They also make it possible to broaden the spectrum of susceptible cells and alter the transduction efficiency, allowing for stable and long-term gene delivery.

The ORF coding sequences of ten TFs were cloned into LV#1000-hPGK (Figure A2, Appendix), and these constructs were used to produce the lentiviral vectors (lentiStria), that were subsequently used for infection of human embryonic fibroblasts (hEFs).

In order to test the biological functionality and correctness of the lentiStria constructs, these were validated on 293T cells. For this purpose, we assessed their ability to produce a dose-dependent increase in the expression of the TFs assayed by qRT-PCR. The produced lentiviruses, carrying the transgene of interest, were used to infect 293T cells at three different increasing concentrations (1, 3 and 5 μL), individually, followed by gene expression analysis.

During the first attempt to validate the constructs, we used primers that were able to amplify a fragment spanned between the transgene and WPRE (viral) sequences. This kind of primer allows distinguishing between transgenes that are virally delivered or endogenously expressed. qRT-PCR using these primers demonstrated that seven of the ten transcription factors (FoxG1, Gli1, Gsx1, Gsx2, Otx2, Isl1 and Nolz1) yielded a dose-dependent increase in transgene expression, when transduced in 293T cells (Figure 9). No expression was detected in untransduced 293T cells. A second qRT-PCR round was performed, which allowed confirming the previous observations.

In the case of the three remaining constructs, no dose-dependent increase in the expression of the TFs Dlx2, Dlx5 and CTIP2 was observed. Additionally, some expression of Dlx2 and Dlx5 was observed in the untransduced 293T cells.

Since it was not possible to observe an increase in the expression of those factors (Dlx2, Dlx5 and CTIP2), a different type of primers was designed. These primers hybridized exclusively within the coding sequence and did not recognize any viral sequences. Even not distinguishing between virally delivered and endogenously expressed transgenes, these primers would be able to detect a dose-dependent increase in the expression of the TFs, specially in relation to the uninfected control cells. In this attempt to validate the three constructs, it was still not possible to detect a dose-dependent increase in the expression of CTIP2 by using these primers. Expression of CTIP2, Dlx2 and Dlx5 was detected by qRT-PCR in the control (untransduced) 293T cells, indicating that these cells probably were not suitable for the validation of these three constructs.

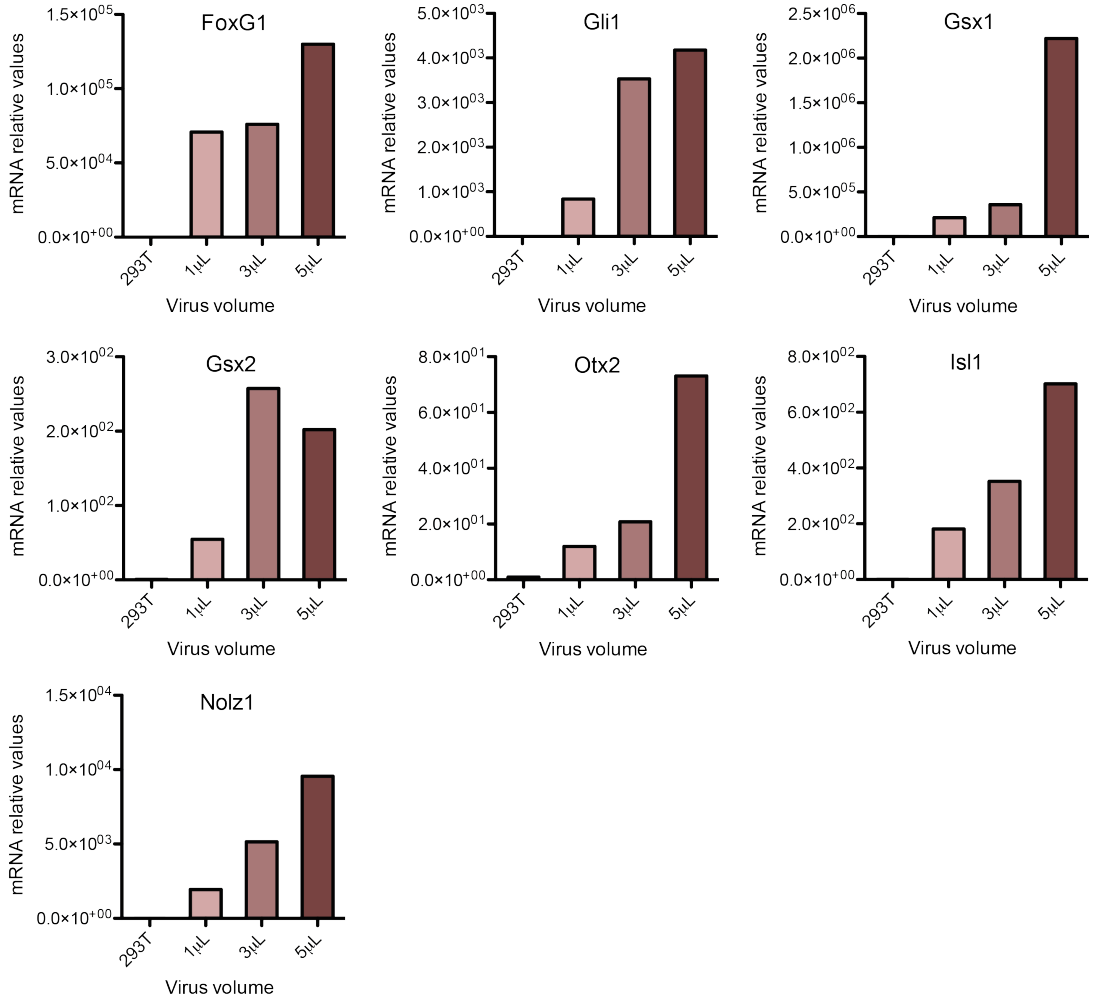


Figure 9 - Validation of seven LentiStria vectors by qRT-PCR, in 293T cells.
The graphs show the dose-dependent increase in the expression of the transcription factors in 293T, normalized to the control (untransduced) and in relation to the housekeeping genes GAPDH and β-Actin. The vectors carrying the TFs' transgenes FoxG1, Gli1, Gsx1, Gsx2, Otx2, Isl1

and Nolz1 were validated through qRT-PCR, using primers that could detect both part of the transgene sequence and the viral sequence WPRE.

3.1.2 Validation of two of the three remaining lentiStria vectors by qRT-PCR, in NSE14 cells

After validating seven of the lentiStria constructs using 293T cells, three of them remained to validate. Since there was detection of Dlx2 and Dlx5 expression in the untransduced human 293T cells, we then proceeded to overexpress these two TFs in a mouse cell line that do not express them, the NSE14. It was possible to observe a dose-dependent increase in the expression of Dlx2 and Dlx5, with no expression in the untransduced NSE14 cells. These observations confirmed that the two constructs effectively allow the TFs' overexpression in the transduced cells, as well as a dose-dependent increase in their expression (Figure 10). By doing so, they were considered as validated in NSE14 cells.

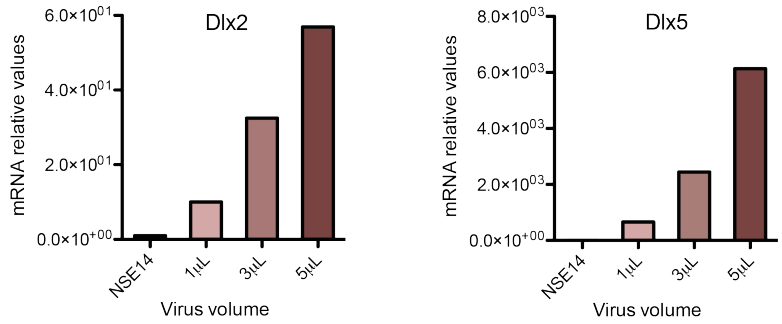


Figure 10 – Validation of the constructs containing the Dlx2 and Dlx5 transgenes by qRT-PCR, in NSE14 cells.

The graphs show a dose-dependent increase in the expression of the transcription factors in NSE14 cells, normalized to the control (untransduced) and in relation to the housekeeping genes GAPDH and β-Actin. The vectors were validated by qRT-PCR, using primers that could detect only the transgene sequence of the transcription factors.

3.1.3 qRT-PCR studies did not allow for the validation of the LV#1000-CTIP2 construct

In an attempt to validate the CTIP2 transgene-carrying construct, NSE14 cells were also used. Three different additional primer pairs were designed for this validation (described in table IV, 2.3.3).

A high background expression on the untransduced NSE14 cells was detected and again, no dose-dependent increase in the expression was verified. We then proceeded to verify the correctness of the cloned construct, by digesting it with three different restriction enzymes (BamH1, SalI and MluI) and then comparing the resulting bands with the expected restriction digest pattern. According to this, it was possible to observe the expected pattern of bands in the different digestions (Figure A3, Appendix). Thus, it seems that the CTIP2 construct's sequence is correct. The validation of this construct remains to be completed and will be addressed in future experiments.

3.2 Direct conversion of hEFs into hiN cells

3.2.1 Experimental design

To investigate the *in vitro* conversion process of the hEFs and the contribution of the TFs selected to perform this screening, thirteen TFs were organized in four different groups according to their functions and patterns of expression (see table A1, Appendix) where these aspects are summarized for each TF individually).

We then designed a strategy in order to address the importance of the groups for the conversion of the hEFs. This contribution was determined by infecting the hEFs with different combinations of lentiviruses containing the TFs' transgenes. First, each one of the groups of factors was used to infect the hEFs individually (Figure 11, A) as well as a combination in which all the groups of TFs were included (Figure 11, B) and then, different combinations of three groups per condition, in which one of the groups was lacking in the pool, were tested individually (Figure 11, C). For each one of the conditions, 3 technical replicates (3 wells per condition) were made, and 15 days after transgene activation, converted cells were quantified by using Immunocytochemistry assays.

3.2.2 Generation of hiN cells from hEFs using a subtractive screening approach

Through the analysis of the expression of the neuronal marker βIII-tubulin (βIII), it was possible to determine the number of iN cells being formed, by quantifying the number of βIII-positive cells per mm^2. The generation of GABAergic hiN cells was also quantified, through the expression of GABA in the converted cells. In order to assess a

possible expression of striatal markers in the GABAergic hiN cells, DARPP-32 expression was also investigated. This molecule is localized to neurons containing dopamine

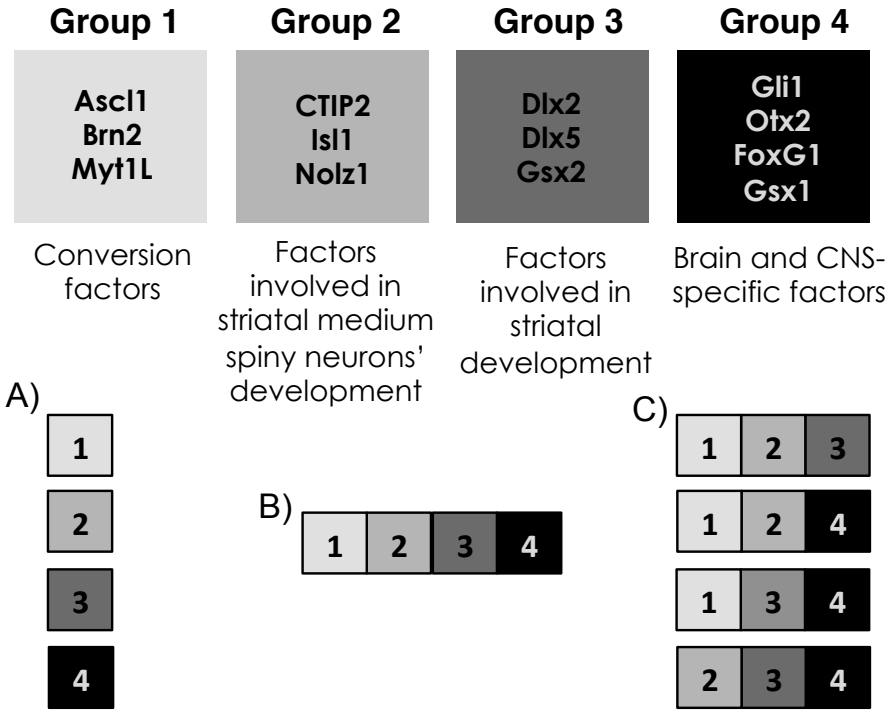


Figure 11 – The transcription factors' combination.
After grouping the transcription factors into four different groups (Conversion factors, factors involved in striatal medium spiny neurons' development, factors involved in striatal development and brain and CNS-specific factors), they were used in different combinations: (A) each one of the groups was tested individually; (B) a combination containing all the groups together was also tested; (C) different combinations of three groups per condition were made.

receptors (Gould and Manji, 2005), and is a commonly used marker for striatal neurons.

Due to technical reasons, the analysis of βIII and GABA expression in the cells was performed in only one of the three replicates, per condition. No or very low levels of GABA- or βIII-expressing cells were found in the negative controls (less than n=0.04). No DARPP-32 expression was detected in any of the tested conditions.

When analyzing the number of βIII-positive cells/mm^2, the highest value was obtained in the condition where all the thirteen TFs were used to transduce hEFs (n=115.0). Group 1 also gave rise to a high number of converted cells per mm^2 (n=88.9) and when excluding these transcription factors (ABM) from the infection pool, almost no conversion was detected (n=0.07) (Figure 12, B). None of the other individual groups of transcription factors were able to convert hEFs in the same extent than Group 1 (n=0,14,

n=0,09 and n=0,08, for groups 2, 3 and 4, respectively). These results showed that when Group 1 was present in the infection pool, a robust hiN generation was obtained in different conditions, with similar levels of βIII-positive cells.

We also set out to determine if another quantification method could be used to access the efficiency of this conversion. In order to explore this question, the average percentage of converted cells, relatively to the number of DAPI$^+$ cells was also calculated. According to this, we verified that even though the proportions were not exactly the same, similar observations could be made, by using this method (Figure 12, C).

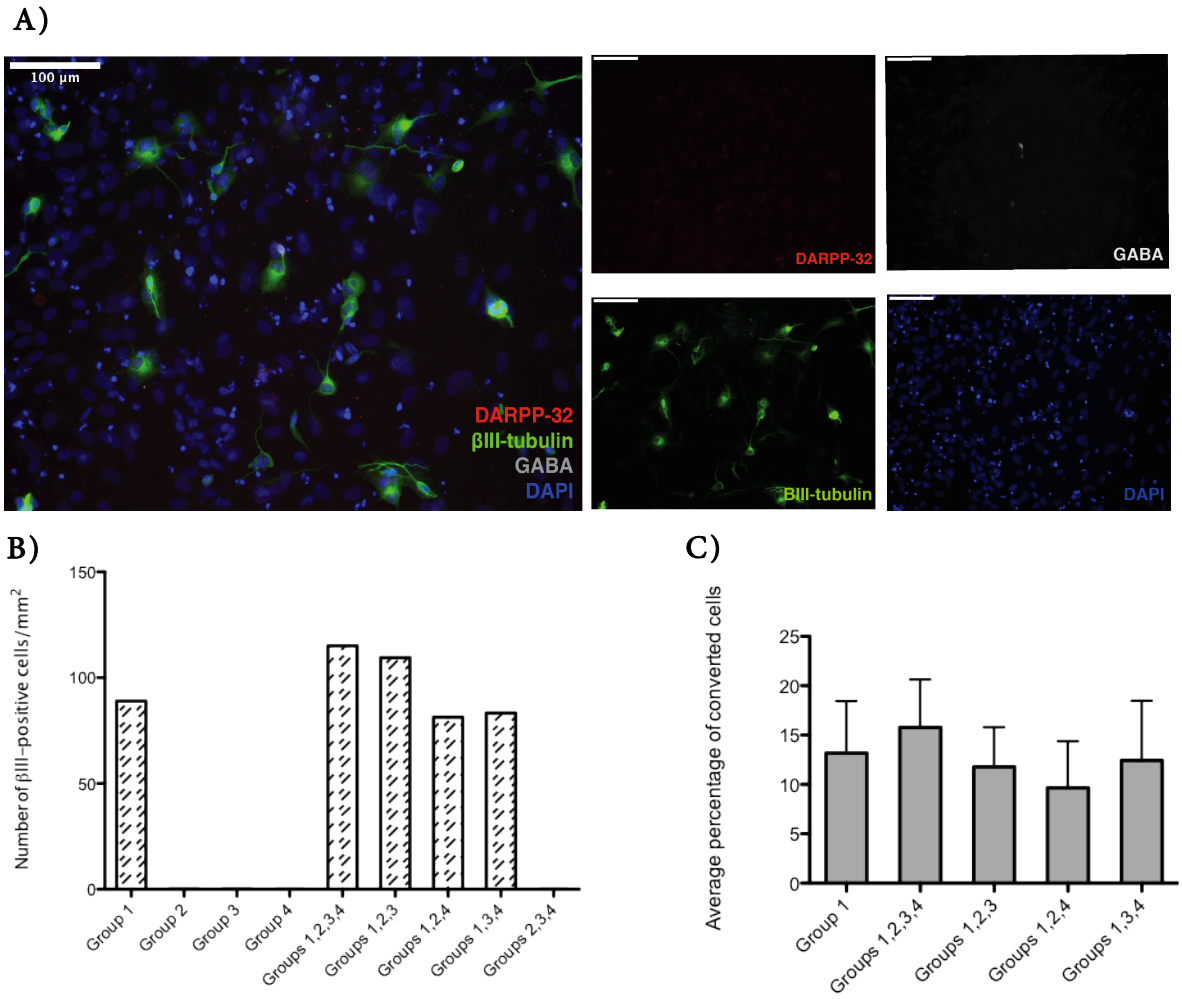


Figure 12 – First attempt to convert hEFs into striatal hiN cells by using a subtractive screening approach.

(A) Fluorescence microscopy image (20x) showing a GABA-expressing cell (grey), among several βIII-tubulin-expressing converted cells (green) (Groups 1,2,3,4); (B) Number of βIII+ cells per mm2, per condition. 30-36 representative pictures were taken per well (condition), the total number of βIII+ cells was counted in these images and the total number of neurons per well was determined, as well as the number of neurons/mm^2. In the case of the conditions comprising the

individual groups of transcription factors 2,3,4, the exact number of βIII+ cells was determined, due to a very low number of converted cells; (C) Average percentage of converted cells (Mean± SD). 30-36 representative pictures were used to determine the percentage of βIII+ cells per picture, relatively to the number of DAPI+ cells.

3.2.3 Effect of different TF groups on GABAergic hiN formation

After analyzing the βIII expression in the generated hiN, the GABA expression was also determined in these cells. According to this, the combination that allowed for the generation of the highest number of GABA-expressing cells was the one that comprised the four groups together (n=49). All the groups, except group 4 (n=0), showed an ability to generate GABA-expressing cells individually. When having the three-group combination per condition, the only condition in which no GABA-expressing cells were detected was the one where group 1 was missing. When omitting group 4 from the infection, the greatest decrease (n=8) regarding total number of GABAergic hiN cells compared to the combination of groups 1-4 (n=49) was observed (Figure 13, B). A smaller decrease in total GABAergic hiN was found when leaving out either group 3 or group 2. All the previously determined values also allowed us to determine the percentage of generated GABA-positive neurons out of converted βIII-positive cells (Figure 13, C). The obtained percentages were in the range of 0.04% (in the absence of TFs from group 4) and 0,24% (in the presence of all the groups of TFs).

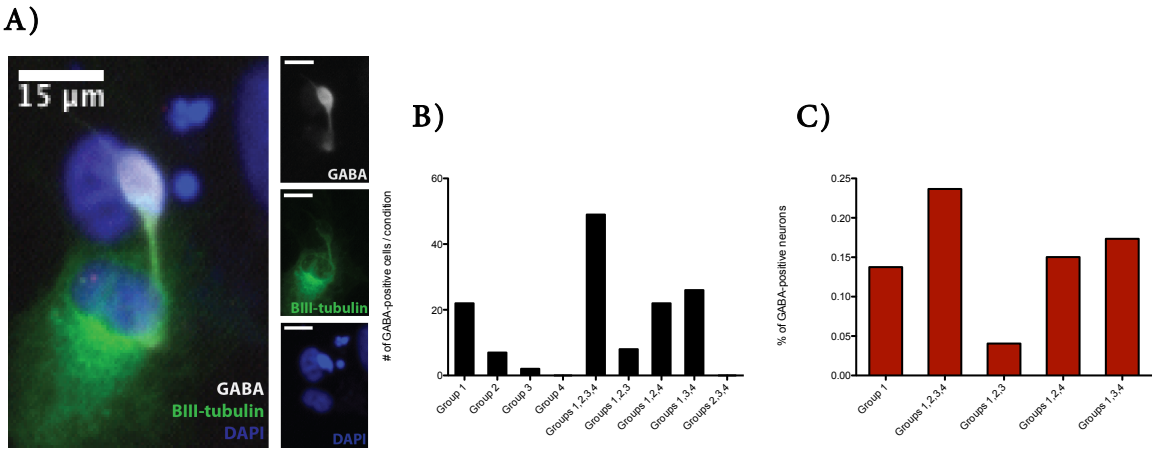


Figure 13 – GABAergic hiN cells' quantification (first round of infections).
(A) Fluoescence microscopy image (20x, cropped), showing a GABAergic hiN cell (Groups 1,2,3,4); (B) Number of GABA-positive cells, per condition; (C) Percentage of GABA-positive cells relatively to the number of βIII+ cells, per condition (n=1).

3.2.4 A high variability affected the reproducibility of the results

To access the reproducibility of the protocol and the obtained results, a second round of experiments was carried out according to the previously described screening approach and the quantification of the βIII- and GABA-expressing cells was determined according to previously described quantification methods, per condition. Additionally, in order to investigate a possible striatal phenotype in the generated hiN, the expression of Isl1, which is expressed in all differentiating striatal neurons until birth (Stenman et al., 2003), was also investigated.

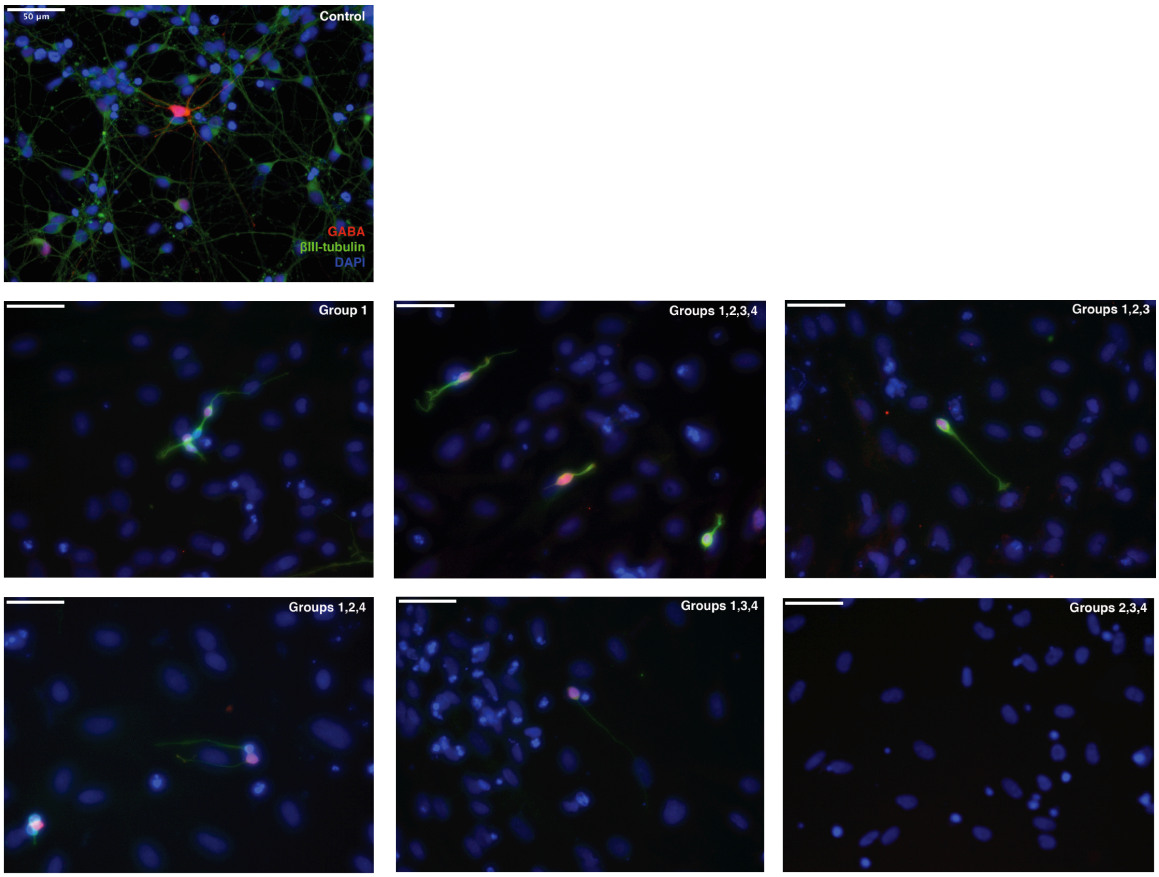
For each one of the studied parameters, two of the replicates were used for quantification. Similarly to the previous round of experiments, rare or non-existing βIII- and GABA- expressing cells were observed in the untransduced controls. No Isl1-expressing cells were detected.

A high variability was observed in the values obtained through the quantifications among different wells within the same conditions. Additionally, the levels of βIII- and GABA-expressing cells appeared to be very different from the ones obtained in the previous infections' round.

Fifteen days after transgene activation, the expression of βIII in the reprogrammed cells was quantified. Contrarily to what was verified in the previous round of experiments, the condition that gave rise to the highest number of βIII-positive cells per mm^2 was the one in which group 1 was used to reprogram the cells individually (n=10.5±4.2). When group 1 was not present in the combination, no conversion was detected (n=0) (Figure 14, B).

The analysis of GABA expression in the generated hiN cells revealed that the highest number of GABA-expressing cells was obtained when all the groups of factors were present in the pool (n=300.0±144.2) (Figure 5, C). When the TFs from group 4 were not present, the highest decrease in the generation of GABAergic cells was achieved (n=139.5±31.8) (comparatively to the one obtained with groups 1,2,3,4). Similarly, the only condition that did not show to be able to generate GABA-positive cells was the one where group 1 was missing (Figure 15, B).

A)


50 µm
Control
GABA
βIII-tubulin
DAPI
Group 1
Groups 1,2,3,4
Groups 1,2,3
Groups 1,2,4
Groups 1,3,4
Groups 2,3,4

B

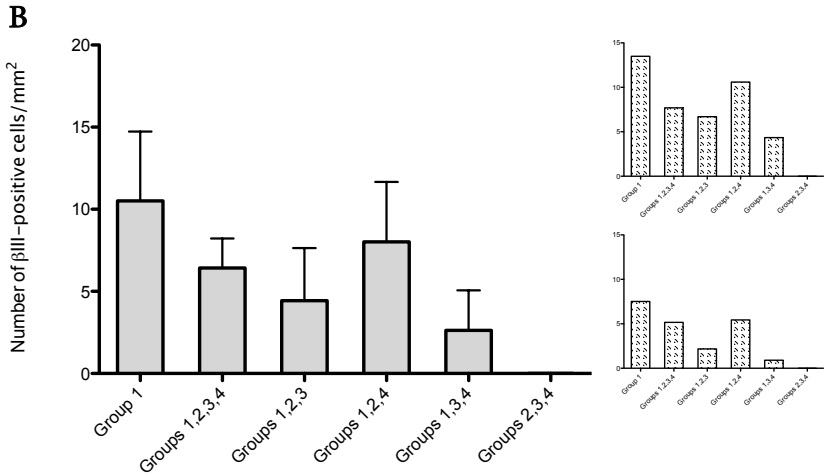

Number of βIII−positive cells/mm2
20
15
10
5
0
Group 1
Groups 1,2,3,4
Groups 1,2,3
Groups 1,2,4
Groups 1,3,4
Groups 2,3,4

Figure 14 – Second subtractive screen for striatal hiN cells.
(A) Representative images of nine different TFs group combinations used to generate βIII- and GABA-expressing hiN cells (40x). As a reference for neuron morphology and expression of the markers, a primary culture of human cortex was used (control); (B) Number of βIII-positive cells per mm2. Combined results obtained from two wells (Mean±SD).

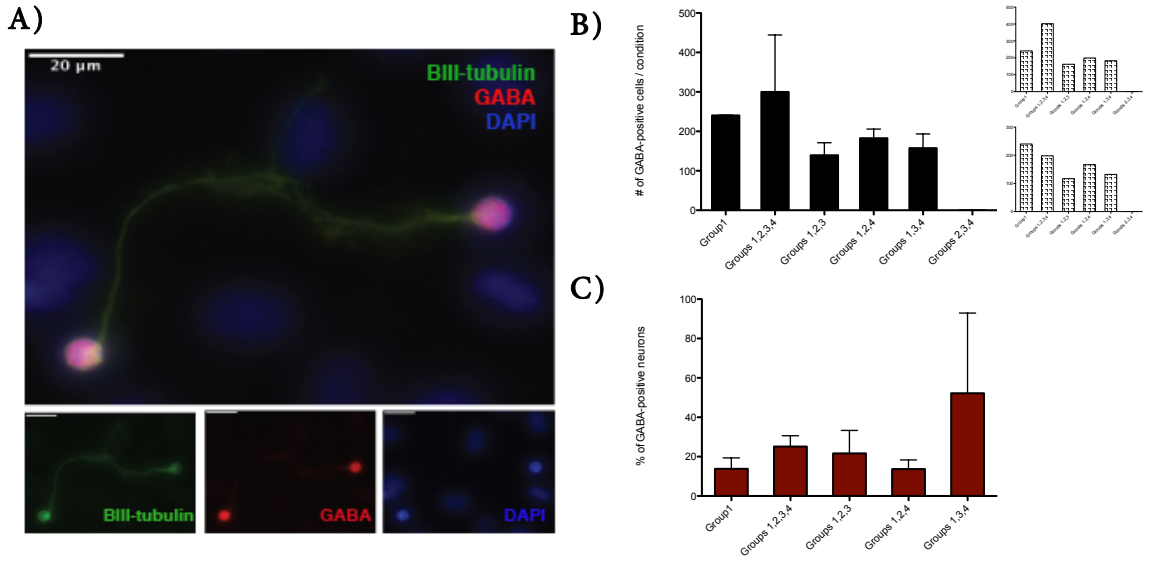


Figure 15 – GABAergic hiN cells' quantification (second round of infections).
Fluorescence microscopy picture (40x, cropped), showing two GABAergic hiN cells (Group1); (B) Total number of GABA-positive cells, per condition. Combined results obtained in 2 wells (Mean±SD); (C) Percentage of GABA-positive cells, relatively to the total number of converted cells (βIII-positive) (Mean±SD, n=2).

The results from the first round of infections suggested that group 1 was required for the generation of cells expressing the neuronal marker βIII. We decided to additionally combine the different groups of TFs pairwise, always including group 1 in the combination. The obtained results did not give us any additional information, but demonstrated that when group 1 was combined with group 4, the highest number of GABA-expressing cells was obtained (Figure 16, C), confirming the presence of potentially important genes in pool 4.

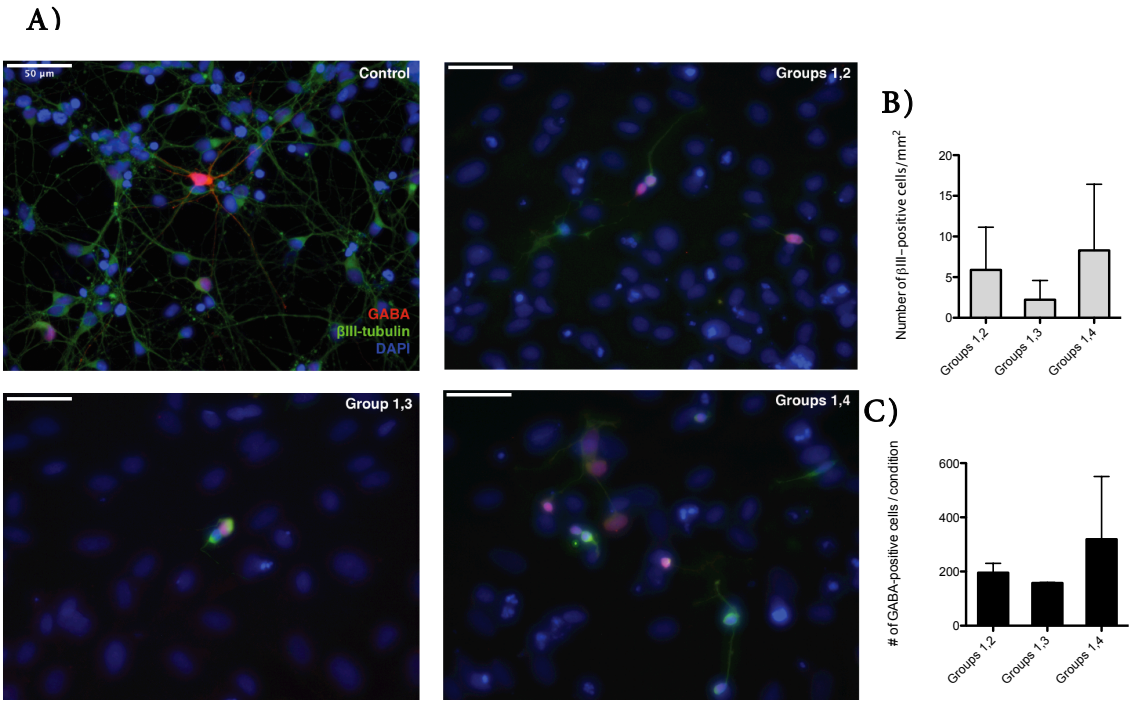


Figure 16 – Pairwise combination of the groups containing the thirteen transcription factors.

(A) Representative images of the wells, where different pairwise combinations were used to infect hEFs, generating βIII- and GABA-expressing hiN cells (40x). As a reference for neuron morphology and markers' expression, a primary culture of human cortex was used (control); (B) and (C) Number of βIII-positive cells per mm^2 and corresponding total number of GABA-positive cells, obtained in the pairwise combinations of the TF groups (Mean±SD, n=2).

3.3 Investigating the efficiency of different differentiation protocols in the generation of hiN cells

3.3.1 Experimental design

As the previously presented results show, we could successfully convert hEFs into hiN cells. However, the generation of those cells was variable in frequency and sometimes conversion was low, which led us to ask whether it would be possible to promote an increase in the stability of conversion and in the yields of generated converted cells, by using a different protocol. Therefore, five different protocols were tried to access their potential in the generation of hiN cells from hEFs.

The first condition includes the use of the SM and a characteristic cocktail of neurotrophic factors, as described by Ladewig et al. (Ladewig et al., 2012). In order to compare the yields of generated cells, we also included a condition with the delayed

doxycycline protocol (Condition II) used in the previous experiments of this project, and a condition in which a protocol with no delay in doxycycline was used, similarly to previous studies performed in the lab (Pfisterer et al., 2011a) (Condition IV). Finally, we then combined the protocols used in condition I and II, aiming to investigate if any synergistic effect of the use of these two protocols together could be observed (conditions III and V). The difference between these two conditions is that they involve the use of a different cocktail of neurotrophic factors, along with the use of SM and a delay in doxycycline delivery to the cells.

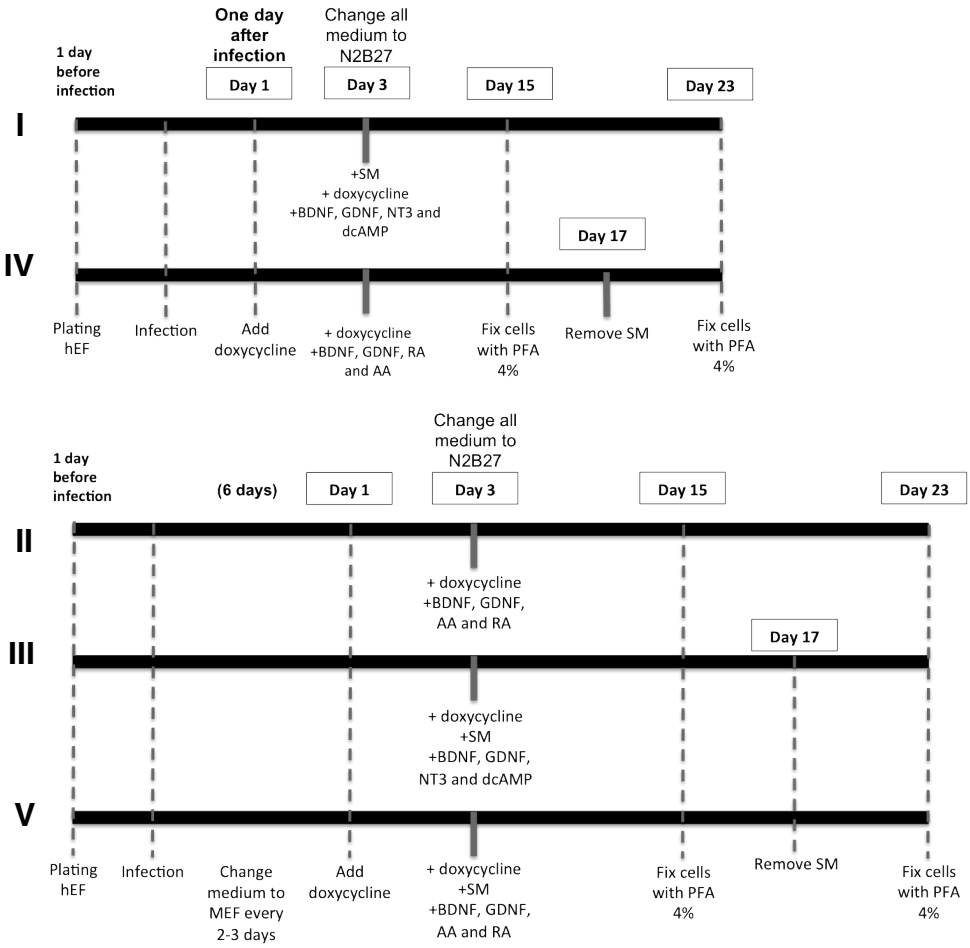


Figure 17 - Schematic representation of the timelines for the five different differentiation protocols (conditions).

Here, the conditions I and IV are grouped together, since they last the same (30 days). For the same reason, the conditions II, III and V, which include a delay of six days until the adding of doxycycline, are also grouped (they last 36 days).

We tested the five different protocols in cells infected either with the conversion factors ABM or with AN (except for condition V, in which the factors AN were not tested).

For each one of the conditions, three replicates were made (except for infections with AN, in which n=2) and the converted cells were quantified by high content analysis (described in 2.7) following Immunocytochemistry assays. Similarly, the protocols were also tested in untransduced cells (negative controls). Two different timepoints were analyzed: day 15 and day 23, post transgene activation. For the conditions II, III and V, which involve a longer period of culture due to the delay in doxycycline, the cells from the second timepoint were analyzed at day 21 instead of 23, due to observed cell death.

3.3.2 Different effect of the tested protocols in the generation of hiN cells

Eleven days after infection of the hEFs with the factors ABM and AN, it was already possible to observe some differences in the cultured infected cells. Under the microscope, cells that resembled neurons were observed, and when looking to the cells infected with ABM, clear differences in the number and morphology of the cells were visible (Figure 18).

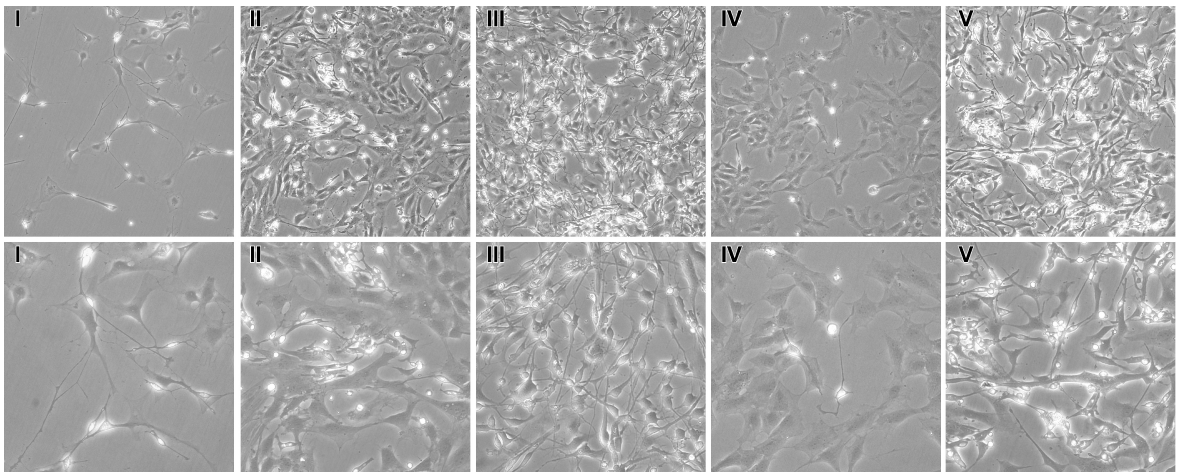


Figure 18 – Bright field microscope pictures of ABM-infected hEFs, eleven days after infection.

The hereby shown pictures were taken eleven days after infection, i.e., for conditions I and IV, ten days after transcription activation, and for conditions II, III and V, four days after transcription activation. Cells infected with ABM (upper, 10x; lower, 20x magnification).

Fifteen days after transgene activation, hEFs treated according to conditions I-V were fixed and stained for further analysis and quantification. Through the expression of

the neuronal marker MAP2, it was possible to analyze the number of generated hiN cells (MAP2-expressing), and to determine the percentage of converted cells in culture. Additionally, and since changes in cell numbers during the process of conversion may contribute to the overall conversion efficiency, we also determined the conversion efficiency as suggested by (Vierbuchen et al., 2010), which allows determining the percentage of MAP2-positive cells in relation to the initial number of plated cells. The three replicates were used for this purpose. Similarly, in one of the replicates, the generation of GABAergic hiN cells was quantified through the analysis of GABA expression in these cells, when infected with ABM. For technical reasons, it was not possible to analyze the expression of GABA in hiN converted from hEFs through infection with AN.

Some MAP2-positive and GABA-positive events were detected in the untransduced controls (an average of 2.5% and 0.2% of the total, respectively).

When observing the cells through fluorescence microscopy, it was possible to observe not only differences in the cell number, but also in cell morphology, when comparing the different conditions (Figure 19, A). Condition III gave rise to the highest percentage of MAP2-positive cells (of the total DAPI-positive), either when infecting the cells with ABM or AN. However, the infection with ABM gave rise to the highest percentage of MAP2-positive cells, when compared with AN, already on day 15 (n=22.9%±3.2% for ABM and 11.3%±0.0% for AN) (Figure 19, B and C). It seems that the use of the factors ABM in the infection of the hEFs, in general, leads to a higher efficiency in the hiN generation, for all the conditions. When analyzing the conversion efficiencies (in relation to the number of plated cells) obtained in condition III, at day 15 after transgene activation, that becomes also clear (n=121.7%±21.5% for ABM and 56.3%±0.0% for AN) (Figure 20).

The results obtained in condition III show that the combination of the protocols used in conditions I and II gives raise to a synergistic effect in the generation of MAP2-positive hiN cells. None of the two conditions, by themselves, was able to give rise to the same yield of converted cells than condition III.

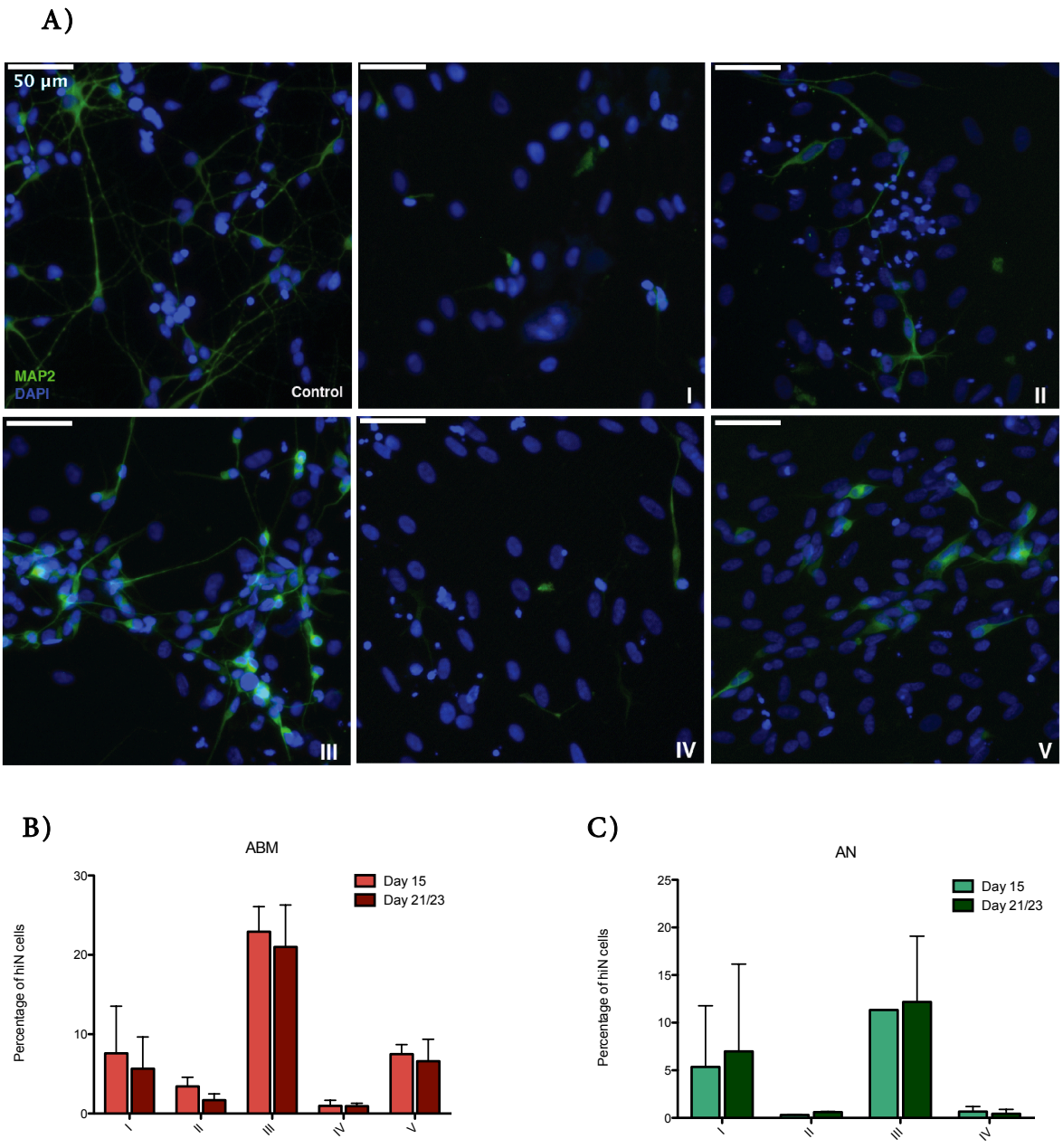


Figure 19 – Different effect of the tested differentiation protocols, in the generation of hiN cells.

(A) Fluorescence microscopy pictures, from the different tested conditions (I-V) and positive control (human cortex). Cells infected with ABM and fixed 15 days after transgene activation (20x magnification). For all the conditions, it was possible to observe hiN cell generation (MAP2-positive cells); (B) and (C) Percentages of MAP2-positive cells, out of the total number of cells in culture (DAPI-positive), per condition (Mean ± SD), when hEFs were infected with ABM and AN, respectively.

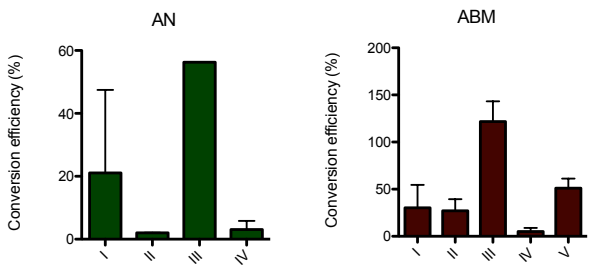


Figure 20 – Conversion efficiencies (%), per condition.
Conversion efficiencies obtained for the conditions I-V, when hEFs were infected with ABM (on top) or AN (bottom), calculated for cells fixed 15 days after transcription activation (Mean±SD).

The lowest percentages of MAP2-positive cells and conversion efficiencies were obtained when testing the protocols of conditions II and IV. The percentages of hiN cells were only slightly superior for condition II (delayed doxycycline, no SM) (n=3.4%±1.1%) when comparing with condition IV (no delayed doxycycline, no SM) (1.0%±0.7%), upon infection with ABM (Day 15) (Figure 19, B).

When comparing conditions which differed only in the cocktail of neurotrophic factors used along the differentiation protocol, condition III shows to be able to generate a higher percentage of hiN cells, either with ABM (n=22.9%±3.17%, for day 15 after adding doxycycline) or AN (n=11.3%±0.0%, day 15), than condition V (n=7.5%±1.2% for ABM and n=0.7%±0.5% for AN, day 15). The conversion efficiencies also show these differences (Figure 20).

During the experiment, from the first to the second timepoint, an overall decrease in cell number was observed, especially when the protocols involved the use of SM. When comparing the total number of cells between the first (day 15) and second timepoints (day 21/23), a decrease in the range of 43.7% and 56.1% (data not shown) for all the conditions involving the use of SM (condition I, III and V), was observed when infecting the cells with ABM. When using the TFs AN, these were in the range of 41.2% and 49.4% (conditions I and III). In conditions that do not involve SM in the protocol, the decrease in cell number was lesser (n=8.44% for condition II, with ABM; for condition IV with ABM and II with AN, there was an increase in the overall number of cells, between the two timepoints).

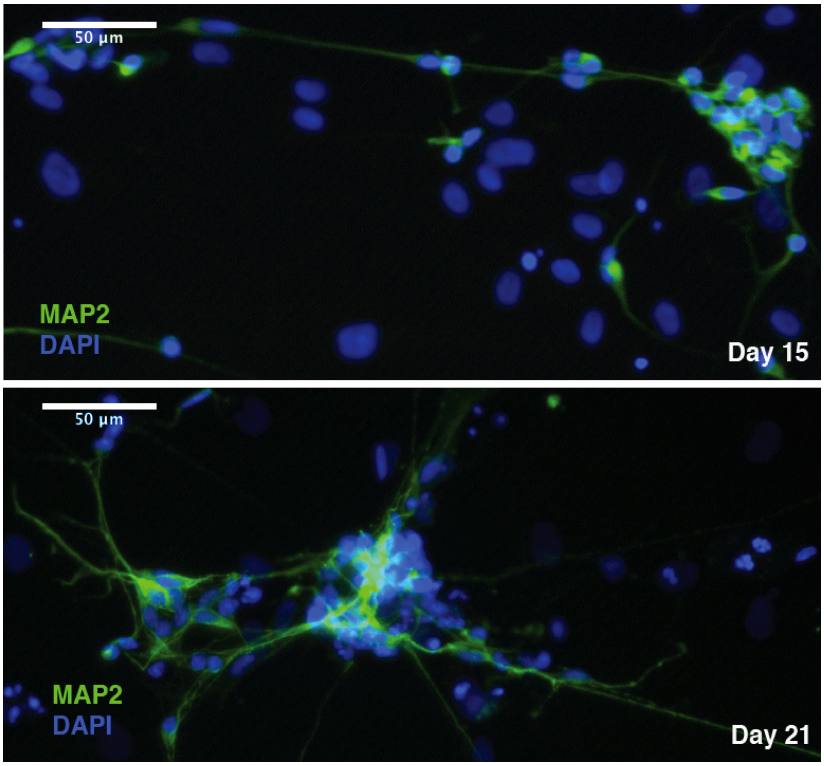


Figure 21 – Differences in hiN cell morphology in two different timepoints.
Fluorescence microscopy pictures (20x), showing hiN cells (MAP2-positive) generated through the use of the protocol according to condition III, and ABM for the infection, in two different timepoints (day 15 and day 21).

Despite the cell death that occured, when comparing the morphology of the obtained hiN cells, in two different timepoints, the cells that were fixed 21/23 days after culture seemed to have longer branches, and to resemble a more mature neuron morphology, when observed through fluorescence microscopy (Figure 21).

3.3.2 Different protocols give rise to distinct numbers of GABAergic cells when infected with ABM

When analyzing the expression of GABA in the converted hiN cells obtained from ABM-infected hEFs, $GABA^+/MAP2^+$ cells were observed in all the tested conditions (I-V). In Figure 22 (A), an example of GABAergic cells among the converted MAP2-positive cells is shown.

Moreover, the numbers of GABAergic hiN cells yielded by the different conditions were different. Already at day 15, the results obtained in conditions III (n=1651) and V (n=3659) showed that the use of SM along with the delay of doxycycline, highly increases the number of GABA-expressing hiN cells. Contrarily, conditions I, II and IV, yielded

lower numbers of GABAergic hiN cells (n=440, n=358 and n=55, for respectively) (Figure 22, B). When comparing the two different timepoints, there is an overall decrease in the number of converted GABA-expressing cells, except for condition IV, in which it was possible to observe an increase in the number of those cells, from day 15 to day 23 (n=233.8).

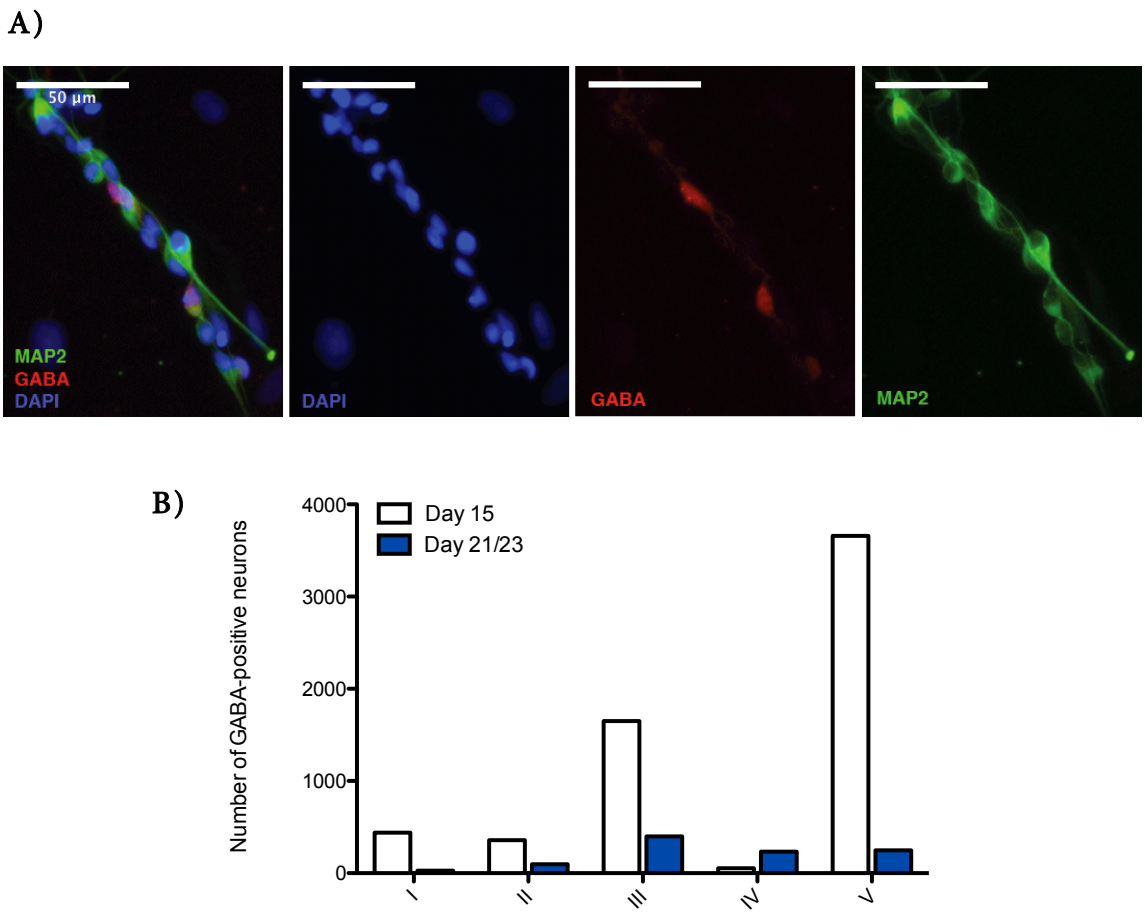


Figure 22 – GABAergic hiN generation when using different differentiation protocols.

(A) Fluorescence microscopy picture (20x) showing GABAergic cells (red), among several MAP2-positive hiN cells (day 15, condition III; resulting from ABM-infected hEFs); (B) Number of GABA-positive neurons, per condition, in two different timepoints (Mean; n=1).

The results from this experiment suggest that the combination of a delay in the doxycycline-regulated transcription activation of the transgenes and the use of SM highly increases the yields of hiN cells and GABAergic hiN cells. The combination of factors ABM seems to be more efficient in the conversion of the cells than AN.

Chapter IV – Discussion

4.1 Validation of LentiStria vectors by qRT-PCR

During this project, nine of the ten lentiviral vectors carrying the transgenes that encode the striatal transcription factors were successfully validated, by overexpression in both human 293T and mouse NSE14 cells, and by analysis of a dose-dependent increase in their expression by qRT-PCR. However, and for reasons that remain unknown, the construct carrying the CTIP2 transgene could not be validated by using this same approach.

Our results indicate that the construct sequence is correct and therefore, due to the fact that background expression was detected in the untransduced human 293T control cells, a different cell line was transduced, the NSE14 cell line (mouse). The same problem was found, which led us to question the specificity of our primers and therefore to align the mouse and human CTIP2 sequences. The result from this alignment (data not shown) demonstrated that the two sequences are highly similar, not allowing for the design of primers with high specificity for the human sequence, and therefore, there is the possibility of a problem in the detection of the transgene. However, special attention to the design of several different primer pairs was paid, and no improvement in the detection of an increase of CTIP2 expression was observed. Also, a possible explanation for the lack of dose-dependent expression of this factor could be in the fact that the amount of viruses used to transduce the cells was not enough for its detection.

Those questions remain unanswered, and future experiments need to be done to assess the problem found during validation of the construct. In this attempt, a possible next step would be to analyze in a gel, after PCR, the different cDNA samples, that were synthesized for use in the validation qRT-PCR assay, from the different transduced and untransduced 293T and NSE14, and see if it is possible to detect a CTIP2 fragment.

4.2 Generation of hiN cells and screening for factors that could direct them towards a striatal neuron phenotype

To investigate the use of thirteen transcription factors in the possible generation of neurons with a striatal phenotype, a group-subtractive screening approach, which has proved to be effective in other study (Kim et al., 2011), was applied. The use of such approach allows for screening the factors based on their biological functions and patterns

of expression, since the factors are grouped according to those features, and for a faster and less virus-consuming screening than the one that could be made by removing the factors one by one from the infection pool.

During the screening, the important role of the ABM factors (Group 1) for generation of hiN cells was clear. In both rounds of experiments, when excluding Group 1 from the infection pool, no or almost no neuron formation was detected, and when included, it gave rise to one of the highest detected portions of neurons in both rounds of experiments.

The use of an additional cocktail of transcription factors with the ABM factors seems to have a positive effect in the generation of GABAergic hiN cells, since there was an increase in the number of those cells, when comparing only with ABM.

Already in the first round of infections, Group 4 seemed to have an important role in the generation of GABAergic cells, since the lack of these factors in the infection pool, led to the highest decrease in the number of GABA-positive neurons. The significance of this group was reinforced by the additional data from the second round of experiments. When the groups were organized pairwise, the combination of groups 1,4 was able to generate the highest number of GABAergic cells, compared to the values obtained with the combinations 1,2 and 1,3.

The transcription factors from Group 1 (Ascl1, Brn 2 and Myt1L) are proved to be involved in processes related with neuronal commitment and differentiation (www.ncbi.nhm.nih.gov/gene/429), regulation of gene expression during neurogenesis (Atanasoski et al., 1995; Schreiber et al., 1993) and found at early stages of neuronal differentiation (Armstrong et al., 1995; Kim et al., 1997). While the transcription factors from group 4 (Gli 1, Otx2, FoxG1 and Gsx1) are related with forebrain development, gene regulation during proliferation and differentiation, and they are involved in brain development. It seems likely that functions related to these processes are important for the generation of GABAergic hiN cells.

Even not resulting in the generation of neurons with striatal markers' expression (DARPP-32 and Isl1), these experiments were very valuable, leading us to the determination of which transcription factors could be more important for the generation of GABAergic hiN cells. Similarly to the direct conversion of human fibroblasts into dopaminergic neurons, in which these iDA cells were rare events, in initial screens, also

the possibly arising striatal GABAergic cells could be. Therefore, a high level of GABA-positive events also increases the probability of striatal markers' occurrence.

Future studies involving gene expression analysis in the generated cells would also be necessary to confirm the observed phenotypes and to exclude the fact that striatal markers are not expressed at all in these cells. It would also be interesting to understand which genes are being up- and down-regulated during the process of reprogramming, using the selected cocktail of factors.

Due to the high variability among the results, we cannot draw conclusions concerning the screening for determining the minimal number of TFs for the generation of striatal hiN cells by using factors belonging only to groups 1 and 4. More experiments would be needed to address the reasons and possible solutions for such variability. It is likely that the use of different lentivirus batches in the two experiments could affect the conversion of the hEfs and possibly, the resulting data.

4.3 Investigation of the best protocol for converting hEFs into hiN cells

After successfully converting hEFs into hiN cells, we next performed a comparative study, involving different differentiation protocols, in order to investigate which one could lead to an improvement in the generation of these iN cells.

A protocol that combines the use of a delay in doxycycline, responsible for the transgenes activation in the cells, and SM (CHIR, SB and Noggin) with the use of a specific cocktail of transcription factors, as suggested by (Ladewig et al., 2012) (Condition III), gave rise to the highest percentage of MAP2-positive hiN cells. The results strongly suggest that there is a synergistic effect when combining the two different protocols (conditions I and II), since none of these protocols was able to generate similar percentages to those observed in condition III. Furthermore, when observing the cells obtained in condition III at the microscope, they seemed to be more developed morphologically, presenting a higher number of branches.

When comparing the results obtained in the conditions that gave rise to the lowest percentages of hiN cells, conditions II (with delay in doxycycline, no SM) and IV (no delay in doxycycline, no SM), it seems that the delay in doxycycline does not add a significant effect in the generation of hiN cells, since there is only a slight increase in the percentage

of MAP2-expressing hiN in condition II, when compared with condition IV. By using a delay in doxycycline, an increase in the conversion efficiency is expected though, since the transduced cells keep proliferating for six days, increasing the number of cells that express the genes that will allow for conversion, when transcription is activated.

The use of the cocktail of neurotrophic factors suggested by (Ladewig et al., 2012), which includes the use of NT3, BDNF, GDNF and d-cAMP , also seems to positively affect the survival of generated converted cells, which can be observed when comparing the results obtained in conditions III and V.

When analyzing these results (described in 3.3), and the results from previous infection rounds (performed for screening of striatal TFs, described in 3.2), and comparing the obtained conversion efficiencies (Figures A4, A5 and 20), the effects of using the protocol of condition III also become clear. In the first and second round of infections, conversion efficiencies of approximately 80% and below 10%, respectively, were obtained (Figures A4 and A5). And when using the protocol of condition II for conversion of hEfs into hiN with the ABM factors (Figure 20, upper graph), a conversion efficiency of approximately 27% was achieved. Therefore, by comparing those values with the conversion efficiencies obtained by applying the protocol of condition III, a great improvement was made. Furthermore, this protocol also allows for an increase in the number of observed GABAergic hiN cells.

An analysis of the average number of DAPI-positive cells found in the two different studied timepoints (day 15 and 21/23) (data not shown) revealed that there was a decrease in the number of cells in a range between 40 to more than 50% in conditions involving the use of SM (conditions I, III and V). However, the reasons for this decrease are still unknown. This could be due to cell death or detachment, or simply due to the occurrence of less proliferation.

Taken together, the results show that the most advantageous protocol for hiN generation is the one from condition III, and that this protocol is more effective when using the TFs ABM for infecting the hEFs. By analyzing the conversion efficiencies of these conversions, which relate the number of hiN cells with the number of plated cells, it is possible to observe values close to 100%, while with the conversion factors AN, conversion efficiencies below 60% were observed.

Chapter V – Conclusions

Although some complementary studies must be done and repetition of the assays is needed, it is possible to draw some main conclusions from the studies performed during the course of this project.

First, the different groups of TFs selected to perform the screening for striatal GABAergic projection neurons' formation were all able to generate hiN cells, but none of them generated yields of converted cells as the ones obtained with the conversion factors factors ABM (group 1). And through the analysis of the results obtained in both rounds of infection, it is possible to conclude that the TFs ABM are essential an efficient generation of hiN cells from the infected fibroblasts. Furthermore, the results obtained during the evaluation of different protocols for the generation of the hiN cells showed that infecting the cells with the TFs ABM is more effective than with another combination of TFs, also able to generate neurons (AN).

Second, the addition of striatal TFs to the ABM combination did not result in hiN cells that express the striatal markers DARPP-32 or Isl1, which was analyzed by Immunocytochemistry assays, but affects the yields of GABA-expressing hiN cells, increasing them.

Third, the TFs present in group 4 (Gli1, Otx2, FoxG1 and Gsx1) revealed to have an important role in the generation of GABAergic cells, since the lack of these factors in the infection pool led to the highest decrease in the number of GABAergic cell, compared to the values obtained when other groups were lacking. The importance of this group was revealed already in the first round of infections, and reinforced in the second, when analyzing the results from the pairwise combination of the four different groups of TFs.

Also, the use of a differentiation protocol for the conversion of hEFs into hiN cells that includes both a delay in the deliver of doxycycline in the cells and the use of small molecules, as well as a specific cocktail of neurotrophic factors, is more effective in the generation of hiN cells as well as in the generation of cells with GABAergic phenotype, than the ones that do not include a delay in doxycycline and/or the use of small molecules. Therefore, future studies involving direct conversion of fibroblasts into neuron-like converted cells could benefit from the use of this new protocol.

Chapter VI – References

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VII - Appendix

Table A1 – The transcription factors selected to perform the screening, their biological roles and patterns of expression.

Thirteen transcription factors involved in neuronal development and differentiation of striatal neurons, with distinct patterns of expression were sorted out into four different groups.

In Group 1 are the **conversion factors**, Ascl1, Myt1L and Brn2, known by their capacity to convert fibroblasts into neurons (Vierbuchen et al., 2010). In **Group 2** were grouped CTIP2, Isl1 and Nolz1, which are involved in the **striatal medium spiny neurons' development** specifically, while in **Group 3**, Dlx2, Dlx5 and Gsx2, TFs that seemed to be related to the **striatal development.** Finally, on **group 4**, the TFs Gli1, Otx2, FoxG1 and Gsx1 that were grouped together since they are considered as **specific and related to early CNS development**.

Official name		Also known as	Superclass/class	Involved in
Group 1	**Ascl1**	Ash1; Hash1; Mash1; bHLHa46	Basic-helix-loop-helix (bHLH)/bHLH	Neural commitment and differentiation (http://www.ncbi.nlm.nih.gov/gene/429).
	POU3F2	Brn2; Oct7; Otf7; POUF3	Helix-turn-hélix/Homeo domain	Mammalian neurogenesis through the regulation of their diverse patterns of gene expression (Atanasoski et al., 1995; Schreiber et al., 1993).
	Myt1L	Myt1-L; NZF1; KIAA1106.	Zinc-coordinationg DNA-binding domains/Cys2 His2 zinc finger domain	Development of neurons in the mammalian CNS, suggested by the developmental expression and localization of this protein. Found in neurons at early stages of differentiation (Armstrong et al., 1995; Kim et al., 1997).
Group 2	**BCL11B**	CTIP2; CTIP-2; RIT1; ZNF856B; hRIT1-alpha	Zinc-coordinationg DNA-binding domains/Cys2 His2 zinc finger domain	Controls the differentiation of medium spiny neurons and the establishment of the cellular architecture of the striatum (Arlotta et al., 2008).
	Isl1	Isl-1; Islet1; Islet-1	Helix-turn-helix/Homeo domain	Generation of motor neurons (Pfaff et al., 1996). Without it, there is no neural tube motor differentiation (http://www.ncbi.nlm.nih.gov/gene/3670). It has been reported in the developing striatum. Expressed in striatal precursors (Wang and Liu, 2001).
	ZNF503	Nolz-1; MGC2555; FLJ45745	Zinc-coordinating DNA binding domains	Striatal neurogenesis through the regulation of retinoic acid signaling (Urban et al., 2010). Expressed in differentiating progenitors of striatal projection neurons (Chang et al., 2004).
Group 3	**Dlx2**	Tes-1; Tes1; Dlx-2	Helix-turn-helix/Homeo domain	Forebrain and craniofacial development (http://www.ncbi.nlm.nih.gov/gene/1746). Characteristic protein in the developing striatum and striatal neural stem cells (Willaime-Morawek et al., 2006). Its expression

				defines distinct stages of basal forebrain differentiation (Eisenstat et al., 1999). Dlx1 and 2 and Ascl1 control striatal patterning and differentiation through parallel and overlapping pathways (Long et al., 2009).
	Dlx5		Helix-turn-helix/Homeo domain	Forebrain and craniofacial development (http://ncbi.nlm.nih.gov/gene/1746). Its expression defines distinct stages of basal forebrain differentiation (Eisenstat et al., 1999).
	Gsx2	Gsh-2; Gsh2	Helix-turn-helix/Homeo domain	Development of GABAergic neurons (Kurokawa et al., 2005). Its function has been reported to be essential for maintaining the molecular identity of early striatal progenitors (Toresson et al., 2000). Expressed in precursor cells, from which, during the embryonic period, many olfactory bulb neurons arise from the lateral ganglionic eminence (Vergano-Vera et al., 2006).
Group 4	**Gli1**	Gli	Zinc-coordinating DNA-binding domains/Cys2 His2 zinc finger domain	Development and it is a known oncogene (Liu et al., 1998), regulating stem cell proliferation. Expressed during mouse embryo development in forebrain, midbrain and cerebellum (Hui, 1994; Walterhouse et al., 1993). Some of the downstream gene targets of human Gli1 include regulators of the cell cycle and apoptosis (Yoon et al., 2002).
	Otx2	MCOPS5; MGC45000	Helix-turn-helix/Homeo domain	Brain and sensory organ development. A similar protein in mice is required for proper forebrain development (http://www.ncbi.nlm.nih.gov/gene/5015). Expressed in the developing mammalian brain (Rath et al., 2006).
	FoxG1	BF1; BF2; QIN; FKH2; HBF2; HFK1, among others	Helix-turn-helix/Fork head/winged helix	Regulation of the expression of some genes that are involved in cellular growing, proliferation, differentiation and longevity. Important for embryonic development (Tuteja and Kaestner, 2007). It may play a role in the development of the brain and telencephalon (http://www.ncbi.nlm.nih.gov/gene/2290).
	Gsx1	Gsh-1; Gsh1	Helix-turn-helix/Homeo domain	Brain development, suggested by its expression in several discrete domains of the developing brain, including the diencephalon, which gives rise to the thalamus and hypothalamus. Its transcription is restricted to the CNS (Valerius et al., 1995).

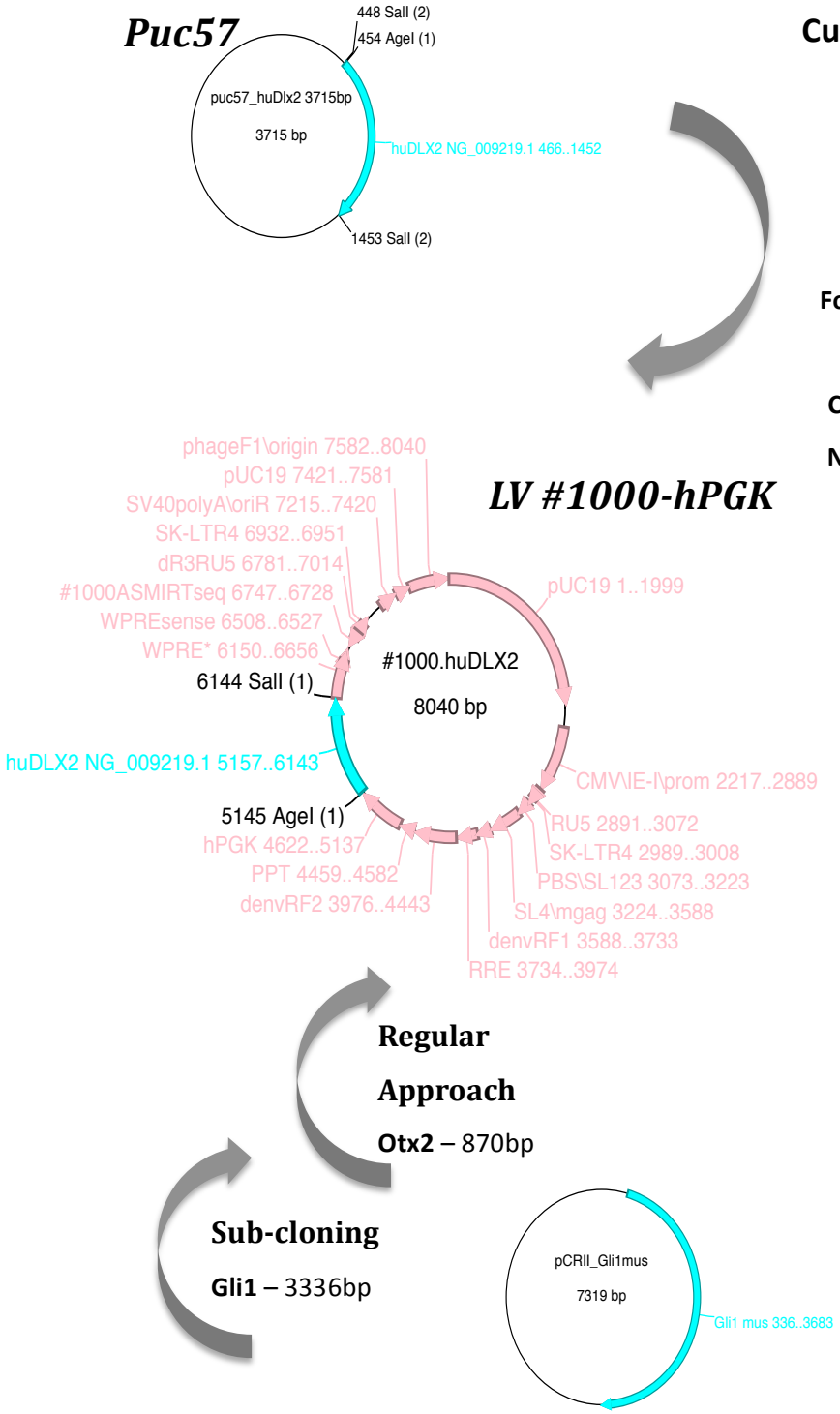

Figure A2 - Schematic representation of the cloning strategy for the 10 striatal Transcription Factors (TFs).

The ten TFs were cloned into the LV #1000-hPGK. The ORFs of eight transcription factors (Dlx2, Dlx5, Gsx1, Gsx2, FoxG1, Isl1, CTIP2 and Nolz1) were cut with the proper restriction enzymes and directly ligated into the LV#1000-hPGK vector plasmid. The Otx2 ORF sequence was amplified by PCR, gelpurified and then ligated into pCRII vector. Chemically competent bacteria were transformed with the cloning plasmid and suitable clones were identified by EcoRI restriction. After identifying the correctness of the insert by sequencing, Otx2 was cut out by SalI/BamH1 restriction and subsequently ligated into the linearized LV #1000-hPGK with

corresponding ends. Gli1 was sub-divided into a larger fragment (Gli1 upper), containing SalI/BamH1 compatible ends and a smaller fragment (Gli1 lower) with two BamH1 compatible ends. Gli1 upper was cloned into the LV #1000-hPGK thereby reducing the number of BamH1 restriction sites and subsequently, Gli1 lower was inserted into the same plasmid, following a sub-cloning strategy.

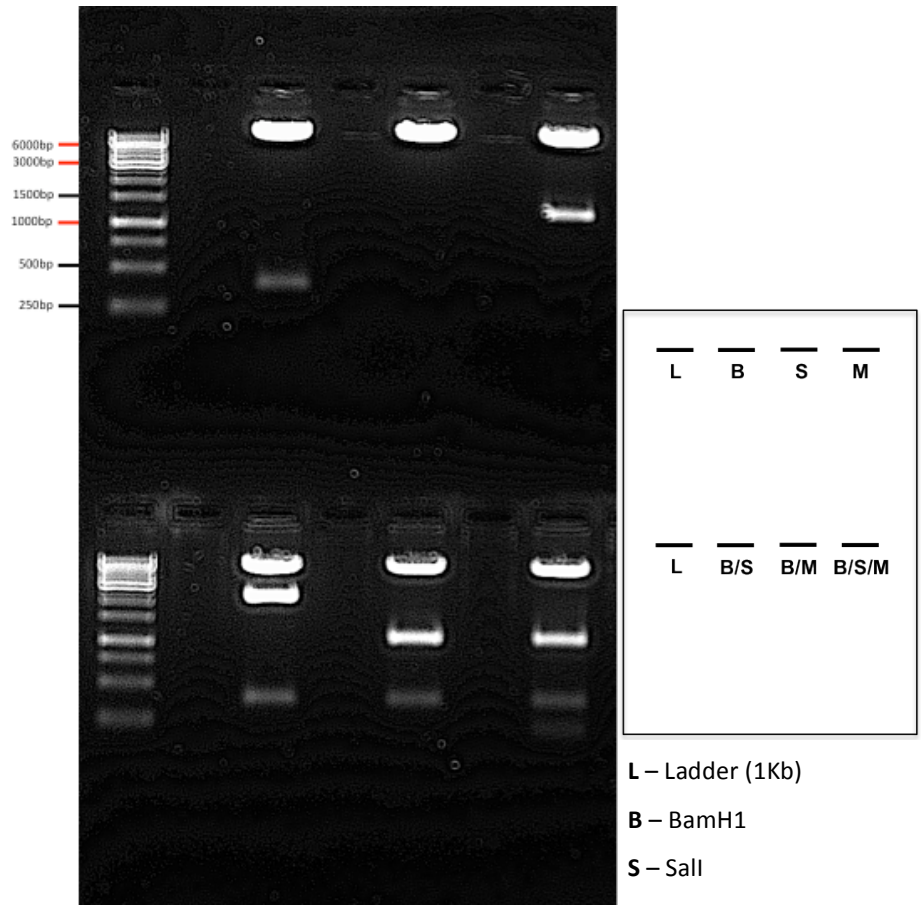

Figure A3 – Gel picture showing the bands resulting from the digestion of the LV#1000-hPGK-CTIP2 construct (9738bp) with the restriction enzymes BamH1, SalI and MluI.

The expected fragments for each digestion were: B (9374bp and 364bp), S (9738bp), M (8631bp and 1107bp), B/S (7035bp, 2339bp and 364bp), B/M (7229bp, 1107bp, 1038bp and 364bp), B/S/M (7035bp, 1107bp, 1038bp, 364bp, 194bp).

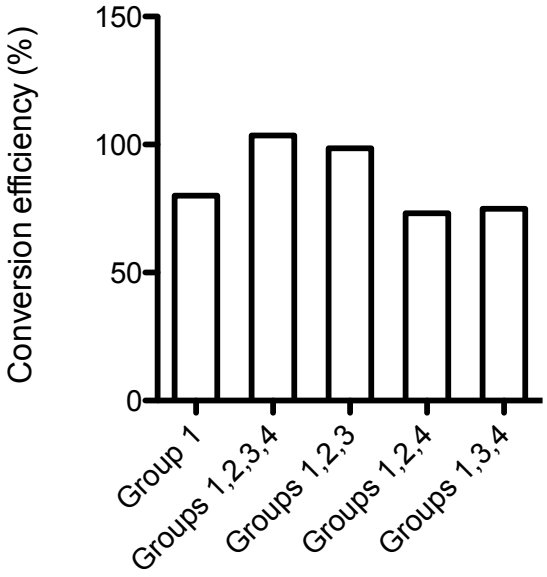

Figure A4 – Conversion efficiencies obtained in the first subtractive screen for striatal hiN cells.

The presented conversion efficiencies were calculated by relating the number of converted neurons with the number of plated cells, in each of the tested conditions (Mean; n=1).

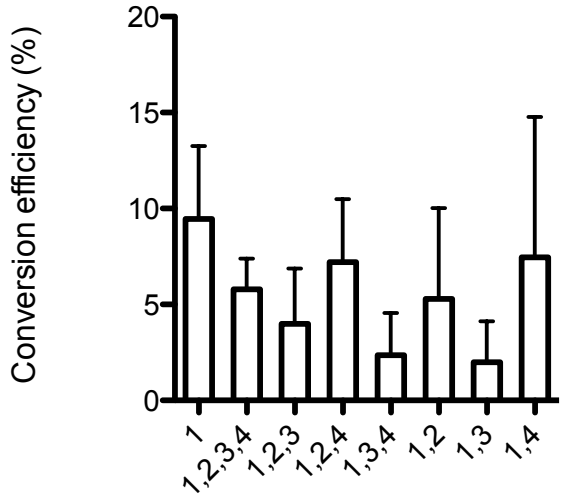

Figure A5 – Conversion efficiencies obtained in the second subtractive screen for striatal hiN cells.

Similarly to the first screening round, also the presented conversion efficiencies relate the number of converted cells with the number of plated cells (Mean±SD; n=2).