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Review

Cell reprogramming and neuronal differentiation applied to neurodegenerative diseases: Focus on Parkinson's disease

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ABSTRACT

Adult cells from patients can be reprogrammed to induced pluripotent stem cells (iPSCs) which successively can be used to obtain specific cells such as neurons. This remarkable breakthrough represents a new way of studying diseases and brought new therapeutic perspectives in the field of regenerative medicine. This is particular true in the neurology field, where few techniques are amenable to study the affected tissue of the patient during illness progression, in addition to the lack of neuroprotective therapies for many diseases.

In this review we discuss the advantages and unresolved issues of cell reprogramming and neuronal differentiation. We reviewed evidence using iPSCs-derived neurons from neurological patients. Focusing on data obtained from Parkinson's disease (PD) patients, we show that iPSC-derived neurons possess morphological and functional characteristics of this disease and build a case for the use of this technology to study PD and other neuropathologies while disease is in progress. These data show the enormous impact that this new technology starts to have on different purposes such as the study and design of future therapies of neurological disease, especially PD.

1. Introduction

- Stem cells. Definition and classification. The notion of pluripotency.
- Pluripotent stem cells: embryonic and induced pluripotent stem cells.
- Embryonic stem cells: origin and applications.
- Induced pluripotent stem cells: origin, current and future applications.

Stem cells have two main characteristics: the ability of self-renewal and the capacity to differentiate into specialized cell types. These two features confer them the possibility to generate differentiated cells that could be used in basic and translational research and cell therapy strategies. Cell potency is defined as the cell's ability to differentiate into other cell types [66].

Stem cells can be divided into three main groups according to their potency: totipotent stem cells which have the capacity to differentiate into all cell types including extra-embryonic cells; pluripotent stem cells which have the potential to differentiate into all somatic cell types and multipotent stem cells, such as adult stem cells, which can only generate specialized cells types of their own specific lineage [29].

Embryonic stem cells (ESCs) are pluripotent cells found in the inner cell mass of the blastocyst, originated by the union of the oocyte with the spermatozoid. These cells originate all cell types of an organism, e.g., more than 200 cell types in humans [46]. ESCs can be cultured and expanded *in vitro* [83]. This possibility has enthused numerous research groups to use ESCs as cellular sources of differentiated cells, the required cellular substrate for regenerative medicine [17,29]. However, fertilized oocytes are the source of human ESCs, which is a matter of ethical concerns in some countries. This issue, the availability of the starting material and possible immunogenicity differences between the ESC-derived cells and the recipients that could potentially lead to immunological rejection, have hampered the successful exploitation of this type of stem cell for the benefit of patients [77].

The way a cell possesses or acquires pluripotency capacity as been a topic of intense research. Physiologically, cytoplasmic factors of the oocyte are capable to modify (or reprogram) the nucleus of a spermatozoid to originate a fertilized egg, which will become a blastocyst, the prime source of ESCs. Experiments of somatic cloning and cell fusion between oocytes and adult cells generated

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pluripotent cells by reprogramming the expression pattern of the adult nucleus, supporting the idea that factors present in the oocyte cytoplasm were able to provide pluripotency properties to adult cells ([20] reviewed in [75,89,45,29]).

In 2006, Takahashi and Yamanaka, based on information provided by more than 40 years of previous research (reviewed in [52]), selected 24 genes as candidates to mediate cell reprogramming to a pluripotent state. In a seminal work that paved the way for the winning of the 2012 Nobel Prize of Medicine and Physiology, they proved that the exogenous expression of four genes (Oct4, Sox2, c-Myc and Klf4) was able to convert fibroblasts into pluripotent stem cells [82]. These pluripotent stem cells originated from adult cells were called induced pluripotent stem cells (iPSCs) and were able to originate all three germ lines (mesoderm, endoderm and ectoderm) and contribute to germ line transmission in the mouse [82,75].

The development of this new method, which allows the production of stem cells with similar pluripotency properties than ESCs, represents the beginning of a new era for basic and translational research with great perspectives in clinical treatment [89].

iPSCs have the potential to provide all specialized cell types of a given organism for regenerative therapies without the previously mentioned shortcomings of ECSs. Moreover, since iPSCs can be obtained from the same patient, immunological issues would be prevented using this strategy [10,59]. Alternatively, since donors can be selected beforehand according to their HLA signature, a country-specific collection of iPSCs, compatible to that country population, can be generated as a source of HLA-compatible cell substrates [85]. For example, it has been calculated that 50 homozygous HLA types selected from 24000 donors could match 90.7% of the Japanese population [48]. Similarly, as few as 10 homozygous cell types, selected from 10000 donors, could provide compatible cell substrates at the HLA-A, HLA-B and HLA-DR levels for transplantation of 37.7% of the UK population [85]. In Argentina, it has been calculated that 33 and 106 selected donors could provide histocompatible cells to 50% or 90% of the population, respectively (C. Gamba, personal communication). Furthermore, a global initiative to enable the interchange of HLA-typed iPSCs worldwide in resemblance of the Bone Marrow Worldwide Registry is underway and Argentina is part of it. We believe that this initiative will have a major impact in the next-generation, iPSCs-based cell therapies to come since it can provide HLA-matched cells worldwide for allogeneic transplantation [72].

Still, many bottlenecks remain for most of these foreseeing therapeutic interventions. In particular, for some specialized cells, cell culture protocols are lacking the needed efficiency and GMP-compatible procedures to be translated to a clinical setting.

Notwithstanding the expected high impact of this technology on future treatments, one immediate application for iPSCs came from the realization that specialized cells originated from patients using reprogramming technology possess characteristics of the disease affecting that patient, in particular if the disease has a genetic component [15]. In other words, by cell reprogramming and differentiation, we can model and study diseases that were not amenable to research before. This holds true especially for neurological diseases of genetic origin where neurons from patients have been shown to mimic pathological features of the diseases.

Translationally, this technique allows identifying potential targets for future therapies in these *in vitro* models of disease. Moreover, iPSCs cells from patients can be generated and differentiated into specialized cells of interest and used as platforms for drug assessment, where screening tests of several compounds will give relevant data about toxicity and therapeutic efficacy of each drug. Thus, this new approach could improve the accuracy of drug development and therefore enhance the probability of successful therapies [89,29].

In this review, we will discuss critical issues for the translation of the iPSC technology to the benefit of neurological patients, i.e., the reprogramming techniques themselves, the advantages and shortcomings of available protocols for neuronal differentiation from iPSCs and its application in neurological disease modeling. We will focus on the evidence obtained from iPSC-derived neurons from PD patients and the validation of this technology to study PD and other neurological disorders.

2. Methods of cellular reprogramming

In order to achieve optimal iPSCs various cellular reprogramming techniques were developed, including the use of viral vectors (Moloney leukemia virus (MLV)-retroviral, lentiviral, adenoviral, Sendai virus-derived vectors, among others), non-integrative plasmids, proteins, miRNA, mRNA, small molecules, etc [44,29,18]. In the present review we will be focusing on two types of reprogramming systems widely used for academic purposes (MLV-retroviral and lentiviral vectors) and two systems, more amenable to be used in a clinical setting (non-integrative plasmids and proteins).

2.1. Retroviral vectors I: MLV-derived

The first reprogramming protocol described used 4 MLV-derived retroviral vectors to express each of the 4 nowadays called Yamanaka reprogramming factors in murine cells. This approach yielded 0.02% of ES-like cells from MEFs after introduction of the four reprogramming factors selected [82,54].

Using this delivery system, the first hiPSCs generation from adult human fibroblasts was achieved in 2007 by S. Yamanaka's group with iPSC colonies appearing between 25 and 30 days post-infection with a reprogramming efficiency of 0.01–0.02% [81,44].

The first years after reprogramming was described, much of the research effort was focused on increasing reprogramming efficiency. Later, it was realized that, even using the less efficient MLV-derived reprogramming protocols, enough iPSCs clones could be obtained for further characterization. In the practice, the selection and characterization of bona fide hiPSCs is cumbersome and time-consuming and therefore, a surplus of iPSCs is usually obtained even when using low efficiency protocols.

The amount of each reprogramming factor expressed determines several features of the iPSCS generated [25,43,24]. This variable is difficult to control using 4 retroviral vectors for each reprogramming gene. The capacity of MLV-derived vectors to accommodate only a limited size of transgenes (aprox. 6 kb) precludes the expression of all 4 genes in one vector backbone [1]. This limitation was overcome by the group of G. Mostoslavsky by developing a system based on another type of retroviral (lentiviral) vectors, which can express the 4 factors simultaneously (STEMCAA).

2.2. Retroviral vectors II: lentiviral vectors

A lentivirus-derived system was designed by G. Mostoslavsky's group to express a single multicistronic transcript which contains all the reprogramming [25,74] factors (see Fig. 1). This new vector named, "stem cell cassette" (STEMCCA), allows iPSCs generation with a minimal numbers of viral integrations which increase the safety of reprograming method and the reproducibility of the amount of expression of each reprogramming factor [74]. This strategy appears to be more efficient and safer, since previous reports using the multiple vectors system have required more than 15 viral integrations to accomplish cellular reprogramming. This

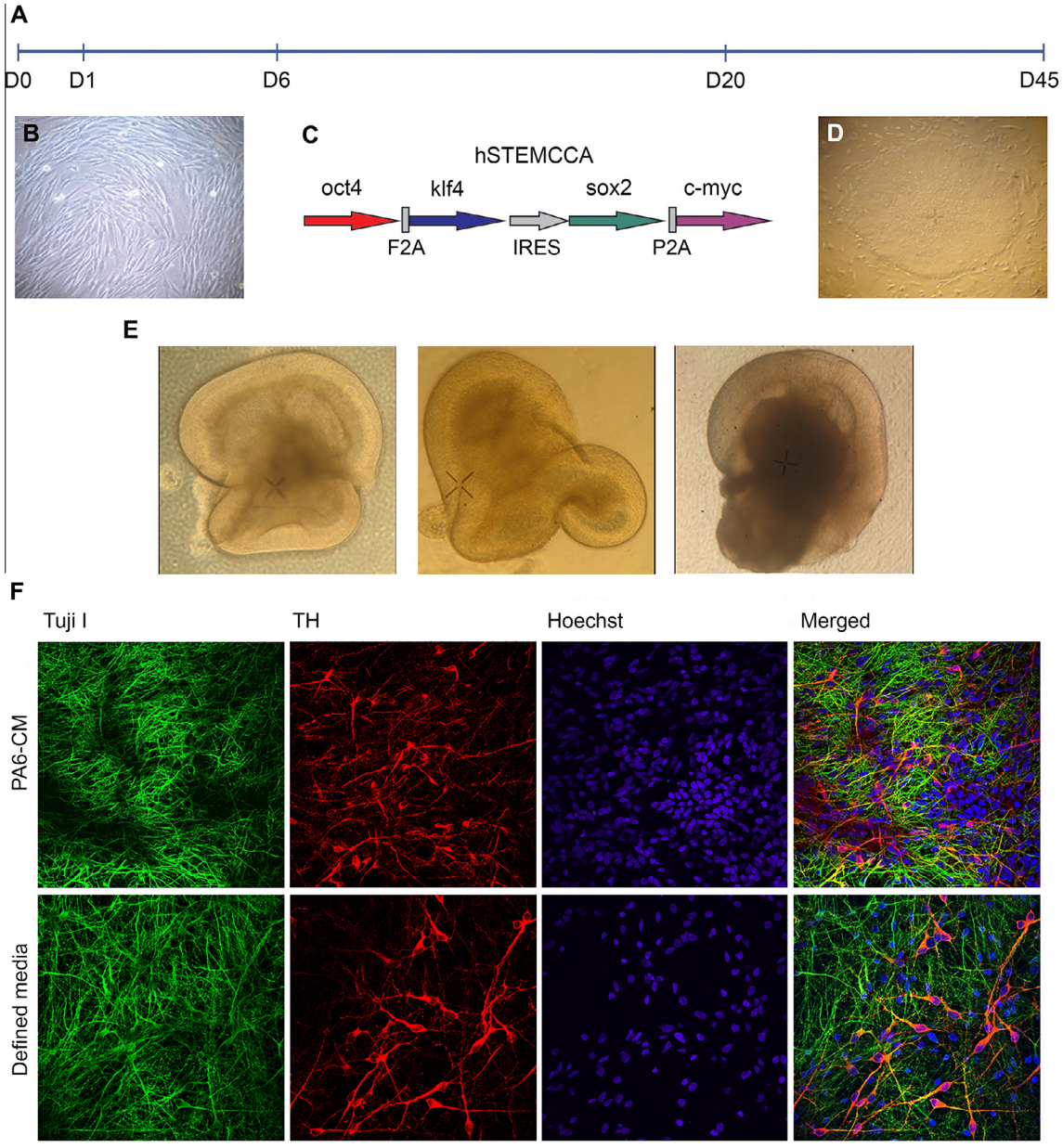

Fig. 1. Illustration of steps and cellular products of the reprogramming and the differentiation process towards neuroepithelium or DA neurons. (A) Time schedule of cell reprogramming. D0, fibroblast seeding, D1 cell transduction with lentiviral vectors, D7, cell passage, D20 to D45, manual selection of iPSC clones according to morphology. (B) Representative photograph of fibroblasts (40×). (C) Scheme of the STEMCAA vector. (D) iPSC colony obtained according to Sommer et al. [74]. (E) Representative images illustrating a neuroepithelium culture obtained according to Lancaster et al. [36]. (F) Representative images illustrating DA differentiation of hNSCs using PA6-CM or defined medium according to [42,55] Neurons and dopaminergic neurons are seen by TUJ-1 and TH immunofluorescence analysis, respectively. Hoechst staining indicates the nucleus. Magnification: 40×.

strategy allowed us and other groups to obtain higher levels of reprogramming efficacy [74] and nowadays the STEMCCA vector is widely used with reprogramming efficiencies of 0.1–1.5% [44,29] (see Fig. 1 for illustrative purposes).

Another advantage of the system is based on the fact that lentiviruses allow the transduction of both dividing and non-dividing cells, being therefore a more suitable system for reprogramming of quiescent cells [74].

Nevertheless, this system has several disadvantages inherent to all retroviral-based methods. The risk of insertional mutagenesis is a major concern when iPSC-derived cells are being considering for clinical applications, providing increased difficulties at the regulatory level. In order to increase the safety of the iPSC obtained, a STEMCCA version was engineered with LoxP sites. Using this vector, iPSCs free of reprogramming factors were obtained by transient transduction with a defective adenovirus or a episomal plasmid which expresses Cre-recombinase. This strategy does not solve the regulatory problem entirely, since it leaves a bit more than 200 bp of the original STEMCAA sequence in the host genome [73,2,44].

2.3. Plasmid constructs

Recently, seeking to establish an approach more prone to clinical translation, several research groups have sought new reprogramming methods by transferring non-integrative constructs such as expression plasmids containing the 4 reprogramming genes.

In 2008, Okita and colleges were the first to reprogram mouse embryonic fibroblasts (MEFs) with repeated transient transfections of two plasmids, one of them containing Oct3/4, Sox2, and Klf4 cDNAs and the second one expressing c-Myc. Expression of endogenous pluripotent genes and teratoma development analysis of the iPSCs obtained with this method suggested that non-integrating vectors can also successfully induce cellular reprogramming [53,76,44]. This represents an advantage since the possibility of genomic integration is lower than other methods. Moreover, transient transfections are technically less complicated and safer than viral vector manipulation.

However, reprogramming efficiencies are lower (0.001–0.0001%) than the ones obtained by integrating vectors (0.1–1%). This low efficiency could be partially caused by insufficient time of expression of the reprogramming factors, which is needed to complete the epigenetic modifications instrumental for cellular reprogramming. Even with the implementation of several rounds of transfection, the reprogramming efficiencies values remained low.

A year later, Kaji and colleagues have reprogrammed murine and human fibroblasts using a Cre/loxP-based system. The construction was a single plasmid which contained a cassette with the four reprogramming factors and 2A peptides, flanked with loxP sequence. This strategy allows transgenes excision after cellular reprograming through Cre transfection. Analysis for endogenous pluripotency markers demonstrated that the loss of exogenous reprogramming factors did not affected the reprogrammed state of iPSCs cells [30,54]. In fact, it has been shown that after pluripotency is achieved by the expression of the exogenous reprogramming factors, the host cells shuts down the exogenous expression and induces the endogenous expression of the reprogrammed genes [75]. This endogenous expression is crucial to maintain long-term pluripotency in culture of the iPSCs generated and allows the correct, unbiased differentiation of these cells towards the desired phenotype.

Alternatively, other systems were designed, such as the episomal vector oriP/EBNA1 (Epstein Barr nuclear antigen-1). This construction, derived from Epstein Barr virus, expresses the four reprogramming factors, the transgene Lin28 and SV40 large T antigen [50]. However, this new alternative had low reprogramming efficiency in human fibroblasts. Nevertheless, this system allows the generation of iPSCs from peripheral blood or CD34 positive cells from cord blood, with efficiencies of 0.02 and 0.009% respectively on day 14 post-transfection, suggesting that this technique could be useful for cell reprogramming of these specific cells [44,50].

For clinical applications, safer methods are required to minimizing alterations of iPSCs genome and possibilities of tumor formation. The last described, non-viral techniques are interesting options to achieve this goal.

2.4. Protein cell reprogramming

Another approach is based on cellular reprogramming with proteins as reprogramming factors. As a proof-of-principle, MEF cells were reprogrammed using a fusion protein which contained the four reprogramming factors with a poly-arginine protein transduction domain [92].

A new alternative based on recombinant proteins from an *Escherichia coli* expression system was used on murine and human fibroblasts. However, this method had showed low reprogramming efficacy [44]. An important factor is related to the protein expression system selected for reprogramming factors synthesis. Mammalian expression systems are capable of producing correct protein folding, assembly and post translational modification. Kim and colleagues generated iPSCs using recombinant reprogramming factors obtained from a stable HEK293 human cell line. However, it required several rounds of transduction and 8 weeks to achieve cellular reprogramming [32,39].

Other system, based on cell-permeable reprogramming proteins, was used on human dermal fibroblasts. Stem cell-like colonies which resembled embryonic stem cells features were generated. However, they failed to expand as iPSC or ESC lines [41].

Disadvantages such as low reprogramming efficiency together with technical issues related to the system of protein expression selected, large-scale production, protein purification and characterization suggest that more studies are needed in order to analyze the potential application of protein-based reprogramming approaches.

In relation with cell differentiation and reprogramming methods, Rhee and colleagues analyzed cell death and senescence in neural precursors (NPs) derived from iPSCs generated by different methods of cellular reprogramming. Interestingly, they observed that NP cells from iPSCs obtained by cell reprogramming based on direct delivery of poly-arginine-tagged reprogramming factors were expandable for several passages without detection of senescence. In contrast, residual expression of exogenous reprogramming genes was detected in NP cells derived from iPSCs generated by retroviral or lentiviral cell reprograming methods. They also observed cell death and senescence in NP cells at low passages. However, tumor formation was observed in one condition of cell transplantation of mature dopaminergic neurons derived from iPSCs generated by poly-protein-cell reprogramming technique. This was related to a high number of undifferentiated cells detected into the graft [61].

As a cautionary note, independently of the reprogramming method use, it is important to notice that bona fide iPSCs need to pass several quality control tests in order to be defined as such. The burden impose to the dynamics of the cell genome, inevitable creates unstable cells that need to be discarded from downstream applications. These controls include tests for transgene silencing and integration, stemness, pluripotency, normal and stable karyotype, viability after freezing and thawing, maintenance of stemness and genomic stability after long-term culture, among others. In addition, epigenetic memory, i.e., the remaining epigenetic signature from the donor cell could be problematic, especially if iPSCs are used at early passages [4,19]. In addition, not all iPSCs colonies are fully reprogrammed and some can still express the exogenous transgenes after several passages. The constant expression of the reprogramming genes that gave pluripotency to the cells in the first time could in theory result in a problematic issue when iPSCs are driven towards the differentiation of a specific cell phenotype. Therefore, several issues need to be considered to finally decide if the iPSCs obtained are amenable for downstream applications.

In summary, an ideal reprogramming method should provide: fully reprogrammed iPSCs, medium to high reprogramming efficiency, iPSCs generated not longer than 1 month after gene delivery, a vector/expression system cheap, easy to produce and with straight forward quality control tests available, reliable expression of the 4 Yamanaka factors whose expression should shut down after reprogramming is achieved. In addition, the method should be clinically compatible (integration-free) if required (see Table 1).

In reality, a scenario starts to appear where, as it was for the case of gene therapy, a particular reprogramming method would be ideal for a given cell and not for another. In addition, the final use of the iPSC determines the reprogramming method of choice since the easiest, more efficient and more reliable reprogramming methods (retrovirus-derived vectors) are the approaches of choice for academic purposes but not for clinical applications due to potential risks represented by the usage of genome-integrating viruses. In other words, it takes two to dance the desired tango

Table 1
Features of an ideal reprogramming method.

The ideal reprogramming method should:
✓ Generate fully reprogrammed iPSCs
✓ Allow medium to high reprogramming efficiency
✓ Achieve fast reprogramming of cells (less than 1 month after gene delivery)
✓ Include a vector/expression system:
o Easy to produce
o Cheap
o With straight-forward quality control tests available
o Able to express reproducible amounts of the 4 Yamanaka factors in all experiments
o Allowing the shutdown of the reprogramming genes after reprogramming is achieved
✓ Be clinically compatible (integration-free) if required

(clinical or academic purposes): the gene delivery method and the cell used as starting material.

Once the challenge of generating a bona fide iPSC is met, an even more complicated task is awaiting: the differentiation to the desired cell type.

3. iPSCs and cell differentiation

As mentioned before, ESCs and iPSCs can potentially generate the more than 200 different, human cell types. During development, several factors such as transcription factors, growth factors, and the extracellular environment direct specific genetic programs to originate a particular cell type. The task of mimicking such conditions in the lab is formidable monumental. Due to the plethora of data coming from developmental biology, embryology and other areas of research studying the phenomenon of cell differentiation and the studies conducted with hESCs, it is now sometimes possible to drive the iPSC to a pathway that ends in a desired cell type. However, not all cell types can be obtained nowadays from iPSCs or ESCs. For example, functional hepatocytes are still not available from these cells [65].

We will focus our analysis on neuronal differentiation since it allows us to discuss both established protocols and procedures that have room for improvement. For example, nowadays, it is possible to generate neuroepithelium with high (>90%) efficiency. At the same time, specific neuronal populations, such as dopaminergic (DA) neurons (DAn), are harder to obtain. Therefore, neuronal differentiation allows us to discuss both established protocols and procedures that have room for improvement.

3.1. iPSCs-derived neurons

IPCs-derived neurons represent a useful tool that could provide a first understanding for physiological and pathological processes of the central nervous system. To achieve this complex goal obtain neurons from iPSCs, several research groups had developed differentiation protocols to obtain diverse neurons phenotypes by manipulation of culture conditions, such as type of coating and specific combinations of differentiation factors. In this case, the efficiency to obtain the desired neuronal phenotype is crucial in order to perform downstream experiments: i.e., preparations with low number of the required neurons will serve for imaging analysis or single cell studies, whereas cultures with over 90% of homogeneity could be utilized for robust molecular biology techniques.

3.1.1. Neuroepithelial culture

It is nowadays feasible to obtain a neuroepithelial culture with consisting of over more than 90% of neurons [13,36,47] and own unpublished data). Most of these neurons are glutamatergic and GABAergic and less than 10% of dopaminergic and serotonergic neurons are also routinely present ([36] and own unpublished data)). In addition to culturing these cells in a monolayer, it is possible to generate them in suspension, in a 3D fashion. This culture system allows studying developmental processes [13,36,47]. Moreover, from human iPSCs, Lancaster and colleagues were able to generate cerebral organoids, a three dimensional structure that contains areas which resemble specific independent brain regions such as cerebral cortex ([36] and Fig. 1)). Using this model, neuronal differentiation analysis from patients with microcephaly could be performed [36]. The facts that these regions contain neuronal progenitors which can reach a mature state allow the opportunity for example to study cell–cell interactions, time course of cell differentiation in both normal and pathological conditions. It is foreseen that this technical development will facilitate the comprehension of the processes involved in various brain diseases by simplifying the variables associated with the animal models traditionally used.

3.1.2. Specific neuronal differentiation

As stated above, specific neuronal phenotypes can be obtained with less efficiency. Both types of cortical neurons, glutamatergic and GABAergic cortical neurons were successfully derived from human iPSCs. A preparation was obtained with 70–75% of glutamatergic and 10–20% of GABAergic neurons, respectively [5,58,86]. This is an attractive approach for *in vitro* studies of physiological and pathological mechanisms involving the cerebral cortex cortical neurons [70,5,58,86].

Shimada and colleagues reported differentiation to serotoninergic neurons from PSC with 80% of efficiency. The study and characterization of this neuronal type could be useful to understand the development of psychiatric conditions, such as obsessive–compulsive disorder, depression and impulsivity [71,58].

Motor neurons are the other type of neuron derived from iPSCs which could represent a useful *in vitro* model for understanding physiological process involved in motor system. Additionally, a disease-modeling-approach could be used for development of effective therapies. Furthermore, it has opened opportunities to cell therapy strategies for motor neuron disease [23,58].

3.1.2.1. Methods of dopaminergic differentiation. Dopaminergic differentiation from PSCs, resembling bona fide A9 neurons, is instrumental to study Parkinson's disease and for the development of therapies against this pathology. Dopaminergic differentiation from PSCs was has already been achieved by several research groups [90,16,35,61,8,42,58].

One strategy for dopaminergic differentiation involves the generation of embryoid bodies (EBs) followed by selection and expansion of nestin-positive cells and treatment with sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8). This is a complex multiple-step method which could generate up to 30% of tyrosine hydroxylase (TH) (the limiting enzyme in dopamine synthesis)-positive cells [37].

Cho and colleagues have obtained mature and functional DAn from homogeneous spherical neural masses (SNMs) derived from hESCs with an efficiency of 86%. The authors stated that DA differentiation protocol based on SNMs usages have various advantages such as: SNMs can be expandable for several passages and DA differentiation can be achieved in 14 days. Also the protocol developed did not use any type of genetic modification or co-culture with feeder cells. This represents a safer method for cell transplantation. However, histological analysis of grafts showed that after 12 weeks post transplantation, only 2.7% of the surviving cells were TH positive cells from which a small population were positive for DAT (dopamine active transporter) suggesting that the maturation state of the majority of cells present in the graft was incomplete at the time selected for this study [16].

Other DA-differentiation protocols are based in co-culture systems with meningeal cells; glial cells or stromal cells [31,6,22] (see Fig. 1 for illustration). Co-culture with stromal cell lines such as PA6 or MS5 cells, which have a stromal-derived inducing activity (SDIA) is a simpler and faster method than the EBs strategy [31]. Using this protocol dopaminergic differentiation from PSCs was successfully reported with efficiencies between 54% and 87% [31,90,91,87] (see Fig. 1 for illustration).

In order to elucidate the molecular mechanism of DA induction, various research groups tried to establish which factors were involved in DA differentiation. For example, GDNF, a survival factor for dopamine neurons was able to duplicate the efficacy of DA-differentiation using the PA6 co-culture system in hESCs [6]. It was also observed that cell-surface components and soluble factors had DA activity [31]. It has been shown that generation of DAn from hESCs could be obtained using PA6-conditioned medium (CM) with fewer efficiency than PA6 co-cultures methods [87]. Interestingly, the addition of heparin to PA6-CM increased the percentage of dopaminergic neurons generated, suggesting that the biological activity of the soluble factors required for survival and dopaminergic differentiation of ESCs could be stabilized and/or activated by heparin addition [22,87]. Related to this fact, it was demonstrated that while PA6-CM is not able to induce dopaminergic differentiation in ESCs with high efficiency, it could differentiated neural stem cells (NSCs) to the DA phenotype with a 25% of efficiency [78]. This observation suggests that PA6-CM contains the necessary factors for dopaminergic induction from NSCs, while the cell survival factors required for ESCs could be active only with heparin addition. Based on results obtained from microarray analysis from the PA6 line and NSCs, dopaminergic differentiation from NSCs was achieved with a protocol based on a treatment with Shh/FGF8 factors and subsequently, with BDNF and GDNF-supplemented medium [78]. Optimization of the PA6-CM differentiation method includes addition of BDNF and GDNF which favors cell survival and dopaminergic differentiation [80]. In addition, it was found that Amiodarone can selectively kill ESCs but spare DA precursors, increasing DA differentiation efficiency [21].

While these works suggested that PA-6 co-culture or PA6-CM are simple and fast strategies, they are not totally compatible with the development of good manufacturing practices (GMPs) protocols, which are mandatory for the generation of clinical-grade DA neurons. Related to that issue, X. Zeng's research group was capable to obtain functional dopaminergic neurons under defined media, a protocol compatible with a GMP-compliant process [79].

Reports from Kirks and colleagues suggested that Lmx1a and FoxA2 expression is an important factor for the generation of dopaminergic neurons with A9/A10 phenotype [35]. Based on this fact, Liu and colleagues optimized cell culture conditions in order to increase the percentage of DA cells expressing Lmx1A and FoxA2. This strategy allowed DA differentiation in a shorten period of time with less variability [42]. Using this protocol and also the PA6-CM method, we were able to obtain TH/TuJI positive cells from hNSCs (Fig. 1).

Two key, mostly neglected issues need to be solve in order to move forward to a clinical setting: the viability after thawing and the definition of the degree of maturity of the dopaminergic precursor to be transplanted.

The transplantation of fully mature DAn into the brain has resulted in very poor survival [3]. This fact is supposed to originate from the damage to fully differentiated neurons with relatively long processes when they are harvested from the culture dish and the inability or extreme difficulty of mature neurons to integrate into the host parenchyma. Therefore, there is a consensus that DA precursors should be transplanted and allow their final maturation step *in vivo*. However, few studies have addressed this issue. Peng and colleagues have determined that DA precursors harvested 14 days after the beginning of the differentiation protocol can mature, survive and ameliorate motor symptoms in a rat model of Parkinson's disease [56].

In a fundamental study, frozen and cryopreserved dopaminergic precursors were tested in cell transplants on a 6-OHDA animal model of Parkinson's disease. The results obtained showed similar motor behavior recovery for both types of cellular preparations using a specific protocol. Moreover, it was observed that frozen-DA precursors were able to differentiate to a mature state equally to fresh preparations of DA precursors [42,56]. Therefore, these publications indicate that it is feasible to prepare DA precursors preparations at a differentiation step that allowed survival and amenable to freezing and thawing. This last step is crucial since, most of the cases, it is envisaged that the place of DA precursors production could be at a long distance from the site of cell transplantation into the patient.

4. Cellular reprogramming based models of human neurological disorders

iPSCs-derived neurons represent a potential tool for disease modeling, and identification of genes as therapeutic targets. Likewise, this technology opens a new perspective for drug discovery, since it allows the possibility to generate iPSCs from patients which could serve as a specific disease model for compounds screening.

Various neurological disease models have already been developed using iPSC technology. Research works for iPSCs generation and neuronal differentiation were reported from Alzheimer's disease (AD), Rett syndrome, Huntington's disease, spinal muscular atrophy, amyotrophic lateral sclerosis, schizophrenia, Down's syndrome, Parkinson's disease, etc. [14]. The model of AD includes iPSCs differentiation not only towards neuronal phenotypes such as cortical and cholinergic neurons and neural precursor cells but also astrocytes [28,34,88,26].

Also, a consortium for Huntington disease (HD) has developed 14 iPS cell lines from HD patients in order to obtain neuronal cells and characterize cell vulnerability and alterations in metabolic and electrophysiological features that could be related to HD [9].

In addition, Li and colleagues had generated 22 iPSCs lines from patients with familial amyotrophic lateral sclerosis (fALS), which includes several types of mutations being therefore a useful model for analyze molecular pathways involved in fALS development [40].

More than 10 publications are available describing the functional and morphological characteristics of iPSCs-derived DA neurons from PD patients. This prolific activity allows an in-depth comparison of the results obtained which are relevant to drug discovery strategies for this disease. To obtain a better understanding of the implications of those results, a brief introduction to PD is provided.

4.1. Parkinson's disease and in vitro models using iPSC-derived DA neurons

PD is a neurodegenerative disorder that affects more than 1% of people over the age of 60. The principal feature of this disease is the progressive loss of DAn of the nigrostriatal system, causing the motor symptoms observed in these patients [33,49]. At the moment, approved treatments include pharmacological replacement of dopamine and electrical inhibition of specific areas such as the sub-thalamic nucleus. Oral intake of DA precursors or agonists can control motor symptoms in PD patients for several years, but thereafter many motor and non-motor complications arise. In addition, there is no treatment that protects DAn from cell death and halt disease progression. Thus, there is an urgent need to develop new drugs and novel therapeutic strategies that prevent the progressive loss of DAn [38,51,57].

An example of the use of iPSC for drug screening was developed by X. Zeng's research group. In order to generate a platform of DAn for drug screening, hNSCs were obtained from hESCs and hiPSCs. To validate this system, a set of several candidate neuroprotective agents were tested in a model of cell neurotoxicity by MPP+, a specific neurotoxin for DAn. The results of this study showed that from a total of 44 compounds tested only a third were neuroprotective. Interestingly, molecules which were reported previously as cytoprotective *in vitro* but failed in clinical trials, did not have neuroprotective action in this model, suggesting that DA neurons derived from PSCs may represent an accurate system for drug testing [55].

iPSCs-derived dopaminergic neurons from patients with sporadic and genetic forms of PD were generated in order to study molecular and cellular alterations that could lead to the development of this neuropathology [91,7,49,68,10,27,63,60,62,64,67,69].

Vulnerability and/or an increased response to oxidative stress, alpha-synuclein accumulation, alterations in autophagy mechanism, changes in mitochondrial morphology and function are all previously known features of DAn pathology. These features were studied in the iPSC-derived models. A summary of this effort is summarized in Table 2. Susceptibility to oxidative stress and/or an increased response to oxidative stress was proven in 5 of the studies (4, 6, 8, 10 and 11); alpha-synuclein accumulation was observed in 7 studies (1, 2, 4, 5, 8, 9 and 10); changes in autophagy or lysosome function was detected in 2 publications (1 and 2); alterations in mitochondrial function, in 5 manuscripts (3, 6, 7, 8, and 9) and morphological alterations in 2 (2 and 5).

The studies from iPSCs-derived neurons from PD patients can be divided according to the form of PD studied (sporadic or genetically-related to genes such as alpha-synuclein, LRRK-1, PARK-2 or PINK-1), where they focused on a spontaneous or treatment-triggered phenotype and there was an attempt to test a therapeutic intervention on these cells (see Table 2). Some interesting features emerge from individual as well as by comparing several studies.

Investigating iPSC-derived DA neurons from idiopathic PD patients, Sanchez-Danes and colleagues discovered that a spontaneous phenotype was not observable at 30 days of culture [63]. However, if cells were left to grow for 75 days, spontaneous alterations typical of PD such as increased apoptosis and accumulation of alpha-synuclein emerged [63]. It is remarkable that time in culture can favor the occurrence of pathological landmarks of PD while aging is the highest risk factor for PD. This concordance may indicate that aging in culture can be used to study a chronic process in the cells that leads to morphological and functional alterations.

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene is the most prevalent in the genetically-linked PD as well as in sporadic forms [84]. However, its function is not fully understood. DAn from iPSCs of patients with mutations in the LRRK2 gene (LRRK2mut) showed an increment in mitochondrial DNA damage together with mitochondrial dysfunction, and cellular vulnerability. Also, genes involved in oxidative stress pathways were upregulated in DAn carrying the LRRK2-G2019S mutation [49,64].

Mutations in gene coding for the ubiquitin ligase Parkin (PARK2) are linked to familial Parkinson's disease. One of the main functions of Parkin is to maintain mitochondrial function and integrity. First, a decrease in the efficacy of dopaminergic differentiation between NSCs of PD patients carrying the mutations and control subjects was observed. Then, alterations in mitochondrial volume fraction and molecular pathways related with mitophagy (degradation of mitochondria by autophagy) were also detected in DAn derived from iPSCs-PARK2mut, which is consistent with the biological function of PARK2 [69].

Other iPSC-derived PD model is based on PINK1 (PTEN-induced putative kinase 1) gene mutations which are also related to PD development. PINK1 is a mitochondrial protein which function is related to cellular protection against stressors agents that could cause mitochondrial damage. DA neurons were generated from iPSCs from patients carrying the *PINK1*-Q456X mutation (iPSCs-PINK1mut). Cellular vulnerability to different stressors agents were increased in DAn derived from iPSCs-PINK1mut. This was accompanied by increased levels of mitochondrial reactive oxygen species and alterations in mitochondrial functions. Moreover, pharmacological treatment with antioxidant compounds prevented cellular susceptibility to stressors agents [11].

Taken all these results together, these models using different reprogramming and differentiation methods are validated by these common findings. Once validated, they serve as a cellular experimental set-up to address new questions on PD pathophysiology, target discovery and drug screening. This approach is certainly providing a new way to understand this and other diseases that adds the traditional post-mortem analysis of brain samples or disease animal models with the advantage of a study conducted while the disease is progressing in the patient, providing a window of opportunity for, sooner or later, therapeutic interventions.

5. Conclusions and perspectives

Considering that S. Yamanaka's seminal work on murine cell reprogramming was published in 2006, the Nobel Prize for Medicine or Physiology of 2012 to his work was one of the fastest to be conceded. Certainly, the other Nobel award winner of that year, Dr. J. Gurdon, conducted an instrumental contribution to cell reprogramming more than 40 years ago, highlighting the importance of previous basic research efforts in the field. We believe that the initial data already generated with this technology has proven that the Nobel Prize committee was right in honouring these scientists for their discoveries.

First, cell reprogramming has been reproduced by several groups worldwide using a variety of methods. As discussed before, we believe that the field will come to a conclusion similar to the gene therapy field: there will be an ideal reprogramming (gene transfer) method for a given cell, but not for all cells.

Second, iPSCs have arisen much hope concerning future iPSC-based cell therapies. Indeed, a first clinical trial for macular degeneration using iPSC-derived retinal pigmented cells has started in Japan, showing the developmental speed of the field [12]. Nevertheless, clinical translation is inevitably long and requires a tight regulation. In order to preserve the patient's safety and hopes, each step of clinical protocol must be deeply discussed. Thereby, each step achieved would have a high chance to provide valuable evidence, without the risk of producing a negative impact on the field. Again, lessons could be learned from the gene therapy field where adverse effects or deviations from the approved protocol in three clinical trials out of several thousands, have put a cone of shadow in that field for several years. In particular, the stem cell therapy field is already primed with offers of unproven, ineffective and potentially dangerous stem cell treatments, raising the hope of patients and the hype of media. In this context, a critical discussion of future steps is mandatory so that clinical application of iPSC-derived treatments reaches a safe harbour.

Third, notwithstanding the future impact of this technology on cell therapy, a first, clear and potent effect on neurological diseases arises from the application of this technology to model diseases in a dish. The ability to have neurons available in the laboratories from neurological patients was unrealistic ten years ago. Nowadays, the generation of neuroepithelium from iPSCs is relatively simple and straightforward. Obtaining the desired neuron

Table 2
PD models using iPSC-derived DA neurons.

Disease	Source	Cell type	Cell characterization	Spontaneous disease phenotype	Disease phenotype after treatment	Treatment tested	Refs.
Parkinson's disease (PD)	GBA mutation PD iPSC Non-PD control	DAn	TH+; Foxa2+; NURR1+; GIRK2+ and VMAT2+ cells	Increased levels of glucosylceramide and a-synuclein. Alterations of autophagic and lysosomal mechanism. Dysregulation of calcium homeostasis. Cell vulnerability	–	Genetic repair of the GBA mutation reduced patological features	[1]
	LRRK2 G2019S mutation PD iPSC Idiopatic PD Non-PD control	DAn	bIII-tubulin+ and TH+ cells	Morphological alterations, anormal accumulation of α-synuclein and disfuntional autophagic clearance mechanism in PD-iPSC-derived DAn	–	–	[2]
	LRRK2 G2019S mutation PD iPSC LRRK2 R1441C mutation PD iPSC Non-PD control	Neuroprogenitor cells and neural cells	Synapsin+; alpha-synuclein+; GABA+; TH+ and VMAT2+ cells	Mitochondrial DNA lesions	–	Genetic repair of the LRRK2 G2019S mutation reduced mtDNA damage	[3]
	LRRK2 G2019S mutation PD iPSC Non-PD control	DAn	bIII-tubulin+; TH+; Foxa-2; Pitx3; and Nestin+ cells	Higher expression of oxidative stress response genes and α-synuclein in DAn derived from LRRK2 G2019S iPSCs	Increased susceptibility to H_2O_2 or 6-OHDA treatments in DAn derived from iPSCs of LRRK2 G2019S	–	[4]
	LRRK2 G2019S mutation PD iPSC Non-PD control	DAn	MAP2+;TH+ and Foxa-2	Alterations in neurite outgrowth. High level expression of α-synuclein, TAU, MAPT and pERK	Increased cell vulnerability to 6-OHDA or rotenone treatments in DAn derived from iPSCs of LRRK2 G2019S	ERK inhibition lead to cytoprotection of DAn derived from iPSCs of LRRK2 G2019S and neurite outgrowth	[5]
	PINK1 Q456X mutation PD iPSC LRRK2 G2019S mutation PD iPSC LRRK2 R1441C mutation PD iPSC Non-PD control	Neural cells including DA neurons	bIII-tubulin+; TH+ and Foxa-2 cells	Differential vulnerability of human neural cells, neurons and DAn carrying PD associated mutations Mitochondrial alterations in neural cells derived from iPSCs of LRRK2 mutated form	Vulnerability to cellular oxidative stress in neural cells derived from iPSCs of PINK1 mutated form. Cellular stressors used: valinomycin; MPP+; 6OHDA; concanamycin A; H_2O_2; MG132; CCCP	Coenzyme Q10, rapamycin or the LRRK2 kinase inhibitor GW5074 showed protective effect	[6]
	PARK2 mutation PD iPSC Non-PD control	DAn	bIII-tubulin+; TH+ and Foxa-2 cells	Decrease of TH+ cells numbers in iPSCs with PARK2 mutation. Mitochondrial alterations	–	–	[7]
	PARK2 deletion PD iPSC Non-PD control	DAn	bIII-tubulin+ and TH+ cells	Increased ROS levels. Mitochondrial morphological alterations. Accumulation of α-synuclein	–	–	[8]
	PINK1 Q456X mutation PD iPSC PINK1 V170G mutation PD iPSC Non-PD control	DAn	bIII-tubulin+ and TH+ cells	Alterations in mitochondrial biogenesis and increases in mitochondrial copy number	mtDNA alterations after loss of mitochondrial membrane potential induced by valinomycin	–	[9]
	A53T α-synuclein mutation PD iPSC Non-PD control	DAn	bIII-tubulin+; TH+; Girk2+ and Lmx1a+ cells	Accumulation of oligomerized α-synuclein and Lewy neurite/body-like deposition	Accumulation of ROS/RNS to treatment with mitochondrial toxins (H_2O_2; MPP+;paraquat; rotenone; manganese ethylenebisdithiocarbamate)	Inhibition of NOS protects DAn derived from A53T α-synuclein iPSC from cell death. Screening of several compounds	[10]
	SNCA triplication (Trpl-HDF) PD iPSC Non-PD control	DAn	bIII-tubulin+; TH+; Girk2+; Foxa-2 and Lmx1a+ cells	Accumulation of α-synuclein, increase of oxidative stress markers	Cell vulnerability to oxidative stress induced by H_2O_2	–	[11]

1, [67]; 2, [63]; 3, [64]; 4, [49]; 5, [60]; 6, [10]; 7, [69]; 8, [27]; 9, [68]; 10, [62]; 11, [7].

with an efficiency required for most studies is still cumbersome in some cases. Levelling this up to the production of clinical grade cells requires specific expertise that is usually not in the comfort zone of basic research scientists and therefore this crucial step calls for a team effort. Nevertheless, due to the plethora of data obtained in the last decades, several differentiation protocols have already been established and valuable data have been obtained for many diseases. Especially the studies on PD, have provided irrefutable evidence that this technology can be indeed used for disease modeling.

In conclusion, cell reprogramming came here to stay and move forward the neurology field as no other time in the last century. Hopefully, the coordinated action of scientists, regulators, physicians, legislators and bioethicists will provide a clear pathway to translate the enormous potentiality of this technology into real, clinically-relevant results in terms of cell therapy, identification of therapeutic targets or drug discovery for neurological diseases.

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