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TRANSLATING STEM CELL RESEARCH TO THE CLINIC: A CONSTANT CROSSTALK BETWEEN BASIC AND APPLIED RESEARCH

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Abstract

Pluripotent stem cells hold great promise for the generation of patient-specific cells for disease modeling and regenerative medicine. Focusing on a recent study reporting the successful generation of midbrain dopaminergic neurons and their efficient grafting in animal models of Parkinson's disease, I discuss how crosstalk between basic and applied stem cell research more generally paves the road toward clinical translation.

Keywords

• Cell replacement therapy • Disease modeling • Induced-pluripotent stem cells • Neuronal subtypes • Neuronal specification • Translational research.

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Introduction

Stem cell research has made rapid and significant progress over the last decade. In the context of CNS repair, two lines of research are currently being investigated extensively for the development of neuronal replacement therapies, i.e. the recruitment of endogenous neural stem cells and the transplantation of exogenous stem cells. The first line of research emerged from the observation of a persistent neurogenesis taking place in discrete regions of the adult brain [1], although its relevance in humans is still debated. The second rapidly evolved due to recent advances that allow deriving, culturing and directing the fate of pluripotent stem cells (PSCs) from several mammalian species, including humans.

Both approaches, although experimentally different, are conceptually related. They are both guided by advances in our basic understanding of how the brain develops and influence each other in a bidirectional manner. This necessary cross talk between basic and more applied fields of research is particularly well illustrated by a recent study of Studer and co-workers. They demonstrated the derivation of midbrain dopaminergic neurons from human pluripotent stem cells and their successful engraftment in animal models of Parkinson's disease [2].

Pluripotent stem cells: from induced pluripotency to regenerative neurosciences

"Pluripotent stem cells (PSCs)" are cells that can self-renew and are capable of generating all cell types in an organism, with the exception of trophoblasts of the placenta. The classical method for isolation of PSCs has long been their derivation from early embryos. This approach has allowed the isolation and development of numerous embryonic stem cell lines, of both murine and human origin, that are currently being used in many laboratories around the world. Recent years have seen the development of alternative approaches for the production of PSCs, in particular, the development of protocols to induce pluripotency of more differentiated cells, e.g. somatic cells. A major breakthrough in the establishment of induced-PSCs (iPSCs) has been the demonstration that the introduction of a limited number of transcription factors (TFs) is sufficient to reset the epigenome and induce pluripotency in rodent [3,4], as well as human somatic cells [5,6]. Whereas these original experiments relied on the use of viral constructs for delivery of the TFs, the following years have seen intense research aimed at developing non-viral means to produce iPSCs. Among other methods, non-integrating viruses, naked plasmids, peptides or small molecules have successfully been used, although with variable efficiency [7]. While these findings are of great significance, this field of research is still in its infancy and several concerns remain for iPSC research [8]. For example, differing methods of producing iPSCs might not all equally induce pluripotency. Thus, has previously observed for embryonic stem cells, iPSCs produced from cells of different origins [9], by different protocols [10] or exposed to different microenvironments [11], vary in their gene expression signature and differentiation potentials. In addition, recent studies suggest genetic and epigenetic alterations in iPSCs that may arise from culturing or reprogramming [8].

The aim of human PSC (hPSCs) research was first thought to be the development of sufficient cell material to model diseases. In this context, a notable advantage of iPSCs is their possible derivation from individuals suffering from a specific disease, allowing the generation of patient-specific cells for high-throughput drug screening [12]. In addition,

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the autologous nature of iPSCs makes them suitable for transplantation. The feasibility and potential benefits of PSC transplantation have recently been highlighted in a number of studies [13] which used animal models of several neurodegenerative disorders. Among them, Parkinson's disease presents several advantages to test PSC-based cell replacement therapies. First, the pathophysiology of this disease is relatively simple with the loss of catecholaminergic neurons, in particular dopaminergic (DA) neurons of the *substantia nigra*, leading to motor and cognitive dysfunctions. In addition, due to its long history of fetal DA progenitors transplantation in the caudate-putamen starting in the 1970's [14], Parkinson's disease is a prime illustration of the regular progress made in this field of research, allowing the establishment of new neuronal replacement strategies to be guided by previous attempts. Early reports of fetal transplant survival and motor function improvement in Parkinson's patients [15] have provided a solid ground for the pursuit of this research, despite development of side effects in some patients [16]. In particular, methods to obtain more uniform and defined midbrain DA neurons from PSCs have been extensively explored.

Generating transplantable midbrain DA neurons from pluripotent stem cells

The use of hPSCs (i.e. human ES or iPS cells) for drug screening or transplantation purposes relies on the development of protocols to efficiently and homogeneously differentiate them into defined cell types. Early studies have shown that PSCs differentiate by default towards an anterior neuroectodermal fate when grown in a serum-free suspension culture [17], a process that can be promoted by dual inhibition of BMP and TGF-β signaling [18]. Subsequent exposure of the obtained neural precursors to patterning factors defining anterior-posterior and dorso-ventral identity during CNS development, can successfully direct differentiation towards specific neuronal fates [19]. For example, neural precursor cells can be instructed to acquire a cortical fate by using protocols containing the dorsalizing factor retinoic acid [20,21]. Specification toward a floor plate fate relies on early exposure to the ventralizing factor sonic hedgehog (Shh) [22,23].

The study of Kriks et al. represents a significant new step toward the development of protocols aimed at generating specific neuronal subtypes from PSCs, and is particularly illustrative of the evolution of this field of research. By refining previously established protocols, they report the efficient generation of neurons from human PSCs that show a molecular and phenotypic signature of midbrain DA neurons [2]. Three protocols, consisting of sequentially exposing the cells to one or several morphogens, were tested and their efficiency to induce a midbrain dopaminergic fate directly compared. Control cells not exposed to morphogens differentiated by default to a dorsal forebrain precursor fate. In contrast, and in agreement with their pattern of expression in the developing telencephalon, exposure to activators of the Shh-signaling pathway and to FGF8 resulted in a "ventro-rostralization" of the cells and acquisition of a hypothalamic precursor fate. A subsequent timely co-activation of the Wnt-signaling pathway by exposing the cells to GSK3β inhibitors resulted in their "caudalization" and in the generation of midbrain DA neuron precursors. Acquisition of a midbrain dopaminergic fate was accompanied by a reduction in acquisition of other alternative fates, as illustrated by lower number of cells acquiring a serotonergic or GABAergic fates.

In a second part of the study, although systematic comparison of DA neurons produced by these different protocols was not performed, the authors evaluated the capacity of PSC-derived midbrain DA neurons to differentiate and functionally mature. By performing various measurements, they could show that these neurons differentiate more efficiently and completely when compared to DA neurons obtained by a neural rosette intermediate, the currently most widely used strategy for deriving DA neurons from PSCs [24]. Finally, they showed that grafting of their PSC-derived midbrain DA neurons at a stage when they express the post-mitotic marker NURR1 resulted in efficient long-term graft survival (i.e. up to 4.5 months) with no sign of transplant overgrowth in intact and parkinsonian immune-deficient mice. This was accompanied by a complete rescue of the amphetamine-induced rotation behavior, which contrasted with the absence of functional recovery, poor survival and extensive overgrowth observed in rosette-derived grafts. Survival and function of midbrain DA neuron grafts was confirmed by behavioral testing in immunosuppressed adult parkinsonian rats. Lastly, transplants were performed in the caudate/putamen of two immune-suppressed rhesus monkeys after MPTP (a neurotoxin precursor) treatment, a classic primate model of Parkinson disease, demonstrating scalability of this approach.

From basic developmental principles to lineage specific PSC differentiation, and back again

These results are significant in several ways. First, they illustrate the current evolution of this field of research. In particular, they demonstrate how basic knowledge acquired in developmental biology serves as milestones for establishing novel PSC differentiation protocols. This knowledge acquired over decades in rodents, chickens and frogs allowed the defining of general principles and the identifying of key patterning molecules involved in the regionalization of the developing brain. This allowed the development of protocols used to generate neuronal subtypes as diverse as cortical neurons [21], motoneurons [25], basal forebrain cholinergic neurons [26] and dopaminergic neurons [2] (for a recent review see ([12,27]).

Furthermore, this study illustrates how advances in molecular biology, in particular in transcriptional profiling of divergent cellular lineages and of the distinct CNS cell types that they give rise to (see for example [28]) have impacted stem cell research. Public access to this vast amount of data, as well as the development of search engines and freewares to analyze them [29] offers unique opportunities to identify key transcriptional programs involved in neuronal subtype specification. These transcriptional programs can be used as readouts to assess appropriate

specification of PSCs exposed to various differentiation protocols, a resource elegantly compiled and applied in the study of Kriks and collaborators. By systematically performing microarrays at defined time points during differentiation, the authors were not only able to confirm the identity of PSC-derived midbrain DA neurons, but also to monitor their progression from proliferative progenitors to post-mitotic neurons, a crucial step for defining the optimal timing for engraftment of these cells (see below). The availability of this raw data (NCBI's GEO database, dataset GSE32658) will prove to be a precious resource for future development and refinement of other differentiation protocols.

Interestingly, the gene expression analysis performed by Kirks et al. also led to the identification of new transcription factors not previously associated with midbrain DA neuron development, illustrating how different domains of research can inform each other in a bidirectional manner. This identification of new transcription factors potentially involved in midbrain DA neuron generation may offer alternatives to the use of patterning molecules to induce lineage selection of PSCs, as, for example by forcing expression of carefully selected transcription factors [30]. In the context of DA neuron specification, previous research has shown that expression of Lmx1a is sufficient to trigger dopamine cell differentiation [31], which transplantation promote recovery in an animal models of Parkinson's disease [32]. Identification of key transcriptional regulators may also allow direct reprogramming of somatic cells into DA neurons [33], a technique that might circumvent some of the concerns associated with induced-pluripotency (see above).

Timing of cells engraftment: balancing safety with cell integration

Although it has not yet been systematically studied in neurodegenerative diseases the timing of engraftment in relation to disease progression is likely to have a very important role in determining the success of this procedure, as previously shown in other tissues. Equally important however, is the timing of engraftment regarding the stage of differentiation of the transplanted cells. Accumulating evidences indicate that these cells must have exited cell cycle to prevent risks of teratoma formation, but must remain immature enough to show optimal integration in the receiving tissue. Prior to the study of Kriks et al. several attempts had been made to use PSCs of murine or human origin for replacing lost dopaminergic neurons in animal models of Parkinson's disease. They relied on the use of ES cell lines, and more recently on the demonstration that iPSCs can also be used to this end [34-36]. In general, while these studies have demonstrated some partial functional recovery following transplantation, some reported overgrowth of the transplants [34]. These observations raised important and long-lasting safety concerns, and emphasized the need to develop methods to purify hPSC-derived neurons from reminiscent PSCs prior to their transplantation [37].

The study of Kriks et al. suggests that efficient and homogenous hPSC differentiation might be sufficient to eliminate the need for this purification step. Their study indeed shows a more complete and homogenous differentiation of PSCs when using more complex differentiation protocols, with minimal Nestin or Ki67 (i.e. proliferating) progenitors remaining both *in vitro* as well as in the grafts *in vivo*. Furthermore, their systematic gene expression analysis allowed the authors to optimize the timing of engraftment, which was determined by expression of Nurr1, a postmitotic marker and downstream target of Neurog2 previously used to identify transplantable dopamine precursors [38]. With this approach, they did not detect any graft overgrowth after transplantation in 3 animal models. The incidence of teratoma formation following hPSC transplantation into rodents being related to the degree of immunosuppression [39], graft overgrowth after autologous cell transplantation in humans remains a major concern [40]. In respect to this, it is noteworthy that the authors did not detect graft overgrowth in a mouse strain particularly sensitive to xenograft overgrowth.

While these observations illustrate the need for efficient and homogenous differentiation of hPSCs prior to grafting, they also highlight important considerations on the timing of hPSC transplantation for their optimal integration within the host tissue. Both the anatomical and functional analysis made in this study support the optimal integration of the transplanted neurons. In rodent as well as primate hosts, a halo of TH-positive fibers was observed around the graft questioning the well-known "inhibitory nature" of the adult CNS for axonal outgrowth. Impressive graft integration has been previously reported for embryonic cortical neurons in the cortex of neonatal [41,42] and adult animals [43]. In the later study, transplanted embryonic cortical neurons were capable to differentiate in projecting pyramidal cells that extended long-distance projections in the mature host brain to appropriate cortical and subcortical targets. This normal integration was only observed for homotopic neurons, as embryonic neurons from the visual cortex failed to correctly integrate [43], and was proposed to explain the functional recovery observed in previous studies where similar strategies were employed [44]. These striking observations suggest that early PSC-derived neurons express receptors that allow them to recognize molecular cues still maintained in the adult brain or re-expressed following lesion. At the same time, they suggest that the same neurons do not yet express the receptors or signaling machinery necessary to respond to growth inhibitory molecules of the adult CNS [45].

Altogether, these observations imply the existence of a narrow window of opportunity for optimal grafting of PSCs that has not been systematically studied so far.

Potential relevance for endogenous adult NSC research?

Although PSC research has greatly benefited from advances made in developmental neurosciences (see above), it might in turn instruct future research aimed at recruiting endogenous neural stem cells (NSC) for brain repair. Establishment of efficient PSC-differentiation protocols identifies the minimal signals necessary for neuroectoderm induction and neuronal subtype specification

in conditions where cells are isolated from their environment. Although it is unlikely that these protocols will be directly compatible with the more complex *in vivo* situation, it is tempting to speculate that this research will inspire the development of strategies aimed at recruiting endogenous adult NSC to sites of neuronal loss.

Although some studies have shown that NSC can be recruited into the denervated striatum in rodent models of Parkinson's disease, their subsequent differentiation in therapeutically relevant DA neurons has not been realized [46,47]. These findings suggest that the appropriate instructive signals are absent in the adult forebrain that would be necessary to promote the required specification and maturation of neuroblasts recruited to the site of injury. Small molecules activating specific signaling pathways such as those used in PSC studies might be relevant to test in this context. Thus, recent research identified Shh as a potent ventralizing factor in the adult murine lateral ventricle [48]. Interestingly, ectopic activation of the Shh signaling pathway in dorsal NSC leads to differentiation of their progeny to deep granule interneurons and calbindin-positive periglomerular cells, a fate normally acquired by ventral NSC [48,49]. Thus these observations suggest that some levels of plasticity exist for endogenous NSC, rendering them capable of acquiring alternative fates. Future studies aiming at "activating" such plastic potential of endogenous NSC will likely be guided by PSC research.

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