Review

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Stem cell programming – prospects for perinatal medicine

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Abstract: Recreating human cell and organ systems *in vitro* has tremendous potential for disease modeling, drug discovery and regenerative medicine. The aim of this short overview is to recapitulate the impressive progress that has been made in the fast-developing field of cell programming during the past years, to illuminate the advantages and limitations of the various cell programming technologies for addressing nervous system disorders and to gauge their impact for perinatal medicine.

Keywords: cellular reprogramming techniques; disease models; regenerative medicine.

Introduction

The advent of the pluripotent stem cell (PSC) technology has provided entirely novel prospects for biomedicine. With the availability of human embryonic stem cells (ESCs) and their controlled *in vitro* differentiation into somatic derivatives, it became possible to generate human cells of a large number of tissues and organs in the laboratory [1]. This approach is particularly relevant for cell types of non-regenerative tissues, which typically do not contain sufficient numbers of adult stem cells or are hardly accessible to cell retrieval. Such tissues include, among others, the nervous system, heart and insulin-producing islets of the pancreas – coincidentally tissues that are primarily affected by diseases associated with the current demographic change.

Less than ten years later, the pioneering work by Shinya Yamanaka and his team rang in the next level of PSC research: the generation of induced pluripotent stem cells (iPSCs) from somatic cells, such as skin fibroblasts, by overexpression of defined transcription factors [2, 3]. This reprogramming approach opened the door to the generation of patient-specific PSCs and their subsequent differentiation into various tissue-specific cells – in virtually unlimited numbers. With this development, iPSCs became not only a resource for cell therapy development but also an exciting tool for modeling diseases *in vitro*. Based on this, tissue-specific cells generated from patient-derived iPSCs further found entry in drug development, offering a route to assess drug effects on disease-relevant patient cells at an early stage of compound development. These approaches were further boosted by the enormous advances in the field of genome editing. In particular the CRISPR-Cas technology allows to either repair patient-specific mutations in iPSCs or to introduce such mutations into PSCs derived from healthy donors, with both approaches resulting in an ideal isogenic control scenario, which significantly reduces the experimental noise in cell-based disease modeling that is typically observed using control cells from different genetic backgrounds [4].

Naturally, developments did not stop there. Soon the idea of using pioneer transcription factors to switch cell identities was applied to directly inter-convert somatic cell types of different germ layers without traversing through a pluripotent state. Using this rationale, fibroblasts could, e.g., be directly converted into neurons [5]. Along a similar line, fate-instructing transcription factors were used to rapidly differentiate PSCs into distinct somatic fates, a strategy referred to as ‘forward programming’ (reviewed in [6]).

This steadily increasing methodological tool box makes it challenging to navigate in this fast-moving field and to select appropriate cell sources for individual biomedical applications. The following paragraphs aim at addressing this challenge using the central nervous system (CNS) as exemplar.

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Extrinsic factor-driven neural differentiation of PSCs

During physiological CNS development, neural subtypes evolve from distinct stem and progenitor cell populations. These cells divide and specialize in response to diverse extrinsic factors. While specific growth factors mediate the proliferation of precursor cells, which is essential for building an expanding structure, morphogens play a key role in instructing regional specification, thereby defining cell populations located in specific compartments of the CNS [7]. It was thus a logical choice that growth factors and morphogens were first used to mimic these developmental processes for differentiating PSCs into distinct regional fates and neural subtypes.

A classic approach along this line is the generation of stably expandable neural stem cell (NSC) populations from PSCs, e.g., by safe-guarding the continued proliferation of neural precursors evolving during undirected differentiation of PSCs by supplementation of the growth factors FGF2 and EGF [8, 9]. Once generated, such long-term self-renewing neuroepithelial stem cells (lt-NES cells) can be kept for numerous passages. Upon growth factor withdrawal, they generate stable fractions of neurons and glia, which largely facilitates standardization of the differentiation process. Human lt-NES cells have been successfully used in a number of applications including disease modeling [10, 11] and preclinical neurotransplantation [8, 12–14], in which their neuronal derivatives were shown to establish synaptic connections with the host brain [15]. Over the years, alternative protocols for the establishment of PSC-derived NSCs were established, including a paradigm based on dual-SMAD inhibition [16] and small molecule-based protocols exploiting, e.g., induction of SHH and Wnt signaling [17]. In addition, later stage NSC populations have been established and used for the generation of glial cells [18]. While such PSC-derived NSCs provide expandable and cryopreservable intermediates for robust generation of neurons and glia, they also come with some limitations. In particular, they are mostly restricted with respect to regional differentiation. Specifically, directing PSC-derived stable NSCs toward distinct forebrain phenotypes has proven difficult and requires sophisticated protocols [19, 20]. When it comes to the generation of very defined neuronal subpopulations, recapitulation of their developmental trajectory can yield remarkably authentic cells. A paradigmatic example is the generation of midbrain dopamine neurons, where a combination of SHH activation, canonical Wnt induction and timed delivery of FGF8b has been successfully used to generate this floorplate-derived population *in vitro* [21–23].

Forward programming: from morphogens to transcription factors

Guiding the differentiation of PSCs with morphogens and small molecules modulating cell specification-relevant pathways remains a versatile approach for mimicking cell fate acquisition during development. However, cells from different genetic backgrounds can show subtle differences in their response to extrinsic factors, which might require protocol adaptations for individual PSC lines. In case full maturation into a functional phenotype is required, longer *in vitro* differentiation times may further complicate the derivation process. Considering the power of transcription factors, which was impressively illustrated with the reprogramming of somatic cells into iPSCs, it was a logical next step to move from extrinsic factor-based differentiation paradigms to transcription factor-mediated approaches.

Indeed, overexpression of a few or even single transcription factors can suffice to force PSCs into differentiation trajectories yielding forebrain excitatory or inhibitory neurons within a few days [24, 25]. While initial approaches mostly relied on viral vectors, genome editing has facilitated the development of highly controlled forward programming systems, such as those making use of inducible transgene cassettes that are stably inserted into genomic safe harbor loci in order to avoid variability in transgene expression levels due to positional effects at different insertion sites [26]. Forward programmed neurons generated in this manner can become functional within three weeks of *in vitro* maturation on mouse astrocytes [27], and combining defined fractions of excitatory and inhibitory neurons even enables to 'tune' the activity levels of *in vitro*-assembled neuronal networks [28]. Furthermore, evolving neurons can still be efficiently cryopreserved. Importantly, the concept of forward programming has meanwhile been extended to numerous CNS cell types including also astrocytes and oligodendrocytes [29, 30], altogether providing a versatile resource for setting up disease modeling and drug screening platforms.

Traditionally, neurotransplantation approaches were based on immature neural precursors, which are considered particularly well suited for integrating into a host CNS environment. On the other hand, later stage neuronal progenitors derived via extrinsic factor-based differentiation of PSCs have been successfully grafted [31–35] and have even shown superior intracerebral migration compared to earlier stage neural precursors [36]. However, since only little is known about the survival and integration capacity of

forward programmed neurons at later stages of differentiation (reviewed in [6]), it remains to be explored how these cells fare in neurotransplantation compared to donor cells generated by more traditional methods. One attractive prospect here is the development of 'designer grafts' composed of defined fractions of specific forward programmed cell types.

Cell programming for the direct conversion of somatic cell types

Following the seminal discovery of Shinya Yamanaka and his team that fibroblasts can be reprogrammed into iPSCs, the question of how transcription factors could be used to directly switch one somatic cell type into another gained new attention. This question was not entirely new, as previous studies had already shown that somatic-to-somatic cell conversion is feasible for lineage-related cell types [37]. Now, with Yamanaka's discovery, it seemed possible to extend such conversion paradigms even to switches between different germ layers. Indeed, several papers soon reported that fibroblasts forced to overexpress ASCL1 in combination with either BRN2 and MYT1L [5, 38] or, e.g., NGN2 [39, 40] can turn into 'induced' neurons (iNs).

Notably, such a direct conversion has not only advantages with respect to time and reduced experimental complexity. Several studies confirmed that iNs, in contrast to neurons generated from iPSCs, maintain their epigenetic and cellular age [40–43] – a property that could offer significant advantages for modeling diseases of old age [44, 45]. On the other hand, iNs are postmitotic cells, which renders the generation of large numbers of clonally defined cells for biomedical applications almost impossible. However, subsequent studies demonstrated successful conversion of blood cells into proliferative neural stem and progenitor cells [46, 47]. These cells can indeed be clonally expanded, cryopreserved and, upon growth factor withdrawal, differentiated toward different neuronal and glial subtypes. Furthermore, they have been shown to be suitable for *in vitro* disease modeling and *in vivo* neurotransplantation [46].

The possibility of transcription factor-based cell conversion finally raises the question whether cells resident within the CNS can be interconverted, too. Indeed, already earlier work by Magdalena Götz and others had shown that astrocytes can be converted into neurons both *in vitro* and *in vivo* [48–52]. Subsequent studies refined this approach toward the generation of distinct therapeutically relevant neuronal subtypes such as dopamine neurons [53]. With concomitant advances in the field of *in vivo* gene transfer in the context of gene therapy, such approaches could in the end provide prospects for *in situ* cell replacement without cell transplantation (reviewed in [54]).

Standardization and automation as key requirements for biomedical applications

Several requirements need to be met in order to move the latest developments in stem cell research toward therapeutic application. A key aspect is standardization of cell production, which necessitates robust protocols enabling reproducible and scalable cell programming and differentiation. Here, automation becomes crucial in order to reduce variable outcomes due to manual processing and subtle interindividual differences of experimenters. Significant progress has been made with respect to bioreactor development, enabling scaling of both, pluripotent and somatic stem cells [55, 56]. However, the execution of complex protocols encompassing multiple distinct handling steps has remained a challenge.

While a number of automated modules for specialized cell culture steps have been established and exemplarily used for automated iPSC generation [57], their assembly into larger automation platforms remains a bottleneck. Here, developments such as the StemCellFactory® provide new prospects. Constructed for automated cell reprogramming, this system covers the entire process, including clone picking, expansion and in process quality control steps [58], and is designed to parallelize cell reprogramming – a key prerequisite for generating larger numbers of cell lines, e.g., from different donors. Notably, since cells from different donors and tissues can show subtle differences in reprogramming efficiency and proliferation speed, cell culture automation systems cannot only rely on fixed protocols and holding patterns, such as in automobile industry, but require smart systems including artificial intelligence-trained algorithms, e.g., for the assessment of cell morphology and density [59–61]. Yet, since many of the handling steps in cell reprogramming are also used during genome editing, such system developments are expected to enable parallelized generation of genetically modified PSCs.

Prospects for the use of human PSCs in perinatal medicine

As for any disease, the potential use of PSCs in perinatal medicine extends to both, disease modeling and drug

discovery as well as cell therapy and cell-based gene therapy. Modeling neurodevelopmental disorders or neurotoxic insults *in vitro* represent a particularly interesting avenue. Here, human PSC-derived brain organoids might provide fascinating prospects. As self-organizing 3D structures recapitulating key aspects of the cytoarchitecture and cell type composition of a variety of human brain regions, cerebral organoids were successfully employed to model developmentally-relevant diseases such as microcephaly and lissencephaly (for further reading see review by [62]).

On the other hand, direct application of human PSC derivatives could be a therapeutic modality for a number of diseases. Already in 1999, we could show that glial precursors generated from mouse ESCs can restore myelin when transplanted into the CNS of neonatal rats deficient in proteolipid protein, an animal model of Pelizaeus-Merzbacher disease [63] – the first example of using ESCs for therapeutic application in an animal model of a human disease. In subsequent studies, we and others used mouse ESC- and human iPSC-derived neural precursors engineered to overexpress arylsulfatase A for reduction of cerebral sulfatide load in a mouse model of metachromatic leukodystrophy [64–66]. While alternative gene therapy approaches are developing with promising results that have been observed, e.g., upon transplantation of arylsulfatase A-overexpressing hematopoietic progenitors for the treatment of metachromatic leukodystrophy [67], neural cell-based therapies offer the additional prospect of *bona fide* structural repair such as replacement of oligodendrocytes and lost myelin. This also sets them apart from broadly advertised cord blood and mesenchymal stromal cell treatments with their often ill-defined mode of action.

However, a key challenge of neural cell-based treatments of disorders affecting large areas of the CNS is their limited distribution after intracerebral transplantation. For example, attempts to translate neural precursor cell transplantation into clinical treatment of Pelizaeus-Merzbacher disease patients revealed very poor distribution of the cells and no evidence of substantial graft-related myelination [68]. While transplantation studies in rodents have shown impressively widespread distribution of neural precursors upon multifocal neonatal transplantation [69, 70] or after intrauterine transplantation into the lateral ventricles [71, 72], it remains to be explored whether the spread of cells is sufficient to cover much larger territories in the human brain. Further hurdles that need to be overcome on the route to the clinics include safety concerns relating to the tumorigenicity and immunogenicity of PSC derivatives [73], as well as regulatory considerations relating to genetic modification of human cells. Still, transplantation of human neuronal and/or glial progenitors remains a promising approach for the development of novel treatment regimens for a number of diseases in perinatal medicine, including Pelizaeus-Merzbacher disease [68], Canavan's disease [74], metachromatic leukodystrophy [65, 66], Niemann-Pick disease [75, 76] and Krabbe disease [77, 78], as well as perinatal brain damage resulting from neonatal encephalopathy and periventricular leukomalacia [79–81] (for further reading see reviews [82–86]). It remains to be explored how such approaches can be further enhanced by the use of human PSC-derived and forward programmed donor cells as well as *in situ* cell fate conversion strategies.

In sum, recent advances in stem cell technology and cell programming hold great promise for a variety of applications in perinatal medicine, ranging from *in vitro* disease models to cell and gene therapy. Considering the intricacies of these approaches, sound investigation of mode of action, efficacy and clinical safety is mandatory to further develop this field, which is increasingly confronted with a plethora of unproven therapies [87–89].

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