What do we know about the neurogenic potential of different stem cell types?

O que sabemos sobre o potencial neurogênico de diferentes tipos de células-tronco?

Guilherme Lepski

ABSTRACT

Cell therapies, based on transplantation of immature cells, are being considered as a promising tool in the treatment of neurological disorders. Many efforts are being concentrated on the development of safe and effective stem cell lines. Nevertheless, the neurogenic potential of some cell lines, i.e., the ability to generate mature neurons either *in vitro* or *in vivo*, is largely unknown. Recent evidence indicate that this potential might be distinct among different cell lines, therefore limiting their broad use as replacement cells in the central nervous system. Here, we have reviewed the latest advancements regarding the electrophysiological maturation of stem cells, focusing our attention on fetal-derived-, embryonic-, and induced pluripotent stem cells. In summary, a large body of evidence supports the biological safety, high neurogenic potential, and in some diseases probable clinical efficiency related to fetal-derived cells. By contrast, reliable data regarding embryonic and induced pluripotent stem cells are still missing.

Key words: fetal neuronal stem cells, embryonic stem cells, induced pluripotent stem cells, electrophysiology, neuronal differentiation.

RESUMO

Terapias celulares, baseadas no transplante de células imaturas, têm sido consideradas ferramentas promissoras no tratamento de doenças neurológicas. Muitos esforços têm sido concentrados no desenvolvimento de linhas de células-tronco seguras e eficazes. No entanto, o potencial neurogênico de algumas linhagens celulares, ou seja, a habilidade de gerar neurônios maduros, in *vitro* ou *in vivo*, ainda é altamente desconhecida. Dados recentes sugerem que esse potencial é distinto entre diversos tipos celulares, o que limitaria o largo emprego como células restauradoras no sistema nervoso central. Neste relato, revisaram-se os avanços recentes relacionados à maturação eletrofisiológica de células-tronco, com foco em células derivadas de tecido fetal, células embrionárias e células pluripotentes induzidas. Em resumo, há evidências que apontam para segurança biológica de células fetais, com alto potencial neurogênico e, em se tratando de algumas doenças, provável eficiência clínica. Ao contrário, ainda não há dados confiáveis acerca de células embrionárias e pluripotentes induzidas.

Palavras-Chave: células-tronco neuronais fetais, células-tronco embrionárias, células-tronco pluripotentes induzidas, eletrofisiologia, diferenciação neuronal.

The management of the majority of neurological diseases, specially the degenerative subset, or the entities whose natural history runs to a degenerative fate, e.g., the vascular disorders, brain or spinal cord injuries, still imposes a great challenge for Medicine. Yet, most therapeutic interventions, whether clinical or surgical, focus on the consequence rather than on the cause of such disorders, thus providing palliative amelioration, sometimes by means of highly invasive interventional techniques, or resulting in insufficient or no functional recovery.

In face of this scenario, with the advent of stem cell therapy as a promising tool to actuate in the cause, or as an intermediate between the cause and the outcome, many efforts have been concentrated in the last decades for the purpose of conquering safety and effectiveness of stem cells for the treatment of neurological disorders. Despite the advances already achieved, not only in the manipulation of the stem cells itself, but also the mechanisms of diseases they helped to elucidate, many issues regarding the neurogenic potential of these cells are to be answered before they become real therapeutic possibilities.

We define neurogenic potential as the ability of stem cells to differentiate, under specific conditions, from cells with electrophysiological properties of mature neurons. Furthermore, once differentiated, these cells shall fire action potentials and create effective synapsis, not only

Department of Neurosurgery, Eberhard Karls University, Tübingen, Germany.

Correspondence: Guilherme Lepski; Hoppe-Seyler-Strasse 3, D-72076; Tübingen - Germany; E-mail: lepski@gmail.com

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among themselves, but also with the host tissue when implanted, hence permitting functional and structural restoration of the impaired nervous system. As stated by Liebau et al.[1], the ability to establish and to maintain polarized excitatory synaptic contacts would be one of the basic requirements for intercellular communication and functional integration into existing neuronal networks.

In the present work, we have aimed at reviewing the most important achievements to our understanding of the neurogenic potential of stem cells. For this, we direct the discussion to two cell subtypes, which have already been proved to generate morphological and functional mature neuronal cells, namely the embryonic (ESCs), and neural stem cells (NSCs). In order to provide a rational and comprehensive analysis of the neurogenic potential of these cells, based on the current scientific knowledge, we have structured our review in two major subjects: the morphological and functional *in vitro* characterization, and the *in vivo* integration and synaptogenesis. We have previously demonstrated that noninduced adult mesenchymal stem cells present a very limited neurogenic potential compared to NSCs[2], although they may acquire some neuronal fate, therefore they were not included in the present review.

IN VITRO EVIDENCE

Fetal neural stem cells

Pioneer studies have demonstrated the enormous capacity of fetal grafts to reverse some motor signals observed in the animal model of Parkinson's disease (PD)[3]. In further studies, some specific properties of neural progenitor cells, like expression of the intermediate neurofilament nestin[4], were described. This knowledge permitted in a following phase the isolation of NSCs from the fetal and adult rodent brains, and consequently the generation of neurons and astrocytes *in vitro*[5]. Since then, several authors have confirmed the importance of epidermal grow feature (EGF) and b fibroblast glow factor (bFGF), for proper isolation and expansion of neuronal precursors[6]. In this regard, EGF seems to be especially important to induce and maintain an undifferentiated state[7], whereas bFGF seems to induce the expression of neurogenic genes[8]. Although NSCs can be expanded for prolonged periods, still maintaining certain ability to generate neuronal cells[9], other evidence suggests that the neurogenic potential is reduced, according to the expansion time[10].

Although numerous studies report the successful generation of mature neuronal cells based on the expression of neural genes and proteins, and acquisition of typical neuronal morphology, very few evidence is available concerning the electrophysiological maturation of NSCs-derived cells. Indeed, our data have showed a chronological discrepancy between expression of neuronal proteins and development of electrical activity in maturating cells from human fetuses[11]. This emphasizes the importance of electrophysiological methods to ensure acquisition of neuronal fate. Thus, the aim of the present review was to summarize the latest evidences supporting electrophysiological maturation of neuronal precursors.

The importance of specific neurotrophic factors and signaling molecules, such as brain-derived neurotrophic factor (BDNF)[12], NT3[13], or Wnt[14], for functional maturation of NSCs has been emphasized by many authors. Several pieces of evidence suggest that BDNF causes differentiation of neuronal progenitor cells *in vitro*[15] and *in vivo*[8], probably by inducing expression of Na^+ and K^+ channels[12], as well as by promoting synaptic maturation and increasing synaptic transmission[16]. Using BDNF in some experiments, we could observe that 60% of GABAergic neurons in the culture system resulted from the differentiation of human neural fetal cells[11]. Similarly, the effect of NT3 on inducing neuronal differentiation has also been demonstrated[13]. Other lines of evidence suggest that the cAMP-pathway, via phosphorylation of cAMP response element binding (CREB) protein, is particularly involved in adult neurogenesis[17]. Moreover, electrical activity was proved to be essential for proper maturation of progenitor cells *in vitro*[18], since chemically-induced depolarization at high potassium concentrations and glutamate in culture medium increased the number of microtubule-associated protein 2 (MAP2)-positive neurons derived from neural progenitor cells (NPCs). Also, the presence of astrocytes or immature cells in the culture medium seems to play a central role on accelerating neuronal maturation[19].

Additionally, calcium is believed to be involved not only in controlling cell survival and death[20], but also in neuronal differentiation[18]. It was observed a strong correlation between intracellular Ca^{2+} signals and regulation of neuronal gene expression[21]. Ca^{2+} may activate numerous transcription factors, such as CREB, C/EBTβ, MEF-2, NT-ATc4, NFκB, and c-fos[22]. In cortical neurons, Ca_v1-mediated Ca^{2+} influx stimulates the expression of various genes that regulate neuronal survival and plasticity through CREB phosphorylation[23]. This evidence supports the concept that Ca^{2+} signaling is a central requirement for proper neuronal differentiation. According to these observations, Ca^{2+} currents have been recorded in neuronal progenitor cells[24].

Embryonic stem cells

ESCs have introduced new hope in the field of neural repair, because they can be expanded for prolonged periods without loosing neurogenic potential, and, under appropriate conditions, they give rise to mature neurons *in vitro* and *in vivo*[25]. Nevertheless, due to their high mitotic activity, these cells tend to form malignant teratoms.

Spiliotopoulos et al.[26] reported a high yield of electrophysiologically confirmed neuronal cells derived from ESCs,

using a different medium comprised of increasing concentrations of BDNF and decreasing concentrations of bFGF for 21 days. Under these conditions, they observed 80% of GABAergic neurons after 21 days *in vitro*. ESCs acquire neuronal characteristics once in presence of all-trans-retinoic acid, and the maturation into GABAergic and glutamatergic phenotypes has been described previously[27]. Other authors have also dedicated efforts in creating better cell culture conditions with improved yield of neuronal cells by cultivating cells onto bioengineered polyamide nanofibers, with an average diameter of 280 nm. Under these conditions, the authors described neurons derived from ESCs, which were able to fire action potentials and presented significantly greater Na^+ and Ca^{2+} currents[28].

In spite of the improved conditions already reached by novel methodologies applied to the standard two-dimensional culture systems, they still only allow the formation of neurospheres with limited expansion life span. With the purpose of increasing reliability of the culture design, Preynat-Seauve et al.[29] have created an air-liquid interface culture system for human ESCs, which allowed three-dimensional cell expansion and neural differentiation. The tissue obtained after a three-month culture period formed immature tubular structures, which were constituted by niches of cells resembling germinal layers of the human fetal brain. The analysis of this tissue revealed a dense network of neurons, astrocytes, and oligodendrocytes able to produce electrical activity. It is noteworthy that such results were obtained in the absence of growth factors.

Continuing by this venue, other authors[30] transduced by retroviral infection ESC-derived glial precursors to overexpress polysialic acid, a carbohydrate polymer attached to the neural cell adhesion molecule (NCAM). As a result, the transfected cells showed enhanced migration in monolayer cultures and an increased penetration of organotypic slice cultures.

Recently, new methodologies of reprogramming mature glia into immature neural progenitors were described. By applying transforming growth factor alpha (TGF-alpha) in astrocyte cultures, Sharif et al. were able to obtain neural progenitor cells. These NPCs generated cells with morphological and electrophysiological properties of neuroblasts. Keeping such culture conditions for longer periods enabled the conversion for a neural phenotype even more immature, whose characteristics resembled NSCs, i.e., they could be clonally derived from a single cell, formed self-renewing floating spheres, and underwent, upon proper stimulation, differentiation into a neuronal lineage[31].

Induced pluripotent stem cells

In 2006, Yamanaka group demonstrated that a mature somatic cell can be reprogrammed to acquire immature stem cell fates after transfection with certain neurogenic and oncogenic genes, specifically Oct4, SOX2, Klf4, and c-Myc[32]. Using a similar strategy, Caiazzo et al. were capable of generating functional dopaminergic neurons directly from mouse and human fibroblasts, without reverting to a progenitor cell stage, by means of three transcription factors – Mash1, Nurr1 and Lmx1a.[33]. Until now, at the best of our knowledge, there are very few evidence proving electrophysiological differentiation of induced pluripotent stem cells (IPS) into mature neurons, so that no conclusion can be driven related to the neurogenic potential of these cells in comparison with other stem cell types.

IN VIVO EVIDENCE

Fetal neural stem cells

Studies with engrafted stem cells into the developing or adult central nervous systems are particularly important not only because they have been contributing to elucidate how these cells may interact with the host tissue and how they will be allowed to exert their neurogenic potential in a non-controlled environment, but also to establish the safety of transplantation protocols. In this setting, the local host microenvironment plays a decisive role in cell viability and compromising. The concept of stem cell niches that maintain an endogenous pool of stem cells of a living organism highlights the complex interrelationships between native stem cells and the architectural space, as well as the signaling interactions at the interface of stem cells and niche or

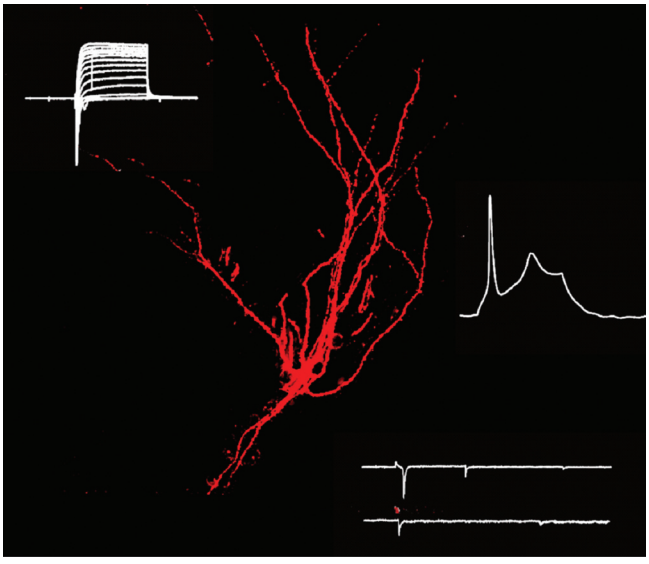

Figure. Confocal picture showing a mature neuronal cell derived from a human fetal neural stem cell, extracted from a nine-week-old embryo, differentiated under brain-derived neurotrophic factor (BDNF) for five weeks *in vitro*, and stained for MAP2ab (microtubule-associated protein 2, a mature neuronal marker, revealed with AlexaFluor 594). The traces around the cell represent voltage-clamp traces that show inward and outward currents (above, left), a single action potential (on the right), and spontaneous synaptic currents (below, right).

descendent cells, paracrine and endocrine signals from local or distant sources, neural input, and metabolic products of tissue activity[34]. Particularly, the nervous system participates of the niche expanding its boundaries: in the case of a distant injury, for example, a recruitment of the physiological stem cell pool is known to occur, thus contributing to the local repair[35].

A vast body of evidence confirms the great neurogenic potential of fetal-derived NSCs and NPCs (Figure). Additionally, human tissue was already implanted in controlled randomized clinical trials[36]. Postmortem studies have successfully demonstrated that NSCs survive for many years after transplantation into the diseased brain[37], and they are able to differentiate into mature neurons[38], to produce neurotransmitters like dopamine, and to reverse some neurological deficits accompanying PD[36], Huntington's disease[39], spinal cord injury[40], stroke[41], and brain injury[42].

The malignant degeneration of fetal tissue was not reported so far. Notwithstanding, the difficulty in obtaining human fetal tissue for scientific purposes, the prohibitive regulations in some countries, and ethical issues, have limited the broad usage of these cells in the clinical field. Furthermore, many intracellular signaling molecules like cAMP, Ca^{2+} or GSK3 were reported to be important for *in vivo* neurogenesis and early neuronal development[43]. In particular, it has been reported that the inhibition of phosphodieserase 4 (PDE4) by rolipram strongly increases cognitive functions and the number of newly generated neurons in the hippocampus[44].

Previous studies have demonstrated that the transcription factor CREB is expressed by neuroblasts during maturation[45], and that its downregulation or deletion leads to defects in neuronal progenitor cell expansion, neuronal survival, and differentiation[46]. It has been demonstrated in previous studies that GABAergic afferences through the perforate pathway promote functional maturation and synaptic integration of neuroblasts in the subgranular zone of the dentate gyrus[47]. These findings corroborate with the mentioned *in vitro* evidence, which have shown the central role that electrical excitation promotes on neuronal maturation. In this regard, gap-junctional coupling between young and mature cells is also considered to be extremely relevant. It is accounted as an essential step in the functional integration of grafted murine and human NSCs into the host neural circuitry, even before mature electrochemical synaptic communication has been established. In addition, gap junction formation apparently prevents death of host neurons and inhibits gliosis, thus yielding stem cells to exert a protective effect on host cells[48].

In the *in vivo* microenvironment, many molecules play important roles in the homeostasis of endogenous stem cells. Tenascin C, an extracellular matrix glycoprotein that participate in the neural embryonic development, is highly expressed in the sub-ventricular zone (SVZ) in mice, and it is responsible for switching NSC responsiveness from the fibroblast growth factor to the epidermal one by regulating the expression of the EGF receptor[34]. Furthermore, Wnt, N and Hedgehog pathways are required for the self-renewal and the differentiation of stem cell progeny in a variety of systems[49].

Other studies have also reported successful integration of transplanted stem cells in different central nervous system (CNS) structures, whether in the hippocampus[50] or in the spinal cord[51]. The latter was assumed to barely support neuronal differentiation and integration, due to the tendency of glial fate commitment by the spinal cord white matter. Nevertheless, more recent studies have contributed to pull down the paradigm that the spinal cord only offers a hostile environment for stem cells. Yan et al.[19] grafted human fetal spinal cord NSCs into the lumbar cord of normal or injured adult nude rats. These authors have also observed large-scale differentiation of these cells into neurons that formed axons and synapses and established extensive contacts with host motor neurons.

Most interestingly, the microenvironment seemed to actively participate in the fate choice, since centrally located cells predominantly underwent neuronal differentiation, whether those under the pia mater persisted as NSCs or underwent astrocytic differentiation. In accordance with these observations, we demonstrated, by fluorescence-guided patch clamp, complete functional maturation and synaptic integration of implanted NSCs into the normal hippocampus of rats[50]. Physiological neurogenesis in the hippocampus is supposed to be involved in mnestic processes, since cell turnover facilitates the incorporation of new information and enhances learning performance[18]. At which extent, artificially implanted cells into the hippocampus are able to interfere with learning, and behavioral performance of host animals remains an intriguing issue yet to be clarified.

Embryonic stem cells

It has been demonstrated that ESCs can restore functional deficits in the animal model of PD[52], Huntington's disease[53], spinal cord injury[54], and stroke[55]. These cells retain large proliferative capacity, and thus can be expanded in culture over longer periods. Nevertheless, the high proliferative capacity confers a trend to malignant degeneration, since it is difficult to control their *in vivo* growth after transplantation[52]. In fact, these authors reported the occurrence of malignant teratoms in 25% of the animals implanted with such kind of cells. Some strategies are currently being considered to overcome this problem, like the previously selection of cells with slower growth rate[56]; nevertheless, these approaches must be validated by the scientific community before ESCs can be safely used in human trials.

Considering the importance of the stem cell niches, *in vivo* studies help to provide insights in how native stem cells or engrafted ones may interfere or be influenced by the

delicate and complex balance encountered in the niche microenvironment. In this direction, Joannides et al.[57] found that transplanting human ESCs into the diseased brain, the microenvironment accelerated the development into a mature neuronal phenotype.

Not less important is the response of the host immune system to the engrafted cells. Because local trauma and inflammation are inevitable in transplantation surgery, it is possible that inflammation and immune reaction after transplantation of NPCs influence survival and differentiation of grafted cells. By transplanting allogeneic ES cell-derived NPCs, Ideguchi et al.[58] have reported accumulation of microglia, macrophages, and lymphocytes around the graft and that immunosuppression promotes actually neuronal differentiation rather than survival of grafted NPCs. Furthermore, they found that the ratio of neurons to astrocytes was higher in the grafts of immunosuppressed mice. They also cultured these cells *in vitro*, and by the addition of interleukin-6 (IL-6), a decrement in the neuron/astrocyte ratio was observed. Conversely, Oh et al.[59] reported an enhancement of the neurogenic potential of rat adult hippocampal progenitor cells when they were exposed to IL-6. Such contradictory results demand further investigation in order to determine the actual role of IL-6 in neurogenesis.

Regarding the safety use of ESCs in the clinical practice, the efforts must be directed towards growth control and development of strategies to reduce the incidence of tumor formation after implantation. In this regard, promising solutions have been considered. Cho et al.[56], for instance, developed a method to pre-select nononcogenic cells, based on the formation of neural rosettes during expansion. Other authors are working on the overexpression of pro-neurogenic genes, like Nurr1, which supposedly can also reduce tumor formation[60]. Time will show if this category of cells will be valuable for clinical transplantations.

Induced pluripotent stem cells

Wernig et al.[61] successfully demonstrated the induction of a mature neuronal fate after implantation into the fetal mouse brain, and amelioration of motor deficits in the rat model of PD. However, these promising results need to be reproduced by other authors. Additionally serious concerns about the biological safety of these cells still remain; first, c-Myc is a known oncogenic gene, which certainly favors occurrence of teratoms and possibly other malignant tumors; second, the lentniviral transfection necessary during construction of this cell line increases the biological risk in human subjects. Trying to solve these issues, some authors have developed a similar cell line created without the oncogenic gene c-Myc, at the expense of a considerably lower efficiency rate[62]. Additionally, they are trying to generate IPS cells based on plasmid vectors, dispensing with the incorporation of new genetic material by the modified cell[63]. These strategies represent encouraging solutions that might enable the use of IPS cells for brain repair.

The CNS is recognized as the most complex and specialized system of a living organism. This complexity relies on its intrinsic cellular components and interactions between itself and the whole organism. Although much is still to be clarified about the idiosyncratic mechanisms that govern the homeostasis of neuronal populations, including an infinite range of molecular components, networking and communication subsystems, altogether orchestrating the *modus operandi* of the nervous system, the advancements provided by the stem cell investigation have substantially contributed to our current understanding of the neurogenic potential of the *in vivo* and *in vitro* cells.

Fetal NSCs cells still present the best results when compared to other stem cell types in terms of neurogenic potential, survival, and safety in *in vivo* models. More studies, however, are required in order to compare, in the mentioned parameters, the efficiency on generating functional neuronal cells from various stem cell systems.

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