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Neurodegenerative Diseases: "*In vitro*" Culture of Neural Progenitor and Neural Stem Cells Challenges and Hopes

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The State of Art

It is widely recognized by the scientific community that there is a serious health problem in developed societies due to the increased prevalence in neurodegenerative diseases. Diseases such as Parkinson´s, Alzheimer´s, ALS, etc., require more than one therapeutic approach depending on the pathogenesis of the disease, among them, regenerative therapy of the nervous tissues is needed in an urgent and safe way.

Nowadays, regenerative medicine has arisen great expectations for the treatment of diseases in which the cells suffer degeneration and finally they die. In order to repair damaged cells, usually pluripotent stem cells are produced from adult cells, multiplied, differentiated into several cells lines, and finally are transplanted into patients or disease animal models. Pluripotent stem cells are those cells capable of self-renewal and differentiation into all three germ layers. To date, the best clinical results are obtained with embryonic and human fetal cells, but its use is restricted because extraction is a very complex process with ethical objections. In view of this, Yamanaka [1] attempted to develop an unlimited source of stem cells with a radically new vision, using fibroblasts from adult individuals he introduced four transcriptional factors (Oct3/4, Sox2, Klf4, and c-Myc) with retroviruses. He succeeded and the fibroblasts were transformed into cells very similar to embryonic stem cells (ESC). In this work Yamanaka et al. dedifferentiated adult cells to pluripotent stem cells which they named induced pluripotent stem cells (iPSCs); he was awarded the Nobel Prize in 2012.

Since then, an explosion of studies has been produced following this strategy, trying to figure out the minimum factors necessary to induce different cell lines from adult or stem cells. However, although the resources being devoted worldwide are enormous and paramount advances have been obtained, currently, important obstacles still prevail:

1. Lack of functional maturation of the induced cells.
2. The efficiency of production has to increase in order to use autologous transplantation.
3. The incorporation of the transcription factors has usually been made with retrovirus which is not acceptable from a clinical standpoint.
4. Formation of tumors in the transplanted animals.
5. Systematic immune rejection, except when embryonic or fetal cells are used.

It should be pointed out that contrary to the paradigm long time accepted, in the central nervous system there are neural progenitor cells (NPCs) which can regenerate neural cells, these are localized in the Subventricular zone of the Lateral Ventricle and Subgranular zone of the Hippocampus and could be manipulated with regenerative proposes [2]. Besides fetal and embryonic cells, NPCs are the most promising cells. NPCs are multipotent cells with nearly unlimited capacity of multiplication and they can differentiate into various types of nervous cells.

Although this overview focuses on "*in vitro*" cellular studies, we must not forget that there is a growing evidence for a common pathogenesis of most relevant neurodegenerative diseases. An abnormal protein structure is implicated (f. i. β amyloid in Alzheimer´s, α synuclein in Parkinson´s and superoxide dismutase SOD in ALS) and its transmission from cell-to-cell is similar to that described in prion diseases [3]. If this is the case, as suggested by sporadic cases of these diseases in which genetic alterations are not found, additional approaches should be taken besides regeneration or transplantation of the degenerated tissues.

"*In vitro*" Multiplication and Differentiation of Progenitor and Stem Cells into Neural Cells

The production of neural progenitor cells NPC "in vitro" has enormous interest due to the limited capacity of regeneration in the nervous system "*in vivo*" and this is reflected in the scientific literature where many studies have this objective. Given the enormous number of publications we will focus only in those studies done with human cells. The vast majority of research studies have been done with hiPSCs derived from different tissues, which later on are differentiated into neural stem cells (NSCs). The most used cells have been the fibroblast [4], bone marrow mesenchymal stem cells (BM-MCs) [5,6] and umbilical cord cells [4].

However, other cells from adults have also been successfully differentiated like pericytes from the capillaries of the cerebral cortex, assayed in Germany by Karow et al. [7], from olfactory ecto-mesenchymal stem cells in France by Nivet et al. [8], carotid body in Spain by Platero-Luengo et al. [9], human urine epithelial cells (hUC) in China by Cheng et al. [10], stem cells from human dental pulp by Zhang et al. [11], etc.

Improvements in cell growing conditions and reduction of transcriptional factors have achieved important advances. In a study published in 2015 by Bardy et al. [12], glial and neuronal cells were derived from human fibroblasts without the need of producing iPSC. They directly differentiated fibroblasts into neural cells (induced neural cells, iNC) by using two transcriptional factors Ascl1 (Mash1) and Ngn2 (Nurr1). Also in this work, the authors developed a new defined culture medium superior to the rest of the media traditionally used for growing and maturing nervous cell lineages. The authors claimed that neuronal cells were fully functional and mature enough not to revert into tumoral cells.

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However, a crucial step towards avoiding retroviruses was taken earlier in 2014 by Cheng et al. [10] by using a cocktail of small molecules (SMs) which includes only three chemicals (Valproic acid, Repsox and CHIR 99021). Human epithelial cells extracted from urine (hUC) were derived into neuronal cells. Conceptually these SMs are essentially inhibitors, that is, Valproic acid inhibits deacetylation of histones, Repsox inhibits glycogen synthase kinase 3β (GSK3β) and CHIR 99021 inhibits epidermal growth factor (EGF). They also achieved other important milestones, mimicking physiological conditions of oxygen partial pressure in the Central Nervous System, cells with high plasticity (potential of neuronal conversion) were cultured under the beneficial hypoxic conditions (5% O_2). This strategy has been further improved by Li et al. making it more efficient [13], by using a cocktail of six SMs (Cyclic pifithrin-a, CHIR99021, A-83-01, Thiazovivin, PD0325901 and NaB) and coculture of hUC with autologous feeder, they increase 170 times the reprogramming efficiency. Interestingly, this procedure could be applied to other non-neuronal cells of epithelial origin, such as endometrial menstrual cells in women which have already been transformed into iPSC using Yamanaka´s procedure by Ding et al. [14].

Cheng et al. in 2014 advanced the possibility of applying these chemicals directly in patients with Parkinson´s. In 2015 other crucial forward step was reported by them obtaining "in vitro" direct conversion of astrocytes into neurons (dopaminergic neurons, GABAergic neurons, glutamatergic neurons and motor neurons). They cleverly dissected the essential factors, in their initial work from three SMs (Valproic acid, Repsox and CHIR 99021) to two (Valproic acid and Repsox). Then, they replaced SMs by drugs already authorized for other indications, which should simplify the authorization process for the subsequent neural cell therapy. Valproic acid, an anticonvulsant drug, and Tranilast, antiallergic drug and inhibitor of EGF, were used in newborn mice astrocytes with successful results "*in vitro*" [15].

Important implications can be envisaged in neurodegenerative diseases derived from this study, for example in Parkinson´s and Alzheimer´s patients. The astrocytes localized in the Subgranular and Subventricular zones, behave as neurogenic stem cells in adult mammals [16]. This peculiar characteristic makes them the ideal target cells for regenerative proposes "*in vitro*" and "*in vivo*". Therefore, hopefully the treatment with Valproic acid and Tranilast could favor the differentiation of astrocytes into neurons whenever and wherever required.

The use of Valproic Acid in Parkinson´s patients could result in additional advantages; besides its antidepressant therapeutic effect other mechanism can be relevant, that is, it's positive effect on the expression of galectin 1 [17]. Galectin 1, a galactose binding lectin implicated in cellular proliferation, is a neuroprotective agent in neural cells, for instance exogenous galectin 1β promotes "*in vitro*" axonal regeneration [18]. On the other hand, strong evidences support that α-synuclein is propagated by exocytosis-endocytosis from cell to cell. In fact, human BM-MCs cocultured with hNSCs produce Galectin 1, which in turn, behaves as a competitive protein in the cell-to-cell transmission of α-synuclein by endocytosis. Importantly, the N-methyl-D-aspartate receptor (NMDA receptor) implicated in the endocytosis of pathogenic α-synuclein fibril was competitively inhibited by galectin 1 in hNSC. Moreover, in parkinsonian mouse model galectin 1 improved neuron survival and motor function [19].

Studies in Alzheimer´s models made "*in vitro*" and "*in vivo*" have also supported the implication of NMDA receptor in the cellular internalization of β-amyloid fibrils. In fact, BM-MCs injected in the tail vein of mice increase β-amyloid clearance and the survival of hippocampal neurons [20], probably by the same mechanism described for α-synuclein. Therefore, in α synucleinopaties and diseases with β-amyloid fibrils, the treatment with VPA alone or with Tranilast can have beneficial effects in clinical therapies.

Glycobiology should study the action of galectin 1 in the glycocalyx of the NSC and NPC and its regulation by the appropriated carbohydrates. In rodents the patterns of specific glycans in CNS have been studied with lectins in Subgranular and Subventricular zones and all types of neuronal cells could be identified [21]. In fact, using lectins, Hamanoue and Okano [22] isolated NPC and deciphered the roles of N-acetylglucosamine and its enzymes on NPCs functions [23]. These studies should also be done in other animals; for example, in chickens, intracerebral injections of 2-deoxigalactose induce a reversible amnesia which is reverted when co-injected with galactose [24]. Interestingly, in chicken β-amyloid structure and its regulatory mechanism are very similar to humans [25]. These results underscore the important role of the chemical structure of carbohydrates and their functions in the CNS. On the other hand, it is possible to culture neural progenitor cells "*in vitro*" from chick embryos [26] and to study the glycan composition and their carbohydrate effects. If we want to advance into a successful tissue replacement with NSC and NPC, more glycan studies are needed. Taken together all these facts emphasize how important the implication of glycoproteins and their carbohydrates are and how many challenges glycobiology engineering has ahead [24].

Since Yamanaka´s [1] procedure publication, many advances have been made in terms of avoiding tumor formation and efficiency production. To this end, Haus et al. [27] developed a xeno-free culture system starting with hESC and enriched with magnetic sorting cells CD133+/CD34-. In only ten days 300 million of hNSC can be produced and no teratomes were induced in mice.

The objective to repair the neuron, functional cell of the nervous system, has been the priority. Unfortunately, a great amount of studies have evidenced that not only the neurons are affected by the diseases but also the astrocytes, oligomicroglia and even progenitor cells. Moreover, there is evidence in ALS´s patients that oligomicroglia cells degenerate before motor neurons do. Therefore, the regenerative approach probably should include more than one lineage of nervous cells. Others challenges are controlling epigenetic memory and final differentiation of grafted cells.

To end this exposition on *the state of art* of nervous tissue replacement with hopes for neurodegenerative diseases, it should be noted that the results of NSCs derived from human fetuses transplantation into Parkinson's and ALS´s patients, have been more than encouraging [28-32]. In Parkinson´s patients after twenty four years post-implantation of fetal ventral cells from Mesencephalon, in the Putamen, where the cells were injected, there was a complete recovery of the innervation with still functional dopaminergic neurons [29]. Beneficial effects of transplantation included; motor symptoms recovery and withdrawal of the treatment with LDOPA. In fact, the deaths of some patients had nothing to do with the disease [28]. In ALS´s patients, transplantation into spinal cord of NSCs (spinal stem cells from human fetuses) [30] truly have positive results, during the 9 months of the clinical trial the progression of the disease stopped [31]. In ALS, for the first time, a treatment has achieved recovery of symptoms in patients [32].

Future Directions

Nowadays, human embryonic and fetal cells are becoming available in many countries around the world [33-35]. Consensus sets of robust protocols for multiplication and differentiation of NSCs and iPSCs,

good practice codes and standards (FDA's GPR, EU's GMP), disclosed specs for the starting somatic cells, and robust cryopreservation procedures of cells, etc. should be agreed upon as soon as possible. This would guarantee a fruitful outcome for the enormous cooperative efforts being made on this research field.

We must not forget that the detection of abnormal prion proteins with reliable diagnosis test is crucial in the early stages of neurodegenerative diseases. One of the most reasonable strategies would be to reduce the levels of pathological proteins in the patients. This should be done before or concomitant with regenerative tissues therapies. Otherwise, newly implanted cells could be affected by the pathological levels of prion-like proteins in the recipient patients, jeopardizing their recovery.

Undoubtedly, there is still a long way to walk before nervous tissue replacement therapies are optimized and fully implemented in daily clinical practice. The main aim in future studies should focus on which are the key elements for a neural fetal cell transplant to be beneficial and non-rejectable. It is a long way, but the joint efforts from so many labs around the world are getting us closer and closer.

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