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Further remarks on potential implementation of the ESC and iPSC technology in clinical practice

Jarosław Lewandowski and Maciej K Kurpisz*
Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

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***Correspondence:**
Dr. Maciej Kurpisz, MD, PhD
Institute of Human Genetics, Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznan, Poland, Telephone: +48 61 6 579 202/212, E-mail: kurpimac@man.poznan.pl

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ABSTRACT

The first clinical trials subdued optimistic forecasts of the rapid use of iPSC technology across a large spectrum of diseases. However, positive safety results originating from administration of differentiated ESCs to first patients can be treated as the promise to future of this regenerative medicine branch. Advances in non-viral methods for pluripotency induction, including episomal and mRNA strategies as well as microRNA switches robustly purifying heterogeneous differentiated PSCs may accelerate further progress. In the present decade the development of an effective genome editing including CRISPR/Cas9, novel therapeutic approach and intensive research in 3D in vitro culture systems including tissue engineering can further expand the potential of iPSCs. The herein mini-review article extends and updates the current approaches in state-of-the-art techniques of human embryonic stem cell and induced pluripotent stem cell derivation.

Introduction

The iPSC technology, initiated in 2006[1], is still waiting in line for its wide use in clinical practice. In brief, commonly used protocols for pluripotency derivation, described previously[2], are presented in Figure 1. The novel genetic engineering techniques and wide repertoire of molecular biology tools that have been developed in the present decade as well as ongoing first clinical trials give a hope to accelerate the potential lying behind the personalized pluripotent stem cells administration. Examples demonstrated below indicate a wide spectrum of undertaken research.

Advances in safe non-viral iPSC derivation methods

After 10 years of iPSC research, according to the survey carried out by networking site, regmednet.com concerning iPSC production, the most frequent viral method of reprogramming used has been a non-integrative Sendai-virus vector which is two times more popular than genome-integrating lentiviral technique, respectively applied in 45% vs. 27% currently performed studies. For safety reasons and clinical applications non-viral reprogramming is more often used but still less efficient, not well-established, not reproducible, time-consuming while labor-intensive protocols still limit their use. Analysis of iPSC lines obtained this way revealed one more advantage – compared to retroviral vectors there was detected a lower chance for aneuploidy and copy number variation[32]. Popularly conducted now are episomal plasmids (28%) and then both RNA and miRNA transfections (22%).

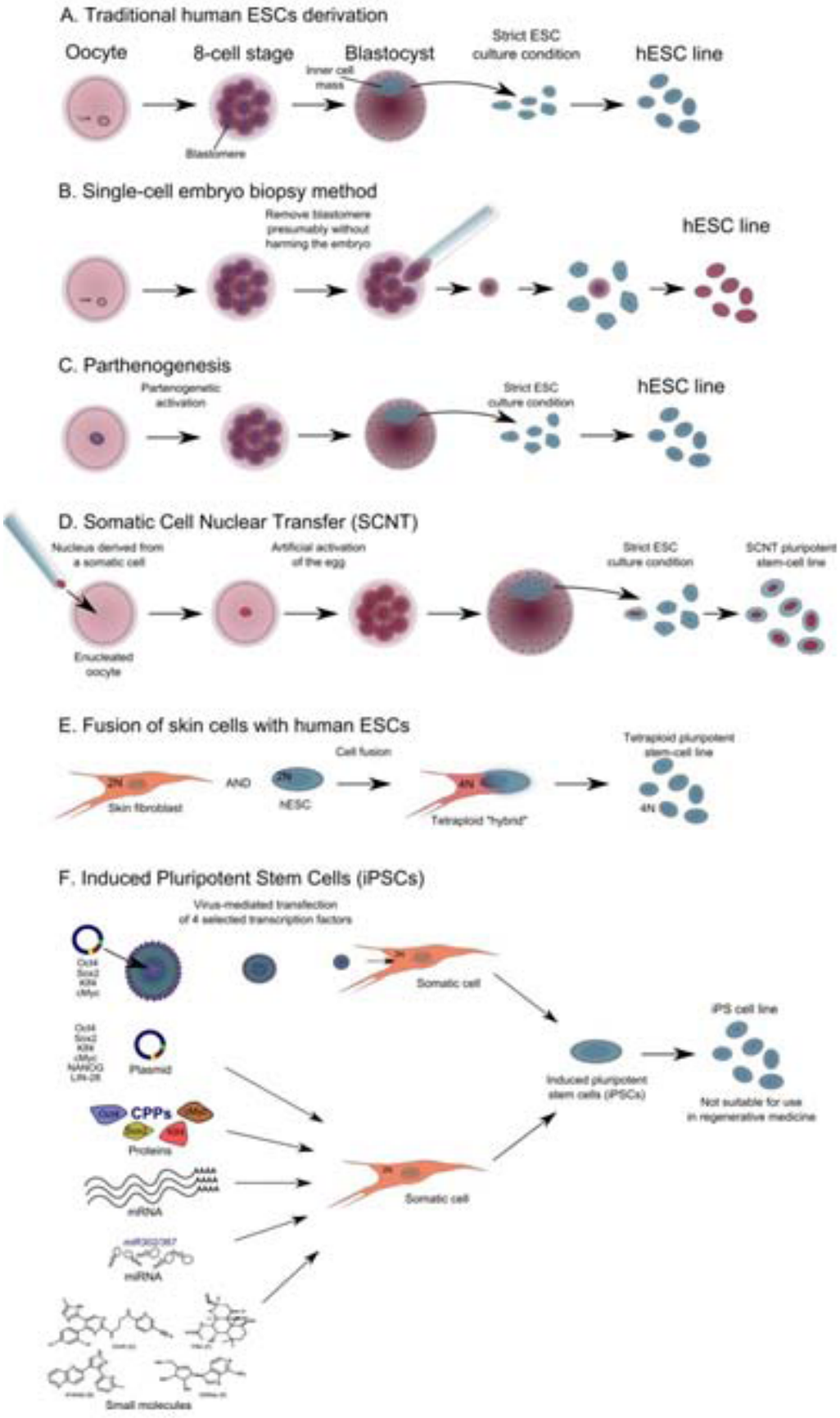

Figure 1. Selected techniques for pluripotent stem cell derivation[2].

Episomal strategy could be an useful alternative with fast vanishing reprogrammed transgenes and GMP compliant (xeno-free and feeder-free) *in vitro* culture. Recently, there was improved integration-free episomal vector (EV) system in order to induce pluripotency by nucleofection of adult peripheral blood (PB). A combination of four factors: OS (*OCT4*, *SOX2*) + M (*cMYC*) + K (*KLF4*) + B (extra anti-apoptotic factor *BCL-XL*) is a competitive solution to Sendai virus efficiency but significantly less costly[33]. Due to the importance of the main factors used, Wen et al. proposed equine rhinitis A virus (E2A) to link *OCT4* and *SOX2* genes with 2A peptides which ensures their equimolar expression. Therefore M and K components were delivered in two individual plasmids. This change improved a 100-fold peripheral blood reprogramming (1,000–3,000 iPSC colonies per 1 × 10^6 cultured cells). The authors omitted also shP53 and SV40 big T protein to reduce a risk of aneuploidies. Additionally, it is worth noting that this is another study highlighting the superiority of peripheral blood cells as the most accessible cell source for genetic reprogramming over commonly used fibroblasts which require longer (2-3 weeks) *in vitro* culture and non-sterile procedure of invasive skin biopsy. Fibroblasts are also more prone to mutations owing to environmental insults like UV light[34].

Derivation of mRNA-iPSCs is characterized by greater efficacy than other non-viral methods. However, this technique is insufficient for successful differentiation of all the cell types, e.g. blood cells[32]. Nevertheless, there has been a progress in developing new robust differentiation protocols, eg. towards retinal lineages (optic vesicle-like structures[35]), based on chemical transfection of synthetic mRNAs[36] inducing pluripotency. Next step is to extend the use and safety of the method and make it more accessible by non-synthetic mRNA development[37]. There was proposed capping 2′-O-methyltransferase enzyme derived from *V. virus* which builds a cap1 structure and yields a higher translation efficiency in shorter time compared to previous cap-analog approaches. Polymeric transfection was not associated with human IFNα or IFNγ release so there is no need to use immune suppression.

Another appearing method in this field is a strategy of chemical reprogramming without using any external vectors but only small molecule compounds of epigenetic function. Low cost, easy to optimize, reversibility without cell permeabilization and non-immunogenic elements are in its favor. However, after first hopeful report about generation of chemically induced iPSC (ciPSC) from mouse embryonic fibroblasts following administration of 7 chemical modulators instead of Yamanaka's master pluripotency factors (OCT4, SOX2, KLF-4, c-MYC) revealed efficiency reaching up to 0.1%[38], for the time being. There was not developed successful protocol for human ciPSCs. Nonetheless, as applied chemical substances act in broad range of epigenetic factors, modulators of metabolism and the modifiers of cell signaling (including wingless and integration site growth factor) open a prospect for generation of small molecule cocktail for human cell purpose which should come soon.

Instead, serious alternative approach of direct cell reprogramming developed recently for neural stem cells (NSCs) has been performed with patient-specific cells. Only three chemical compounds (VPA – an inhibitor of HDACs; C, CHIR99021 – an inhibitor of GSK-3 kinases and R, Repsox – an inhibitor of TGF-β pathways) were sufficient to chemically induce neural precursor cells (ciNSCs) from mouse embryonal fibroblasts (MEFs), mouse tail-tip fibroblasts (TTFs) and human urinary cells (HUCs)[39]. In the process of reprogramming endogenous Sox2 expression was transiently detected in so called intermediate cells, also under hypoxic conditions. Obtained ciNSCs had similar gene expression and differentiation potential into neural cell types as mouse brain-derived NSCs. In subsequent studies, neurobiologists established even a mixture of chemicals compounds to efficiently and directly produce mature functional neurons from dermal fibroblasts[40,41]. These results prove that, in order to acquire desired cell types, trans-differentiation strategies applying extracellular chemical developmental cues and triggering development-related signaling pathways[42] may be competitive for iPSC technology, which still presents a risk of tumorigenicity.

Effective purification of PSC-differentiated cells by microRNA switch approach

Apart from previously established small molecules inhibiting PSC-derived teratoma[43] and applying of an oleate synthesis inhibitor[44], also new techniques appeared for purification of heterogeneous cell population using microRNA switches. This methodology could reduce clonal variety and improve quality of cells differentiated from iPSCs for clinical purposes[45]. The method applies differences in microRNA (miRNA) influence towards cells differentiated from iPSCs and non-target ones. Miki's group designed synthetic mRNAs encoding fluorescent proteins and sites for particular endogenous miRNAs (in 5' UTR) expressed in cells of interest. Specific switches were detected for cardiomyocytes (miR-1-, miR-208a-, and miR-499a-5p-switches) and other cells in respect to their purification. Another element encoding miR-Bim-switch, the apoptosis inducer for non-target cells can be promoted easily enriching differentiated cardiomyocyte population without cell sorting. The purification efficiency was higher than by $SIRPA^+$ or $VCAM1^+$ selection (more than 95% due to miRNA switches compared to range of 60% and 85% of cTNT+ cells). Synthetic modRNA switch (miRNA switch) has ability to selectively induce apoptosis in cells contaminating desired cell population. miRNA effectively

purifies not only cardiomyocytes but also hepatocytes (with miR-122-5p-switch), insulin-producing cells (miR-375-switch) and endothelial cells (miR-126-switch) derived from iPSCs. mRNA degrades after 48 hours from transfection which is also beneficial. This technique may one day become an important part of the cellular material preparation for future therapeutic applications.

iPSCs as a strategy for correcting genetic defects

Some reports have raised the importance of pluripotency induction to bypass genetic abnormalities. Human iPSC and ESC derived skeletal myogenic progenitors originated from Duchenne muscular dystrophy (DMD) patients restored not only dystrophin expression but also improved regeneration and enhanced muscle contractility in dystrophin-deficient mouse, in explanted *in vitro* culture[3]. The procedure may promote potential therapeutic applications in variety of genetic diseases but it requires utilization of safe integration-free transient vectors[4], chemical compounds or other pharmaceutical complements[5] (myogenic progenitors are commonly obtained *via* Pax7-lentiviral overexpression). Moreover, Goudenege et al. developed another protocol for successful myogenic differentiation inducing first PSCs out of DMD patients which resulted in cells forming abundantly muscle fibers (expressing dystrophin) and fusing with the host cells after intramuscular transplantation into immunodeficient mouse[6].

Nevertheless, there is also widespread interest in editing tools for correcting mutations using iPSC lines obtained from individuals with hereditary diseases. Increasingly common genome editing technology with bacterial interspaced short palindromic repeats, CRISPR - associated protein 9 (Cas9)[7], is prone to be efficient alternative for nuclease effectors TALENs and nuclease zinc fingers (ZFNs)[8]. Modification of specific genome location occurs by recognizing target DNA through RNA guide instead of proteins. This procedure may be potentially used in personalized medicine to correct genetic defects of diseased patients at the iPSC stage and, after differentiation towards desired cell type, modified autologous cells could be applied back to recipients.

For the time being, the most attractive iPSC technology concerns patient-tailored disease modelling. CRISPR-CAS9 facilitates knowledge of the disease pathogenesis by reversing genetic alterations in iPSCs (repair of putative causative alleles or knocking in disease alleles to wild type cell line) and further analyzing differentiated cells. There have been reported a gene correction reversing impaired genotype in differentiated cells[9, 10, 11]. In 2015, iPSCs obtained from patients suffering from Hemophilia A were edited by CRISPR/Cas9 nuclease technique to revert large chromosomal rearrangements in F8 gene. As a result, differentiated endothelial cells transplanted to mice revealed functionally rescued factor VIII deficiency[12]. Also iPSCs with sickle cell anemia were edited to correct homozygous missense point mutation affecting adult β-globin proteins[13]. The same strategy may promote a treatment of beta-thalassemia (β-Thal) patients[14]. In turn, addition of the missing exon to DMD-associated iPSC genome resulted in restoration of dystrophin protein. The authors implied that the developed method may be useful in iPSC-based gene therapy for genetic disorders[15]. Yet, there are still unresolved obstacles related to CRISPR/Cas9 technology; uncontrollable side effects (eg. random point mutation), high costs of the procedure, inability to use in genetic diseases with unrecognized mutations or risk variants[16].

iPSC-derived organoids may better represent intercellular interactions

3D organoids are, in turn, a new approach of tissue engineering that may give a chance to make iPSC platform even more profitable cellular resource for stem cell-based therapy. Human iPSC-derived organoids as brain, lung, liver, kidney or intestine have been already well-developed and cell 3D organization may allow investigation of cell-to-cell interactions in physiological conditions. Thus, observations in spatiotemporal context can be conducted at the organ level and not at a level of single cells. This is advantageous in respect to studies in conducted 2-D *in vitro* cell cultures. Currently, 3D structures have been used for disease modeling and developmental processes, however, the therapeutic compounds and cells derivatives can be transplanted[17], e.g. for a treatment of intestine conditions[18]. This useful tool for drug modeling still must be refined in terms of reproducibility and efficiency which are still much worse than in 2D cultures. The development of new standardized techniques (e.g. miniaturized spinning bioreactors promoting forebrain organoid formation[19] and shaking culture platforms for better nutrient supply due to a lack of vascularization system in organoids[20]) gives a hope to fasten research for new drug discovery, development in regenerative medicine and may even significantly reduce the number of required animal models. As a proof of principle, in mice studies, human iPSC-derived liver organoids gave rise to functional human liver-like tissues in transplanted animal recipients[20].

New possibilities for the PSCs in immunotherapy and haemotherapy

The current applications of ESCs and iPSC derived cell types in regenerative medicine, drug screening, disease modeling and basic developmental research may be further extended. Recent immunotherapeutic approaches pave the way to iPSC new applications. Until recently, practical use of T cells derived from pluripotent stem cells encountered

the main obstacle associated with rearrangement of T cell receptor (TCR) that occurs randomly. In fact, there is no possibility to control T cell receptor repertoire[21]. However, the reprogramming of the mature cytotoxic T cells yielded TCRs having specific functional antigen binding with particular epitope[22]. Another approach contributed to an increase of TCR repertoire[23], however, it still extorts autological cellular therapy due to incompatibility reasons. A successful strategy may be to develop effective therapy with iPSC-derived T cells bearing chimeric antigen receptor (CAR) which is meant to be functionally independent from HLA. In first attempts there were applied iPSC lines with inserted CAR targeting the antigen CD19 present in B cells. Differentiated T cells demonstrated successful cytotoxic activity against CD19-positive lymphoma cells. This may provide foundation for future personalized cancer therapy[24].

It is also worth mentioning another iPSCs interest area, namely, red blood cell generation for the need of transfusions. On the one hand, generated erythrocytes should not express antigens triggering immune response but on the other hand blood antigens act at functional and structural roles and they are ligands for many receptors. For optimal protocol many iPSC clones have been established to represent commonly documented red blood cell phenotypes. Kappler-Gratias et al.[25] concluded that 15 iPSC clones would be sufficient for the purpose. Moreover, epigenetic memory of particular cell type, no burden with nuclear and mitochondrial DNA mutations, easy to handle isolation and well characterized cord blood samples collected may prevail in decision that human cord blood-derived CD34+ hematopoietic stem cells may constitute a better initial cell population for genetic reprogramming into red blood cells[26] than iPSC of personalized origin. As a matter of fact, epigenetic factor only marginally improved the expansion and erythroid differentiation. Large-scale cell amplification is still a hurdle and certainly erythroid-specific genes, proliferation-associated transcription factors or modification of growth factors in hematopoietic and erythroid progenitors must be still tested[27]. Nevertheless, in recent years the first protocols for red blood cells[26], lymphocytes B and T[28,29], megakaryocytes[30], NK cells[31], were established which may be a start for potent iPSC-based therapies with circulating blood elements.

PSC clinical trials

There is no credible consensus among scientists and clinicians regarding acceptable quality and safety standards of iPSC-derived cells used for transplantation. Undoubtedly, the proof of the full genetic reprogramming can be detected by genes and protein pluripotency markers, including DNA methylation as well as successful assay for differentiation potential into three germ layers. However, at high cell division rates, the genetic instability may be still a barrier for clinical trials which do not progress beyond I/II phase.

Most clinical trials in the field of regenerative medicine involve adult stem cells as mesenchymal stem cells (374 registered clinical trials with MSCs[46]), mobilized bone marrow cells, umbilical cord blood cells and adipose-derived stem cells. Relating to pluripotent stem cell therapy, in 2016 amongst ongoing clinical trials only 8 concerned ESCs and 1 iPSC-derived cell transplantion[17].

Treatment of eye degenerative diseases seems to be the closest one to be implemented due to readily accessible tissue to be healed, easy differentiation protocol and small number of required retinal epithelial cells[47].

Recently, pioneering research in Japan has proven iPSC differentiation potential in treatment of eye disease. 70-year-old female in September 2014 suffering from exudative age-related macular degeneration (AMD) was treated with sheet of autologous iPSC-derived retinal pigment epithelial cells. At that stage, the primary goal was to assess the safety of the therapy. The procedure seems to meet this requirement. There were no negative effects of transplantation, including potential tumorigenesis. Admittedly, visual acuity has stabilized after the surgery but it could be also the effect of the removal of the neovascular tissue. However, further treatment of the other patients was suspended even before the results were announced. For iPSCs of the next patient a mutation in oncogene was detected by whole-genome sequencing analyses which posed a serious safety issue. It was not revealed whether it occurred during genetic reprogramming or it pre-existed in a source of the cells.

In a new clinical research project launched in 2017, allogenic cell suspensions of retinal pigment epithelial (RPE) cells made from iPSCs of a healthy donor were transplanted to five matched (in terms of the human leukocyte antigens) recipients with the wet type of macular degeneration. The approach was to reduce costs and the time associated with preparing transplantable RPE cells. Perhaps, in future, cellular therapies for more common diseases as acute myocardial infarction, autologous personalized iPSC therapy will be discontinued because of its high cost, and long time required to thorough validation of each reprogrammed cell line. This may be not practical for a large number of patients in an acute phase of the disease.

Banking iPSCs for allogenic therapies may bring down the overall costs and new gene editing tools may contribute to create universally accepted donor cells and induce immunotolerance for transferred cells. Indeed, in Japan Kyoto University's Center there was developed umbilical cord blood-derived iPSC bank. By 2020 collected 75 clinical-grade iPSC lines are to cover an immunological

match for about 80% of Japanese population[48]. The iPSC store from HLA-homozygous donors, which reduces a risk of immune rejection, could be further used for therapeutic purposes (after differentiation of appropriate pluripotent cell line and transplanting iPSCs- derived cells to the recipient having the same HLA haplotype).

Another cell banks in different countries are set up to be used for research in variety of diseases. The largest repository is going to be established in the United States at California Institute For Regenerative Medicine comprising of 9,000 rigorously qualified lines from 3,000 individuals, governed by Cellular Dynamic. In the European Bank for Induced Pluripotent Stem Cells there are approx. 1000 quality-controlled iPSC lines available for generation of differentiated cells. Their delivery and maintenance is compliant with commonly accessible Standard Operations Procedures (SOPs). Both Cellular Dynamics and Lonza (Switzerland) have started already producing GMP iPSC lines for therapeutic purposes.

Now it seems that ESC-oriented cell therapy may be implemented at the earliest for eye, pancreas, neural degenerative disorders, Parkinson's disease, amyotrophic lateral sclerosis (ALS) or spinal cord injury. The private sector is increasingly involved in the development of therapy. Transplantation of embryonic stem cell-derived retinal pigment epithelium to Asian patients with dry age-related macular degeneration or with Stargardt macular dystrophy improved visual acuity and turned out to be safe and devoided of side effects[52]. The similar promising clinical trials take place in USA and Israel[53].

In turn, in France cardiac progenitor cells derived from ESCs are tested in humans. Purified (with immunomagnetic sorting for Isl-1 and SSEA-1) and differentiated cells were delivered to 68-year-old patient with severe heart failure into the infarct area. After 3 months functional heart performance improved, e.g. left ventricular ejection fraction increased and no complications like arrhythmias were reported[54]. Meanwhile ViaCyte (USA) runs the phase II clinical trial with developed ESC-based protocol for pancreatic precursor cells (PEC-01™ cells) encapsulated in selectively porous cell-impermeable membrane which implanted under the skin are expected to mature in functional beta cells. The company reported in 2016 promising preliminary results suggesting that islet cell replacement therapy (VC-01™) could find its application for type 1 diabetes[55].

ESCs are also used for ongoing clinical trials as the source of differentiated cells for cellular therapy of subacute thoracic cervical cord injury[56]. In 2016, Asterias Biotherapeutics presented positive interim efficacy data. 90 days after transplantation of oligodendrocyte progenitors derived from human embryonic stem cells (AST-OPC1) at least one motor level of improvement (in ISNCSCI neurological classification scale) was observed in 5 treated patients with loss of all motor and sensory functions giving them a hope for regaining the ability of daily activities due to reusing their own hands or arms. No serious adverse events proved the safety of the procedure[55].

Distant prospects for a broad use of PSCs in cellular therapy

Subsequent reports indicate an age of the donor as one more factor that must be taken into account to consider iPSCs as a good source for cell-based therapy. Kang et al.[49] showed that accumulated somatic mitochondrial genome mutations in skin and blood cells from older donors remain also after pluripotency transduction. Disturbances in genetic integrity related to age, including both point mutations and larger rearrangements (therein subchromosomal copy number variations[34], exome mutations[50] or lose heterozygosity[43]), may influence metabolic status of iPSCs (mainly respiratory capacity), the immunogenicity of iPSC derivatives[51] and argue for allogeneic iPSC sources from young donors. Also iPSC generation or extended *in vitro* culture *per se* may contribute to further mitochondrial mutations. These issues do not apply in respect to ESCs and despite ethical concerns they are main pluripotent cells used for generating particular cell types in ongoing clinical trials.

The first clinical trials temper optimistic forecasts for the rapid use of iPSC technology across a large spectrum of disease and injury. Namely, satisfactory results on animals have not yet found a clear cut effects after delivery to humans. One of the reasons for these discrepancies may be that transplanting human cells to animal model does not accurately reflect human pathophysiological conditions, e.g. PSC derived human cardiac myoblasts in a regeneration of post-infarcted mouse heart cannot be treated as an adequate model[57]. Besides, an obstacle to overcome the immature phenotype of iPSC-originated cell types has been a big challenge. This may have its implications for malfunctioning, e.g. human PSC-cardiomyocytes are maintained at fetal stage and exhibit underdeveloped morphology and contractile apparatus structure affecting impaired contractile performance[58] while iPSC-beta islet cells abnormally respond to glucose[59]. There is also no clear improvement in applied PSC-derived differentiated cells and adult stem cell therapy due to poor cell retention and survival. Yet unresolved issue is connected with reliable safety confirmation and efficacy of protocols for differentiated cell generation.

Additional co-administering of immunosuppressive and pro-survival agents, cardioprotective factors delivered in exosomes (instead of the cells), tissue engineering problems with tumorigenicity, immunogenicity and

arrythmogenicity (in heart) still must be solved to overcome current obstacles[60]. Certainly, advances in the practical use of new pluripotent stem cell techniques, including successful clinical translation of stem cell therapies, are slower than was expected. Vigorous research in many fields, however, leads us to utilize their powerful potential.

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Conflict of interests statement

The authors declare no conflict of interests.

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