Clinical and ethical use of induced pluripotent stem cells

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Ricevuto il 14 marzo 2018; Accettato il 25 maggio 2018

ABSTRACT

Since its discovery, more than a decade ago, induced pluripotent stem cells (iPS) have had a prominent relevance in the environments of biomedical research and, at the same time, their origin has been related to the search for an ethical alternative to use of the stem cells obtained from internal mass of the human embryo.
In this article we intend to give an overview of its possible applications in the advancement of biomedicine and its relationship with bioethics. From its possible application to regenerate tissues, after proceeding to their differentiation; testing of drugs for different conditions; or their use in models of diseases, among which the neurological ones stand out. Also, its application in obtaining germ cells and human embryos.
The situation of the first clinical trial to regenerate a tissue from the subject's own iPS cells, and the recent allogeneic transplantation in Japan, suggest advances in the clinical translation of these cells. On the other hand, the production of germ cells from iPS cells and the new cells called extended pluripotent stem cells (EPS), obtained by genetic reprogramming through a chemical cocktail, that give rise not only to the tissues of the embryonic layers, but also extra-embryonic, are a new path to making clonation by another route.

RIASSUNTO

Uso clinico ed etico delle cellule pluripotenti indotte.

Fin dalla sua scoperta, per oltre un decennio, cellule staminali pluripotenti indotte (iPS) hanno un'importanza notevole nella ricerca biomedica ambienti e, allo stesso tempo, la sua origine è legata alla ricerca di un'alternativa etica all'utilizzo le cellule staminali ottenute dalla massa interna dell'embrione umano.
In questo articolo diamo una panoramica delle possibili applicazioni nell'avanzamento biomedicina e la loro relazione bioetica. Dalla sua possibile applicazione di rigenerare il tessuto, quindi procedere alla differenziazione; la sperimentazione di farmaci per diversi disturbi; o il suo uso in modelli di malattie, tra cui spiccano quelle neurologiche. Così come la sua applicazione nell'ottenere cellule germinali e embrioni umani.
La situazione del primo studio clinico per rigenerare un tessuto da cellule iPS e proprio trapianto recente del soggetto in Giappone rappresentano passi nella traduzione clinica di queste cellule. Inoltre la produzione di cellule germinali dalle cellule iPS e nuove cellule chiamate cellule staminali pluripotenti estesi (EPS), riprogrammando geneticamente da un cocktail chimico, causando non solo ai tessuti degli strati embrionali, ma extraembrionali anche costituire un nuovo percorso verso la clonazione di un altro itinerario.

Parole chiave: cellule staminali pluripotenti indotte, differenziazione cellulare, riprogrammazione cellulare, cellule staminali totipotenti.

Keywords: induced pluripotent stem cells, cell differentiation, cellular reprogramming, totipotent stem cells.

1. Introduction. What are Stem Cells?

The stem cells are those that possess three properties that make them different from other cells in an organism. First, they are undifferentiated cells, that is, they do not have the phenotypic characteristics of the cells of each tissue of an adult organism. Second, they self-perpetuate, that is, they can divide themselves to produce cells identical to the original, indefinitely. Third, they are capable, under certain natural or experimental physiological conditions, of giving rise to other cells that will produce already differentiated or specialized cell lines for a given function. The stem cells have the possibility of performing an asymmetric division to give rise to a cell that is a replica of the original and another with the ability to differentiate itself [1-3].

The zygote is the first cell of the organism, the one that gives rise to the others, the mother of all cells. When talking about stem cells, we speak of stem cells of embryonic origin and stem cells from specific tissues or adult stem cells. From stem cells, various types of cells have been obtained in the laboratory, from blood cells to neurons, which has led to the possibility of curing, with them, various diseases.

According to their differentiation capability to originate specialized tissues, the stem cells can be:

– totipotent: they give rise to both the entire embryo and extraembryonic tissues. They can originate a complete individual as it happens with the zygote that is implanted in the uterus;

– pluripotent: they give rise to tissues of the three embryonic layers: ectoderm, mesoderm and endoderm. This happens with the internal cell mass of the blastocyst, whose cells can give rise to all types of somatic and germ cells, if they are injected into a blastocyst, but do not originate the trophectoderm. Embryonic stem cells originate from this internal cell mass;

– multipotent: they give rise to single-layer embryonic cell lines. Adult stem cells are often considered multipotent but there is evidence of greater plasticity. Actually, during embryonic development they gradually lose their potential: the multipotent cells come from the pluripotent cells;

– unipotent: they give rise to a cell line: a single type of differentiated cells, because they have lost the plasticity to originate other tissues and have what is called lineage commitment. However, it has been verified that some of them retain their pluripotency [4-5].

There are authors who only speak of totipotentiality and progenitor or precursor cells with different degree of undifferentiation [6-7].

Currently, stem cells are used in a few already proven therapies such as hematological, immune and tumoral diseases [8]; transplant of corneal limbus stem cells to repair ulcers [9]; therapy with mesenchymal stem cells to treat graft-versus-host disease in children [10]; therapy with stem cells derived from allogeneic expanded adipocytes (Cx601) in patients with Crohn's disease and perianal fistulas [11]. In addition, as we recently revealed in a research article, therapies that have not yet been reliably demonstrated are offered, which constitutes an important ethical breach [12].

2. A new kind of pluripotent stem cells: induced pluripotent stem cells (iPSCs)

In 2006, a new technique emerged, having the team of S. Yamanaka as its author. These researchers carried out an experiment in mice that consisted in the reprogramming of somatic cells by means of a group of genes transferred via a retrovirus (lentivirus). After many tests with 24 possible genes that are associated with embryonic stem cells, Yamanaka was left with four: Oct3/4, Sox2, Kfl4 and cMyc, which allowed him to obtain cells with stem characteristics. Because of the obtaining method, they were classified as induced pluripotent stem cells (*induced Pluripotent Stem cells*: iPSCs) [13]. This confirmed that the differentiation process was not irreversible.

This technique was born because of a series of previous investigations, such as the studies in which Gurdon demonstrated the acquisition of pluripotency by cell reprogramming in cloning experiments by nuclear transfer [14], and the production of the Dolly sheep for which Wilmut proved that the cloning could take place in mammals [15]. Secondly, Weintraub's research showed that fibroblasts could be converted into myoblasts by transduction with the *MyoD* gene [16]. The third line of research was the development of embryonic stem cells from mouse by Evans and Martin [17-18]. Smith identified many essential factors for pluripotency [19] and Thompson generated human embryonic stem cells [20]. Pluripotency can be induced by transcription factors or by modulation of key pathways with microRNAs, proteins or small molecules, which has caused drastic changes in the field of research with iPSCs [21].

3. The iPSCs cells and their approach to therapeutic use

Due to the importance of the discovery of the iPSCs, which won the Nobel Prize, it seems appropriate to review their current and future applications. Induced pluripotent stem cells begin their path to therapeutic use. A clear utility of the iPSCs is the possibility of having disease models. [22-27]

Although the greatest concern in the use of iPSCs in future therapies is the possibility of forming teratomas, there is no doubt that, by the Yamanaka method, iPSCs can be obtained from practically any patient. These cell lines are very valuable to try new therapies. That is why the industry invests more and more in this technology [28]. In a combination of reprogramming without the cMyc transgene and the enzymatic dissociation of the residual iPSCs, beta-pancreatic cells have been obtained without producing tumors when transplanted [29].

Since Yamanaka and his colleagues discovered the iPSCs, there were researchers who argued that they were not equivalent to the cells of embryonic origin and that these are the most appropriate for the development of research and new therapies. However, a work that compares both types of cells concludes that they are almost equal and functionally indistinguishable and that, if there are some genetic variations, these are due to the original cells of the skin, from which the iPSCs were obtai-

ned and not to the reprogramming process [30]. Researchers identified 49 genes whose activity differs from embryonic stem cells and iPSCs. Then they evaluated two of them associated with the absorption and digestion of glucose. The result was that iPSCs are as efficient as the embryonic ones and functionally equivalent in terms of the activity of these genes [31]. Previously, the production of live and fertile animals by tetraploid complementation from iPSCs cells had demonstrated their pluripotency [32]. It has also been verified that the iPSCs fulfill what has been called "*gold standard*", that is: when transplanted to a mouse embryo in the gastrula phase, they are incorporated without difficulty and develop normally without producing tumors [33].

A study has been conducted to find out if iPSCs accumulate mutations when they are grown in the laboratory and, as a result, could cause cancer. For this reason, it would be unethical to use them until this risk is eliminated. To do this, the mutation rate between blood cells and iPSCs originating from these same cells was compared. The result was that the mutation rate of the iPSCs was 10 times lower than that of the blood cells. It is important to note that none of the mutations occurred in genes related to cancer. With this work, a better understanding of the process of mutation of somatic cells and cancer disease can be reached [34]. According to a study, it is necessary to monitor mutations in the mitochondrial DNA (mtDNA) of the iPSCs, especially if they come from the elderly because they could affect the therapeutic value of these cells [35].

4. Experimental therapeutic applications

In disorders ranging from the loss of dopaminergic neurons in Parkinson's disease, to hematopoietic stem cells in aplastic anemia or beta cell in type I diabetes [36], as well as the need to restore endothelial function in patients with vascular disease, among other pathologies, the differentiation of iPSCs towards cells of the tissue destroyed by the disease, would theoretically be a valid application of this technique [37-39]. Obtaining iPSCs also opens new perspectives for basic research and drug discovery for hereditary skeletal muscle diseases, since skeletal myocytes have been obtained from iPSCs that are electrophysiologically and structurally equivalent to their embryonic counterparts [40]. Subsequent works related to bioengineering show the possibility of manufacturing tissues and re-cellularize acellular structures of hearts subjected to a washing process with detergents, to which cardiac muscle cells from the patient are added, obtained from iPSCs [41].

A study with neurons obtained from iPSCs of patients helps to understand the cause of a certain type of hereditary dementia that represents 50% of the cases of dementia of people under 60 years old and affects the cortical neurons of the frontal and temporal lobes of the brain. The investigation served to determine that the Wnt signaling path is defective [42]. It has also been possible to obtain serotonin-producing neurons from iPSCs. This is important because this type of substances intervene in psychiatric disorders such as depression, bipolar disorder or schizophrenia, in addi-

tion to regulating appetite, pulse, breathing, sleep, anxiety and emotions [43].

In people with a weakened immune system, T cells can undergo genetic reprogramming and thus transform them into iPSCs. Subsequently, these iPSCs can be differentiated again into T cells, with long-life characteristics and maintaining their capacity to recognize pathogens. This is of singular importance in viral diseases such as HIV and also in some cancers [44]. It would be legitimate to use all these applications in case their safety and efficacy are proven.

5. Drug development

The generation of induced pluripotent stem cells offers an interesting alternative for its use in the development of drugs. These cells with unlimited proliferation capacity in undifferentiated state remain genetically stable. Under suitable growing conditions, they can be directed towards a variety of types of the different germ layers. In this manner, clones of iPSCs that will give rise to specific cells in which to test new drugs can be obtained from somatic cells of a patient [45-46].

The first studies have focused on four types of cells: cardiomyocytes, hepatocytes, neurons and pancreatic islet beta cells. Development biologists have studied these types and much is known about the molecular and biochemical signals that lead to their differentiation *in vivo*. The human cells of these tissues are particularly difficult to grow and expensive to obtain and in limited quantity as well. For companies dedicated to obtaining new drugs, these cells are vital since many drugs are not being developed due to the presence of cardiotoxicity or hepatotoxicity. These studies are also very important because neurodegenerative diseases and diabetes are an increasing cause of morbidity and mortality.

The development of new drugs is a costly and slow process in which 90 percent of drugs are not approved after a clinical trial, due to efficacy or safety issues. Preclinical studies are limited by available cell lines or animal models and functional trials relevant to the disease are lacking. That is why iPSCs have many advantages over traditional methods. Several experimental models of diseases obtained from iPSCs have shown improvement in the phenotype in response to therapeutic agents. From these models, a more sensitive and accurate evaluation of the compounds under test can be provided. This has been done in dopaminergic neurons derived from iPSCs with substances of neuroprotective properties, as a treatment strategy for Parkinson's in its early stages. By this procedure, therapies are being evaluated for diseases of the central nervous system [47-48].

To identify the genetic bases of hypertension and the responses to drugs, pharmacogenomics uses biological models. The iPSCs of hypertensive patients provide the possibility of having smooth muscle cells and better knowing the response to drugs [49]. In the case of bipolar psychiatric disorder, which is characterized by phases of mania and depression, lithium acts as a stabilizer, but is not effective in all patients. With the iPSC technique, neurons of the dentate gyrus of the hippocampus were produced, both from patients who responded to treatment with lithium and

from patients for whom lithium was not effective. Mitochondrial abnormalities and hyperexcitability were discovered, which was only reversed by lithium in those neurons from patients who had reacted favorably to lithium treatment. This leads to the conclusion that hyperexcitability is an early endophenotype of bipolar disorder and that models with iPSCs can be useful for the discovery of new therapies [50].

A disease for which an *in vitro* model has been produced by means of human iPSCs is the Jervell and Lange-Nielsen syndrome, which produces a serious heart rhythm disorder and can lead to sudden death in young patients. The cause has its origin in homozygous mutations of recessive genes. This leads to cardiomyocytes presenting electrophysiological defects. With this model, we can better understand the mechanisms of recessive inheritance and, at the same time, check the effectiveness of certain medications [51].

For a better understanding of the pathophysiology of Amyotrophic Lateral Sclerosis (ALS), motor neurons were obtained from iPSCs and the effectiveness of a drug was verified by verifying an improvement in the activity and excitability of these neurons when administering it [52]. From iPSCs of glaucoma patients, it has been possible to obtain retinal ganglion cells, which provides a model for this disease and facilitates the search for drugs for this pathology [53]. From the ethical point of view, the new drug cannot be prescribed until its verification as a safe and effective drug.

6. First clinical trial

The first clinical trial with iPSCs began in Japan to treat patients with macular degeneration due to age [54; 55, p. 17]. Masayo Takahashi, of the Riken Center for Developmental Biology in Kobe, Japan, used retinal cells derived from iPSCs obtained from the patient's skin in a first experiment in a Japanese woman of 70 years, hoping that they do not produce rejection [56-57]. Later this trial was suspended due to the detection of mutations produced, apparently, in the cell reprogramming process. The worry of uncontrolled cell growth with possible tumors led to this decision [58-59]. It would be unethical to expose a person to a serious risk. Recently, the first allogeneic transplant was reported with cells derived from iPSCs from a donor to treat macular degeneration [60].

7. Experimental models of neurological diseases based on iPSCs

Because of its importance and the difficulty of obtaining cell lines for the study of these pathologies, the advances in the use of iPSCs in experimental models of neurological pathologies and their applications in cell therapy are discussed below. Part of what is discussed below is based on the topics analyzed in a review work by Okano and Yamanaka [61-62]. Ethics also leads us to look for alternatives that avoid harm to humans when investigating various pathologies.

The possibility of obtaining iPSCs opens a new panorama to count on experimental models of neurological pathologies,

since it is possible to derive from them cells with the genotype of the disease. With these models, we can learn more about the beginning and development of the pathology, as well as try new drugs in the cells from the patient [63].

IPSCs have been obtained from endothelial cells of the umbilical cord vein, from which cells of the nervous system lineage are derived with high efficiency and which present the morphology and physiology of neurons, astrocytes and glial cells. With them, we can study the development of the nervous system as well as the pathophysiology of various neurodegenerative diseases and possible new medications [64]. The advantage of this procedure is that it is not invasive and decreases the probability of mutations compared to other cells, such as fibroblasts, resulting from aging and exposure to UV rays. It also has a high reprogramming efficiency and fast kinetics [65].

In people with neurodegenerative diseases, it is difficult to access affected sites and animal models do not necessarily reflect human pathology. Biological or biochemical changes have been known from post-mortem brain analysis. With the development of iPSCs, it is possible to obtain pluripotent stem cells from somatic cells and thereby reproduce *ex vivo* the phenomena that occur in *in vivo* patients, particularly disorders of the nervous system and to better understand their pathophysiology [66]. This technology has begun to study various neurological diseases such as, among others, amyotrophic lateral sclerosis, [67-69] spinal muscular atrophy, [70] Friedreich's ataxia, [71] Alzheimer's, [72] Parkinson's, [73] Huntington's disease, [74] fragile X syndrome, [75] adrenoleukodystrophy [76] and schizophrenia [77-78]. The safety, sensitivity and toxicity tests of new medicines can be accelerated by this technique.

7.1 Modeling Parkinson's disease with neurons obtained from iPSCs

The dopaminergic cells derived from the iPSCs, obtained from cells of patients with Parkinson's, could serve as models of the disease to investigate the changes that occur in time since the beginning of the pathology. Recently, it has been possible to obtain a model of family Parkinson's generally severe with these cells [79]. In neurons obtained from iPSCs of a certain type of Parkinson's disease, the existence of these alterations was confirmed, as well as the accumulation of alpha-synuclein, as was found in the analysis of corpses of patients [80].

The main risk factor for Parkinson's disease is the heterozygous mutation of the glucocerebrosidase gene that encodes the lysosomal enzyme. In a research work with brain cells obtained from iPSCs from the skin of Parkinson's patients with the genetic mutation GBAN370S, a link is established between the GBAN370S mutation and the accumulation of alpha-synuclein [81]. It has been possible to characterize the dopaminergic neurons of the mesencephalon, from the differentiation of human iPSCs from a Parkinson's patient, proving their physiological function in the synthesis, release and reuptake of dopamine. This constitutes a magnificent model for the study of the disease [82]. The monogenic

forms of Parkinson's are particularly interesting, because they are very similar to the most common form of this pathology. They also facilitate research through neurons derived from iPSCs [83]. By inducing monogenic mutations in iPSCs derived from healthy subjects, by means of genome-editing technologies (using adenoviral vectors), it is possible to accurately analyze the pathogenic mechanisms attributable to a single gene.

7.2 Demonstration of genotype-phenotype causal relationships

Because of the discovery of last generation sequencers following the sequencing of the human genome, many mutations related to some pathology and polymorphism of a single nucleotide have been identified. In most diseases, there is no formal proof about a causal relationship between the genetic mutation and the disease phenotype. This can be verified by genome-editing technologies such as nucleases associated with short, grouped and regularly interspersed palindromic repeats (CRISPR) [84-90]. These techniques can be used for the realization of experiments in which the genetic defect is corrected or the introduction of disease-related mutations in iPSCs control [91]. More recently, we have discovered the way to edit a single base, both in DNA and in RNA [92]. This could reveal genotype-phenotype causal relationships. At the moment, it only applies to monogenetic diseases [93-94].

8. Harmonize standards and protocols

Because of its characteristics of pluripotency, the management of these cells entails greater complexity because they must undergo extensive growth processes, and a long process of differentiation to generate the desired phenotypes and eliminate the defective ones, in addition to residual pluripotent cells; for that reason various authors, including Ian Wilmut and Shinya Yamanaka, request to harmonize the norms to produce clinical therapies from pluripotent stem cells [95]. Although the biochemical and biological techniques for reprogramming are already established, there are new bioengineering instruments for reprogramming, isolation, differentiation [96] and the expansion of iPSCs [97]. The differentiation protocols to get the right cell from the iPSCs lead to obtain a heterogeneous population. The selection of the appropriate cell type is made, up to now, with the surface antigens, but it is not always effective, so a new, more efficient biotechnology has been sought using microRNA switches that consist of synthetic RNA sequences [98]. Good Manufacturing practices (GMP) to obtain iPSCs are currently necessary to ensure compliance with international regulations regarding tissue supply, manufacturing, testing and storage [99]. This recommendation improves safety, which it is a necessary condition for a procedure to be ethical.

9. An ethical way of doing research that can be misused: iPSCs and EPS

In order that an investigation adheres to ethics, it must first be scientifically valid.

In the previous discussion, we observe the seriousness and good practices of the discoverers of this type of cells and how they have been obtaining procedures that involve advances in biomedicine to cure various pathologies.

The origin of the iPSCs has an ethical basis, as Gámez [100] and Aznar [101-102] state, since it is about avoiding the use of *left over* embryos from *in vitro* fertilization. In addition, the discoverers take into consideration the previous scientific theories and open the possibility of experimental cell de-differentiation. Pre-clinical studies in animals and clinical trials should be done with due respect and considering the absolute dignity of the human person. Yamanaka acts with these criteria and tries to extrapolate, as far as possible, the data obtained with animals to the human being, avoiding the use of cells of embryonic origin. Only as a last resort, and starting from existing cell lines, he does approve the comparison of iPSCs with embryonic cells. On the other hand, he warns about misuse of his discovery to obtain oocytes and sperm that may give rise to human embryos.

Despite having a clear biological utility, the destruction of human embryos to obtain cells from the internal mass of the embryo ethically invalidates, in our opinion, any use of cells of this origin. Therefore, iPSCs are an excellent ethical alternative to the use of embryonic stem cells, especially for clinical purposes. The development of new breeding techniques could avoid disadvantages such as teratogenic capacity and certain genetic anomalies.

However, the possibility of producing germ cells from iPSCs and, with them, living embryos opens a new ethical dilemma [103-106]. In recent years it has been reported that it would be possible to manufacture primordial human germ cells, precursors of ovules and spermatozoa [107-109]. It is not justifiable to obtain human beings with this procedure, nor would it be to use the technique of cell reprogramming to obtain totipotent cells, from which generate human blastocysts. In this line, apparently, some researchers are working, as stated in a publication about the so-called *extended pluripotent stem cells* (EPS) that could give rise not only to any tissue of the three embryonic layers, but also extraembryonic [110]. It would be something like cloning by nuclear transfer and, therefore, ethically unjustifiable. «During embryonic development, both the fertilized ovum and its initial cells are considered totipotent, since they can give rise to all the embryonic and extra embryonic lineages. However, the capture of stem cells with such in vitro development potential has been a major challenge in stem cell biology» says Professor Izpisua Belmonte, «this is the first study that reports the derivation of a stable type of stem cells that shows a potential for biological development similar to totipotentiality for both embryonic and extra-human lineages» [111]. After the fertilization of the ovule, in very early stages, specialization takes place either towards embryonic tissues, or extra-embryonic, so that until now it is not possible to maintain the possibility of obtaining both types of tissues. The cocktail discovered by these researchers allows the cells to remain with that potentiality. Teams of the Salk Institute and Peking University found that, by combining four chemical compounds and a growth factor and applying this cocktail,

the pluripotent cells could move to a more immature state and this facilitates the formation of human-animal chimeras to generate transgenic animals, as well as obtaining organs for transplant, which leads to another ethical problem [112].

On the contrary, the possibility of generating gametes obtained by genetic modification of somatic cells of people with some inherited disease to give rise to healthy children could be admitted. If *in vitro* fertilization is used, it would entail the ethical problems of this technique.

In conclusion, the initial idea that iPSCs would avoid the use of embryonic cells has not yet been consolidated and the possibility of using iPSCs to obtain human gametes and embryos has arisen, which overshadows their impeccable starting point. Basically, this happens in many scientific advances: its suitability also depends on the purposes for which it is used. In short, it will not be enough to provide norms for the proper use of technology with iPSCs, but it will be necessary to promote the ethical training of people and institutions so that they can use it correctly.

Acknowledgment

My gratitude to Doctors José Luis Pérez Requejo, Justo Aznar Lucea and Damián García Olmo for their guidance and advice in this work.

I would also like to thank Drs. César Gutiérrez, Hans Vasquez and Pedro E. Romero for their valuable suggestions in the revision of the manuscript.

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