Chapter 24

Induced Pluripotent Stem Cells: Therapeutic Applications in Monogenic and Metabolic Diseases, and Regulatory and Bioethical Considerations

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Additional information is available at the end of the chapter

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1. Introduction

The potential use of stem cells in advanced therapies such as tissue engineering, regenerative medicine, cell therapy and gene therapy by virtue of their significant therapeutic potential and clinical applications has aroused keen interest among scientists [1,2]. Cell therapy is based on the transplantation of living cells into an organism with a view to repairing tissue or restoring a lost or deficient function. Stem cells are the most frequently used cells for such purposes given their ability to differentiate into other more specialized cells [3].

The chief defining feature of stem cells is their capacity for self-renewal and their ability to differentiate into cells of various lineages. Stem cells can be classified on the basis of their potency and their source into (i) Totipotent stem cells (zygote and 2-4 cell embryo), since these cells are capable of giving rise to the entire organism (both embryonic and extra-embryonic tissues); (ii) Pluripotent stem cells (embryonic stem and embryonic germ cells), which can give rise to derivatives of all three germ layers (embryonic tissues only, but not the extra-embryonic ones); (iii) Multipotent stem cells (adult stem cells) [4,5].

Adult stem cells are undifferentiated cells that provide a natural reservoir that is available to replace damaged or ageing cells throughout the lifetime of the individual. They can be found in virtually any kind of tissue including bone marrow, trabecular bone, periosteum, synovium, muscle, adipose tissue, breast gland, gastrointestinal tract, central nervous system, lung, peripheral blood, dermis, hair follicle, corneal limbus, etc. [6]. The clinical application of this type of cell is associated with potentially better prospects than that of embryonic stem cells since use of adult stem cells does not raise any ethical conflicts nor does it involve immune rejection problems in the event of autologous implantation.

The possibility to generate induced pluripotent stem cells (iPSCs) by reprogramming somatic stem cells through the introduction of certain transcription factors [7-12] is radically transforming received scientific wisdom. The pluripotency of these cells, which enables them to differentiate into cells of all three germ layers (endoderm, mesoderm, and ectoderm), makes them an extremely valuable tool for the potential design of cell therapy protocols. iPSC technology can indeed allow the development of patient-specific cell therapy protocols [13] as the use of cells like iPSCs, which are genetically identical to the donor, may protect the individual from immune rejection. Furthermore, unlike embryonic stem cells, iPSCs are not associated with bioethical problems and are considered a "consensus" alternative that does not require use of human oocytes or embryos and is therefore not subject to any specific regulations. Lastly, iPSCs are very similar to embryonic stem cells as far as their molecular and functional characteristics are concerned [14-15].

Although research into iPSCs is still at an early stage, interesting results have already been obtained in a number of monogenic and polygenic diseases of different etiologies: cardiovascular and liver diseases, immunologic, infectious, metabolic diseases, rare diseases and cancer [16-19]. Researchers have also looked into the application of iPSCs to toxigological and pharmacological screening for the presence of toxic and teratogenic substances [20].

Stem cell therapy is emerging as a new concept of medical application in pharmacology. For all practical purposes, human embryonic stem cells are used in 13% of treatments, whereas fetal stem cells are used in 2%, umbilical cord stem cells in 10%, and adult stem cells in 75% of cases. The most significant treatment indications for gene and cell therapy have so far been cardiovascular and ischemic diseases, diabetes, hematopoietic diseases, liver diseases and, more recently, orthopaedics [21]. For example, over 25,000 transplants of hematopoietic stem cells are performed every year for treatment of lymphoma, leukemia, immunodeficiency disorders, congenital metabolic defects, hemoglobinopathies, and myelodysplastic and myeloproliferative syndromes [22].

Each type of stem cell has its own advantages and disadvantages, which vary depending on the different treatment protocols and the requirements of each clinical condition. Thus, embryonic stem cells have the advantages of being pluripotent, easy to isolate and highly productive in culture, in addition to showing a high capacity to integrate into fetal tissue during development. By contrast, their disadvantages include immune rejection and the possibility that they may spontaneously and uncontrollably differentiate into inadequate cell types or even induce tumors. Adult stem cells have a high differentiation potential, are less likely to induce an undesirable immune response and may be stimulated by drugs. Their disadvantages include that they are scarce and difficult to harvest, grown slowly, differentiate poorly in culture and are difficult to handle and produce in adequate amounts for transplantation. In addition, they behave differently depending on the source tissue, show telomere shortening, and may carry the genetic abnormalities inherited or acquired by the donor.

At least three different strategies are available for proper use of stem cells. The first one is stimulation of endogenous stem cells by growth factors, cytokines, and second messengers, which must be able to induce tissue self-repair. The second alternative is direct administration of the cells so that they differentiate at the damaged or non-functional tissue sites. The third

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possibility is transplantation of cells, tissues, or organs taken from cultures of stem cell-derived differentiated cells. The US Food and Drug Administration defines somatic cell therapy as the administration to humans of autologous, allogeneic or xenogeneic living non-germline cells, other than transfusion blood products, which have been manipulated, processed, propagated or expanded *ex vivo*, or are drug-treated.

The most significant applications of cell therapy as a whole are expected to be related to the treatment of organ-specific conditions such as diabetes —a typically metabolic disease—, liver and cardiovascular conditions, immunological disorders and hereditary monogenic diseases such as haemophilia. As one of the key advanced therapies —together with gene therapy and tissue engineering— cell therapy will require a new legal framework that affords generalized patient accessibility to these products and that allows governments to discharge their regulatory and control duties. In this respect, the main advantage of iPSCs lies in the fact that their use does not raise bioethical questions, which means that regulatory provisions governing their use need not be overly stringent.

2. Induced pluripotent stem cells technology and general clinical applications

iPSCs are obtained through the reprogramming of an individual's somatic stem cells by the introduction of certain transcription factors. Their chief value is based on their pluripotency to differentiate into cells of all three germ layers, which makes them an useful tool for the discovery of new drugs and the establishment of cell therapy programs.

iPSC technology makes it possible to develop patient-specific cell therapy protocols as they are genetically identical to the donor and thus prevent the occurrence of an immune rejection in autologous transplantations. Moreover, unlike embryonic stem cells, they are not associated with any ethical controversies and therefore regulatory conditions governing their use are much less stringent.

Induced pluripotent stem cells were generated for the first time by Shinya Yamanaka's team [8] from murine and human fibroblasts by transfecting certain transcription factors (Oct4, Sox2, c-Myc, and Klf4) by means of retroviral vectors. (Figure 1). Thomson *et al*. replicated Yamanaka's experiments with human cells and two additional factors: Nanog and Lin28, which rendered the reprogramming process more efficient [9].

The same group developed an alternative reprogramming method using non-integrating episomal vectors derived from the Epstein-Barr virus (oriP/EBNA1), which may be maintained in a stable form in transfected cells by pharmacological selection [23]. Nonetheless, it was later reported that only two transcription factors (Oct4 and Klf4) are needed for generating the iPSCs from neural stem cells that endogenously express high Sox2 concentrations [24].

All of these strategies require transfection through retroviral vectors and integration for *in vitro* and *in vivo* modeling, which precludes their clinical use because of the potential risks involved. This is the reason why several research teams have looked into the reprogramming

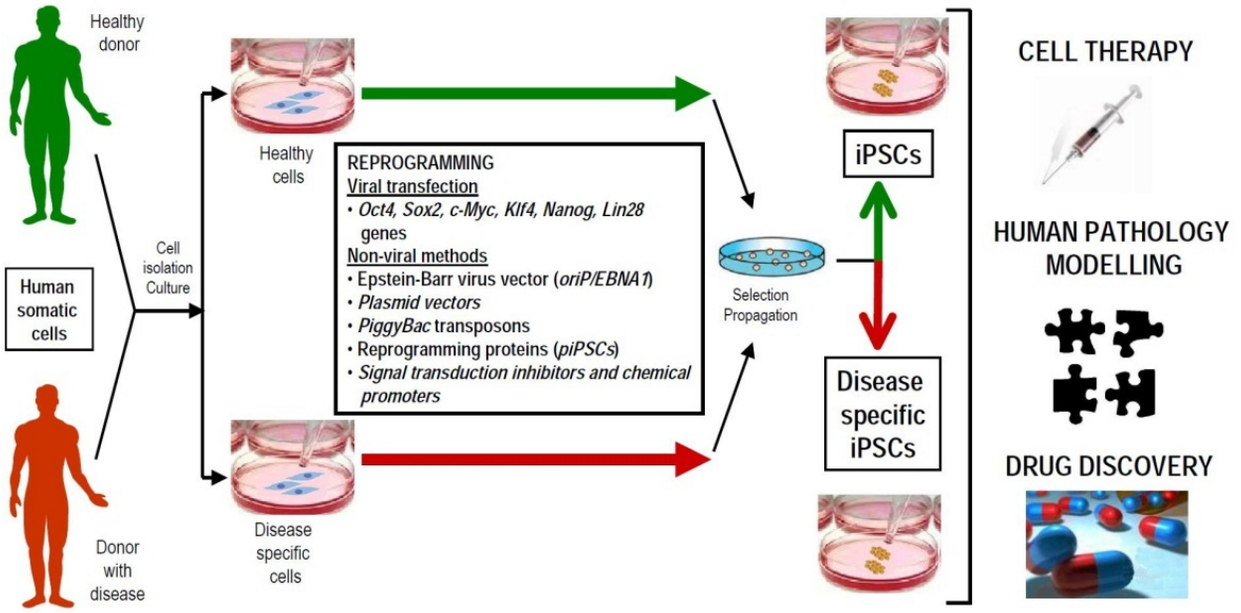

Figure 1. Generation of human induced pluripotent stem cells for use in cell therapy, in vitro human pathology modelling and in drug discovery. Reprogramming of human somatic cells can be induced by: Viral transfection of Oct4, Sox2, c-Myc, Klf4, Nanog and Lin28 genes; non-viral methods using a nonintegrating episomal vector derived from Epstein-Barr virus (oriP/EBNA1), plasmid vectors or piggyBac transposon/transposase systems; direct delivery of the reprogramming proteins (piPSCs) and signal transduction inhibitors and chemical promoters cell survival.

of cells using plasmid vector rather than viral vector transfection [10-12]. Although reprogramming efficacy with plasmid vectors is lower —as is also the case with non viral gene therapy— this method significantly increases the safety of the procedure, which makes it clinically applicable and also constitutes a source of valuable cell material that can be used for research into reprogramming and pluripotency.

Another promising strategy consists in the direct release of reprogramming proteins through modified versions of reprogramming factors in some of their molecular domains. These protein-induced pluripotent stem cells (piPSCs) bind to the membrane of cells reaching their nucleus [25]. Ding *et al.*, have also shown that the addition of two signal transduction inhibitors and certain cell-survival promoting chemicals (e.g. thiazovivin) can induce a 200-fold increase in reprogramming efficacy [26].

As explained above, iPSCs technology makes it possible to establish patient-specific cell therapy protocols [13]. On the one hand, this reduces the risk of immune rejection in autologous transplantations by virtue of gene identity. On the other, it provides treatment that is customized to the specific characteristics of each patient and takes into account the etiology and severity of the condition. Moreover, induction of pluripotency has been developed for a great variety of tissue types [9,24,27] as it is a relatively straightforward procedure and —as mentioned above— subject to fewer regulatory constraints [28].

Important as these advantages are, there are still a few uncertainties that need to be resolved. One of the most pressing ones is related to determining the likelihood that these iPSCs may undergo genetic aberrations further to the reprogramming process [29].

In order for the clinical application of these cells to become a reality both for diagnostic purposes and for the design of cell therapy protocols, a few methodological hurdles must still be resolved in connection, as is often the case with pharmacological products, with their safety profile [30]. This means basically that efforts must be directed at removing the genome in the integrating viral vectors, eliminating the risk of tumor formation and establishing more efficient reprogramming and differentiation protocols. Clearly our knowledge on the reprogramming mechanisms leading to pluripotency are still insufficient to understand and more importantly control the adverse events that could potentially occur. Therefore the most important goal for research in this field will be to study genetic modifications in animal models by means of large-scale genome sequencing programs. This task will require sharing cell lines with other researchers, with appropriate confidentiality protections and, eventually, patenting scientific discoveries and developing commercial tests and therapies. It will also be necessary to fully ascertain and confirm that pluripotency confers iPSCs with functions similar to those of embryonic stem cells regardless of the initial source of somatic cells used [14,15].

Undoubtedly, the most attractive application of this type of strategy is the production of patient-specific or healthy individual-specific iPSCs for replacement of damaged non-functional tissue. Thus for example skin fibroblast-derived iPSCs have been shown to possess a high potential to differentiate into islet-like clusters and to release insulin, which is highly relevant for diabetes [16]. Such developments are also relevant for amyotrophic lateral sclerosis (Lou Gehrig´s disease) [17]; adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Parkinson disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus and Down syndrome (trisomy 21) [31]; spinal muscular atrophy [19]; and in toxicology and pharmacology for screening toxics for embryo and/or teratogenic substances [20].

The great promise of iPSCs (Figure 1) is associated to their role in the investigation of the phyisiological mechanisms related with the biology of stem cells themselves; in the modeling of different pathologies; and, fundamentally, in the development of therapies for human diseases and in drug screening. In fact, since they were discovered in 2008, almost one-hundred-and-fifty iPSCs have been established from nearly thirty fibroblast cell lines related to over a dozen conditions, including some complex diseases such as schizophrenia and autism and other genetic or acquired disorders such as cardiovascular or infectious diseases. Numerous types of functional cells have already been derived from iPSCs including neurons [17,32], hematopoietic cells [33], and cardiomyocytes [34,35].

Taking into account the far-from-trivial fact that iPSCs can be obtained from individuals affected by a disease and that they are indefinitely self-renewable and fully of human origin, it could well be that these cells, obtained from several individuals suffering from the same disease and presenting with similar clinical manifestations, may provide highly valuable information about certain predisposing genes —as in the case of diabetes mellitus— and therefore allow physicians to provide well-grounded genetic guidance.

Human iPSCs have the potential to be used in regenerative medicine for the design of individualized therapies and also in the field of research and development. However, it is still necessary to optimize iPSC protocols, particularly with respect to the possible modifications

to their genome, and to increase the efficacy of the transfection process leading to iPSC reprogramming [36,37]. The present state of the art of reprogramming mechanisms —viral transfection of Oct4, Sox2, c-Myc, Klf4, Nanog and Lin28 genes; non-viral transfection using a non-integrating episomal vector derived from the Epstein-Barr virus (oriP/EBNA1), plasmid vectors or piggyback transposon/transposase systems; direct delivery of the reprogramming proteins (piPSCs); and signal transduction inhibitors and chemical promoter cell survival— will allow safe integration and the removal of ectopic transgenes, improving the efficiency of iPSC production using a minimally invasive strategy.

3. Advanced therapies for monogenic and metabolic diseases

The progression of the different areas of biology, biotechnology and medicine leads to the development of highly innovative new treatments and pharmacological products. In this regard, advanced therapies based on the by-products of gene therapy, cell therapy and nanomedicine/tissue engineering are of great importance for their potential to radically improve treatment of a large number of conditions. The different schools of thought that advocate the emerging concept of advanced therapies agree that the latter must be used for the treatment of diseases (both hereditary and non-transmissible) caused by the anomalous behavior, or complete lack of function, of a single gene (also called monogenic hereditary diseases) or by an anomaly in several genes (polygenic diseases).

Metabolic diseases, or congenital metabolic errors, are conditions highly amenable to be treated by the new advanced therapies as such treatments have been shown to restore mutation-induced alterations of gene products. Proteins are the most commonly affected gene products, although messenger RNA is also a usual victim. Alterations affect gene products, i.e. proteins, most of which are enzymes but there is also a group of other proteins fulfilling all kinds of different functions (structural proteins, transport proteins and signal cascade activation proteins). Of particular interest are the proteins that participate in homeostasis and exert their functions outside the cells that synthesize them. This is the case of coagulation factors VIII and IX (FVIII and FIX), whose deficiency results in the development of haemophilia A or B, respectively. Another member of this class of proteins is antitrypsin, also of hepatic origin and secreted into the bloodstream, whose function is to prevent the digestion of pulmonary alveoli by proteolytic enzymes. Lastly, mention should be made of proteins with such diverse functions as transcription factors, oncogenes, tumor-suppressing genes and even some hormones and their receptors, the latter being specifically related with diabetes mellitus, a typically metabolic disease.

The nature of the monogenic or metabolic disease is the main factor that determines whether a treatment that can eradicate or at least mitigate its clinical consequences is possible or not. Before the concept of advanced therapies came to be applied to these (wide ranging) conditions, many of them were treated using both conventional/classical and more advanced approaches.

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Advanced therapies are applied following three basic approaches: replacement of a deficient gene by a healthy gene so that it generates a certain functional, structural or transport protein (gene therapy); incorporation of a full array of healthy genes and proteins through perfusion or transplantation of healthy cells (cell therapy); or tissue transplantation and formation of healthy organs (tissue engineering). In this context, induced pluripotent stem cells can play a very significant role and hold an enormous therapeutic potential in the fields of cell therapy and tissue engineering.

4. Advanced therapies and induced pluripotent stem cells in the treatment of haemophilia

Haemophilia is a recessive X-linked hereditary disorder caused by a deficiency of coagulation factor VIII (haemophilia A) or IX (haemophilia B). The disease is considered to be severe when factor levels are below 1% of normal values, moderate when they are between 1 and 5% and mild when levels range between 5% and 40%. Haemophilia A is four times more common than haemophilia B and, in terms of severity for both types, 35% of patients have the severe form, 15% the moderate form and 55% have mild haemophilia. Incidence of the disease is 1:6,000 males born alive for haemophilia A and 1:30,000 for haemophilia B [38].

The etiopathogenesis of the disease is related to different kinds of mutations (large deletions and insertions, inversions and point mutations) that occur in the gene expressing the deficient coagulation factor. The clinical characteristics of both types of haemophilia are very similar: spontaneous or traumatic hemorrhages, muscle hematomas, haemophilic arthropathy resulting from the articular damage caused by repetitive bleeding episodes in the target joints, or hemorrhages in the central nervous system. In the absence of appropriate replacement treatment with exogenous coagulation factors, these manifestations of the disease can have disabling or even fatal consequences thus negatively impacting patients' quality of life and reducing their life expectancy [39].

At present, patients with haemophilia benefit from optimized treatment schedules based on the intravenous systemic delivery of exogenous coagulation factors, either prophylactically or on demand. The current policy in developed countries is in general to administer a prophylactic treatment (2 or 3 times a week) from early childhood into adulthood [39]. Such prophylactic protocols result in a clear improvement in patients' quality of life on account of the prevention of haemophilic arthropaty and other fatal manifestations of the disease as well as a reduction in the long-term costs of treatment because of a decrease in the need of surgical procedures such as arthrodesis, arthroplasty or synovectomy [40].

Conventional treatment of haemophilia [41,42] is currently based on the use of plasma-derived or recombinant high-purity coagulation factor concentrates. The former are duly treated with heat and detergent to inactivate lipid-coated viruses [43], and the latter are a recently developed product that does not contain proteins of human or animal origin [44,45]. Both kinds of factor boast high efficacy and safety profiles, at least for the inactivation-susceptible pathogens

known to date. The choice of one product over the other is usually based on the clinical characteristics of the patient and on cost and availability considerations [46,47].

Now that infections by pathogenic viruses (HIV, HCV) that were common a few decades ago have been eradicated, the most distressing adverse effect observed when using either product is the development of antibodies (inhibitors) against the perfused exogenous factors [48,49]. The appearance of inhibitors renders current treatment with factor concentrates inefficient, increasing morbidity and mortality, leading to the early onset of haemophilic arthropathy and disability and to a consequent reduction in patients' quality of life. Lastly, inhibitors result in higher costs as treatment must be provided both for bleeding episodes and inhibitor eradication (immune tolerance induction). The incidence of inhibitors is around 30% in haemophilia A and 6% in haemophilia B.

The immunologic mechanism whereby these neutralizing antibodies are generated is highly complex and involves several messenger molecules (tumor necrosis factor, interleukins…), and cells (T-lymphocytes B-lymphocytes, macrophages...). They are directed at certain regions in the factor molecule that interact with other components of the coagulation cascade and, depending on their titre level and on whether they are transient or persistent, will bring about greater or lesser alterations in the said cascade. The causes that influence inhibitor development may be genetic, i.e. inherent in the patients themselves [48], such as ethnicity, familial history, type of mutation or certain changes in some of the genes involved in the immune response; or non-genetic, i.e. environmental [50], such as age at first factor infusion, breastfeeding, stimulation of the immune system by other antigens or the treatment regimen used (prophylactic vs. on demand). Whether the factor concentrate used is plasma-derived or recombinant does not have a significant influence on the inhibitor incidence rate [51].

Short and medium-term perspectives for the treatment of haemophilia strongly rely on the current research efforts directed at increasing the safety levels of (especially) plasma-derived factors. Such research focuses on the detection and subsequent inactivation of emerging blood-borne pathogens in donors such as the prions causing variant Creutzfeldt-Jakob disease, or other potential emerging agents [52-54]. It is also important to increase the efficiency of recombinant factors increasing their half-life (by PEGylating the factor molecule or using fusion proteins [55-58] and attenuating their immunogenic capacity to produce inhibitors, by chemically modifying them [59] or by developing recombinant factors of human origin [60].

In the long term, efforts must be directed at the development of advanced therapies, particularly strategies in the field of gene therapy (using of adeno-associated viral vectors) and cell therapy (using of adult stem cells or induced pluripotent stem cells). The chief goal of these new strategies will be to address some of the shortcomings associated with current treatment options such as the short *in vivo* half-life of administered factors, the impending risk of a pathogen-induced infection and the development of inhibitors. Another goal of the advanced therapies (cell therapy) will be palliative treatment of the articular consequences derived from haemophilic arthropathy [40].

Haemophilia is optimally suited for advanced therapies as it is a monogenic condition and does not require very high expression levels of a coagulation factor to reach moderate disease

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status (Figure 2). For this reason, significant progress has been possible with respect to these kinds of therapies: cell therapy has broken new ground with the use of several types of target cells and gene therapy has shown particular promise with the use of viral and non-viral vectors. In fact, haemophilia is now recognized as a condition amenable to gene therapy [61-64]. Strategies available include use of lentiviral (LVV) [65] and adeno-associated (AAV) [66] vectors in adult stem cells and autologous fibroblasts, in platelets and in hematopoietic stem cells; transfer by means of non-viral vectors; and repair of mutations with chimeric oligonucleotides. The studies published so far have, in the most part, not reported any severe adverse effect resulting from the application of such strategies in the clinical trials performed.

Specifically, gene therapy trials in haemophilic patients have shown adeno-associated vectors to represent the most promising treatment option given their excellent safety profile, even if on occasion they may create immune response problems. Efforts are currently centered on minimizing the incidence of immune rejection and increasing efficacy and expression time. In this connection, several studies have been published with a view to optimizing the use of this type of viral vectors. Among them, in a landmark study on patients with severe haemophilia B (<1% FIX), Nathwani *et al.* infused their subjects with a dose of a serotype-8-pseudotyped, self-complementary AAV vector that expresses factor IX and can efficiently transduce hepatocytes [66]. Their results showed that factor IX expression ranged between 3 and 11% of normal values. Significant as they may seem, these results must be considered with caution as the expression levels achieved rather than normalize the patient's phenotype convert it to a mild-to-moderate form. Also, concomitant treatment with glucocorticoids is needed to prevent immune rejection and elevation of liver transaminase levels. Due account must also be taken of the fact that the adeno-associated vector has the potential to induce hepatotoxicity. For all these reasons, these undoubtedly encouraging results can only be considered a first step in the development of safe and effective advanced therapies for the treatment of haemophilia.

Non-viral strategies also have a role to play in the treatment of haemophilia as they could in the long term provide a safer alternative than viral vectors which, as we have seen, are fraught with significant biosafety and efficacy-related problems, which have so far limited their clinical application. Sivalingam *et al*. [67] evaluated the genotoxic potential of phiC31 bacteriophage integrase-mediated transgene integration in cord-lining epithelial cells cultured from the human umbilical cord. This non-viral strategy has made it possible to obtain stable factor VIII secretion *in vitro*. Xenoimplantation of these protein-secreting cell lines into immunocompetent haemophilic mice corrects the severe form of the disease. Such implantation could prove extremely useful as a bioimplant in the context of monogenic diseases such as haemophilia.

Our laboratory has advanced the use of nucleofection as a non-viral transfection method to obtain factor IX expression and secretion in adult adipose tissue-derived mesenchymal stem cells [68]. Although it is certainly true that expression efficacy with these types of protocols is lower than when viral vectors are used, it must be underscored that these protocols do offer much higher safety levels, with the additional advantage that increasing factor activity to above 5% of normal values already places the patient in the mild phenotype group.

The use of cell therapy in the treatment of haemophilia has to date consisted mainly in the transplantation of healthy cells in an attempt to repair or replace a coagulation factor defi-

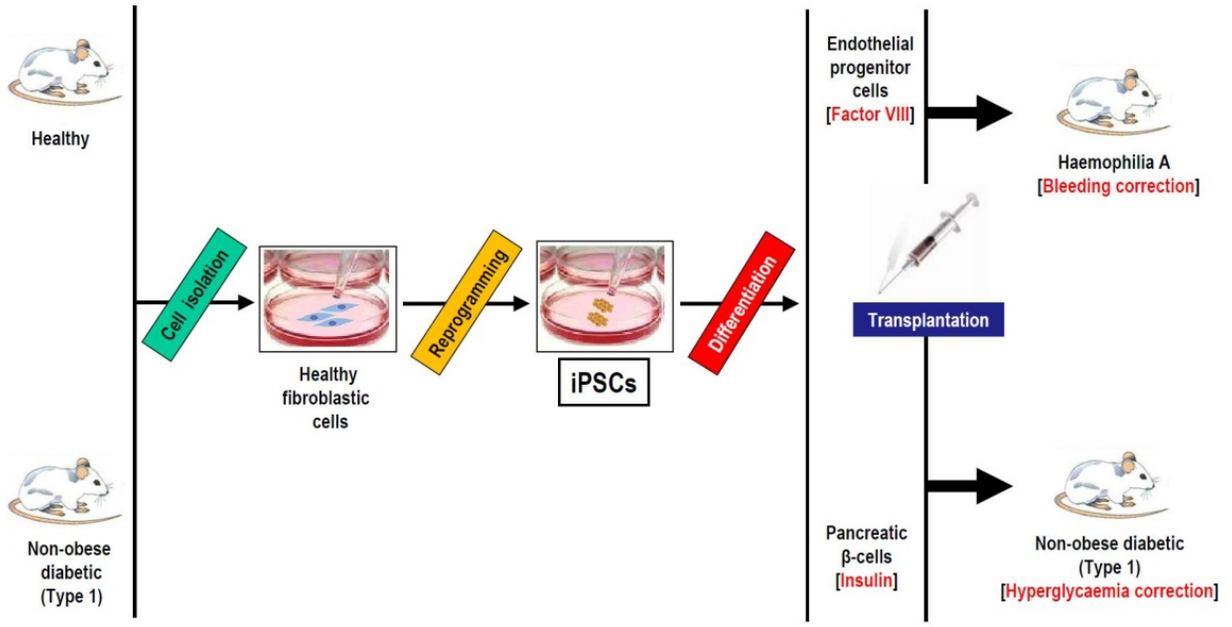

Figure 2. Induced pluripotent stem cells application to the treatment of haemophilia and diabetes mellitus. Autologous transplantation of healthy differentiated cells, obtained from iPSCs, into an animal model with haemophilia or diabetes mellitus type 1, normalizes the corresponding altered function by in vivo production of the deficient protein or hormone.

ciency. These procedures have been conducted mainly with adult stem cells and, more recently, with progenitor cells partially differentiated from iPSCs, albeit in most cases the mechanisms by which transplanted cells (to a greater or lesser extent) engraft and go on to proliferate and function remain unknown.

Aronovich *et al*. [69], have shown that transplantation of embryonic spleen tissue (embryonic day 42 spleen tissue) in immunocompetent mice with haemophilia A attenuates the severity of the disease in the 2-3 months after the procedure. These results would seem to indicate that transplantation of a fetal spleen (obtained from a developmental stage prior to the appearance of T-cells) may potentially be used to treat some genetic disorders. For their part, Follenzi *et al*. [70] reported that once liver sinusoidal endothelial cells were transplanted and successfully engrafted into mice with haemophilia A, they were seen to proliferate and partially replace some areas of the hepatic endothelium. This resulted in a restoration of factor VIII plasma levels and in the correction of the bleeding phenotype. More recently, this same team [71] demonstrated that transplantation of bone marrow cells (healthy mouse Kupffer cells —liver macrophage/mononuclear cells— and healthy bone marrow derived mesenchymal stromal cells) can correct the phenotype of haemophilic mice and restore factor VIII levels.

As far as the use of iPSCs is concerned, the first paper came from Xu *et al*. [72], who reported on the generation of murine iPSCs from tail-tip fibroblasts and their differentiation into endothelial cells and their precursors. These iPSC-derived cells express specific

membrane markers for these cells such as CD31, CD34 and Flk1, as well as factor VIII. Following transplantation of these cells into mice with haemophilia A, the latter survived the tail-clip bleeding assay by over 3 months and their factor VIII plasma levels increased to 8%-12%. Yadav *et al*. [73] studied transdifferentiation of iPSC-derived endothelial progenitor cells into hepatocytes (primary cells of FVIII synthesis). These transplanted cells were injected into the liver parenchyma where they integrated functionally and made correction of the haemophilic phenotype. High levels of FVIII mRNA were detected in the spleen, heart, and kidney tissues of injected animals with no indication of tumor formation or any other adverse events in the long-term. Alipio *et al*. [74] for their part also reported on the generation of factor VIII in a haemophilic murine model one year after transplantation of iPSC-derived endothelial cells.

5. Induced pluripotent stem cells in the treatment of diabetes mellitus

Diseases caused by the destruction or loss of function of a limited number of cells are good candidates for cell therapy. Such is the case of diabetes mellitus (Figure 2).

Diabetes mellitus (DM) is classified into two broad categories: type 1 DM, which is a genetic disease, and type 2 DM, a more generalized variety related with insulin resistance. DM, especially the type 1 form, is associated with microvascular complications, such as retinopathy, neuropathy or nephropathy, as well as cardiovascular problems. Type 1 DM is a T-cell mediated autoimmune disease specifically aimed against pancreatic beta cells, which results in insulin deficiency [75,76].

Symptoms of DM include episodes of lethargy and fatigue, polyuria, enuresis, nocturia, polydipsia, polyphagia, weight loss and abdominal pain. The disorder has a strong genetic component related with the susceptibility to inherit and develop the disease through the HLA complex (HLA-DR and HLA-DQ genotypes) and other loci involved in immunologic recognition and cell-to-cell signaling in the immune system (graft compatibility) [77,78].

Abnormal T-cell activation in susceptible individuals results in both an inflammatory response within the Langerhans islets and a humoral immune response involving the production of antibodies against insulin-specific beta cell antigens, decarboxylase glutamic acid or the protein tyrosine phosphatase [79]. The presence of one or more types of antibodies may precede the appearance of type 1 diabetes and its subsequent development [80,81]. In any case, the final result is the destruction of beta cells and progressive impairment of the blood glucose metabolism [82]. Some patients with type 1 diabetes may show a higher susceptibility to other conditions such as thyroiditis, Graves disease, Adisson disease, celiac disease, myasthenia gravis or to degenerative skin conditions such as vitíligo [83-85].

The greatest incidence of type 1 DM occurs during childhood and in the early years of adulthood with significant variations across different geographies. Diagnosis is usually made

before the age of 20 (between 16 and 18 in 50-60% of cases) [75]. The factors involved in the development of type 1 DM include the so-called familial predisposing factors, gestational status, age and other iatrogenic causes.

Type 2 DM is characterized by a functional deficiency of insulin per se or by a resistance to the hormone resulting from an alteration of the function or structure of the insulin receptor at the level of the membrane or of any of the molecules involved in the intracytoplasmic signal transduction cascade [86]. The metaboilic effects of insulin vary depending on the action of the molecules that participate in signaling pathways to regulate gene expression in striated muscle cells, adipocytes, hepatocytes and in pancreatic beta cells [87-90]. Thus, for example, insulin resistance caused by the impairment of glucose transporter GLUT4 initially results in a metabolic syndrome, type 2 diabetes, lipodystrophy, hypertension, polycystic ovary syndrome or atherosclerosis.

In general, the morbidity and mortality of DM is related with the different long-term cardiovascular complications associated with the disease, also taking into account other proactivating factors such as smoking, obesity, a sedentary lifestyle, hypertension, early onset and prolonged duration of type 1 DM, genetic predisposition and hyperglycemia.

Nephropathy, retinopathy and diabetic neuropathy are the most common microvascular complications of DM. As regards diabetic neuropathy, this can be a focal complication associated with diabetic amyotrophy or with cranial nerve III oculomotor palsy, or a more generalized occurrence that can take the form of a sensorimotor polyneuropathy affecting the autonomic nervous system, gastric motility and cardiac function. Peripheral neuropathy together with peripheral vascular disease may lead to a diabetic foot syndrome, characterized by ulcerations and poor healing in the lower limbs [91]. As a macrovascular complication, cardiovascular disease accounts for 70% of mortality in individuals with type 2 DM, with the incidence of coronary artery disease being higher in women than in men suffering from type 1 DM [92]. Atherosclerotic processes are in turn more common in patients with type 1 DM [93].

Although treatment and diagnosis of diabetes is well-established, there is a constant quest for new drugs that may be more effective at lowering blood glucose levels, controlling their therapeutical management —especially in younger patients—, and preserving patients' long-term quality of life by reducing the incidence of complications resulting from the disease. Current research is centered on unveiling the structure and function of glucose transporters, which may offer significant therapeutic advantages [86], as well as on the development of new fast-acting insulin analogs and more accurate subcutaneous pumps [94-98]. Commendable as these initiatives are, it is difficult to anticipate and control factors that exert a variable influence upon glucose levels such as nutrition, physical activity or stress. These factors alter the glycemic environment and consequently the amount of insulin required at each point in time, which reinforces the need to establish sophisticated artificial pumping systems that may simulate the natural endocrine pancreas.

The continuous advancement of our understanding of the mechanisms that govern the physiopathology of diabetes and gene susceptibility together with the multiple possibilities currently offered by biotechnology have fuelled the researchers' interest in the development

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of all three types of advanced therapies: gene therapy, cell therapy and tissue engineering. In this regard, although we are still at a very incipient stage [99,100], procedures based on transplantation of insulin-secreting cells or islets obtained from stem cell differentiation may hold valuable hope for the future.

The need to justify the human and financial investment made in the development of new advanced therapies is as strong in diabetes as it is in haemophilia. However, in the case of the former justification is even more compelling taking into account that an optimal and efficient treatment is already available for the disease. The discovery of insulin as a therapeutic tool for DM constituted an important milestone in the history of medicine even if administration of this hormone does not fully compensate for the function lost. This is also the case with factor replacement in haemophilia. Moreover, both coagulation factor and insulin treatment are only palliative, never curative, which is the basic idea underlying treatment of DM and haemophilia. Moreover, it is also important to take into account the potential adverse effects of these therapies, and particularly the complications associated with DM, which derive from the fact that it is a long-term disease.

In addition, advances in terms of the clinical transplantation of Langerhans islets have not met with the expected success as a result of the inadequate number of donors available and the incidence of immune rejection of the newly transplanted beta cells [101]. This has intensified efforts aimed at developing insulin-producing cells from stem cells. iPSC technology could turn the tide in this respect as such cells may be induced to form endodermal structures, pancreatic and endocrine progenitors and, naturally, differentiated insulin-producing cells [102-104].

Built upon the knowledge gained from studies on embryonic cells about the differentiation process, the first studies on iPSCs, whereby human cells were reprogrammed to become *in vitro* differentiated insulin-producing cells, showed great promise [105,106]. However, as only partial cell differentiation was achieved, those studies failed in their attempt to enrich insulin-producing cell lines or assess their function.

Drawing on current knowledge on the embryonic development of the pancreas, Zhu *et al*. [107] recently reported on the generation of insulin-producing pancreatic cells from iPSCs obtained from a rhesus monkey [108]. These authors established a quantitative cytometric method to evaluate the efficacy of cell differentiation. In addition, they increased the level of precision in the assessment of the competence and function of the iPSCs from a rhesus monkey by means of transplantation into immunodeficient mice. These cells were induced to form endodermal structures, pancreatic and endocrine progenitors and insulin-producing cells. By means of a TGF-β inhibitor, generation of endocrine precursor cells capable of generating insulin-producing cells that respond to glucose stimulation *in vitro* was undertaken. Transplantation of these cells into a type 1 DM murine model decreased blood glucose levels in 50% of the mice. These results show the high efficacy that can be achieved by obtaining iPSCs from a superior animal model as well as the capacity of iPSCs to be transformed into insulin-producing cells, which opens up the possibility for carrying out autologous transplantations in the future.

Along the same lines, Jeon *et al*. [109] studied the functionality of iPSC-derived insulin-producing cells generated from pancreas-derived epithelial cells in non-obese diabetic mice. The insulin-producing cells obtained in this way express different pancreatic β cell markers and secrete insulin in response to glucose stimulation. Transplantation of these cells into non-obese diabetic mice (a model of autoimmune type 1 DM very similar to the human form) results in a kidney graft with a functional response to glucose stimulation and a consequent normalization of blood glucose levels (Figure 2).

Until recently, iPSC generation from patients with type 2 DM had not been reported in the literature. However, Ohmine *et al*. [110] described not long ago the generation of iPSCs from keratinocytes of elderly patients with type 2 DM. These cells were reprogrammed by lentiviral transduction with human transcription factors OCT4, SOX2, KLF4 and cMYC, telomere elongation, and down-regulation of senescence and apoptosis-related genes, and were subsequently differentiated into insulin-producing islet-like cells. Reprogramming of keratinocytes from elderly type 2 DM patients produces efficient iPSCs with a "privileged" senescence status that allows them to transform into insulin-producing islet-like cells, which may lead to the development of a versatile strategy for modeling the disease as well as an advanced therapy for treating it.

Generally speaking, several problems must yet be resolved before iPSCs can be applied clinically, specifically to the treatment of haemophilia or diabetes. In the first place, it is essential to optimize the reprogramming process so that it provides maximum safety assurances against the potential risks derived from undesirable genetic changes in iPSCs [111]. Recent studies have revealed significant chromosomal changes that take place during the long-term culture of iPSCs as well as variations in the number of copies of certain genes and point mutations, which could clearly be related with the reprogramming of somatic cells and result in damage to the DNA [112-115].

The second hurdle that must be overcome is the high variability that exists between the different cell lines in the context of differentiation into pancreatic lineages [16]. The epigenetic and functional trials that should be performed in this respect are complicated by the fact that iPSCs have a high epigenetic content [116]. The third obstacle has to do with the purification of iPSC-derived β cells to prevent the transplantation of undifferentiated cells, which could result in the formation of teratomas. Moreover, it is necessary to develop new reagents to make direct differentiation of pancreatic progenitors into functional β cells more efficient and to design highly specific surface markers for these cells so that a more precise fluorescence analysis can be performed in order to isolate homogeneous populations of this kind of cell so that their function can be rigorously controlled.

6. General regulatory and bioethical issues

Cell therapy, as one of the bedrocks of the advanced therapies —together with gene therapy and tissue engineering—, requires a new legislative framework in order to guarantee that patients can avail themselves of the products they need and provide governments with a robust

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protection, control and regulation mechanism. The existing framework regulating advanced therapies will have to be adapted fast in order to keep pace with the proliferation of new knowledge in this rapidly developing field. However, desirable that this may be, the pace of legislative reform is unfortunately slow and inevitably lags behind the development of new science.

The aspects to be regulated include mainly those related with controlling the development, manufacturing and quality of release and stability testing programs; non-clinical aspects such as promoting research on biodistribution, cell viability and proliferation levels and ratios, and the persistence of in vivo function; clinical aspects such as dose-specific characteristics, risk stratification; and aspects specifically connected to pharmacovigilance and traceability.

The guidelines for therapeutic products based on human cells must be drawn up by the drug agencies of the different countries [117,118] both as regards the development of clinical and preclinical trials and with respect to pharmacovigilance, taking in all cases a multidisciplinary perspective.

For any product based on cells or on tissue, it should be made compulsory to verify that the desired physiological functions are preserved after the preparation process, both in isolation and in combination with other non-biological components, as many of these products will be used with a metabolic purpose [119,120]. Nevertheless, many things remain to be learned about the procedures that should be followed to guarantee the safety and efficacy of cell therapy products, especially with respect to the biology of stem cells, their self-renewal and differentiation potential and, above all, the evaluation and prediction of potential risks.

Most cell therapy products are not controversial from a bioethical point of view. The exception to this is therapy with human embryonic stem cells, which raises moral and bioethical problems [121,122]. Such consideration refer to the donor's informed consent and to problems associated with the harvesting of oocytes and the destruction of human embryos. In this regard, the guidelines used by the different countries range from total prohibition to regulated authorization. In general, there is an international consensus that the results obtained in stem cell research should be applied to humans without prior bioethical scrutiny, with the understanding that scientific research and the use of scientific knowledge must respect human rights and the dignity of the individual in accordance with the Universal Declaration of Human Rights and the Universal Declaration of the Human Genome [123].

The main advantage of induced pluripotent stem cells is that their use, unlike that of embryonic stem cells, does not raise moral or bioethical issues as the scientific community, as well as society at large, consider it a valid alternative for the generation of pluripotent stem cells without the need to use human oocytes or embryos. Furthermore, these cells have shown themselves to be functionally and molecularly similar to embryonic cells, but without their bioethical problems, which means that their use in humans will not require an overly stringent regulatory framework. The importance of this cannot be overstated as, in many instances, and in some countries more than in others, legislation can hinder the development of science and, consequently, the application of new knowledge and new therapeutic strategies.

7. Concluding remarks

iPSCs offer an unprecedented alternative for basic, clinical and applied biomedical research. The most significant applications of these cells to the field of cell therapy are related to the treatment of such organ-specific conditions as diabetes —a typically metabolic disease—, hepatic and cardiovascular diseases, immunological disorders and monogenic hereditary conditions in general such as haemophilia.

However, many aspects remain to be unveiled about the safety of iPSCs and about their reprogramming mechanisms, although no-one denies that this technology offers new, until-recently-unimaginable possibilities for correcting alterations in a large number of conditions, particularly in monogenic and metabolic diseases [124]. Also, some technical problems will also have to be resolved such as finding a way to produce these cells using risk-free viral vector transfection as well as safer alternative methods such as viral vector-mediated reprogramming.

Other more general, though no less important, issues that remain to be addressed include optimal extrapolation to humans of the high levels of safety and expression obtained in animal models and finding out whether it is adult mesenchymal stem cells or iPSCs that constitute the best and most easily applicable alternative for the administration of combined cell therapy/gene therapy.

For the reasons mentioned it is imperative not to create false expectations in patients suffering from a disease that is amenable to advanced therapies, specifically cell therapy, as these strategies are still in their "infancy". In the longer term, once the challenges mentioned above have been overcome, both cell and gene therapy will become plausible alternatives. Optimism is in order, but fantasy is best avoided.

As far as haemophilia is concerned, the first article discussing the benefits of gene therapy for the treatment of the disease was published a decade ago. At that time, experts in the field anticipated that a cure for haemophilia would be found by the first decade of the 21st century [125], a prediction that did not come true because of multiple problems related to biosafety. Although many steps have been taken in the right direction with respect to gene therapy, cellular reprogramming of iPSCs and the safety of transfer vectors, efforts must continue in order to resolve problems related to immune response, insertional mutagenesis, efficacy and expression time, the collateral (particularly hepatotoxic) damage caused by viral vectors and the risk of teratoma and neoplasia derived from the application of certain cell types. Sight should not be lost of the difficulties inherent in recruiting patients for clinical trials and in the large-scale production of vectors and cell lines, needed to facilitate optimal and efficient implementation in the clinical setting.

One of the first things that must be addressed when doing research into advanced therapies is whether the expected benefits of such therapies will be able to offset the investment needed. In the case of haemophilia, the answer is clearly in the affirmative as it is a chronic disease that requires high-frequency life-long treatment, very costly in patients on prophylaxis, and which poses a potential risk of infection by emerging pathogens. The second question is whether advanced therapies are at all feasible. In this regard, haemophilia is considered an optimal

candidate for such treatments for several reasons: it is a monogenic disease; the expression of low levels of coagulation factor (1-5%) can result in a moderate phenotype; a large variety target cells can be applied; there is no need to regulate factor expression, and a large amount of animal models are available for experimentation. In this regard, application of strategies that are less demanding in terms of efficacy, i.e. level of protein expression, but that afford much greater safety, may be an alternative for this condition, taking into account that both physicians and patients are highly sensitive to the special immunologic situation of the haemophilic population and that viral infections (HIV/HCV) have had lethal consequences for these individuals in the past [76].

As regards diabetes as a typically metabolic disease, advances in the understanding of its physio- and etiopathology, together with the greater biotechnological possibilities available, have made new alternatives possible as a result of the development of advanced therapies to treat it. Transplantation of insulin-secreting cells or of islets obtained a from differentiation of stem cells could hold some hope in the long term.

As in haemophilia, in diabetes it is also necessary to justify the investment of human and financial resources required for the development of new advanced therapeutical strategies, taking account of the fact that patients with this condition also benefit from an optimal and efficient treatment at present. The justification for the said investment is that diabetes gives rise to vascular and neurological complications in the long term and that transplantation of Langerhans islets has not achieved the success that scientists hoped for because of the dearth of donors and the high rate of immune rejection that characterizes diabetic patients.

In a nutshell, iPSCs technology has the potential to produce an about-face in the way we conceive cell behavior as iPSCs can be induced to form hormone-producing differentiated cells. In this regard, several authors have reported on the generation of insulin-producing pancreatic cells from iPSCs from rhesus monkey and murine models which, after transplantation, are capable of producing insulin *in vivo* in response to glucose stimulation. Nonetheless, some general issues affecting iPSCs remain to be resolved before these cells can be used clinically in the treatment of diabetes. Prominent among these are optimizing the reprogramming process as well as their genetic safety, controlling the high differentiation variability of the different pancreatic lines by means of epigenetic trials and enhancing the purification, isolation and characterization of homogeneous populations of iPSC-derived insulin-producing β cells.

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