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Potential Clinical Applications of Embryonic Stem Cells

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1. Introduction

Embryonic stem cells (ESC) have been reported for different mammalian species (i.e. hamster, rat, mink, pig, and cow), but only murine ES cells have successfully transmitted their cell genome through the germline. Recently, interest in stem cell technology has intensified with the reporting of the isolation of primate and human ES cells.
In developing this chapter, some conventions have to be established to describe consistently what stem cells are, what characteristics they have, and how they are used in biomedical research. Also, we intend to describe and distinguish the details of foetal and adult stem cells. In between lie important information describing what researchers have discovered about stem cells and a newly developed autologous ES cell–like stem cells, called induced pluripotent stem (iPS) cells. These reprogrammed stem cells (iPS) could be generated from any patient, thus removing both ethical and immunological issues at one time.
A stem cell is a special kind of cell that has a unique capacity to renew itself and to give rise to specialized cell types. Although most cells of the body, such as heart or skin cells, are committed to conduct a specific function, a stem cell is uncommitted and remains uncommitted, until it receives a signal to develop into a specialized cell. Their proliferative capacity combined with the ability to become specialized makes stem cells unique.
Stem cells can originate from embryonic, foetal, or adult tissue and are broadly categorized accordingly.
Embryonic Stem Cells (ESCs) are commonly derived from the inner cell mass (ICM) of a blastocyst, an early (4–5 days) stage of the embryo. Embryonic germ cells (EGCs) are isolated from the gonadal ridge of a 5–10 week foetus.
Adult stem cells differ from ESCs and EGCs in that they are found in tissues after birth, and to date, have been found to differentiate into a narrower range of cell types, primarily those phenotypes found in the originating tissue. An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found, because they are able to self-renew and yield differentiated cell types.
They are thought to reside in a specific area of each tissue (called a "stem cell niche"). Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated

by a normal need for more cells to maintain tissues, or by disease or tissue injury. Typically, there is a very small number of stem cells in each tissue, and once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Today, donated organs and tissues are often used to replace those that are diseased or destroyed. Unfortunately, the number of people needing a transplant far exceeds the number of organs available for transplantation. Adult stem cells, such as blood-forming stem cells in bone marrow (called hematopoietic stem cells, or HSCs), are currently the only type of stem cell commonly used to treat human diseases.

Scientists in many laboratories are trying to find better ways to manipulate them to generate specific cell types so they can be used to treat injury or disease. Pluripotent stem cells offer the possibility of a renewable source of replacement cells and tissues to treat a myriad of diseases, conditions, and disabilities including Parkinson's disease, Amyotrophic Lateral Sclerosis, spinal cord injury, burns, heart disease, diabetes, and arthritis. This pluripotency represents both advantages and disadvantages in cell-based therapies. In fact for culture *in vitro*, their ability to generate the large number of cells often required for therapies, as well as their potential to yield whichever phenotype may be of interest, is considered beneficial. For implantation *in vivo*, however, the concern arises that these same attributes will either allow ESCs to proliferate limitlessly and form teratomas or differentiate uncontrollably into undesirable cell phenotypes.

Several are the applications of ESCs in human medicine: tissue repair, gene therapy, drug discovery and toxicological testing.

Stem cells are promising tools for studying the mechanisms of development and regeneration and for use in cell therapy of various disorders as cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, stroke, diabetes and cartilage.

Although hESC are thought to offer potential cures and therapies for many devastating diseases, research using them is still in its early stages.

In late January 2009, the California-based company Geron received FDA clearance to begin the first human clinical trial of cells derived from human embryonic stem cells.

But some scientific hurdles to hESCs application have to be deeply considered:

- the rejection of transplanted tissues (originating from donor embryos);
- the risk of teratoma formation due to any residual rogue undifferentiated pluripotent hESCs in the hESC-derived tissue (after the differentiation process);
- the inadequate number of cells available for treatment (for obtaining a large numbers of cells, large-scale cell production strategies are needed utilizing bioreactors and perfusing systems);
- the safety measures to be taken when a whole cell is administered because a variety of impurities may be administered with it (cells cells must be generated under cGMP current good tissue culture practice conditions using xenofree protocols to prevent the risk of transmission of adventitious agents and rogue undifferentiated hESCs that may induce teratomas);
- the best route and the frequency of administration (direct cell injections into the malfunctioned organ would be preferred to peripheral or portal vein administration to prevent the cells homing in unwanted sites, thus inducing cancers).

For the above reasons a long-term *in vivo* functional outcome after hESC-derived tissue transplantation also needs to be properly worked out.

2. Origin and classification of stem cells

Human stem cells can be classified into many types based on their source of origin. More recently, they have been classified based on the presence or absence of a battery of CD and embryonic stem cell (ESC) markers.

The male and female gonads contain stem cells referred to as spermatogonia and oogonia, respectively. Through their self-renewal and subsequent meiosis they are responsible in producing the cells of the germ line and eventually spermatozoa and oocytes. These two haploid gametes eventually fertilize to establish diploidy and produce the zygote. The zygote remains at the top of the hierarchical stem cell tree, being the most primitive cell, and the germ cells therefore possess the unique feature of developmental totipotency (Yoshimizu T et al. 1999; Pesce et al 1998). The zygote undergoes cleavage in the human through a period of 5–6 days, producing two to four blastomeres on day 2, eight on day 3, fusing or completely fused blastomeres (compacting or compacted stage) on day 4, and blastocyst stages on days 5 and 6 (Bongso et al 2005; Fong et al 2004). Each of the blastomeres is considered totipotent because it has the potential to produce a complete organism, as demonstrated when blastomeres are placed into the uterus of rabbits or mice. The first stem cell to be produced in the mammal is in the inner cell mass (ICM) of the 5-day-old blastocyst. These cells self-renew and eventually produce two cell layers: the hypoblast and epiblast. The hypoblast generates the yolk sac, which degenerates in the human, and the epiblast produces the three primordial germ layers (ectoderm, mesoderm, and endoderm). These germ layers produce all the various tissues of the organism. For this reason hESCs are considered pluripotent and not totipotent because they cannot produce complete human beings but have the potential to produce all the 210 tissues of the human body.

During embryogenesis and fetal growth such embryonic stem cells that have not participated in organogenesis remain as adult stem cells in organs during adulthood. It can thus be hypothesized that the function of adult stem cells residing in specific organs is to be dedifferentiated and be recruited for repair of injury incurred by the specific organ. Unfortunately, such adult stem cells in the organs are few in number.

It has been shown that fetal and adult stem cells, referred to as somatic stem cells or non-embryonic stem cells, are able to self-renew during the lifetime of the organism and to generate differentiated daughter cells. Moreover they could cross boundaries by trans-differentiating into other tissue types and are thus referred to as multipotent [Solter et al., 2006, Bjornson CR, et al 1999; Jackson KA, et al 1999;Clarke DL et al 2000 ; Krause DS et al 2001].

Adult tissues, even in the absence of injury, continuously produce new cells to replace those that have worn out. For this reason, adult stem cells can be found in a metabolically quiescent state in most specialized tissues of the body, including brain, bone marrow, liver, skin, and the gastrointestinal tract. Therefore, multipotency is restricted to those mesenchymal stem cell types that can differentiate into a small variety of tissues.

Those stem cells that are unable to trans-differentiate but differentiate into one specific lineage are referred to as unipotent. An example of such unipotency is the differentiation of bone marrow hematopoietic stem cells to blood. Thus as embryogenesis shifts to organogenesis, infancy, and then adulthood, stem cell plasticity shifts from pluripotency to multipotency.

Recently there has been tremendous interest in the derivation from embryonic, fetal and adult tissues and, more recently, also from extra-embryonic adnexa such as umbilical cord,

placenta, fetal membranes and amniotic fluid.[Zhao et al 2006; McGuckin CP et al. 2005 ; Fong CY ,et al. 2007] . These tissues possess both CD and some ESC markers, and thanks to their "intermediate" properties, are considered useful for transplantation therapy [Fong et al 2007] . The umbilical cord, for example, has three types of stem cells localised in cord blood, in the Wharton's jelly, and in the perivascular matrix around the umbilical blood vessels within the cord itself [Sarugaser et al. 2005] .

3. Stem cells characteristics

The term "stem cell" originated from botanical monographs where the word "stem" was used for cells localised in the apical meristem, and responsible for the continued growth of plants [Kaufman et al 2002]. In mammals, given the vast variety of stem cells isolated from pre-implantation embryos, fetus, amniotic liquid, umbilical cord, and adult organs, it becomes necessary to provide a more general definition for the term "stem cell" and a more specific definition based on the type of stem cell.

In general, stem cells differ from other kinds of cells in the body, and have dual ability to proliferate indefinitely (i.e. self renewal) and to differentiate into one or more types of specialized cells (i.e. potency) [Mimeault and Batra 2006].

Stem cells are capable of dividing and renewing themselves for long periods. Unlike muscle, blood, or nerve cells—which do not normally replicate themselves—stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

Stem cells are unspecialized. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. However, unspecialized stem cells can give rise to specialized cells, including heart, muscle, blood or nerve cells.

Stem cells can give rise to specialized cells. When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by cell's genes carrying coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules within the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

The degree of differentiation of stem cells to various other tissue types varies with the different types of stem cells, and this phenomenon is referred to as plasticity.

The plasticity of stem cells and differentiated cells in the postnatal organism poses important questions concerning the role of environmental cues. What mechanisms allow a stem cell to escape developmental pressures and maintain its "stemness"? What macro- or micro-environmental cues maintain a cell in its differentiated state? Other important questions to solve are related to the developmental origin of postnatal stem cells, to their possible relationships, as well as the role of symmetrical and asymmetrical cell divisions that maintain stem cell compartments but allow for differentiation in the same time [Booth, and Potten 2000; Morris, R. 2000]

4. Embryonic stem cell: hESCs and mESCs

Embryonic stem (ES) cells were first isolated in the 1980s by several independent groups [Cole et al., 1965, 1966; Evans and Kaufman, 1981; Martin, 1981; Bongso et al., 1994; Thomson et al., 1995, 1998; Axelrod,1984; Wobus, et al.1984; . Doetschman et al. 1985]. These investigators recognized the pluripotential nature of ES cells to differentiate into cell types of all three primary germ lineages. Gossler et al. described the ability and advantages of using ES cells to produce transgenic animals [Gossler et al 1986]. Thomas and Capecchi reported the ability to alter the genome of the ES cells by homologous recombination (Thomas et al 1987). Smithies and colleagues later demonstrated that ES cells, modified by gene targeting when reintroduced into blastocysts, could transmit the genetic modifications through the germline [Koller at al 1989]. Today, genetic modification of the murine genome by ES cell technology is a seminal approach to understanding the function of mammalian genes *in vivo*. Successively, interest in stem cell technology has intensified with the reporting of the isolation of primate and human ES cells [Thomson et al., 1995, 1998; Shamblott et al 1998; Reubinoff et al. 2000].

Embryonic Stem Cells (ESCs) continue to grow indefinitely in an undifferentiated diploid state, when maintained in optimal conditions. ES cells are sensitive to pH changes, overcrowding, oxygen and temperature changes, making it imperative to care for these cells daily. ES cells that are not cared for properly will spontaneously differentiate, even in the presence of feeder layers and leukemia inhibitory factor (LIF).

Embryonic stem cells have the advantages of possessing pluripotent markers, producing increased levels of telomerase, and being coaxed into a whole battery of tissue types. On the other side they have the disadvantages of potential teratoma production, their derived tissues have to be customized to patients to prevent immunorejection, and their numbers have to be scaled up *in vitro* for clinical application.

Since the first report of ESC derivation in mice was published in 1981, [Evans and Kaufman 1981] various findings have emerged to explain the basic properties of ESCs. Recent advances in our understanding of ESC biology have included the identification of several master regulators of ESC pluripotency and differentiation. However, intensive study of ESC growth conditions has yet to produce a complete picture of the unique transcriptional and epigenetic state that is responsible for pluripotency and self-renewal in ESCs.

In summary, genuine hESC have the following characteristics: (1) self-renewal in an undifferentiated state for very long periods of time with continued release of large amounts of telomerase, (2) maintenance of "stemness" or pluripotent markers, (3) formation of teratoma containing tissues from all three primordial germ layers when inoculated in SCID mice, (4) maintenance of a normal stable karyotype, (5) clonality, (6) stem cells marker expression (e.g., NANOG), and (7) ability to produce chimeras when injected into blastocysts in the mouse model.

hESCs have many applications in human medicine. First of all the production of hESC-derived tissues in regenerative therapy.

5. Using pluripotent stem cells in clinic issues

A number of scientific and medical issues need to be addressed before stem cells can be considered safe for clinical applications. The first difficulty is the tumorigenic potential of pluripotent cells (hESCs and iPSCs). Because pluripotency is evidenced by the ability to

form teratomas when transplanted in immunodeficient mice, the concern exists that these cells could form malignant tumors in the host. One strategy for dealing with this problem is to select pure populations of more committed cells for transfer. Therefore it is important demonstrating the genetic and epigenetic stability before these cells are used clinically. In fact is imperative that controlled, standardized practices and procedures be followed to maintain the integrity, uniformity, and reliability of the human stem cell preparations. Because in many studies stem cells are both maintained and expanded *in vitro* before transplantation, culture conditions compatible with human administration must be used. Feeder cells and sera of animal origin have to be avoided to reduce the potential risk of contamination by xenogeneic protein and pathogens. Also karyotypic abnormalities, might be at least partially dependent on culture techniques [Mitalipova et al. 2005]. Accordingly hESCs must be produced under current Good Manufacturing Practices (cGMP) quality. That is defined by both the European Medicines Agency and the Food and Drug Administration, as a requirement for clinical-grade cells, offering optimal defined quality and safety in cell transplantation. In Europe, the requirement for cell therapy products is outlined in several directives and guidelines that are pertinent as regards hESCs (Directive 2004/23/EC, Commission Directives 2006/17/EC and 2006/86/EC).

Finally, transplantation of hESCs into patients is also limited by potential HLA incompatibility. Consequently, life-long immunosuppressive therapy, which can lead to infections and organ-based toxic side effects, such as nephropathy, might be required to prevent graft rejection.

In this regard induced Pluripotent Stem Cells (iPSCs) hold great promise because they are histocompatible with the patient from which they are derived and their use avoids one of the major ethical concerns associated with hESCs.

6. ESCs cell therapy *in vivo* and *in vitro*

The NIH funded its first basic research study on hESCs in 2002. Since that time, biotechnology companies have built upon those basic foundations to begin developing stem cell-based human therapies.

Cell therapy, including the disciplines of regenerative medicine, tissue-, and bio-engineering, is dependent on cell and tissue culture methodologies to generate and expand specific cells in order to replace important differentiated functions lost or altered in various disease states (i.e. no insulin production in diabetes). Central to the successful development of cell based therapies is the question of cell sourcing. Thus, advances in stem cell research have a vital impact on this problem.

The use of human ESCs as resource for cell therapeutic approaches is currently performed for several diseases. Among these we are going to describe myocardium diseases and lung disease.

The Landmark's study is the first to document the potential clinical utility of regenerating damaged heart muscle by injecting hESC–derived cardiomyocytes directly into the site of the infarct [Laflamme MA et al., 2007]. Researchers have demonstrated the proof-of-concept of this approach in mice. Mouse embryonic stem cells have been used to derive mouse cardiomyocytes. When injected into the hearts of recipient adult mice, the cardiomyocytes repopulated the heart tissue and stably integrated into the muscle tissue of the adult mouse heart. After that, they have derived human cardiomyocytes from hESCs (GRNCM1) using a process that can be scaled for clinical production. GRNCM1 cells shown normal contractile

function and responded appropriately to cardiac drugs. These cells have been transplanted into animal models of myocardial infarction in which the cells engraft and improve the left ventricular function compared to those animals receiving no cells. The ability of hES cell–derived cardiomyocytes to partially regenerate myocardial infarcts and attenuate heart failure encouraged their study under conditions that closely match human disease.

In 2007 another study showed that intramyocardial injection of hESC-CMs performed few days after infarction in immunodeficient rodents seemed to enhance left ventricular ejection fraction (LVEF) compared to a control group [van Laake LW et al 2007].

Unfortunately, this enhancement was not sustained after 12 weeks of follow-up. Another study suggested that a coinfusion of hESC-CMs and MSCs in mice was of benefit because a ''synergistic trophic effect that enhanced repair of injured host tissue'' was brought about. Importantly, no teratoma was found in animals receiving hESC-CMs [van Laake LW et al 2007 ; Puymirat et al 2009].

Respiratory diseases are a major cause of mortality and morbidity worldwide. Current treatments offer no prospect of cure or disease reversal. Transplantation of pulmonary progenitor cells derived from human embryonic stem cells (hESCs) may provide a novel approach to regenerate endogenous lung cells destroyed by injury and disease. In a study researcher examine the therapeutic potential of alveolar type II epithelial cells derived from hESCs (ATIICs) in a nude mouse model of acute lung injury (Spitalieri P. et al. submitted). The capacity of hES to differentiate *in vitro* into ATIICs was demonstrated together with the ability of the above committed cells to repair *in vivo* lung damage in a pulmonary fibrosis disease models, obtained by Silica inhalation in mice. After injection of committed cells into damaged mice, a significant recovery of inflammation process and fibrotic damage, was obtained and demonstrated by the restoration of lung functionality (measurement of blood oxygen saturation levels).

Up to date in human only one trial based on hESCs has been initiated. During July 2010, the FDA notified the biotechnology company Geron that they could begin enrolling patients in the first clinical trial of a hESC-derived therapy. The phase I of this multi-center trial is designed to establish the safety of using hESCs to achieve restoration of spinal cord function. To do this, they have derived oligodendrocyte progenitor cells (GRNOPC1) from hESCs. GRNOPC1 is a population of living cells containing precursors to oligodendrocytes, otherwise known as oligodendrocyte progenitor cells (OPC). Oligodendrocytes are naturally occurring cells in the nervous system that have several functions, they produce myelin (insulating layers of cell membrane) that wraps around the axons of neurons to enable them to conduct electrical impulses.

In collaboration with researchers at the University of California, Geron have shown in animal models that GRNOPC1 can improve functional locomotor behaviour after cell implantation in the damaged site, seven days after injury. Histological analysis also provided evidence for the engraftment and function of these cells [Keirstead HS et al 2005].

In additional studies, GRNOPC1, when injected into the injury site of spinal cord, migrated throughout the lesion site matured into functional oligodendrocytes that remyenilated axons and produced neurotrophic factors [Zhang YW et al. 2006], resulting in improved locomotion of the treated animals. These above observations served as the rationale for the use of GRNOPC1 in treating spinal cord injuries in humans.

The clinical hold was placed following results from a single preclinical animal study in which Geron observed a higher frequency of small cysts within the injury site in the spinal cord of animals injected with GRNOPC1, respect to previous studies. In response to those

results, Geron developed new markers and assays, completed an additional confirmatory preclinical animal study to test the new markers and assays, and subsequently submitted a request to the FDA for the clinical hold to be lifted.
Another biotech company, ACT, has recently filed the paperwork with FDA to request permission to begin another hESC-derived stem cell safety test. The trial regard the treatment of patients with an eye disease called Stargardt's Macular Dystrophy (SMD), using hES-derived retinal cells.

7. Adult stem cell

For many years, researchers have been seeking to understand the body's ability to repair and replace the cells and tissues of some organs. Scientists have now focused their attention on adult stem cells. It has long been known that stem cells are capable of renewing themselves and that they can generate multiple cell types. Today, there is new evidence that stem cells are present in far more tissues and organs than once thought and are capable of developing into more kinds of cells than previously imagined. Efforts are now underway to harness stem cells and to take advantage of this capability, with the goal of devising new and more effective treatments. What lies ahead for the use of adult stem cells is unknown, but it is certain that there are many research questions to be answered and that these answers hold great promise for the future.
Adult stem cells share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies and specialized functions.
Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiate along a particular cellular development pathway, although this characteristic may not be as definitive as once thought [Marcus A. et al. 2008].
Unlike embryonic stem cells, which are defined by their origin, adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.
In the next part of the chapter we will refer only to fetal and adult stem cells.

8. Fetal Stem Cells

In recent years, foetal stem cells (FSCs) and stem cells isolated from cord blood or extraembryonic tissues have emerged as a potential 'half way house' between ES cells and adult stem cells. FSCs can be found in foetal tissues such as chorionic villus sampling (CVS) blood, liver, bone marrow, pancreas, spleen and kidney. They are also found in cord blood and extraembryonic tissues such as amniotic fluid, placenta and amnion [Marcus A et al 2008]. Their primitive properties, expansion potential and lack of tumorigenicity make them

an attractive option for regenerative medicine in cell therapy and tissue engineering settings. While extraembryonic tissues could be used with few ethical reservations, the isolation of FSCs from abortuses is subject to significant public unease. We review here the characteristics of stem cells from foetal, cord blood and extra embryonic tissues, their application in cell therapy and their potential for reprogramming towards pluripotency.

Fetal stem cells are advantageous for research for some relevant reasons.

First, they could be obtained from minimally invasive techniques during the gestation, for prenatal diagnosis. A number of studies followed, reporting that preparations of amniotic epithelial cells (AECs), amniotic mesenchymal cells (AMCs), and cells collected from amniotic fluid (AFCs), seem to contain cells with certain stem cell properties. These cells possess a high proliferation potential, express markers (such as OCT4) specific to pluripotent stem cells, and display the potential to differentiate *in vitro* into cells of all three germ layers [Alviano F. et al. 2007;De Coppi P. et al. 2007; Ilancheran S., et al 2007 Kim, J. et al 2007; Miki, T et al 2005;Tamagawa T. et al 2007; Zheng Y.B et al. 2008].

Second, fetal stem cells have a higher potential for expansion than cells taken from adults. Mesenchymal cells from umbilical cord blood can be induced to form a variety of tissues when cultured *in vitro*, including bone, cartilage, myocardial muscle, and neural tissue [Bieback et al 2004]. Third, the ability to isolate pluripotent autogenic progenitor cells during gestation may be advantageous for the timely treatment of congenital malformations or genetic diseases in newborns (in utero therapy). Fourth, their use is devoid of the ethical issues associated with embryonic stem cells [Weiss, M.L., and Troyer, D.L.2006]. Recently, a new source of human amniotic fluid stem cells (hAFSC) has been isolated [De Coppi et al 2007]. These cells represent 1% of the population of cells obtained from amniocentesis and are characterized by the expression of the receptor for stem cell factor c-Kit (CD117). hAFSC are multipotent, showing the ability to differentiate into lineages belonging to all three germ layers, and can be propagated easily *in vitro* without the need of a feeder layer. hAFSC express the markers OCT4 and SSEA-4, both of which are typical of the undifferentiated state of embryonic stem cells (ESC). However, hAFSC do not express some of the other typical markers of ESC, such as SSEA-3, and instead express mesenchymal and neuronal stem cell markers (CD29, CD44, CD73, CD90, and CD105) that are normally not expressed in ESC. Therefore, hAFSC can be considered as an intermediate type of stem or progenitor cell between ESC and adult stem cells resident in differentiated organs.

Although AFS cells have been recently discovered and many questions concerning their potential are still open, they appear to harbour specific advantages in comparison to other stem cell populations: (1) they can be easily harvested through amniocentesis, which is a safe procedure routinely performed for the antenatal diagnosis of genetic diseases [Caughey AB et al 2006]; (2) they do not form tumours after implantation *in vivo* [De Coppi et al 2007]; (3) obtaining them during pregnancy is harmful neither to the mother nor to the foetus [Caughey AB, et al 2006;Eddleman KA,et al., 2006; Cananzi M, et al 2009]. Moreover, recent papers have demonstrated that, when injected in models of organ damage and development, AFS cells are able to: integrate into the developing kidney and express early markers of renal differentiation [Perin L et al 2007]; repopulate the bone marrow of immunocompromised mice after primary and secondary transplantation [Ditadi A et al. 2009], and engraft into the lung, differentiating into pulmonary lineages [Carraro G et al 2008] respectively.

A recent study reported for the first time a detailed characterization of the differentiation capability of fetal cells obtained from chorionic villus sampling (CVS) [Spitalieri P et al 2009]. CVSs can be routinely obtained during early pregnancy for prenatal diagnosis

purposes, can be easily cultured *in vitro* and modified by gene targeting protocols for cell therapy applications [D'Alton, M.E. 1994.; d'Ercole, C.,et al. 2003;Sangiuolo, F.et al 2005]. The study investigated whether cells with phenotypic and functional characteristics of stem cells are present within human CVSs harvested from the 9th to 12th week of gestation during routine chorionic villus sampling. Results indicate that human CV cytotrophoblasts contains a cell population expressing typical markers, able to differentiate *in vitro* into derivatives of all three germ layers and also able to populate depleted hematopoietic tissues. Moreover these cells, after injection into mouse blastocysts were incorporated into the inner cell mass and could be traced into several tissues of the adult chimeric mice. Finally no teratoma formation was reported after cell injection into SCID mice, demonstrating their usefulness in cell and gene therapy approach.

9. Adult Stem Cells: Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cell (MSCs)

Specialized connective tissues consist of blood, adipose tissue, cartilage, and bone. It has been generally believed that all cellular elements of connective tissue, including fibroblasts, adipocytes, chondrocytes, and bone cells, are generated solely by mesenchymal stem cells (MSCs) [Ashton BA, et al 1980; Prockop DJ.et al 1997; Pittenger MF, et al 999; Bianco P, et al 2008; Studeny M, et al 2002; Verfaillie CM, et al 2003; Gregory CA, et al 2005], while blood cells are produced by hematopoietic stem cells (HSCs).

Bone marrow (BM) is a complex tissue containing hematopoietic progenitor cells and a connective-tissue network of stromal cells.

The continued production of these cells depends directly on the presence of Hematopoietic Stem Cells (HSCs), the ultimate, and only, source of all these cells.

The term mesenchymal stem cells was coined by Caplan [Caplan AI.et al 1991] in 1991 to describe a population of cells present within the adult bone marrow that can be stimulated to differentiate into bone and cartilage, tendon, muscle, fat [Alhadlaq A., and Mao JJ. 2003; Alhadlaq A., et al 2004; Pittenger MF,et al 1999;], and marrow stromal connective tissue which supports hematopoietic cell differentiation [Dexter TM et al.1976;Friedrich C. et al. 1996]. In addition, controversial data suggest that MSCs may give rise to sarcomeric muscle (skeletal and cardiac) [Wakitani S, et al. 1995; Makino S, et al 1999; Planat-Bénard V, 2004;], endothelial cells [Oswald J,et al 2004] and even cells of non-mesodermal origin, such as hepatocytes [Chagraoui J, et al 2003], neural cells [Woodbury D, et al., 2000] and epithelial cells [Spees JL, et al. 2003; Ma Y, et al 2006] MSCs represent a very small fraction, 0.001–0.01% of the total population of nucleated cells in marrow [Pittenger MF et al., 1999].

Although Bone Marrow (BM) has been represented as the main available source of MSCs [Pittenger MF et al 1999 ; Haynesworth SE et al. 1992], the use of bone marrow-derived cells is not always acceptable because of potential viral exposure and a significant decrease in the cell number along with age. In addition, it requires a painful invasive procedure to obtain a BM sample. Therefore, the identification of alternative sources of MSCs may provide significant clinical benefits with respect to ease of accessibility and reduced morbidity.

The umbilical cord blood (UCB) has been used as an alternative source since 1988 [Gluckman E et al 1989]. The blood remaining in the umbilical vein following birth contains a rich source of hematopoietic stem and progenitor cells (HSCs/HPCs), and has been used successfully as an alternative allogeneic donor source to treat a variety of pediatric genetic, hematologic, immunologic, and oncologic disorders [Broxmeyer HE, et al1989; Gluckman E, et al 1997; Han IS, 2003; Kim SK, et al 2002].

9.1 MSCs and HSCs: Cell and gene therapy

Stem cell therapies utilizing adult mesenchymal stem cells (MSCs) are the focus of a multitude of clinical studies currently underway. Because large numbers of MSCs can be generated in culture, MSCs were thought to be useful for ''tissue-engineering'' purposes [Caplan AI, et al 2001], as exemplified by a number of clinical trials [Dazzi F, et al 2007; Prockop DJ, et al 2007].

MSCs are multipotent cells with the capacity to differentiate to produce multiple types of connective tissue and down-regulate an inflammatory response. MSC are being explored to regenerate damaged tissue and treat inflammation, resulting from cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, stroke, diabetes, cartilage and bone injury, Crohn's disease and graft versus host disease (GvHD) [Phinney DG et al. 2007].

Few years after multipotent MSCs were identified (1980), human trials were commenced to evaluate safety and efficacy of MSC therapy.

MSC transplantation is considered safe and has been widely tested in clinical trials of cardiovascular [Ripa RS et al 2005; Chen SL et al. 2004], neurological [Lee PH et al 2008; Bang OY et al 2005], and immunological disease [Lazarus et al 2005; Ringden O et al 2006] with encouraging results.

Widely described above, MSCs are an excellent candidate for cell therapy because (a) human MSCs are easily accessible; (b) the isolation of MSCs is straightforward and this stem cells can expand to clinical scales in a relatively short period of time [Colter DC et al 2000; Sekiya I et al 2002]; (c) MSCs can be bio-preserved with minimal loss of potency [Lee MW et al 2005; Ripa RS et al 2005]; and (d) human trials using MSCs thus far have shown no adverse reactions to allogeneic versus autologous MSC transplants.

More recently, a new study shows that umbilical cord mesenchymal stem cell transplant (UC-MSCt) may improve symptoms and biochemical values in patients with severe refractory systemic lupus erythematosus (SLE) [Sun L et al 2010]. Authors reported a clinical trials on 16 patients with severe SLE that did not respond to standard treatments [Sun L et al 2010]. After receiving umbilical mesenchymal stem cell transplants, 10 of them completed at least 6 months of follow- up. There was no treatment-related mortality or other adverse events. All patients achieved at least 3 months of clinical and serologic improvement, and for two of them this was achieved without any immunosuppressive drugs. For the first time allogenic UC-MSC transplanatation was shown to be safe and effective, at least short term, in treating patients with severe SLE.

HSCs were successfully employed in gene therapy protocols. An ADA-SCID (Adenosine Deaminase Severe Combined Immunodeficiency) clinical trial was performed on 10 affected childrens [Aiuti A et al 2009]. ADA-SCID is one of the most promising conditions for treatment with combine gene therapy and cell therapy and has been the source of early successes in the field. Autologous CD34+ bone marrow cells transduced with a retroviral vector containing the ADA gene were infused into 10 children with SCID due to ADA deficiency who lacked an HLA-identical sibling donor, after non-myeloablative conditioning with busulfan.

In vivo trials have showed a relevant restored immunity in patients treated by a combination of cell and gene therapy protocol, confirmed in the long-term outcome. After about 10 years, all patients are alive after a median follow-up of 4.0 years and transduced hematopoietic stem cells have stably engrafted and differentiated into myeloid cells containing ADA and lymphoid cells. Eight patients do not require enzyme-replacement therapy because their blood cells continue to express ADA. Nine patients had immune reconstitution with increases in T-

cell counts and normalization of T-cell function. In five patients in whom intravenous immune globulin replacement was discontinued, antigen-specific antibody responses were elicited after exposure to vaccines or viral antigens. Effective protection against infections and improvement in physical development made a normal lifestyle possible. Serious adverse events were reported including prolonged neutropenia (in two patients), hypertension (in one), central-venous-catheter-related infections (in two), Epstein-Barr virus reactivation (in one), and autoimmune hepatitis (in one).

Another clinical trial was reported reviewing long-term outcome nine patients with X-linked severe combined immunodeficiency (SCID-X1), which is characterized by the absence of the cytokine receptor common gamma chain. These patients, who lacked an HLA-identical donor, underwent *ex vivo* retrovirus-mediated transfer of gamma chain to autologous CD34+ bone marrow cells between 1999 and 2002. The immune function on long-term follow-up was also assessed [Salima Hacein-Bey-Abina et al 2010].

Gene therapy was initially successful at correcting immune dysfunction in eight of the nine patients. Transduced T cells were detected for up to 10.7 years after gene therapy but however, acute leukemia developed in four patients, and one died. Seven patients had sustained immune reconstitution and three patients required immunoglobulin-replacement therapy. Sustained thymopoiesis was established by the persistent presence of naive T cells and the correction of the immunodeficiency improved the patients' health.

So, after nearly 10 years of follow-up, gene therapy was shown to have corrected the immunodeficiency associated with SCID-X1.

Another recent study was published reporting the successful application of a gene therapy protocol by using lentiviral β-globin gene transfer in an adult patient with severe β(E)/β(0)-thalassaemia dependent on monthly transfusions since early childhood. About 33 months after the treatment, the patient has become transfusion independent for the past 21th months.

These results are not only important due to the tremendous medical need that exists for thalassemia patients around the world, but also represents a significant step forward for the field of autologous stem cell therapy as an emerging therapeutic modality [Cavazzana-Calvo et al., 2010].

Today, gene therapy may be an option for patients who do not have an HLA-identical donor for hematopoietic stem-cell transplantation and for whom the risks are deemed acceptable even if this treatments are associated associated with a risk of acute leukemia.

10. Induced Pluripotent Stem cells (iPS)

In 2006 researchers at Kyoto University identified conditions that would allow specialized adult murine cells, specifically fibroblasts, to be genetically "reprogrammed" to assume a stem cell-like state, by retrovirally transducting four important stem cell factors (OCT4, SOX2, KLF4 and c-MYC) into them.[Takahashi K et al 2006]. These cells, called "iPSCs" for induced pluripotent stem cells, were in this way genetically reprogrammed by being forced to express genes which themselves regulate the function of other genes important for early steps in embryonic development. These factors were involved in the maintenance of pluripotency, which is the capability to generate all other cell types of the body.

Mouse iPSCs demonstrated important characteristics of pluripotent stem cells: they express stem cell markers, form tumors containing cells from all three germ layers, and are also able to contribute to many different tissues, when injected into mouse embryos at a very early stage during development. After one year the same author, using similar experimental

design and the same four genetic factors, reprogrammed also adult human dermal fibroblasts to iPSCs [Takahashi K et al 2007]. Human iPSCs were similar to embryonic stem cells (ESCs) in numerous ways: morphology, proliferative capacity, expression of cell surface antigens, and gene expression. They could also differentiate into cell types from the three embryonic germ layers both *in vitro* and in teratoma assays. At the same time Thomson and coworkers published a separate manuscript that detailed the creation of human iPSCs through somatic cell reprogramming using four genetic factors, two of which were in common with those reported above[Yu J et al 2007]. These cells met all defining criteria for ES cells, with the exception that they were not derived from embryos.

Despite these common features, it is not known if iPSCs and ESCs differ in clinically significant ways.

First of all, it has to be considered that direct reprogramming was originally achieved by retroviral transduction of transcription factors. Retroviruses are highly efficient gene-transfer vehicles because they provide prolonged expression of the transgene after genomic integration and have low immunogenicity. Successively lentiviral vectors was successfully employed to generate hiPSCs from various cell types, including skin fibroblasts, keratinocytes [Maherali N et al 2008], and adipose stem cells [Wu X et al 2003]. Lentiviruses are a subclass of retroviruses capable of transducing a wide range of both dividing and non-dividing cells [Sun N et al 2009].

While for retroviruses, silencing in pluripotent cells is almost complete and provides a way to identify fully reprogrammed clones [Hotta A et al 2008], lentiviruses seem to escape silencing to varying degrees, depending in part on the species and the promoter sequence. In certain cases, probably due to the site of genomic integration, retroviral vector expression is maintained [Dimos T, et al 2008; Park IH et al. 2008]. Moreover some kind of promoter allowed a continued transgene expression that increases the efficiency of iPSC generation but on the other side severely impairs iPSCs differentiation both *in vivo* and *in vitro* [Sommer CA et al 2010]. Spontaneous transgene reactivation may also occur and lead to tumor formation [Okita K et al 2007]. Partial reprogramming may have arisen from cells that either did not receive all reprogramming factors or expressed the factors with stochiometries or expression levels that did not allow for complete reprogramming.

For the above reasons alternative gene delivery methods were experimented to generate transgene-free iPSCs that are suitable for basic research and clinical applications. Recent study reported the use of a single lentiviral 'stem cell cassette' vector flanked by loxP sites (hSTEMCCA-loxP) in order to accomplish efficient reprogramming of normal or diseased skin fibroblasts obtained from humans of virtually any age [Somers A et al 2010]. Human iPSCs obtained in this way contained a single excisable viral integration, that upon removal generates human iPSC free of integrated transgenes. More than 100 lung disease specific iPSC lines were generated from individuals with a variety of diseases affecting the epithelial, endothelial, or interstitial compartments of the lung, such as Cystic Fibrosis, Alpha-1 Antitrypsin Deficiency-related emphysema, Scleroderma, and Sickle Cell Disease. An high efficiency of reprogramming was obtained, using minute quantities of viral vector. Finally all clones generated with the hSTEMCCA-loxP vector expressed a broad complement of 'stem cell markers'.

Viruses are currently used to introduce the reprogramming factors into adult cells, but this process must be carefully controlled and tested before the technique can lead to useful treatments for humans, because sometimes this integration could causes cancers. The protocol efficiency by using retro/lentiviruses is low, with a reported reprogramming rates

of 0.001% to 1%. [Wernig M et al 2007; Maherali N et al 2007]. The differentiation stage of the starting cell appears to impact directly the reprogramming efficiency: mouse hematopoietic stem and progenitor cells give rise to iPSCs up to 300 times more efficiently than do their terminally-differentiated B- and T-cell counterparts [Emnli S et al 2009]. Also terminally differentiated human amniotic fluid (AF) skin cells were reprogrammed twice as fast and yielded nearly a two-hundred percent increase in number, compared to cultured adult skin cells, probably because these cells may have an embryonic like epigenetic background, which may facilitate and accelerate pluripotency [Galende E et al. 2010]. The ability to efficiently and rapidly reprogram terminally differentiated AF skin cells provides an abundant iPS cell source for various basic studies and a potential for future patient specific personalized therapies [Galende E et al 2010].

Significant progress has been made in improving the efficiency and safety of the reprogramming technique, such as investigating non-viral delivery strategies [Feng B et al 2009; Stadtfeld M, et al 2009; Stadtfeld M, et al 2008; Page RL,et al 2009]

Recent studies have reported on the generation of iPS cells using non viral systems, such as plasmids [Kaji K et al 2009], and transposons [Woltjen K et al 2009], all of which allow for subsequent transgene removal through the Crelox system or transposases. A feasible way is to combine the reprogramming factors into a single polycistronic vector [Utikal J et al 2009], transiently expressing the reprogramming factors required to induce pluripotency. Plasmid vectors [Ko K et al 2009] were successfully used to derivate miPSCs, demonstrating that proviral insertions are not necessary for iPSC generation. For non-integrating delivery systems, the reprogramming rates were very low (approximately 0.0005%). Another possible way to induce pluripotency in somatic cells while avoiding the risks of genomic modifications is through direct delivery of reprogramming proteins. Such a strategy has been reported by different groups [Deng J et al 2009; Doi A et al 2009]. A similar study have demonstrated the feasibility of generating iPSCs by applying recombinant OCT4, SOX2, KLF4 and c-MYC proteins which have been engineered to include a C-terminal poly-arginine sequence. This sequence is capable of mediating cell permeation of the reprogramming protein factors, which, upon entering the cells, could translocate into their nuclei. In combination with valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, these protein factors could induce the reprogramming of mouse embryonic fibroblasts (MEFs) to form iPSCs. [Zhou H et al 2009].

One group even reported that hypoxic treatment can enhance the efficiency of iPSC formation [Yoshida Y et al 2009]. These non-genetic strategies have the advantage of being more readily reversible, possibly facilitating downstream differentiation processes and minimizing any permanent deleterious effects on the cells.

It is widely accepted that the choice of the delivery method will impact the reprogramming efficiency, which is defined as the number of formed colonies divided by the number of cells that were effectively transduced with the reprogramming factors [Colman A et al 2009]. Besides to the delivery method, the overall efficiency of the protocol is subject to other sources of variation that include the transcription factors and target cell type employed, the age of the donor, the passage number of the cells (inversely correlated with efficiency), and whether the specific protocol includes splitting of cells after infection.

Researchers have also investigated whether all factors are absolutely necessary. c-Myc gene known to promote tumor growth in some cases, was eliminated. Three-factors were successfully tested, using the orphan nuclear receptor ESRRB with OCT4 and SOX2. [Feng B et al 2009; Wernig M et al 2008]. In subsequent studies the number of genes required for

reprogramming were further reduced [Huangfu D, et al 2008; Hester ME, et al 2009; Kim JB,et al 2008; Kim JB,et al. 2009; Kim JB, et al 2009] and researchers identified chemicals that can either substitute for or enhance the efficiency of transcription factors in this process [Feng B et al 2009].

Of the original four transcription factor-encoding genes, OCT4 is the only factor that cannot be replaced by other family members and the only one that has been required in every reprogramming strategy in either mouse or human cells. Different cell types have been reprogrammed, including hepatocytes [Scadcfeld M et al 2008], stomach cells [Aoi T, et al. 2008], B lymphocytes [Hannal, et al.2008], pancreatic cells [Stadfeld M et al 2008], and neural stem cells [Emnli S et al 2008] in the mouse; keratinocytes [Aasen T et al 2008], mesenchymal cells [Park H et al 2008], peripheral blood cells [Loh YH et al 2009], and adipose stem cells [Sun N et al 2009] in the human; and melanocytes in both species [Utikal J et al 2009] .

An extensive comparisons between iPSc and ESC to determine pluripotency, gene expression, and function of differentiated cell derivatives were made finding some differences whose clinical significance in the application to regenerative medicine has to be determined yet.

iPSCs appear to be truly pluripotent, although they are less efficient than ESCs regarding the differentiation capacity.

Moreover both iPSCs and ESCs appear to have similar defence mechanisms to counteract the production of DNA-damaging reactive oxygen species, thereby conferring the cells with comparable capabilities to maintain genomic integrity [Armstrong L et al 2010].

Comparative genomic analyses between hiPSCs and ESCs revealed differences in the expression of some genes due to detectable differences in epigenetic methylation status [Chin MH, et al 2009; Deng J, et al 2009; Doi A,et al 2009].

Recently gene-expression profiles performed comparing iPSCs and ESCs from the same species revealed that these cells differ no more than observed variability among individual ESC lines [Mikkelsen TS et al. 2008]. A more recent studies reported a detailed comparison of global chromatin structure and gene expression data for a panel of human ESCs and iPSCs, demonstrating that the transcriptional programs of ESCs and iPSCs show very few consistent differences [Guenther MG et al 2010].

An iPSC may carry a genetic "memory" of the cell type that it once was, and this "memory" will likely influence its ability to be reprogrammed. Understanding how this memory varies among different cell types and tissues will be necessary to reprogram them successfully.

Although much additional research is needed, investigators are beginning to focus on the potential utility of iPSCs which represent patient-specific stem cell lines, useful for drug development, modeling of disease, and transplantation medicine. It is now possible to derive immune-matched supply of pluripotent cells from patient's tissue, avoiding rejection by the immune system. Patients who receive ESC-derived cells or tissues may face the same complications that result from organ transplantation (for example, immunorejection, graft-versus-host disease, and need for immunosuppression). In case of iPSCs, the need for immunosuppressive drugs to accompany the cell transplant would be lessened and perhaps eliminated altogether. Reprogrammed cells could be directed to produce the cell types that are compromised or destroyed by the disease in question. Moreover induced pluripotent cells offer the obvious advantage that they are not derived from embryonic tissues, thereby circumventing the ethical issues that surround use of these materials.

iPSCs have the potential to become multipurpose research and clinical tools to understand and model diseases, develop and screen candidate drugs, and deliver cell-replacement therapy to support regenerative medicine.

10.1 Potential medical application of iPSCs

Easily-accessible cell types (such as skin fibroblasts) could be biopsied from a patient and reprogrammed, effectively recapitulating the patient's disease in a culture dish. The usefulness of iPS cells to model a disease in a culture dish is based on the unique capacity of these cells to continuously self-renew and their potential to give rise to all cell types in the human body [Murry CE and Keller G 2008; Friedrich Ben-Nun I, Benveniscy N 2006]. The potential use of iPSCs as treatments for various disorders has been proposed and tested on *in vitro* and/or *in vivo* animal models, with promising results. Direct injection of (non-autologous) iPSCs into the myocardium of immunocompetent mouse models of acute myocardial infarction led to stable engraftment and substantial improvement in cardiac function [Nelson TJ, et al 2009]. On the other hand, dopamine neurons differentiated from iPSCs have been grafted into the striatum of Parkinsonian rats, showing a motor function recovery [Wernig M et al 2008]. A mouse model of haemophilia A has also been successfully treated by iPSC-derived endothelial cells, which express wild-type Factor VIII, directly injected into the liver [Xu D et al 2009]. Furthermore, neural progenitors differentiated from iPSCs have shown further differentiation into neural and glial cells after transplantation into the cochlea, which suggests potential application in the treatment of hearing loss due to spiral ganglion neuron degeneration [Nishimura K et al 2009].

Thus, iPSCs such as ESCs could provide a limitless reservoir of cell types that in many cases were nor previously possible to obtain. Ideally, iPSC-based therapies in the future will rely on the isolation of skin fibroblasts or keratinocytes, their reprogramming into iPSCs, and the correction of the genetic defect followed by differentiation into the desired cell type and transplantation.

Several disease-specific iPSCs are being generated such as Adenosine Deaminase deficiency-related Severe Combined Immunodeficiency, Shwachman¬Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker Muscular Dystrophies, Parkinson's disease, Huntington's disease, type 1 Diabetes Mellitus, Down Syndrome/trisomy 21, and Spinal Muscular Atrophy [Ebert AD et al 2009; Park I-H et al 2008] in order to use them to model disease pathology. For example, iPSCs created from skin fibroblasts taken from a child with Spinal Muscular Atrophy were used to generate motor neurons that showed selective deficits compared to those derived from the child's unaffected mother [Ebert AD et al 2009]. Another study reported the potential of iPS cell technology to model disease pathogenesis and treatment by creating iPS cell lines from patients with familial dysauronomia (FD), a neuropathy caused by a point mutation in theB kinase complex-associated protein (IKBKAP) gene [Lee G et al 2009]. This mutation leads to a tissue-specific splicing defect that was recapitulated in iPS cell-derived tissues, by showing *in vitro* specific defects in neurogenesis and migration of neural crest precursors, tissues that were previously unobtainable.

Before any iPSC derivatives can be considered for applied cell therapy, the potential for tumor formation must also be addressed fully. Furthermore, in proposed autologous therapy applications, somatic DNA mutations (e.g., non-inherited mutations that have accumulated during the person's lifetime) retained in the iPSCs and their derivatives could

potentially impact downstream cellular function or promote tumour formation (an issue that may possibly be circumvented by creating iPSCs from a "youthful" cell source such as umbilical cord blood) [Haase A et al. 2009].

On the basis of the unlimited capacity to be propagated *in vitro*, iPSCs are good targets for genetic manipulation by gene therapy or gene correction by homologous recombination. Classical gene augmentation therapy has also been applied to iPSCs derived from Duchenne Muscular Dystrophy (DMD) [Kazuki Y et al 2009] and Fanconi Anaemia [Raya A et al 2009]patients. In the former case, a human artificial chromosome (HAC), carrying the full length, wild-type dystrophin genomic sequence [Kazuki Y et al 2009] was introduced into iPS cells generated using retroviral vectors. For Fanconi Anemia disease, gene therapy approach using lentiviral vectors, carrying FANCA or FANCD2 genes, were performed before iPS generation [Raya A et al 2009]. The authors demonstrated that gene augmentation was a pre-requisite for successful iPSC generation, as the genetic instability of the mutant fibroblasts made them non permissive for iPS cell generation. [Raya A et al 2009]. The resultant iPSCs were shown to be phenotypically disease-free, with a functional FA pathway, as well as haematopoietic progenitors derived from these iPSCs [Raya A et al 2009].

Gene targeting by spontaneous homologous recombination has similarly been demonstrated in iPSCs [Hanna et al 2007], by successfully treating the sickle cell anemia mouse model mouse with autologous iPSCs, whose β-globin gene has been corrected by homologous recombination [Hanna et al 2007]. Reprogrammed fibroblasts from an anemic mouse were corrected by homologous recombination, successfully differentiated into hematopoietic progenitors, and subsequently transplanted back into the mouse whose bone marrow has been destroyed by irradiation.

As result of the treatment, a substantial clinical improvement was observed in the various disease phenotypes, providing a paradigm for future preclinical and clinical studies regarding gene targeting in iPSCs. As demonstrated the potential of iPS cell technology is enormous for treating genetic diseases. However it is also mandatory to develop better methods of gene therapy, as genetic integration of lentiviral vectors used for expressing therapeutic transgene maybe oncogenic [Hacein-Bey-Abina S, et al 2008]. Regarding their use in gene therapy protocol, the efficiency of homologous recombination in ES and iPS cells remains extremely low [Zwaka TP et al 2003], in this direction recent advancements were reported with zinc finger nucleases [Zou et al 2009; Hockemeyer D et al 2009].

11. Predictive toxicology and drug discovery

The unique properties of pluripotent-stem cells-based models give them the potential to revolutionize the earliest steps of drug discovery and, in particular, the stages of pathological and toxicology modelling, by providing physiological models for any human cell type at the desired amount. In particular, hepatotoxicity and cardiotoxicity are the principal causes of drug failure during preclinical testing, while the variability in individual responses to potential therapeutic agents is also a major problem in effective drug development [Rubin LL 2004; Davila]C et al 2004]. Currently new drug development continues to suffer for the limited ability to predict the efficacy and toxicity of drugs developed and tested in animal models. As a result, several promising treatments in rodents and non human primates fail in human clinical trials. Differentiated cells and/or tissues derived from human iPS cells can address this issue by providing an unlimited source of

cells to screen drug efficacy and toxicity. The human cellular models used in this field are mainly of two types: primary cells coming from patients' samples, and transformed cell lines derived from tumours or resulting from genetic manipulations. Although these resources have widely demonstrated their utility, they present well-known limitations in terms of supply and relevance respectively. This is because primary human cells are difficult to standardize and to obtain in sufficient number for toxicity testing while human cell lines are often derived from carcinogenic origin and could have different properties than non-malignant cells.

Moreover specific ethnic and idiosyncratic differences in drug action and metabolism can also be evaluated with iPS cells derived from selected individuals thereby making possible customized treatments for individual conditions. Besides the possibility to give rise to high predictive phenotypic models, pluripotent stem cells offer the possibility to explore human polymorphisms associated with drug disposition. Several gene products, including drug-metabolizing enzymes and transporters or transcription factors, are known to be involved in drug disposition, and some of them display well-established associations between genotype and metabolism [Katz, DA, et al 2008]. The advantage of iPS cell technology is that it allows for the first time the generation of a library of cell lines that may represent the genetic and potentially epigenetic variations of a broad spectrum of the population.

Besides the common characteristics and properties that they share with hESCs, iPSCs present the additional advantage that they could be derived from any patient whose disease is to be studied. Therefore iPSCs allow the access both to diseases whose mutation is known and pathologies whose causal mutation is unknown. Pluripotent SCs can be an useful tool to study disease mechanisms, either at the undifferentiated stage or in specific cell types. Moreover, they enable the expression of the pathology in the specific cellular model to be correlated with the patient's symptoms.

They can theoretically provide relevant models for any pathology, including neurological disorders and rare diseases that are difficult to analyse *in vitro*. Moreover, as they are compatible with a miniaturized format, they open the way to screening techniques using genomic resources and chemical libraries.

The use of this tool in high-throughput screening assays could allow better prediction of the toxicology and the therapeutic responses induced by newly developed drugs offering insight into the underlying mechanisms. The net result of this approach would substantially decrease the risk and cost associated with early-stage c1inical trials and could lead toward a more personalized approach in drug administration.

Since the first description of iPS cell generation three years ago, there has been remarkable progress toward clinical implementation of reprogramming technologies. Before iPSCs can be used for clinical purposes, few issues need to be addressed. The recent successes in iPS cell derivation without viral vectors and genomic integration from human cells has brought the realization of the therapeutic potential of iPS cell technology closer than ever. Importantly, however, the suitability of individual iPS cell derivation methods for generating cell populations for cell replacement therapy, disease modeling, and drug discovery remains to be widely demonstrated, and studies assessing the equivalence of different types of iPS cells are ongoing.

Moreover the long term efficacy of iPSCs treatments has to be tested considering as fundamental both the survival and the functional integration of the iPSCs, after introduced them into the patient.

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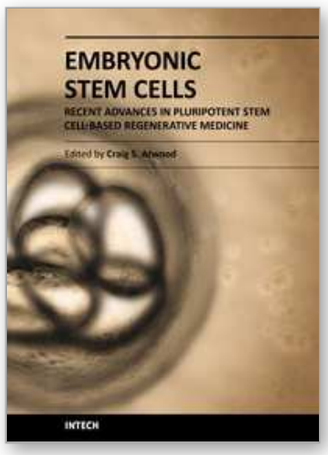

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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