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Taking Stem Cells to the Clinic: Major Challenges

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ABSTRACT

Stem cell therapy offers tremendous promise in the treatment of many incurable diseases. A variety of stem cell types are being studied but human embryonic stem cells (hESCs) appear to be the most versatile as they are pluripotent and can theoretically differentiate into all the tissues of the human body via the three primordial germ layers and the male and female germ lines. Currently, hESCs have been successfully converted in vitro into functional insulin secreting islets, cardiomyocytes, and neuronal cells and transfer of such cells into diabetic, ischaemic, and parkinsonian animal models respectively have shown successful engraftment. However, hESC-derived tissue application in the human is fraught with the problems of ethics, immunorejection, tumorigenesis from rogue undifferentiated hESCs, and inadequate cell numbers because of long population doubling times in hESCs. Human mesenchymal stem cells (hMSC) though not tumorigenic, also have their limitations of multipotency, immunorejection, and are currently confined to autologous transplantation with the genuine benefits in allogeneic settings not conclusively shown in large controlled human trials. Human Wharton's jelly stem cells (WJSC) from the umbilical cord matrix which are of epiblast origin and containing both hESC and hMSC markers appear to be less troublesome in not being an ethically controversial source, widely multipotent, not tumorigenic, maintain ''stemness'' for several serial passages and because of short population doubling time can be scaled up in large numbers. This report describes in detail the hurdles all these stem cell types have to overcome before stem cell-based therapy becomes a genuine reality. J. Cell. Biochem. 105: 1352–1360, 2008.

KEY WORDS: IMMUNOREJECTION; STEM CELL CHALLENGES; TRANSPLANTATION; TUMORIGENESIS

The physiological process of regeneration where remaining tissues organize themselves to replace a lost body part has long been recognized in lower animals. The household gecko can drop its tail on demand when confronted with its predator as a form of camouflage for escape and soon produces a new tail for its body functions. The skinks in the out backs of Australia also drop their tails at will but instead of reproducing one tail they can generate a barrage of tails. Still higher powers of regeneration are seen in the flatworm where if it is cut in half, one part grows into a head and the other into a tail. Although the molecular machinery for regeneration may be still present in the fetus, the only trade-offs that Mother Nature provided in the human are efficient wound healing and regeneration of the liver. The liver therefore is a useful model to study the physiological and pathological powers of regeneration.

It is recognized today that another possible trade-off to regeneration are the mysterious powers of the ''stem cell'' which has the unique properties of self-renewal, differentiation into other specialized cell types, and each new cell type attaining a specialized function. For example, bone marrow stem cells self-renew, differentiate into blood cells, with each blood cell having its own functions. It appears that stem cells are the common denominator for all types of regeneration [Bongso and Richards, 2004].

Human stem cell biology has drawn tremendous interest today as a potential cure for a whole spectrum of incurable diseases. Much of the basic scientific work carried out thus far has broadened our knowledge of stem cell biology, but what is urgent, is an address of the translational issues that could expedite this science reaching the clinic. This article provides an objective view of the issues confronting us as stem cell scientists and where we should be spending much of our efforts to overcome these challenges.

WHAT IS THE TRUE DEFINITION OF A BONA FIDE STEM CELL?

A stem cell possesses properties beyond its classification of self-renewal and differentiation. A bona fide stem cell must pass a full battery of characterization tests of either embryonic stem cell (ESC) or mesenchymal stem cell (MSC) markers or a combination of both. ESC markers include the surface marker antigens (Tra series, SSEA

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series, GCT series, HLA, and CD markers), *OCT4* and other genomic markers for the three primordial germ layers, confirmation of pluripotency by the production of teratomas in immunodeficient mice, stability of normal karyotype with serial culture, alkaline phosphatase positiveness, telomerase production, the ability to differentiate into tissues originating from all three primordial germ layers, confirmation of function, and a full transcriptome profiling to confirm the expression of ''stemness'' characteristics [Bongso et al., 2005]. Through such an analysis it is evident that genomic similarities and differences surface between stem cell lines, sources, and types [International Stem Cell Initiative, 2007; Muller et al., 2008]. Unlike ESCs, MSCs have less specific markers that define them. MSC markers include a complete battery of specific CD markers, the ability to differentiate into a limited number of tissues, maintenance of normal karyotype, and transcriptome profiling to identify gene constructs with ''stemness'' characteristics. Additionally, Abdallah and Kassem [2008] postulated that the ''gold standard'' assay for MSC stemness must be based on the ability of these cells to form ectopic bone and bone marrow microenvironments that support hematopoiesis upon implantation in an open system (subcutaneous transplantation) in severely combined immunodeficient (SCID) mice. Several properties common to stem cells of embryonic or adult origin have been shown through transcriptome profiling. These include active Janus kinase signal transducers and transcription activators (Notch and TGFb signaling), engagement in the cell cycle, sensing of growth factors and interaction with extracellular matrix via integrins, resistance to stress with upregulated DNA repair, remodeling of chromatin brought about by DNA helicases and methylases and histone deacetylases and translation regulation by RNA helicases of the Vasa type [Romalho-Santos et al., 2002]. Recently, the international stem cell initiative (ISCI) characterized 59 human embryonic stem cell (hESC) lines from 17 laboratories derived and grown using different protocols. All hESC lines expressed SSEA 3 and 4, Tra-1-81, GCTM2, GCT343, CD9, Thy1 (CD90), alkaline phosphatase, Class 1 HLA, genomic markers *NANOG*, *OCT4*, *TDGF1*, *DNMT3B*, *GABRB3*, and *GDF3*. However, differences were observed between cell lines for several lineage and imprinting markers. Some female lines expressed detectable levels of *XIST* while others did not [International Stem Cell Initiative, 2007]. The ISCI is now assessing the genetic changes that occur in hESCs after prolonged passage in vitro. This should also highlight differences if any between cell lines derived and grown in different laboratories [Lensch and Daley, 2007].

CLASSIFICATION OF STEM CELLS BASED ON MARKER CHARACTERISTICS

Traditionally, stem cells in the human have been classified based on whether they are embryonic or adult. However given the similarities and differences in the nature, properties and behavior of the stem cell types derived thus far, it would be more relevant to broadly classify them as hematopoietic stem cells (HSC), adult MSCs, and ESCs. Unfortunately, most of the characterization studies on these stem cell types are confined to searching for markers specific to that stem cell type only and not attempting a combination of MSC and ESC markers. Recently, when Wharton's Jelly stem cells (WJSC) were characterized, both MSC and ESC markers were present making this cell type rather unique and useful for clinical application [Fong et al., 2007]. It appears that as development progresses from the embryo to fetus to adult, stem cell plasticity and telomerase levels decrease. Hence, while hESCs are pluripotent and have the potential of differentiating into all 210 tissues in the human body, adult stem cells are multipotent and can differentiate only into a limited number of desirable tissues. While hESCs were programmed by Mother Nature to produce the three primordial germ layers, which in turn generate all the organ systems in the adult, MSCs and HSCs have to be coaxed to differentiate in vitro before transplantation with specific agents or allowed to transdifferentiate in vivo after transplantation. Figure 1 illustrates examples of cell-based therapies practiced in some clinics today. In the left hand column, bone marrow stem cells (HSC) are aspirated from patients, expanded in vitro and then transplanted into the same patient to correct leukemia without immunorejection (autologous) or transplanted into closely matched patients as an allogeneic transplant [Tan, 2005]. In the second column, limbal stem cells from the normal eye of a patient are aspirated, expanded in vitro and then transplanted into the cornea of the other injured eye of the same patient (autologous). Limbal stem cells usually differentiate into corneal cells in vivo [Ang and Tan, 2005]. In the third scenario bone marrow MSCs from the patient are aspirated, expanded in vitro and then transplanted into the injured tendons or ligaments of the same patient. In this scenario, transdifferentiation (change of one cell type to another) is expected to occur in vivo [Goh and Hong, 2005]. In all these three approaches, clinical responses appear to be very encouraging but the results of large multicentric controlled studies have not as yet been undertaken to demonstrate statistically significant benefits. The last column on the right hand side shows the strategy used by hESC scientists where hESC-derived tissues are first produced in vitro before transplantation. Here, transplantation of hESC-derived tissues tantamount to repair rather than regeneration. In this scenario, immunorejection of the hESC-derived tissue is expected as the source of stem cells are from donor embryos (allogeneic). Such hESC-derived tissue therapy has not as yet reached human clinical trials although animal validation studies have been very encouraging [Laflamme et al., 2007; Shim et al., 2007; Yang et al., 2008].

STEM CELL PLASTICITY AND ESTABLISHED PARADIGMS OF EMBRYONIC DEVELOPMENT

CELLULAR MECHANISMS OF TISSUE REPAIR WITH CELL-BASED THERAPIES

Differentiation. Differentiation is a complex physiological process where a less specialized cell acquires the characteristics of a specialized cell. This process takes place continuously after fertilization from the zygote stage to the complex stage of tissue formation. These cell changes are controlled by a series of gene expression events with upregulation and downregulation of genes as cell divisions occur. Cell plasticity can range from unipotency to

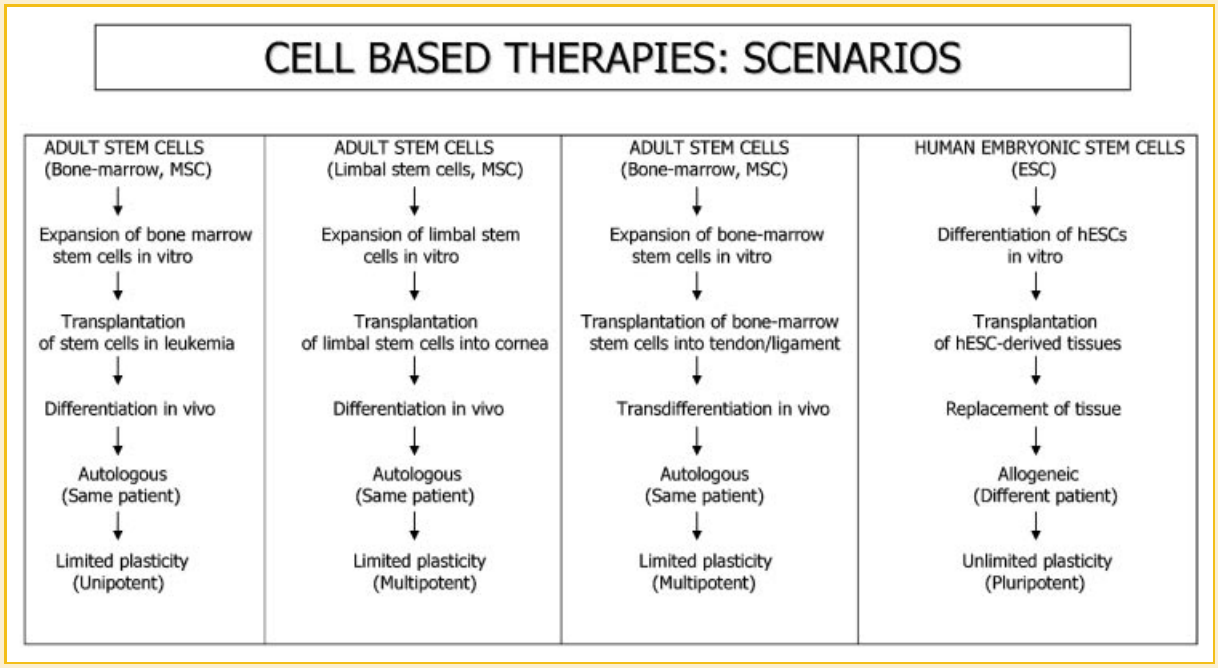

Fig. 1. Four cell-based scenarios illustrating approaches and mechanisms to tissue repair.

multipotency to pluripotency. Differentiation in vitro can be spontaneous or controlled. hESCs differentiate in vitro spontaneously in high-density and suboptimal culture conditions to produce a variety of cell types. Cells from all three primordial germ layers are usually generated and the desired lineage separated, purified, and expanded for cell-based tissue therapies [Reubinoff et al., 2000]. Such spontaneous differentiation is not observed with HSCs or MSCs. Controlled differentiation can be brought about in hESCs by first converting them into embryoid bodies (EBs) and then exposing the EBs to a whole spectrum of protein-based cytokines and growth factors to encourage differentiation along a specific lineage.

Transdifferentiation. Transdifferentiation is the irreversible switch of one differentiated cell into another. Transdifferentiation does not occur naturally in mammals and it is only in salamanders and chickens that removal of the lens of the eye results in the iris cells converting into lens cells. Transdifferentiation is also known to occur in the esophagus of the mammalian fetus where the smooth muscles can transdifferentiate partially or fully into skeletal muscles. The exact molecular mechanisms for transdifferentiation are not known. It has been suggested that it could be a simple repair mechanism in response to severe tissue damage [Phinney and Prockup, 2007]. These authors presented an excellent review on the modes of tissue repair and state of transdifferentiation when MSCs or multipotent stromal cells are used for transplantation. They stated that in trying to decipher the molecular mechanisms that regulate adult stem cell plasticity and developing ways to exploit it for therapeutic interest, much of the efforts have led to publication of many protocols for inducing adult stem cells to transdifferentiate. A large number of studies have assessed the fate of adult stem cells administered in vivo and their effect on disease progression in animal models and human clinical trials. Despite some encouraging results, in vivo studies have shown that adult stem cells typically show low levels of engraftment and transdifferentiation within the malfunctioning tissue and thus do not contribute physically to tissue regeneration significantly. Doubts are thus being cast on the role of adult stem cell plasticity to treat diseases [Phinney and Prockup, 2007]. Recent reports show that the transplanted cells alter the tissue microenvironment by (1) prompting tissue repair by secretion factors (paracrine effects), (2) stimulate already existing stem-like progenitors, (3) decrease inflammation and immune reactions and through these possible mechanisms produce functional improvement rather than transdifferentiation per se [Dimmeler et al., 2008].

Cell fusion. Cell fusion events have explained some of the dramatic responses to functional improvement after transplantation of stem cells from one organ into another. Bone marrow stem cells when transplanted into myocardial infarcts in the human showed fusion of the bone marrow stem cells and cardiomyocytes in biopsies taken from the site after engraftment. The fused cell was tetraploid and whether such cells later reduce to diploidy is not known but the incidence of cell fusion was low [Dimmeler et al., 2008].

Paracrine effects. Transplanted stem or progenitor cells from the bone marrow into the infarcted heart were also shown to release growth factors that promote angiogenesis by acting on mature endothelial cells. Other paracrine effects included arteriogenesis, reduction of cardiomyocyte apoptosis, modulation of inflammation, scar remodeling, and possible activation of cardiac stem cells

either from the atria, ventricles or the pericardium [Dimmeler et al., 2008].

HURDLES TO STEM CELL-BASED THERAPIES THAT REQUIRE URGENT ATTENTION

Three major hurdles need to be urgently overcome before stem cell-derived tissue therapy can enter the clinic. These include the (1) expansion of stem cell numbers as some stem cell types, for example, hESC, have long population doubling times of around 36–48 h; (2) immunorejection of transplanted MSC or hESC-derived tissues in allogeneic settings; and (3) safety of stem cell-based tissue therapy. Safety issues would be the concern of tumorigenesis from systemic administration of MSCs, teratoma formation from rogue undifferentiated hESCs residing in the hESC-derived tissue and the potential transmission of adventitious agents if the stem cell-derived tissues are not manufactured under current good manufacturing practices (cGMP) and according to FDA regulations.

INADEQUATE CELL NUMBERS AND ROUTES OF ADMINISTRATION

Attempts are being made through rotary culture [Carpenedo et al., 2007] and bioreactors to help expand hESC numbers. hESC-derived tissues may have to be first scaled up in large numbers and then frozen before they can be applied to patients given the large number of cells (approximately 1–5 million cells per injection) required for administration for each patient. It is not known whether a single administration or multiple injections would suffice to bring about stable functional improvement over a long time. MSCs cannot be maintained for long periods as stable cell lines unlike hESC, but because of a shorter population doubling time they may be scaled up in primary culture if large numbers are harvested initially from the respective tissues. Homing mechanisms are not definitely known for stem cells and it is not clear as to whether systemic (peripheral, portal, coronary vein), direct injection to organ or under the skin would be the best routes of administration.

IMMUNOREJECTION

Stem cell-based transplantation therapy faces immunorejection problems if the stem cell or its derived tissue is not a close tissue match to the patient. Also, the transplanted stem cells or their derived tissues may require surgical removal should there be a host versus graft reaction [Condic and Rao, 2008]. Several approaches have been suggested to overcome immunorejection. Nuclear transfer (NT) allows personalizing the hESC-derived tissue to the patient by reprogramming the patient's somatic cells to the pluripotent state using mature oocytes of human or animal origin. This approach although recently accomplished for the macaque [Byrne et al., 2007] and the human [French et al., 2008] is fraught with the problems of low efficiency, faulty faithful epigenesis, the influence of mitochondrial DNA, and implications of any remaining spindle apparatus after oocyte enucleation. Some also consider this approach illogical given the paucity of human oocytes and the concerns of viral transmission if animal oocytes are used. Recently, fetal and adult somatic cells were successfully reprogrammed to the pluripotent state using transfection of four pluripotent genes (*OCT4*, *SOX2*, *NANOG*, *LIN28*, *KLF4*, *cMYC*) (induced pluripotent stem cells, iPSCs) [Takahashi et al., 2007; Wernig et al., 2007]. This is a major breakthrough in stem cell biology and alleviates the immunorejection and ethical issues, but it is also fraught with hurdles that need to be overcome such as "partial reprogramming" possibly leading to unstable epigenesis, the implications of the viral vector and the production of teratomas. A critical assessment of this approach raised questions as to whether iPSCs are really indistinguishable from hESCs, whether the somatic cells or another cell population was reprogrammed, and the safety of iPSCs [Liu, 2008].

Alternate Routes to Preventing Immunorejection. One alternate route to preventing immunorejection is the production of panels of HLA typed hESC lines from various ethnic groups. These could be stored in repositories all over the world. It has been debated as to how many actual hESC lines would be needed for a perfect tissue match. Estimates have ranged from hundreds to thousands by different authors [Condic and Rao, 2008]. It has also been debated as to whether the existing number of surplus frozen IVF embryos available in IVF clinics worldwide would satisfy this requirement [Condic and Rao, 2008]. Modifying the histocompatability locus of hESCs to produce universal donor cell lines and encapsulating hESC-derived tissues with immunoprivileged membranes to prevent immunorejection are other possible approaches.

SAFETY OF STEM CELL-BASED THERAPIES

cGMP facilities. Safeguards in ensuring that transplanted cells are safe are usually ratified by the FDA or similar bodies. This applies to all categories of stem cells. The fact that ESCs and iPSCs carry tumor-producing properties raises serious safety issues and makes testing of their derived tissues mandatory. It is very crucial that stem cells in general and tissues derived from them be handled and screened in cGMP facilities to prevent transmission of adventitious agents before being used on humans. A variety of specific cell manufacturing issues have been identified before ESCs or MSCs can be routinely taken to the clinic. These include (1) elimination of animal products in the IVF procedures leading to embryo production, (2) elimination of xenoproteins and xenosupports in the derivation and propagation of stem cells, (3) elimination of DMSO and ethylene glycol as cryoprotective agents in freezing protocols, (4) storage of stem cells in the vapor phase rather than in the liquid phase of liquid nitrogen, (5) elimination of rogue undifferentiated hESCs in hESC-derived tissues, and (6) efficient recording systems, tracking, and shipping [Richards et al., 2002, 2004; Rao, 2008].

Tumorigenesis. Teratoma formation was reported when mouse embryonic stem cell (mESC)-derived insulin producing islets [Fujikawa et al., 2005], mESC-derived cardiomyocytes [Cao et al., 2006], and mESC-derived neurons [Schuldiner et al., 2001] were transplanted into immunosuppressed mice even though there was successful engraftment and functional improvement. When undifferentiated hESCs were injected into the hind limb

muscles or under the kidney capsule of SCID mice, teratomas were readily formed after 8–12 weeks [Richards et al., 2002] but injection of hESC-derived neurons into the brain of immunosuppressed fetal mice did not result in the formation of any teratomas after 8 weeks [Yang et al., 2008]. In another study, successful hESC-derived neuronal engraftment in a Parkinsonian rat model did not yield teratomas after 12 weeks [Ben-hur et al., 2004]. It is therefore tempting to suggest that the brain may be a tumor-privileged site. When hESC-derived osteocytes or cardiomyocytes were transplanted into the bone or heart of SCID mice, there was also no teratoma production within 1 month after injection [Bielby et al., 2004; Laflamme et al., 2007]. The longer hESCs are differentiated in vitro, the risk of teratoma formation seems to be reduced. Other sensitive animal models for testing the proliferative potential of hESC grafts have also been suggested [Lawrenz et al., 2004]. These workers injected defined numbers of mESCs together with non-neoplastic MRC-5 cells into genetically bred nude mice. Up to one million viable mESCs were injected under the kidney capsule or two million mESCs with matrigel were used for subcutaneous transplantation. They concluded that even as low as two mESCs produced teratomas. Nussbaum et al. [2007] also reported that undifferentiated mESCs consistently formed teratomas when injected into normal and infarcted hearts of nude mice. Certain sites appear to favor the growth of teratomas while others do not. In contrast to mESCs it was recently shown that 5,000 hESCs were required to produce teratomas in all animals studied while 50 hESCs completely failed to produce teratomas in any animal. Tumor formation in the lung and thymus had the highest probability of teratoma formation while the pancreas was partially site-privileged [Shih et al., 2007]. More recently, Prokhorova et al., 2008] also demonstrated that the rate of teratoma formation with hESCs in immunodeficient mice was site-dependent (subcutaneous 25–100%; intratesticular 60%; intramuscular 12.5%; and under the kidney capsule 100%).

The pathogenesis of teratoma formation after hESC-derived tissues are transplanted into animal models has not been adequately studied to understand what would actually happen when such tissues are injected into the human. It is also not known whether their pathogenesis would be the same as the naturally occurring germ line teratomas.

Thus far, hESCs generated from surplus human IVF embryos, iPSCs, and non-human primate embryos produce teratomas [Byrne et al., 2007; Aleckovic and Simon, 2008]. It is very likely that hESCs generated via other methods [altered nuclear transfer (ANT), germ cells, parthenogenesis, dead embryos, and blastomeres] will also yield teratomas simply because hESCs are pluripotent. While it is important to tackle the problem of immunorejection we also need to address the issue of tumorigenesis.

The question arises as to how does one ensure that no renegade undifferentiated hESCs are transplanted together with the hESC-derived tissue to prevent teratoma formation? The two most relevant approaches should be (1) to develop reliable methods to eliminate contaminating rogue undifferentiated hESCs and (2) to develop sensitive assays to detect residual hESC contamination in hESC-differentiated tissues prior to clinical application.

APPROACHES TO ELIMINATE ROGUE UNDIFFERENTIATED hESCs AND TUMORIGENESIS

The elimination of rogue undifferentiated hESCs may best be achieved by (1) destroying the remaining undifferentiated hESCs in the differentiated tissue population with specific agents or antibodies, (2) separating or removing the undifferentiated hESCs from the differentiated cell population, (3) eliminating pluripotent cells during the differentiation process, and (4) inducing further differentiation of left-over rogue undifferentiated hESCs. One of the mysterious properties of hESCs is that they are ''social'' cells that remain undifferentiated for long periods of time if propagated in clusters and not as single cells [Bongso et al., 1994, 2005]. In all the conventional teratoma assays using SCID mice, teratomas are produced after injection of clusters of hESCs. Recently, Ellestrom et al. [2007] demonstrated a technique for the facilitated expansion of hESCs by single cell enzymatic dissociation. The hESCs were maintained in an undifferentiated, pluripotent, genetically normal state for up to 40 enzymatic passages. They also showed that a recombinant trypsin preparation increased clonal survival compared with the conventional porcine trypsin, and that human foreskin fibroblast feeder cells were superior to the commonly used murine embryonic fibroblasts (MEF) in terms of their ability to prevent spontaneous differentiation after single-cell passaging. It would thus be very important to evaluate the outcome of injections of single cell suspensions of hESCs at specific transplantation sites in mice with intact immune systems but immunosuppressed (similar to the real human clinical situation) to evaluate the actual numbers of hESCs that produce a teratoma.

It was shown that encapsulation of hESC and mESC with membranes (2.2% barium alginate) prevented the formation of teratomas up to 4 weeks and 3 months, respectively. The mESCs but not the hESCs formed aggregates within the alginate capsules, which remained free of fibrosis [Dean et al., 2006]. These workers concluded that their preliminary work showed that improvements in their encapsulation technique may help to eliminate teratoma formation completely.

Improved safety of hematopoietic cell transplantation with monkey ESCs in an allogeneic setting was reported [Shibata et al., 2006]. Cynomolgus monkey embryonic stem cell (cyESC)-derived hematopoietic cells appeared to contain a residual undifferentiated fraction of SSEA-4 positive cells (38%) that were pluripotent and induced teratoma formation when the differentiated cells were transplanted into the fetal cynomolgus liver at the end of the first trimester. When an SSEA-4 negative fraction was transplanted, the teratomas were no longer observed while the cyESC-derived hematopoietic engraftment was unperturbed. SSEA-4 was therefore a clinically relevant pluripotency marker for primate ESCs. Purging pluripotent cells by magnetic or fluorescent cell sorting with this surface marker may be a promising method for producing clinically safe hESC-derived tissues for transplantation therapy in the human.

Density-based gradients have been widely used for sperm enhancement in various medically assisted conception procedures to separate motile sperm from immotile sperm, cell debris, and microbes. Because of their simplicity, efficiency, rapidness, and

excellent yields they have also become very popular for the separation of cells of various sizes. The Ficoll gradient yielded a twofold more mononuclear cell separation from bone marrow samples compared to Percoll [Cheng et al., 2003] and a two-layer Percoll gradient gave good separation of mESC-derived hepatocytes [Kumashiro et al., 2005]. Because Percoll may not be very safe for clinical application in the human as it is a PVP-coated silica preparation, Puresperm (a silane-coated silica preparation) yielded good separation of motile sperm from dead sperm and cell debris when a three-layer gradient was used [Chen and Bongso, 1999]. Such density gradients alone or in combination with cell sorting may be the useful approaches for the separation of undifferentiated hESCs.

The ceramide analogs (sphingosine fatty acid family) are harmless potent selective apoptosis inducing agents. Bieberich et al. [2004] showed that the expression of prostate apoptosis response-4 (PAR-4) was mediated by ceramide or ceramide analog-induced apoptosis of proliferating EB-derived stem cells. They also concluded that a portion of proliferating Oct-4 stem cells in EB-derived cells can be eliminated by apoptosis by incubation with ceramide or its analogues. Ceramide and other members of the sphingosine fatty acid family need to be evaluated for the induction of apoptosis of undifferentiated hESCs as an approach to prevent teratoma formation.

DEVELOPMENT OF ASSAYS TO DETECT RESIDUAL hESCs

The need for teratoma assays with hESCs is compelling not only to study the elimination of teratoma formation by rogue undifferentiated hESCs but also to evaluate the true pluripotentiality of newly derived hESC lines to confirm their capacity to form the many tissues that specific hESC lines can differentiate into in vitro before preparing terminally differentiated tissues for therapy. As regards the sensitivity of the approach, it was shown that when a minimum of 2 million mESCs were injected into the left flank of nude mice, 60% of the mice developed teratomas [Lawrenz et al., 2004]. Thus, when setting up a sensitive teratoma assay, several important parameters need to be studied such as injection site, route of delivery, dosage, time range, and accurate recording of false positive and negative results with proper positive and negative controls.

Anchorage-independent growth is the hallmark of cancer cells and since hESCs behave like cancerous cells, the anchorage-dependent soft agar assay or suspension culture of hESCs may yield sensitive assay systems instead of the use of SCID mice. If such in vitro assays are sensitive and reliable they would be cost-effective and will not require the use of in vivo animal testing. The lives of many SCID mice can be saved and this would be a cheaper assay. Since it has been claimed that a minimum number of cells residing in an EB is necessary for multi-lineage teratoma formation [Ellestrom et al., 2007] it is not known how many single cells when grown in suspension can eventually produce an EB and how large should such EBs be before they can produce teratomas in vivo. The culture of hESCs in single, pairs, triplets, quadruplets, etc., to form EBs may generate a cut-off point as to how many hESCs are actually needed to generate a proper EB for a given time period and the correlation of such EBs to single or multi-lineage teratoma formation will also improve the sensitivity of the assay system.

ALTERNATIVE SOURCES OF STEM CELLS WITH BOTH ESC AND MSC CHARACTERISTICS

hESC lines from embryonic sources such as blastocysts, morulae, blastomeres, dead embryos, conventional NT, ANT, and parthenogenotes [Rao and Condic, 2008] may be all pluripotent and will thus continue to face ethical challenges and the concern of tumorigenesis. Even the most recent elegant work of iPSC development is fraught with the problem of teratoma formation and the long-term stability of iPSC-derived tissues because of possible faulty epigenesis [Aleckovic and Simon, 2008]. The stability of functional outcome after long-term treatment in large controlled trials of adult stem cell (MSC or HSC) therapy will also be questioned because of the limited multipotency of such stem cells and the mechanisms of tissue repair not being definitely known. Phinney and Prockup [2007] pointed out that if we follow established paradigms of human embryonic and fetal development, cells once differentiated through their journey from fetus to adult cannot be turned back to de-differentiate or transdifferentiate. It is therefore important to consider the benefits of stem cells from other epiblastic sources which are not ethically sensitive, have differentiation potential, have not gone through the journey of fetal and adult development, and do not have the worrying issue of teratoma formation. In this context it is important to examine in depth the potential of stem cells derived from the amniotic membrane (AMSC), subamniotic membrane, and intervascular matrix of the human umbilical cord. Those from the amniotic and subamniotic membrane have been shown to be MSCs and did not exhibit any ESC markers [De Coppi et al., 2007]. Those from the intervascular matrix of the umbilical cord have been named either as WJSCs [Fong et al., 2007] or human umbilical cord perivascular stem cells (HUCPVCs) referring to regions surrounding the blood vessels within the umbilical cord [Baksh et al., 2007]. It may appear that WJSCs and HUCPVCs are one and the same originating from the mucilaginous intervascular matrix surrounding the blood vessels within the umbilical cord. The subamniotic membrane stem cells may be different from WJSCs in that they are derived from the inner surface scrapings of the amniotic membrane and not the mucilaginous intervascular matrix. Even though WJSCs originate from the embryonic epiblast, such cells are non-controversial as they are collected at term when the umbilical cord is discarded at birth. They retain a combination of most of the ESC and MSC markers in primary culture and early passages and can be propagated without major loss of "stemness" for at least 50 passages (Table I). They are therefore self-renewing and are positive for the MSC-CD markers such as CD105, CD90, and CD44 and are also positive for the ESC markers such as SSEA1 and SSEA4, Tra-1–60, Tra-1–81, alkaline phosphatase positiveness, stable normal karyotype and do not generate teratomas in SCID mice [Fong et al., 2007]. Even though 9 out of 10 ESC genomic markers were detectable, these were expressed at low levels. Additionally, they have the advantage of a shorter population doubling time

TABLE I. Differences Between ESC, ESC–MSC, and MSC

Parameter	ESC	ESC–MSC	MSC
Example	hESC	WJSC	ASC
Source	Embryos	Wharton's jelly	Adult organs
Growth in vitro	Prolonged	50 passages	Usually <50 passages
Feeders/matrices	Required	Not required	Not required
CD markers	Positive for some	Positive for many	Positive for many
ESC markers	Positive for many	Positive for some	None
Plasticity	Pluripotent	Widely multipotent	Multipotent
Homing	No	Not known	Yes
Tumorigenesis	Yes	No	No
Doubling time	Long	Short	Short
Cell numbers	Inadequate	Adequate	Adequate
Ethics	Yes	No	No
Animal models	Therapeutic	Therapeutic	Therapeutic
Clinical application	Not yet	Not yet	For some tissues
Hurdles	Immunorejection, tumorigenesis, cell numbers	Immunorejection, efficient differentiation	Immunorejection (if allogeneic), efficient differentiation

compared to hESCs, could be propagated on plastic without the need for feeder cells, and more importantly are multipotent in being able to be differentiated into neurons [Fong et al., 2007], bone, cartilage, adipose tissue, muscle and neural cells [Troyer and Weiss, 2008]. They also appear to be well tolerated by the immune system and preclinical animal work has shown that they are therapeutic via trophic rescue and immune modulation [Troyer and Weiss, 2008]. Further reasons supporting the use of HUCPVCs (WJSCs) for clinical application was recently shown by their ability to express high levels of the MSC marker CD146, high transfection efficiency with nucleofection and liposomal methods, and the demonstration of Wnt signaling pathway genes by gene array analysis [Baksh et al., 2007]. It is thus possible that AMSCs and WJSCs are close siblings to hESCs as they too are truly embryonic and the products of differentiation of the early epiblast and precursors of the amniotic cavity (Fig. 2). In fact, Edwards and Hollands [2007] emphasized some of these facts in their article on the future of the ideal stem cell for stem cell-based therapies. In general, AMSCs, WJSCs, and hESCs all originate from the inner cell mass of the human blastocyst with the major differences being that AMSCs and WJSCs are not ethically controversial unlike hESCs, retain most of the ESC markers while gaining MSC markers, have shorter population doubling times to help scale up cell numbers for therapy, and most importantly are not tumorigenic.

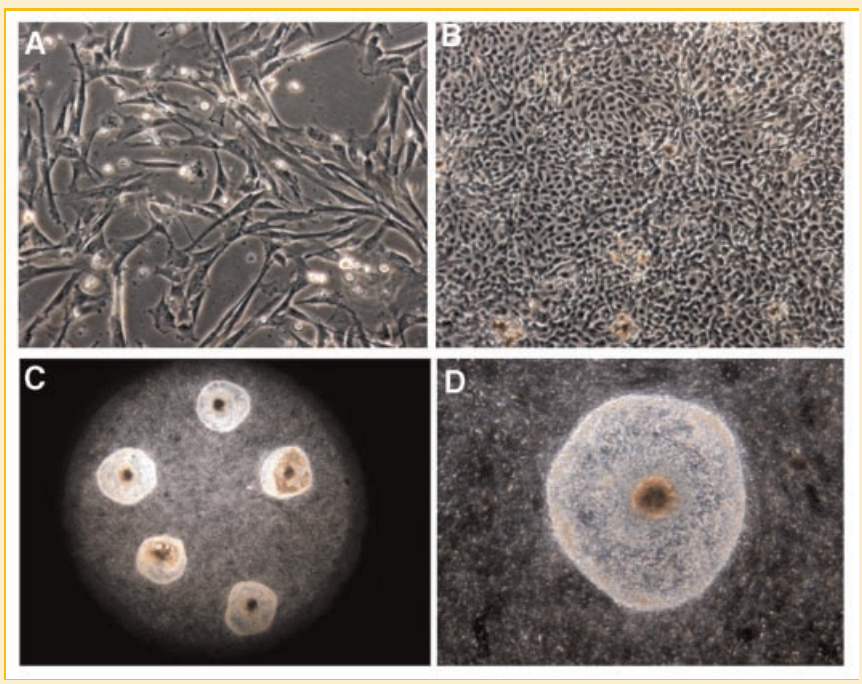

Fig. 2. Typical phenotypes of stem cells. A: Fibroblast-like mesenchymal stem cells (MSC). B: Epitheliod-like stem cells in Wharton's jelly primary cultures (MSC–ESC). C: Low magnification of hESC colonies growing on mouse embryonic feeder cells (ESC). D: High magnification of a hESC colony showing small circular hESCs adhered to each other. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EXPLOITING OTHER BENEFICIAL USES OF hESCs

There is no doubt that after the hurdles are overcome, hESC-derived tissues have a promising future for transplantation therapy given the versatility of these cells. While the focus continues on "tissue-making" for transplantation, the other beneficial uses of hESCs must not be ignored. These include (1) their use as an ideal tumorigenic model to study cancers and cancer stem cells. For example, the hESC-induced teratoma in mice may be a good environment to study the behavior of cancer cells or cancer stem cells tagged with a green fluorescence protein (GFP) and injected into the teratoma. Even though the tissues within the teratoma are disorganized their origins are from all three primordial germ layers and thus close to the human in vivo environment. Anticancer agents can also be investigated in such tumorigenic environments in animal models. (2) Undifferentiated hESCs and hESC-derived tissues are ideal in vitro platforms to screen potential drugs and anticancer agents for therapeutic use. In fact, putative therapeutic agents derived from traditional medicinal herbs can be reliably validated using undifferentiated hESCs and hESC-derived tissues in vitro. (3) Since hESCs are pluripotent they also become useful cells to study early human development and the pathogenesis of congenital defects.

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