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Teratomas From Pluripotent Stem Cells: A Clinical Hurdle

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ABSTRACT

Although basic research on human embryonic stem cells (hESCs) at the laboratory bench has progressed with enviable speed there has been little head way in terms of its clinical application. A look at the Internet however shows several stem cell clinics worldwide offering direct transplantation of undifferentiated hESCs to patients for the cure of a variety of diseases before bona fide evidence-based results can be demonstrated from large controlled studies. This raises concern because reliable protocols have to be first developed to resolve the three major hurdles delaying clinical trials such as inadequate cell numbers, immunorejection and tumorigenesis. Cell expansion methods using bioreactors, rotary culture and mitotic agents have now been developed to generate stem cell derivatives in large numbers. The problem of immunorejection can now be overcome with the development of indirect and direct reprogramming protocols to personalize tissues to patients (human induced pluripotent stem cells, hiPSCs; nuclear transfer stem cells, NTSCs; induced neuronal cells, iN). However, hESC, hiPSC, and NTSCs being pluripotent have the disadvantage of teratoma formation in vivo which has to be carefully addressed so as to provide safe stem cell based therapies to the patient. This review addresses the issue of tumorigenesis and discusses approaches by which this concern may be overcome and at the same time emphasizes the need to concurrently explore alternative stem cell sources that do not confer the disadvantages of pluripotency but are widely multipotent so as to yield safe desirable tissues for clinical application as soon as possible. J. Cell. Biochem. 111: 769–781, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: hESC; hiPSC; NTSC; TERATOMAS; TUMORIGENESIS; WHARTON'S JELLY STEM CELLS; CLINICAL HURDLES

Stem cell biology offers promise for the treatment of a variety of incurable diseases and the enthusiasm in this field continues to be robust. Since the first isolation of human embryonic stem cells (hESCs) from the inner cell mass (ICM) of human blastocysts [Bongso et al., 1994] and the subsequent establishment of the first hESC line [Thomson et al., 1998], research on hESCs has progressed at a rapid pace leading to the derivation of many desirable tissues and their successful engraftment in animal diseased models for the cure of a variety of diseases. However, the clinical translation of hESC-derived tissues to the human has not as yet been a reality largely due to the problems associated with ESCs such as limited cell numbers because of long population doubling times, immunorejection because of their donor origin, and teratoma formation because of their pluripotency. The problem of inadequate cell numbers was recently overcome with the use of rotary culture [Carpenedo et al., 2007], automated systems [Thomas et al., 2009], and mitotic agents [Gauthaman et al., 2010a] and the issue of immunorejection can now be surpassed by personalizing tissues to the patient using reprogramming methods (hiPSCs, NTSCs, iN cells) [Byrne et al., 2007; Takahashi et al., 2007; Yu et al., 2007; French et al., 2008; Huangfu et al., 2008; Vierbuchen et al., 2010]. However, the final obstacle of teratoma formation has not been adequately addressed and remains a major safety hurdle that has to be overcome before pluripotent hESC, hiPSC, or NTSC-derived tissues are taken to the clinic.

Of all the embryonic, fetal, and adult stem cell types studied thus far, hESCs, hiPSCs, and NTSCs appear to be the most versatile and promising, as they all have the unique property of differentiating via the three primordial germ layers (ectoderm, mesoderm, and endoderm) into all the tissues of the human body which is an established paradigm of human development. As such, these ESCs have been aptly called the "mother of all cells." However, their remarkable property of pluripotency also has its disadvantage in that the injection of undifferentiated hESCs, hiPSCs, or NTSCs directly into an in vivo environment (without prior controlled differentiation along a specific lineage) results in chaotic differentiation into benign or malignant tumors referred to as teratomas. The concern therefore has been that in clinical settings

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the accidental injection of rogue undifferentiated cells residing in the differentiated cell populations may induce teratoma formation jeopardizing the benefits of ESC-based cell therapies. We recently showed that before hESCs mature into teratomas, they produce cystic embryoid bodies (EBs) [Fong et al., 2009a] and such cysts have been observed at the site of injection by some groups when hESC-derived tissues were injected into diseased animal models.

Interestingly, the issue of tumorigenesis is not unique to hESCs, hiPSCs, or NTSCs only, as fetal and adult MSCs have also been shown to produce tumors different from teratomas [Houghton et al., 2004; Tolar et al., 2007; Amariglio et al., 2009]. This review aims to highlight this very important safety concern and suggests approaches to help overcome the problem while also discussing the concurrent need to search and evaluate other stem cell sources that may not have such disadvantages so as to bring cell-based therapies to the clinic as soon as possible.

TYPES OF STEM CELLS

A stem cell is a primitive cell from which all other cell types evolve and depending on the different signaling mechanisms various lineages could be produced from them. Stem cells can be broadly classified into ESCs, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), each class recognized by a battery of stem cell surface marker antigens and CD markers.

The ICM of human blastocysts which are essentially in vitro cultured hESCs is apparent around days 5–6 of embryonic development and day 9 is usually associated with hypoblast and epiblast differentiation [Fong et al., 2004]. The epiblast develops into the three primordial germ layers from which the various tissues and organs of the future human being are formed. MSCs usually lie in stem cell niches around the outer walls of blood vessels in most organs and are recruited for repair at the time of injury to that specific organ. HSCs naturally exist in the bone marrow and are committed to producing cells of the hematopoietic lineage. When isolated out of the in vivo environment, ESCs, MSCs, and HSCs can be manipulated in vitro to differentiate along useful lineages, for example, hESCs to neuronal cells [Reubinoff et al., 2000], bone marrow MSCs to cardiomyocytes [Dimmeler et al., 2008], and HSCs to multipotent hemocytoblasts [Zapata, 2009]. Stem cells in general have the capacity to self-renew and possess long life-spans compared to normal differentiated somatic cells and they contribute to the continuous turnover of cells where daily losses are encountered such as in the skin and intestine.

Thus far stem cells have been isolated from many different sources such as preimplantation human embryos [Bongso et al., 1994; Thomson et al., 1998], fetuses [Fiegel et al., 2006], umbilical cords [Fong et al., 2007; Troyer and Weiss, 2008], and adult organs [Weissman, 2000]. These groups of stem cells can also be broadly classified based on "stemness" markers and evolution into (i) primitive stem cells [(hESC), human embryonic germ cells (hEGC)], (ii) intermediate stem cells (stem cells isolated from fetal tissue and extra-embryonic membranes such as umbilical cord, amniotic fluid, amniotic membrane, Wharton's Jelly, and placenta), and (iii) adult stem cells (hematopoietic, mesenchymal, neuronal, intestinal, liver, epidermal, bone, and cartilage stem cells) [Gauthaman et al., 2010b]. Recently, man-manipulated stem cells were produced where differentiated human somatic cells were reprogrammed to the ESC state using a set of defined transcription factors (human induced pluripotent stem cells, hiPSCs) [Takahashi et al., 2007; Yu et al., 2007; Huangfu et al., 2008] or by reprogramming non-human primate somatic cells to the embryonic state by nuclear transfer (NT), using oocytes (nuclear transfer stem cells, NTSC) [Byrne et al., 2007]. Pluripotency was also induced in adult unipotent germline stem cells (gPSCs) [Ko et al., 2009].

STEM CELL PLASTICITY

Plasticity is the ability of a specific stem cell type to produce cells representing other lineages which are entirely different to its original genotype and phenotype. Plasticity depends on the prevailing environmental signals and developmental stage of the stem cell [Martin, 2003; Jang and Sharkis, 2005]. Many different cell types have the natural ability to fuse and therefore a clear demarcation between true plasticity of stem cells and cell fusion becomes important. The in vitro ability for stem cells to differentiate along desirable lineages have been reported for hESCs [Reubinoff et al., 2000; Laflamme et al., 2007; Shim et al., 2007; Yang et al., 2008], hiPSCs [Takahashi et al., 2007; Yu et al., 2007; Huangfu et al., 2008], NTSCs [Byrne et al., 2007], hMSCs [Fong et al., 2007; Troyer and Weiss, 2008], and hHSCs [Zapata, 2009] and these results led to classifying stem cells as pluripotent and multipotent. Pluripotency was reserved for the most versatile stem cells that are able to differentiate into all lineages in the human body [Luong et al., 2008] while multipotency was used for differentiation along some lineages only [Zapata, 2009]. Smith et al. [2009] discussed the various definitions of pluripotency (cellular, molecular, functional, and developmental) and emphasized the need for the establishment of a stringent set of criteria for defining pluripotency for biological studies and clinical application.

Although it is possible to differentiate multipotent MSCs into different lineages in vitro, when MSCs are transplanted autologously in vivo from one site to another (e.g., bone marrow to heart), short-term improved functional outcome is mainly from paracrine factors rather than cell fusion and transdifferentiation [Balsam et al., 2004; Murry et al., 2004; Dimmeler et al., 2008]. Also, treatment of the injured brain with hMSCs is known to amplify the intrinsic restorative process and promote functional recovery and is supposed to trigger off endogenous plasticity [Li and Chopp, 2009]. Tremendous progress has been made thus far in the differentiation of hESCs into desirable cell lineages. Successful functional outcome has also been demonstrated when such hESC-derived tissues are transplanted into animal models [Bongso and Lee, 2005]. The transplanted tissues engraft successfully, enter the in vivo stem cell niche, integrate with the host microenvironment, and improve cell function of malformed organs or tissues [Mummery et al., 2003; Ben-Hur et al., 2004; Roche and Soria, 2005]. Adult hHSCs were shown to transdifferentiate into functional endothelial cells via hemangioblasts, a common precursor to endothelial cells and hHSCs, thereby demonstrating evidence of plasticity [Bailey et al.,

2004]. The remarkable ability of stem cells to undergo plasticity provides a novel and useful tool for the treatment of incurable diseases by producing desirable tissues for the repair of malfunctioning organs by transplantation therapy.

HURDLES TO TAKING STEM CELLS TO THE CLINIC

There are still many hurdles that are delaying the translation of preclinical animal validated stem cell studies to human clinical trials. These include (1) the unavailability of adequate numbers of cells for transplantation therapy, (2) the concern of immunorejection of stem cell-derived cell populations in allogeneic settings, and (3) the concern that rogue undifferentiated hESCs, hiPSCs, or NTSCs residing in their differentiated cell populations may produce teratomas at the host's transplanted site if the cells are injected directly into the organ or that teratomas may develop in extra-transplanted sites if the cells are administered systemically.

CELL NUMBERS

Stem cells in large numbers, genetic stability and culture in serum-free and feeder-free conditions are fundamental prerequisites for all downstream cell-based applications and pharmaceutical screening. It has been estimated that 5×10^9 undifferentiated hESCs are required to derive sufficient cardiomyocytes for transplantation into an infarcted site [Mummery, 2005]. Of the various types of stem cells, the expansion of hESCs in culture is the most challenging due to its long population doubling time of 36–48 h and its "social" and anchorage-dependent growth behavior of having to adhere to each other and grow on xeno-supports such as mouse feeder cells for prolonged undifferentiated propagation. Modifications in hESC culture methods such as use of feeder-free matrices [Xu et al., 2001; Amit et al., 2004], the use of human feeders in the place of mouse embryonic fibroblasts [Richards et al., 2002], and the use of serum-free and animal-free culture media [Ludwig et al., 2006] have helped tremendously in producing clinical grade hESCs for human applications. The Rho-associated kinase (ROCK) inhibitor Y-27632 helped survival and increased proliferation rates of dissociated hESCs in serum-free culture conditions and the use of this agent has contributed significantly in expanding hESC numbers in culture and during hESC freezing [Watanabe et al., 2007; Martin-Ibañez et al., 2008; Gauthaman et al., 2010a]. Recently, nanofibrous scaffolds were also shown to support and increase proliferation rates of hESCs with retention of "stemness" properties [Gauthaman et al., 2010c]. More recently, Steiner et al. [2010] bypassed the requirement of hESCs to be anchorage dependent for continued undifferentiated propagation by culturing hESCs as floating clusters in suspension without EB formation. These authors claimed that their results pave the way for large-scale expansion and controlled differentiation of hESCs in suspension.

However, although all these approaches are important to increase cell numbers, more robust, automated, reproducible, and cost-effective cell expansion systems may be required. High-throughput devices such as the Mcllwain tissue chopper to cut hESC colonies into 200 μm fragments in a reproducible manner [Joannides et al., 2006]; stirred suspension culture systems (Hillex II) using polystyrene microcarrier particles to amplify human feeders; dissociated hESCs and hESC aggregates in a feeder-free culture system that can yield more than threefold cell expansion in about 5 days with retention of "stemness" properties and differentiation potential; are currently being made available [Phillips et al., 2008]. Furthermore, automated culture systems such as CompacT SelecT with robot-accessible incubator and other accessories integrated within a Class II culture cabinet are capable of generating $\sim 2 \times 10^9$ cells in about 8 days [Thomas et al., 2009]. These expansion processes need to be carried out in current good manufacturing practice (cGMP) conditions before they are approved for clinical applications.

IMMUNOREJECTION

Mismatch between the donor and the recipient cells would result in graft versus host disease that may be fatal unless the host immunity is suppressed with the use of immunosuppressive drugs [Condic and Rao, 2008]. The different approaches that may help overcome the problem of immunorejection are (i) establishing global repositories of large numbers of diverse HLA typed stem cell lines in an attempt to obtain the closest tissue match for a specific patient and (ii) personalizing the stem cell or its derivatives to the respective patient by either reprogramming the patient's somatic stem cells to the embryonic state via NTSCs and hiPSCs or by reprogramming the somatic cell directly to the target cell type, for example, induced neuronal cells (iN) (Fig. 1).

Global hESC repositories. Taylor et al. [2005] using blood group and HLA typing on a series of 10,000 consecutive cadaveric organ donors in the United Kingdom as a surrogate for estimating the number of hESC lines that would be required reported that 150 donors would be sufficient to provide a full match of HLA-A, HLA-B, and HLA-DR for approximately 20% of recipients and a beneficial match with only minor mismatches for 37% of HLA-A and 85% of HLA-B. They also claimed that increasing the donor numbers further would offer only a very minimal advantage with respect to HLA matching. Studies by Nakajima et al. [2007] showed that around 200 hESC lines would provide up to 80% beneficial HLA matching of the Japanese population. Condic and Rao [2008] however postulated that 150 donor hESC lines may be inadequate to treat the genetically diverse patient population in the USA. Given the large numbers of surplus frozen embryos that continue to be stored in IVF clinics worldwide today, and the possibility that a fair number of patients may donate such spare embryos for stem cell research, it is very possible that a significantly larger number of HLA typed hESC lines exceeding 200 donors could be made available globally for tissue matching. However, since it has been reported that pluripotent stem cell lines exhibit different differentiation propensities [Adewumi et al., 2007; Osafune et al., 2008], some HLA-matched lines may not produce surrogate cells for a specific disease the patient is suffering from. As such, the realistic number of hESC lines may be much higher.

Somatic cell reprogramming. A second approach to preventing immunorejection is the personalization of ESC-derived tissues to the patient. This is best achieved by reprogramming the somatic cells of the patient to the embryonic state by NT or transfection of the somatic cells with a package of pluripotent genes (hiPSCs).

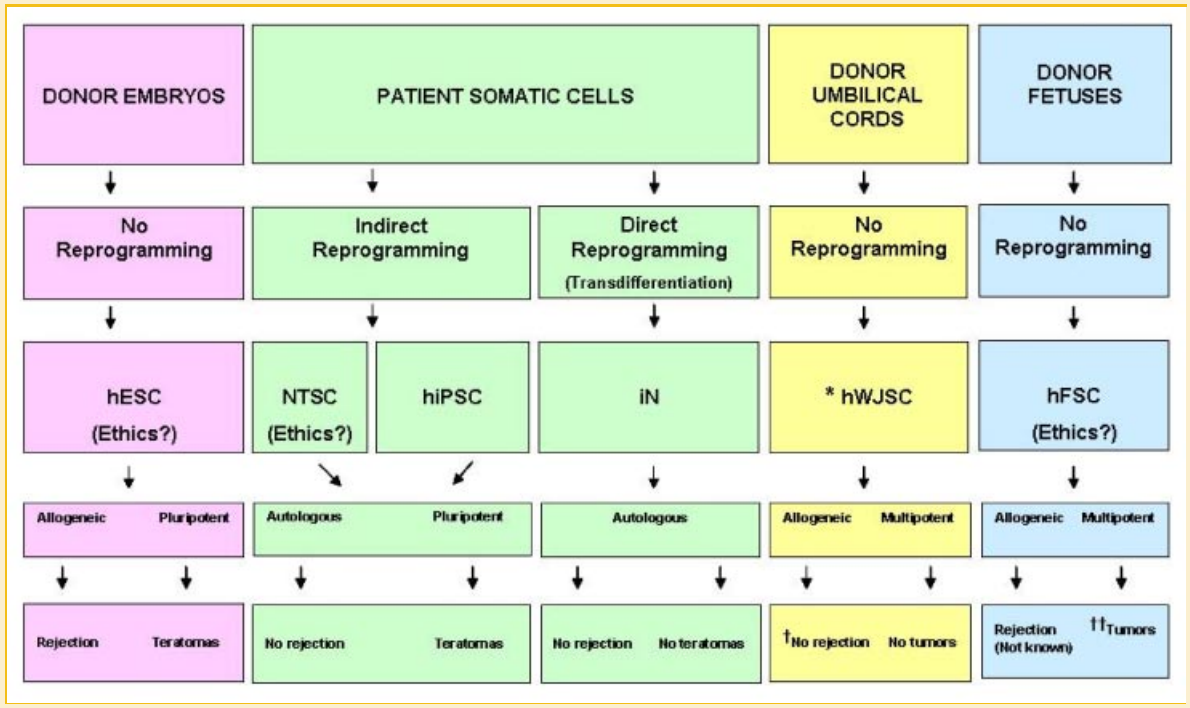

Fig. 1. Routes to production of embryonic/fetal stem cell-derived tissues for transplantation therapy. hESC, human embryonic stem cells; NTSC, nuclear transfer stem cells; hiPSC, human induced pluripotent stem cells; iN, induced neuronal cells; hWJSC, human Wharton's Jelly stem cells; hFSC, human fetal stem cells. Asterisk represents all umbilical cord matrix stem cells; †several reports show no rejection; ††brain tumors reported with fetal neural stem cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Nuclear transfer (NT). In NT, the patient's somatic cell nucleus is first fused with the ooplasm of an enucleated human or animal oocyte by electrofusion and the product then electropulsed to produce a blastocyst from which hESCs are derived. The lineages differentiated from the ensuing hESCs contain the same genome as the patient nucleus and are hence customized to the patient. NT embryos and NTSC lines have been produced in the human and non-human primate, respectively [Byrne et al., 2007; French et al., 2008], but no human NTSC lines reported to date. Although useful, there are some limitations in the use of the NT procedure such as the paucity of human oocytes, low efficiency of stem cell derivation, faulty epigenesis, the influence of mitochondrial DNA from the oocyte, and most importantly the ethical sensitivities with respect to the creation of human embryos and the use of animal oocytes (''human–animal chimeras''). To overcome such ethical sensitivities, parthenogenetic embryos (activated human oocytes with a single pronucleus without involvement of a sperm) have been used to produce hESC lines from which tissues have been derived [Lin et al., 2007; Revazova et al., 2007] but this approach also has its limitations in that human oocytes are scarce.

Human induced pluripotent stem cells (hiPSCs). Development of hiPSCs allows the personalization of cells for cell-based therapies overcoming immunorejection. However, hiPSCs have not as yet proven to have the equivalent differentiation abilities to hESCs [Feng et al., 2010; Hu et al., 2010] and as such human embryos may still be required to generate hESCs. Human adult fibroblasts were reprogrammed to a pluripotent state by transfection and incorporation of 1–4 transcription factors (OCT4, SOX2, NANOG, LIN28, KLF4, and cMYC) into the host's genome under the control of a constitutive promoter giving rise to hiPSCs from which tissues via all three primordial germ layers could be produced. This kind of mutagenesis harbors oncogenic potential and thus a great risk for cellular misbehavior. Transcription factors themselves are oncogenes (c-MYC, KLF4) which on complete silencing may produce various types of cancers in the host. To circumvent these risks excisable vector systems or protein-transduced iPSCs have been reported [Kim et al., 2009; Woltjen et al., 2009; Zhou et al., 2009; Voelkel et al., 2010]. In all the hiPSC studies confirmation of pluripotency was by teratoma formation in immunodeficient mice [Takahashi et al., 2007; Yu et al., 2007; Huangfu et al., 2008]. Recently, hiPSCs were produced by reprogramming cord blood stem cells (CBiPSCs) [Giorgetti et al., 2009], and it was postulated that the use of cord blood stem cells may circumvent the acquisition of genetic mutations in hiPSC derivation from adult somatic cells [Haase et al., 2009].

However, in all the above reprogramming protocols reported thus far, the NTSCs, hiPSCs, or CBiPSCs produced are in a pluripotent state thus conferring teratoma formation which is unsafe and undesirable.

Direct reprogramming. More recently, Vierbuchen et al. [2010] reported transdifferentiation of mouse fibroblasts directly into neuronal cells bypassing the pluripotent state by using transfection with a combination of neural-lineage-specific transcription factors. Nineteen different genes that were specifically expressed in neural tissues and involved in neural development or epigenetic reprogramming were initially selected to infect MEFs from TauEGFP knock-in mice that expressed EGFP specifically in neurons.

Following 32 days of injection, cells of neuronal morphology that were Tuj1-positive and having bright expression of TauEGFP fluorescence were detected, demonstrating that some combination of genes were helpful in converting the MEFs into neuronal cells. Following a series of experiments, a set of five different genes namely, *Brn2*, *Myt1l*, *ZiclOlig2*, and *Ascl1* were used in the transdifferentiation of MEFs and postnatal tail tip fibroblasts into more complex neuronal cell types that were Tuj1-positive, expressed pan-neuronal markers (MAP2, NeuN, Synapsin), formed action potentials, and possessed GABA receptors. *Ascl1* alone was found to be sufficient to derive induced neuronal cells although a combination with *Brn2* and *Myt1l* led to derivation of more mature neuronal cell types that also formed functional synapses [Vierbuchen et al., 2010]. The authors did not inject their induced neuronal (iN) cells into animal models to demonstrate the induction or non-induction of teratomas although teratoma production would not be expected as the iN cells were presumably not pluripotent. This elegant study opens up the possibility of alternate methods of deriving safe patient-specific lineages for clinical application, hopefully without teratoma formation (Fig. 1).

TUMORIGENESIS

Injection of undifferentiated hESC, hiPSC, and NTSCs as well as hESC and hiPSC-derived tissues into normal or immuno-compromised mice has the ability to induce teratomas [Reubinoff et al., 2000; Byrne et al., 2007; Takahashi et al., 2007] (Fig. 1). The etiopathology of teratoma formation after injection of differentiated hESC or hiPSCs is still obscure although one hypothesis is that they may originate from rogue undifferentiated hESCs, hiPSCs, or NTSCs residing in their differentiated cell populations.

It has been hypothesized that hMSCs reside in special perivascular environments (zones) within the various organs known as their stem cell niches and they can migrate across the blood vessel wall to enter the circulation and home in areas of injury to the specific organ [Meirelles et al., 2006]. Almost all organs have such stem cells in their niches [Meirelles et al., 2006] and in vivo these stem cells undergo self-renewal by asymmetrical cell division to produce two daughter cells with different properties, viz. one cell is a copy of the stem cell itself and the other cell is programmed or committed to develop into adult tissue cell types to contribute to the physiological loss. Asymmetrical cell division thus helps to constantly maintain the stem cell pool. However, when the demand is extensive as in disease or other pathological conditions, there is a possibility for the stem cell to migrate out of its special niche and when subjected to a very unfamiliar environment it predisposes itself to tumor formation [Clevers, 2005] suggesting a possible mechanism by which fetal and adult hMSCs induce tumors in their hosts.

PATHOGENESIS OF HUMAN TERATOMA FORMATION IN VIVO

A tumor is defined as an abnormal mass of tissue developed as a result of uncontrolled, progressive multiplication of cells resulting in abnormal physiological function. Tumors can be benign or malignant and there are many different types whose nomenclature usually depend on and represent the kind of tissue they arise in.

The initiation and pathogenesis of teratomas after hESC-derived tissues are transplanted into animal models have not been adequately studied to understand what would happen in the human setting. It is also not known whether their pathogenesis is the same as the naturally occurring germ line teratomas first described in the human. Generally speaking, teratomas are categorized by their developmental potential, cellular origin, and anatomical location. Although conventionally they are of germ line origin they may also arise in extra-gonadal sites that are important to fetal primordial germ cell migration including sites such as the mediastinum. Histologically, they can be classified as mature and benign (containing well-defined adult tissue structures) or immature and malignant (containing embryonal neural derivatives or masses of embryonal carcinoma cells) [Lensch et al., 2007]. Such malignant tumors are commonly referred to as teratocarcinomas [Andrews, 2002]. The majority of naturally occurring teratomas are well-defined benign masses that fail to metastasize, can be removed surgically, and do not recur. Naturally occurring teratomas are neoplastic and carry genetic defects in contrast to experimentally induced teratomas such as those produced from undifferentiated hESCs that are accidently transplanted at growth permissive ectopic sites [Lensch et al., 2007].

Teratomas and teratocarcinomas belong to the class of germ cell tumors and this class of tumor has the unique feature of disorganized arrangement of differentiated tissues originating from the three primordial germ layers, suggesting that they originate from a pluripotent precursor cell [Ulbright, 2005]. Blum and Benvenisty [2008] have provided a comprehensive review of teratomas and teratocarcinomas. Germ cell tumors have been classified into five distinct groups (Gp 1: teratomas and yolk sac tumors in infants occurring in extra-gonadal sites; Gp 2: seminomas and non-seminomas (comprising teratomas and teratocarcinomas occurring mainly in the testes of young males); Gp 3: spermatocytic seminomas occurring mainly in the testes of adult males; Gp 4: dermoid cysts in females; Gp 5: hydatiform moles in females) [Oosterhuis et al., 2007]. It was also hypothesized that teratomas from Gp 1 germ cell tumors originate from an ES-like cell while the teratomas and teratocarcinomas from Gp 2 germ cell tumors originate from a primordial germ-like cell [Looijenga et al., 2007].

It has been suggested that teratocarcinomas can be classified as malignant tumors comprising both somatic tissues and undifferentiated malignant stem cells, whereas teratomas represent only tumors comprising benign somatic tissue and immature fetal precursor cells derived from more than one of the three embryonic germ layers [Damjanov and Andrews, 2007].

Teratoma formation was reported when embryonic stem cell (mESC) mouse-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. When undifferentiated hESCs were injected into the hind limb muscles or under the kidney capsule of severely combined immunodeficient (SCID) mice, teratomas are readily formed after 8–12 weeks [Richards et al., 2002] but interestingly, injection of hESC-derived neurons into the brain of

immunosuppressed fetal mice did not result in the formation of any teratomas after 8 weeks [Zhang et al., 2001]. Also, 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 is not a preferential site and is thus tumor-privileged when it comes to transplantation of differentiated cells hence explaining the absence of teratoma formation in these two studies. However, tridermal tumorigenesis from undifferentiated iPSCS implanted into the brain was recently reported by Kawai et al. [2010]. 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]. It is possible that if the number of undifferentiated cells that escaped differentiation was low, but high enough to produce a teratoma, then teratoma formation may occur well pass 12 weeks. It was hypothesized that the longer hESCs are differentiated in vitro, the risk of teratoma formation after in vivo transplantation is reduced [Brederlau et al., 2006; Laflamme et al., 2007].

INFLUENCE OF CELL NUMBERS AND GRAFT SITE ON TERATOMA FORMATION

Cell numbers appear to influence the growth, size, and time period for initiation of teratomas. Lawrenz et al. [2004] evaluated the outcome of teratoma formation in two groups of immunocompromised nude mice. In one group they injected 1 million viable mESCs under the kidney capsule and in the other group they injected 2 million mESCs subcutaneously in the anterior lower left flank. They concluded that even as low as two mESCs generated teratomas. Shih et al. [2007] showed that the cut-off point was >50 hESCs to induce teratomas in SCID mice. At doses of 1,000 hESCs, 32–40% of animals developed teratomas and at 10,000 hESC doses, 100% of animals developed teratomas. Conventionally, to demonstrate the pluripotency of any ESC, 10 cell clusters (1–5 million cells) are injected into the thigh muscle of SCID mice to generate mature teratomas in 8 weeks that contain lineages from all three germ layers [Reubinoff et al., 2000].

Certain sites appear to favor the growth of teratomas while other sites do not. hESCs and human embryonal carcinoma cells (hECs) grafted in the liver rapidly producing large tumors containing predominantly immature cells in 3–4 weeks, while subcutaneous implants were significantly slower growing and eventually forming tumors composed of differentiated tissues. The authors concluded that the alternative growth patterns between the two graft sites indicated how environmental cues affected stem cell behavior [Cooke et al., 2006]. Recently, Prokhorova et al. [2009] demonstrated that the rate of teratoma formation was site-dependent when hESCs were injected into various sites in immunodeficient mice [subcutaneous (25–100%); intratesticular (60%); intramuscular (12.5%); kidney capsule (100%)]. When the hESCs were injected together with matrigel, subcutaneous teratoma formation was increased from 25–40% to 80–100%. There were no site-specific differences observed at histology but the subcutaneous teratomas were solid tumors as opposed to cystic teratomas at other sites. Differences in the breeds of immunosuppressed mice may play a role in the efficiency of teratoma formation. Differences seem to occur in the number of cells needed to induce tumors from cancer cell lines in different breeds of mice [Quintana et al., 2008]. Thus, differences in teratoma formation due to site specificity may be altered if different breeds of mice are used for testing. A more efficient assay both for assessing pluripotency and potential teratoma formation could be evolved by using different breeds of mice.

POSSIBLE APPROACHES TO ELIMINATION OF TERATOMA FORMATION

The question arises as to how does one ensure that no renegade undifferentiated hESCs or hiPSCs are transplanted together with the hESC or hiPSC-derived tissue thus preventing teratoma formation. To address this issue the two most relevant studies that need to be undertaken are (1) to develop reliable methods to eliminate contaminating rogue undifferentiated hESCs or hiPSCs and (2) to develop sensitive assays to detect residual hESC or hiPSC contamination in hESC or hiPSC-differentiated tissues prior to transplantation.

The elimination of rogue undifferentiated hESCs or hiPSCs could best be achieved by (1) destroying remaining undifferentiated hESCs or hiPSCs in their differentiated tissue populations, (2) separating or removing the undifferentiated hESCs or hiPSCs from their differentiated cell populations, (3) eliminating pluripotent cells during the differentiation process, (4) inducing differentiation of the renegade undifferentiated hESCs or hiPSCs (extended differentiation) and then separation of the undesired cell types.

SINGLE CELL PROPAGATION WITH ENCAPSULATION

One of the mysterious properties of hESCs or hiPSCs is that they are "social" cells that remain undifferentiated for long periods of time only if propagated in clusters (cells adhered to one another) and not as single cells [Bongso et al., 1994]. As such, in all the conventional teratoma assays using SCID mice, teratomas are produced after injection of clusters of hESCs or hiPSCs. Clonally derived hESC lines from single hESCs that maintained pluripotency and proliferative potential for prolonged periods in culture have been reported [Amit et al., 2000]. For clonal derivation, these workers supplemented their culture medium with either serum or serum replacer that was used either with or without human recombinant basic fibroblast growth factor (bFGF, 4 ng/ml). More recently, Ellerström 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.

Although hESC clusters induce teratomas, it is not clear whether single cell injection of hESCs into mice or the human will produce teratomas. Amano and Hagiwara [1976] showed that the presence of

a minimum number of pluripotent cells in an EB was essential for the production of multi-tissue type teratoma differentiation, and Ng et al. [2005] reported that forced aggregation of defined numbers of hESCs into EBs fostered robust, reproducible hematopoietic differentiation. It would thus be very important to evaluate the outcome of injections of single cell suspensions of hESCs or hiPSCs at specific transplantation sites in mice with intact immune systems but immunosuppressed, and also study the correlation between the number of hESCs or hiPSCs required to produce a single EB and the outcome of injection into mice of such EBs generated by specific numbers of hESCs or hiPSCs.

It has also been shown recently that encapsulation of hESCs and mESCs 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.

DENSITY GRADIENTS

Density-based gradients have been widely used for human sperm enhancement in various medically assisted conception procedures to separate motile sperm from immotile sperm, cell debris, and microbes. Because of their simplicity, efficiency, rapidity, and excellent yields they have also become very popular for separation of other cell types of various sizes. Different kinds of gradients have been used for different cell types with varying results. Additionally, the number of layers per gradient (2-, 3-, 4-, and 6-layer discontinuous gradients) also appears to produce different results. The commonly used density gradients have been Percoll, Puresperm, and Ficoll. 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 mouse embryonic stem cell (mESC)-derived hepatocytes [Kumashiro et al., 2005]. The use of Percoll in human laboratory protocols was discontinued as it was claimed to be unsafe for clinical application as it is a PVP-coated silica preparation and instead Puresperm (a silane-coated silica preparation) was tested and shown to yield good separation of motile from immotile sperm when used as a three-layer gradient in human IVF programs [Chen and Bongso, 1999]. We tested the efficiency of Puresperm and Percoll for enrichment and separation of a heterogeneous mixture of undifferentiated hESCs and hepatocarcinoma cells. Puresperm provided good cell separation and enrichment with higher viable cell counts and greater number of fractions than Percoll. Puresperm being safer than Percoll was considered a better alternative for cell separation and enrichment in combination with other methods for stem cell and cancer research applications [Fong et al., 2009b].

SELECTIVE PLURIPOTENT APOPTOTIC AGENTS

The ceramide analogues (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 analogue-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 have not been exhaustively evaluated for the induction of apoptosis of undifferentiated hESCs, hiPSCs, or NTSCs as a method to prevent teratoma formation.

MAGNETIC AND FLUORESCENT-ACTIVATED CELL SORTING (MACS AND FACS)

Improved safety after transplantation of monkey ESC-derived hematopoietic cells in an allogeneic setting was recently reported. Cynomolgus monkey ESC (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 [Shibata et al., 2006]. 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 of primate ESCs. Purging pluripotent cells with this surface marker may be a promising method of producing clinically safe hESC or hiPSC-derived tissues for transplantation therapy in the human. We investigated the use of MACS and FACS to separate labeled undifferentiated hESCs from a heterogeneous population of hESCs and hepatocelluar carcinoma (HepG2) cells that were consciously mixed at ratios of 10:90, 20:80, 30:70, 40:60, and 50:50 so that we could be sure of the actual number of cells separated. hESCs from two different cell lines were labeled in separate experiments for the markers SSEA-4 and TRA-1-60 using primary antibodies. Anti-PE magnetic microbeads that recognize the PE-conjugated SSEA-4 labeled hESCs were added to the heterogeneous cell mixture and passed through the MACS column. The flow-through fraction and the retained labeled fraction were then analyzed using FACS. The maximum efficacy of hESC retention using MACS was $81.0 \pm 2.95\%$ and $83.6 \pm 4.2\%$ for the two hESC lines. Using FACS all the undifferentiated hESCs labeled with the two markers could be removed by selective gating. Both hESCs and HepG2 cells in the flow-through fraction after MACS were viable in culture while by FACS separation only the HepG2 cells were viable. We concluded that MACS and FACS efficiently helps to eliminate undifferentiated hESCs based on their expression of cell surface antigens [Fong et al., 2009c].

TUMOR-PRIVILEGED SITES

It is also not clear whether certain transplantation sites favor teratoma formation over others. As such, it would be very important to clear these ambiguities by evaluating the host response to teratoma formation after injection into common transplantation sites, in single cell suspension versus cell clusters, and with or without encapsulation with membranes such as barium alginate. Prior testing in animal models in statistically large sample sizes before human application will provide information to the existence of tumor-privileged organs that may not induce teratomas providing reassurance for safe clinical application.

ANTIBODIES AGAINST ROGUE UNDIFFERENTIATED EMBRYONIC STEM CELLS

Choo et al. [2008] and Tan et al. [2009] generated monoclonal antibodies against surface antigens of undifferentiated hESCs. One specific antibody (mAB84) was cytotoxic to hESCs and embryonal carcinoma cells (NCCIT) in a concentration dependent, complement-independent manner. This antibody induced cell death of undifferentiated hESCs and not differentiated hESCs within 30 min and immunoprecipitaion of the mAB84-antigen complex showed that the antigen was similar to podocalyxin-like protein-1. When undifferentiated hESCs and NCCIT cells previously exposed to mAB84 were injected into immunodeficient mice, none of the mice showed teratomas after 18–24 weeks. This is a promising approach to eliminating teratoma formation but the results on larger numbers of animals may be required to confirm whether the protocol is foolproof for the complete elimination of teratomas.

DESTRUCTION OF TERATOMAS AFTER ENGRAFTMENT

Although it may not be an ethically comfortable approach, some workers have suggested abolishment of rogue undifferentiated hESCs after transplantation. A hESC line was engineered to carry the transgene viral thymidine kinase (HSV-tk) and on treatment with ganciclovir (an antiviral drug designed to induce apoptosis) the cells carrying the suicide gene were eliminated [Schuldiner et al., 2003]. If the HSV-tk gene was inserted under the control of a constitutive promoter, then the application of ganciclovir may kill all cells (differentiated and undifferentiated). To avoid this, the insertion of HSV-tk under the control of an embryonic promoter element would permit selective ablation of undifferentiated cells only [Naujok et al., 2010]. Cao et al. [2007] transfected mESCs with a lentivirus (containing a red fluorescent protein), thymidine kinase, and firefly luciferase and injected the mESCs into immunosuppressed ischemic rat hearts. The RFP was used to track the location of the mESCs. On treating the rats with ganciclovir the teratomas were abolished. Jung et al. [2007] repeated the same experiments substituting the RFP with a green fluorescent protein (GFP) and injected the transfected mESCs into the central nervous system of SCID mice and obtained the same success. The danger of this suicide gene approach is that the genetically modified cells may themselves undergo transformation and subsequent tumorigenecity.

PROLONGED DIFFERENTIATION IN VITRO BEFORE TRANSPLANTATION

Because of their self-renewing abilities, ESCs possess neoplastic characteristics and on prolonged culture in vitro acquire chromosomal anomalies [Andrews et al., 2005]. Interestingly, these markers (gains in 12p, 17, and X) are similar to those observed in most tumor cells including germ cell tumors. Herszfeld et al. [2006] showed that the CD30 antigen was specifically expressed in such culture-adapted karyotypically abnormal hESCs but not karyotypically normal early passaged hESCs. When these extended culture chromosomally abnormal hESCs were injected into immunodeficient mice, teratoma-like tumors were produced that contained a lesser number of lineages than those seen in normal teratomas produced from chromosomally normal hESCs [Herszfeld et al., 2006; Plaia et al., 2006]. Given this background, some have suggested that the extended culture of ESC-derived tissues may culture-adapt any rogue undifferentiated ESCs and when transplanted may result in the production of immature benign teratoma-like structures only, and this may be an alternate approach to controlling tumorigenesis after transplantation. What is implied is that these tumor-like growths being always benign would be safe and could be surgically removed after transplantation therapy. However, from a patient point of view, this may not be a reassuring and ethically viable approach to negate the concerns of tumorigenesis of cell-based therapies.

DEVELOPMENT OF ASSAYS TO DETECT RESIDUAL hESCs, hiPSCs, AND NTSCs IN VITRO

The need for teratoma assays with hESC or hiPSCs is compelling not only to study the elimination of teratoma formation by renegade undifferentiated hESCs or hiPSCs but also to evaluate the true pluripotentiality of newly derived hESC and hiPSC lines before deriving terminally differentiated tissues for therapy. 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 using proper positive and negative controls.

Anchorage-dependent growth has been considered the hallmark of cancer cells [Hanahan and Weinberg, 2000] and since hESCs behave like cancerous cells, the application of anchorage-dependent soft agar assay or suspension culture of cancer cells [Zhang et al., 2003] may yield sensitive in vitro teratoma assay systems for hESCs and hiPSCs instead of the use of immunodeficient mice. We reported a three-dimensional (3D) anchorage-independent in vitro protocol for the extended growth of EBs from hESCs up to 90 days. We grew hESCs in methylcellulose (MC) in motion culture in the presence of EB medium (EB), EB medium with Matrigel (EB + MAT), bulk culture medium (BCM), and BCM medium with Matrigel (BCM + MAT). All four groups produced EBs which with extended culture to 90 days acquired blood vessels and tissues from all three primordial germ layers. Based on histology and microarray gene expression profiles we could classify the EBs into early EBs, mature EBS, and teratomas. The EB + MAT group produced the highest number of teratomas and their DNA microarray transcriptome profiles suggested the existence of inductive microenvironment niches, and activation of pathways for self-organization, morphogenesis, and growth. Our 3D-MC (EB + MAT) in vitro system required only a few hESCs to generate large numbers of small mature teratomas, could be used to test for pluripotent cells such as hESCs, hiPSCs, and NTSCs, and served as an experimental humanized platform to study cancer cell behavior and the pathogenesis of teratoma formation [Fong et al., 2009a]. If such an in vitro assay can be further refined to be more sensitive and reliable, it would be cost-effective and will not require the use of in vivo animal testing for teratoma formation which is an ethically uncomfortable protocol for some as the in vivo protocol has been considered to be a form of human–animal chimera. Also, the lives of many immunodeficient mice can be saved and an in vitro assay would be cheaper.

NON-INVASIVE MONITORING OF ROGUE hESCs, hiPSCs, AND NTSCs IN VIVO

Once rogue undifferentiated hESCs, hiPSCs, and NTSCs in their derived cell populations are eliminated before injection in vivo, it may be possible to track the presence and behavior of any existing rogue cells non-invasively in the patient using the recently developed serial imaging approaches of Pomper et al. [2009]. These workers induced teratoma formation in SCID mice with the injection of two types of gene-modified hESCs: (1) lentiviral vectors expressing reporter transgenes encoding for luciferase for bioluminescence imaging and (2) HSV1 thymidine kinase for radiopharmaceutical-based imaging. Using an optical imaging system, bioluminescence from the luciferase-transduced hESCs was detected non-invasively in the host mice bearing teratomas long before the tumors were palpable. Single photon emission computed tomography was also used to detect the HSV1-TK hESCs. These workers claimed that non-invasive imaging methods may enable detection of undifferentiated hESCs repetitively in live recipients over long term through the expression of a reporter gene.

NOVEL EMBRYONIC STEM CELL TYPES THAT DO NOT INDUCE TERATOMAS

As much as embryonic stem cells (hESCs, hiPSCs, and NTSCs) have the potential of generating teratomas in vivo after transplantation, fetal and adult human mesenchymal stem cells (hMSCs) have also been shown to induce other kinds of tumors in independent reports [Houghton et al., 2004; Tolar et al., 2007; Amariglio et al., 2009]. Additionally, there have been conflicting data regarding the capability of hMSCs to engraft and differentiate after transplantation, and several studies reporting the short-term benefits of hMSCs suggest that the benefits are mainly attributable to paracrine-mediated effects. Paracrine effects of MSCs however following encapsulation of cells and implantation for limited periods could also reduce if not eradicate the possibility of tumor formation [Heile et al., 2009]. There is also a lack of uniformity of derivation and cell expansion methods and heterogeneity with respect to how subpopulations of hMSCs are characterized [Salem and Thiemermann, 2010]. As such, while attempts are being made to overcome these issues with hESCs, hiPSCs, NTSCs, and hMSCs including the concern of tumorigenesis, there is an urgent need for the concurrent search for stem cell sources that do not have such issues, are safe and do not induce tumorigenecity so as to expedite the approval of stem cell based therapies for human clinical trials.

In our search for such a novel stem cell, we reported the derivation efficiency, growth behavior, ''stemness'' characteristics, freeze–thaw survival rates, and differentiation abilities of stem cell populations in the Wharton's jelly of human umbilical cords [Fong et al., 2010a]. We classified these stem cells as a type lying between ESCs and MSCs based on their presence of low-level expression of ESC markers (SSEA, TRA series, pluripotent molecular markers such as SOX2, NANOG, OCT3/4, and LIN28) and high expression of hMSC-CD markers. Interestingly, based on DNA microarray transcriptome profiles we observed that hWJSCs had their own unique signature of CD markers compared to human bone marrow and umbilical cord blood MSCs [Fong et al., 2010b]. We have shown that hWJSCs can be isolated in large numbers with 100% derivation efficiency from discarded umbilical cords, have high proliferation rates in vitro, have high thaw-survival rates of 80–90%, can be differentiated into neurons, and do not induce teratomas in SCID mice [Fong et al., 2007, 2010a]. Other workers have also shown that hWJSCs are widely multipotent [Troyer and Weiss, 2008; Chao et al., 2009; Hou et al., 2009; Wang et al., 2009]. hWJSCs also have greater expansion rates, faster population doubling times, and prolonged retention of ''stemness'' characteristics in vitro than adult MSCs. Additionally, hWJSCs lack some of the immunosuppression properties of adult MSCs, do not possess class II HLA, synthesize HLA-G, and express a different cytokine profile from adult MSCs [Troyer and Weiss, 2008; Fong et al., 2010b]. As such, hWJSCs are hypo-immunogenic and they or their derivatives may not be rejected in allogeneic settings (Fig. 1). hWJSCs can be painlessly harvested from discarded human umbilical cords and are not ethically controversial unlike fetal MSCs collected from the tissues of human abortuses. There have been no reports thus far of hWJSCs inducing teratomas or other tumors in host animals in which these cells or their derivatives have been transplanted. On the contrary, hWJSCs have been shown to have anticancer effects as they have been able to abolish human breast cancers in vivo in laboratory animal models [Ayuzawa et al., 2009; Ganta et al., 2009]. We showed in pilot studies that they induce apoptosis in human hepatocellular carcinoma, colorectal and ovarian carcinoma cells in vitro (Fong et al., unpublished work). Furthermore, our preliminary studies on the DNA microarray transcriptome profiles of hWJSCs show that they highly express unique tumor suppressor genes and a battery of interleukins that may be involved in immune-mediated anticancer effects [Fong et al., 2010b].

CONCLUSIONS

Basic research in hESC biology has been progressing at a rapid pace but its clinical application has been slow. hiPSCs have been recently produced to help customize tissues to patients to circumvent the problem of immunorejection and bypass the use of human embryos. However, such reprogrammed stem cells have not as yet been proven to have the equivalent differentiation abilities of hESCs derived from surplus IVF embryos. Therefore, it is important that research progresses on both stem cell types (hESC and hiPSC) to generate desirable tissues for transplantation therapy. Although hESC, hiPSC, and reprogrammed stem cells from cloned embryos (NTSC) have the potential to develop into all cell types in the fetal and adult human, the disadvantage of this pluripotency is the generation of benign or malignant teratomas arising from rogue undifferentiated stem cells residing in the differentiated cell population that have not completed the differentiation process. Such rogue cell numbers appear to influence the growth, size, and time period for initiation of teratomas and animal studies show a preference for certain graft sites for teratoma formation and its severity. A variety of approaches have been discussed in this article to help eliminate such rogue cells.

While research on these separation methods are in progress there is an urgent need to explore other candidate ESC sources to enable rapid clinical application. Human Wharton's jelly stems cells (hWJSC) which are widely multipotent, non-controversial, hypoimmunogenic, and do not induce teratomas in vivo, may be one such attractive candidate. Its nature and properties are also discussed in this article.

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