Stem Cells and Cloning: Advances and Applications

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REVIEW

Current protocols in the generation of pluripotent stem cells: theoretical, methodological and clinical considerations

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Abstract: Pluripotent stem cells have been derived from various embryonic, fetal and adult sources. Embryonic stem cells (ESCs) and parthenogenic ESCs (pESCs) are derived from the embryo proper while embryonic germ cells (EGCs), embryonal carcinoma cells (ECCs), and germ-line stem cells (GSC) are produced from germ cells. ECCs were the first pluripotent stem cell lines established from adult testicular tumors while EGCs are generated *in vitro* from primordial germ cells (PGCs) isolated in late embryonic development. More recently, studies have also demonstrated the ability to produce GSCs from adult germ cells, known as spermatogonial stem cells. Unlike ECCs, the source of GSCs are normal, non-cancerous adult tissue. The study of these unique cell lines has provided information that has led to the ability to reprogram somatic cells into an ESC-like state. These cells, called induced pluripotent stem cells (iPSCs), have been derived from a number of human fetal and adult origins. With the promises pluripotent stem cells bring to cell-based therapies there remain several considerations that need to be carefully studied prior to their clinical use. Many of these issues involve understanding key factors regulating their generation, including those which define pluripotency. In this regard, the following article discusses critical aspects of pluripotent stem cell derivation and current issues about their therapeutic potential.

Keywords: pluripotency, stem cells, derivation, human

Introduction

Pluripotent stem cells have been derived from a multitude of embryonic, fetal and adult sources including somatic and germ cells (Table 1). Embryonal carcinoma cells (ECCs) were the first to be identified in the 1960s, from the mouse[1] and subsequently in human tissues.[2] ECCs are pluripotent cells derived from adult testicular teratocarcinomas (or mixed germ cell tumors) from which genetic, immunological and morphological evidence suggest a primordial germ cell (PGC) origin.[3] Building from these studies, pluripotent stem cells have been derived from blastocysts (embryonic stem cells, ESCs); PGCs *in vitro* (embryonic germ cells, EGCs) and more recently from adult germ cells (germ-line stem cells, GSCs) and unfertilized eggs (parthenogenetic pESCs).[4] Of significance are gene discoveries in these stem cells that have led to the ability to produce pluripotent stem cells from differentiated adult cells. These cells, known as induced pluripotent stem cells (iPSCs), have been accomplished by genetic and biochemical engineering of adult and progenitor cells with pluripotent regulators. This review will summarize current issues regarding the derivation and potential clinical applications of pluripotent cells, with a focus on human-derived stem cells (Figure 1).

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Table 1 Types of pluripotent stem cell lines

	Human
Pluripotent stem cell	Source
Embryonic stem cells	32-cell blastocysts 8-cell morula
Epiblast stem cells	Epiblast
Induced pluripotent stem cells	Unipotent fetal and adult cells
Embryonal carcinoma cells	Teratocarcinoma
Parthenogenic stem cells	Chemical-activated unfertilized egg
Germline stem cells	Spermatogonia stem cells from fetal and adult males
Embryonic germ cells	Primordial germ cells
	Mouse only
SCNT-derived ESCs	Mouse blastocysts as a result of inserting the nucleus of an adult mouse cell into an unfertilized egg

Abbreviations: ESC, embryonic stem cells; SNC, somatic cell nuclear transfer.

These unique cell lines share the general properties of pluripotent stem cells including unlimited self-renewal and the ability to give rise to derivatives of all three embryonic germ layers. Pluripotency of these cell types is demonstrated experimentally by producing cell types representing all three germ layers either spontaneously during embryoid body formation, using directed differentiation protocols in culture or in teratomas after injection into adult mice. ESCs, EGCs and iPSCs have also demonstrated the ability to produce representatives of the germ cell lineage.[5–20] The most stringent test for pluripotency involves the ability of these cells to contribute to the development of the embryo proper either partially, in chimeric mice or solely by tetraploid complementation. While most pluripotent cell types have demonstrated their contributions in chimeric mice, ESCs and now more recently, iPSCs have also proven the ability to produce viable offspring through tetraploid complementation.[21,22]

Methodology

Embryonic stem cells

The derivation and maintenance of sustainable human ESCs were first performed in 1998 by James Thomson when his team cultured the inner cell mass of developing blastocysts (embryo proper) from donated embryos received from *in vitro* fertilization (IVF) programs.[23] During this same time John Gearhart derived EGCs from cultured PGCs of the genital ridge.[24] Both research teams developed techniques learned from several decades of prior animal studies deriving pluripotent cell lines from mouse blastocysts.[25,26] Since this time, rapid progress has been achieved in improving culture conditions as more lines are developed. These improvements have primarily addressed two important issues with ESC derivation, the ability to acquire viable starting material and for clinical purposes to derive them under xeno-free conditions. This section will focus on recent updates and novel approaches for deriving and maintaining ESCs. Importantly, advances in ESC derivation will be critical given the shift in the political climate toward expanding stem cell research.

ESCs are isolated from the inner cell mass of 5- to 7-day-old blastocysts and cultured with mitotically-inactivated fibroblast cells. An in depth review of embryo-derived stem cells has been undertaken by Smith,[27] and comprehensive reviews with methodologies can be found in several books.[28–30] Successful derivation of ESCs is limited by the quality and quantity of the inner cell mass obtained.[31] As most lines have been developed from blastocysts for IVF purposes that would have otherwise been discarded, their quality is less than optimal for implantation. Recently, Daley and colleagues have reported an improved method for deriving ESCs from discarded poor-quality embryos from infertility centers.[32] This study utilized hypoxic conditions based on previous studies which show hypoxia was beneficial for preimplantation development, ESC cloning and maintaining pluripotency in culture.[33–35] However, the researchers were careful to state that their experiments were not designed to conclude whether hypoxia was beneficial for derivation. More recently, Yamanaka and colleagues have also reported 40-fold higher efficiency rates in iPSC production under hypoxic conditions compared to controls.[36] Another issue raised by Lerou and colleagues[37] was eliminating the standard immunosurgery and other manipulations normally used to remove the inner cell mass from the blastocysts. As these structures are usually disrupted in poor quality embryos this step would alleviate the stress these procedures apply to the cells. Using this combined strategy their results demonstrated 4% to 6% derivation efficiencies similar to lines derived by frozen embryos.[38]

Optimizing derivation of human ESC lines under animal free conditions is continually evolving as it remains a fundamental concern for their use in cell based therapies. Several reports have demonstrated the ability to derive human ESCs under serum-free and feeder-free conditions but the stability of these cells over long term culture is uncertain.[39,40] Importantly, it will remain to be seen if the approaches that are now employed to enhance iPSC derivation can in fact also enhance the efficiency of human ESC derivation under these conditions as well. For instance,

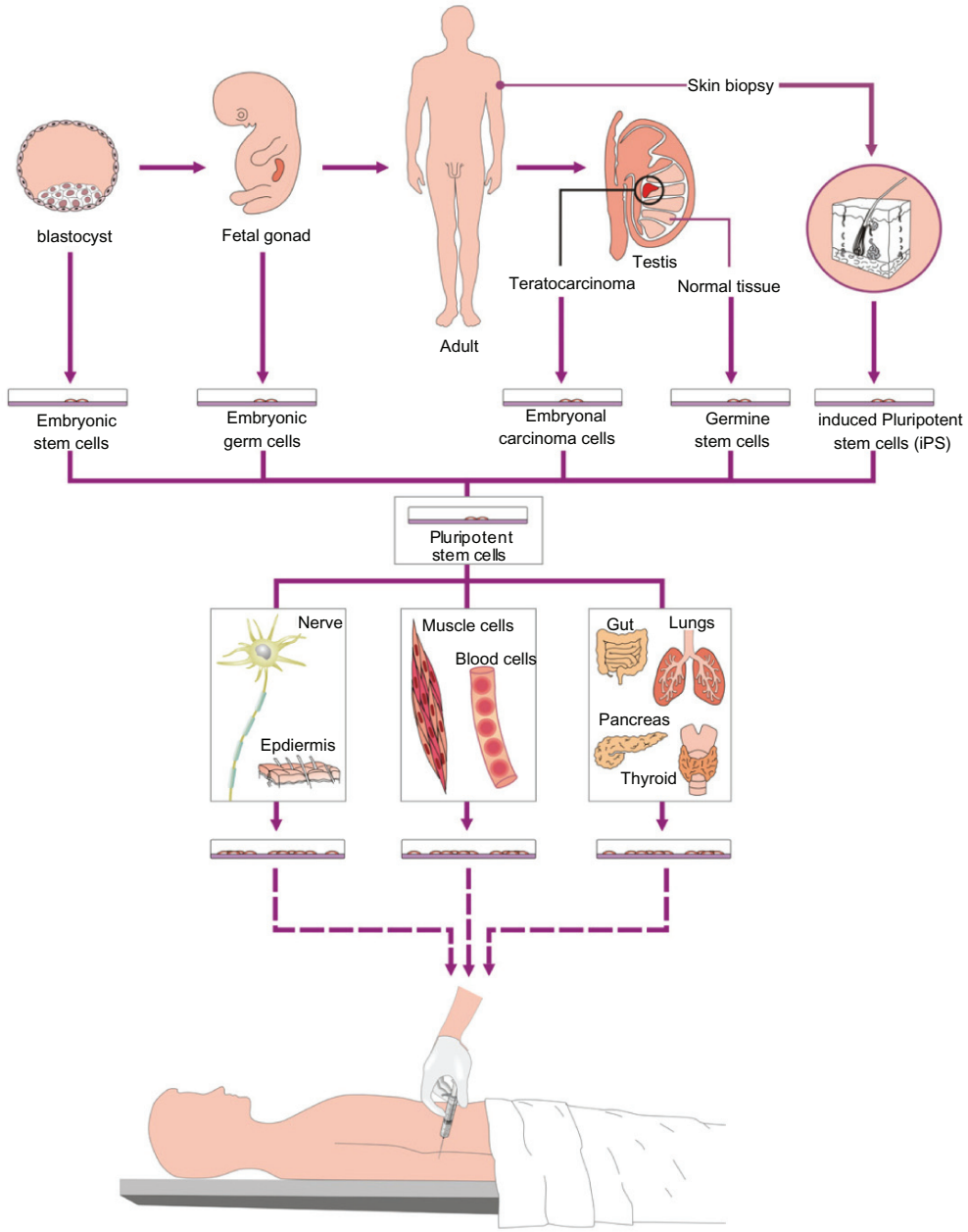

Figure 1 Human pluripotent stem cells include embryonic stem cells cultured from cells of the inner cell mass of normal or parthenogenetic blastocysts, embryonic germ cells generated from primordial germ cells in late embryonic development, embryonal carcinoma cells isolated from adult teratocarcinomas, germline stem cells derived from spermatogonia, and induced pluripotent stem cells generated by reprogramming differentiated adult cells. Pluripotent stem cells exhibit the potential to produce all cell types of the body. Thus, directed differentiation of these cells holds promise for treating a wide variety of diseases and injuries.

a recent study demonstrated that epigenetic modifying reagents like 5-aza-2'-deoxycytidine (AZA) and trichostatin A (TSA) significantly improve efficiency rates in mouse ESC derivation (40% when both AZA and TSA are added compared to 5% in controls).[41] In the mouse, epigenetic modifying reagents have been shown to dedifferentiate ESCs after embryoid formation and prevent ESC differentiation.[42,43] These studies exemplify how defining pluripotency and the factors that regulate self-renewal will be critical for improving pluripotent stem cell derivation as well as for the development of new stem cell types.

Induced pluripotent stem cells

Since the development of the first iPSCs almost 3 years ago, multiple laboratories have reported on the ability to derive iPSCs in mouse, human, rat and monkey cells by genetic and/or chemical manipulation using a small set of transcription factors and in some cases, chemical modifiers.[44] Unlike traditional

methods which utilize viral integration to introduce gene expression, current iPSC strategies focus on reprogramming cells more suitable for clinical applications.[45] These methods include transduction with proteins alone (protein transduction), utilizing small molecules that are able to facilitate expression of reprogramming factors, and by employing nonintegrating vectors that transiently express reprogramming factors. Most studies involve the exogenous expression of known genes regulating pluripotency, such as Oct4, Nanog, and Sox2 (Lin28 and Fbx12) in addition to oncogenic factors such as c-Myc and Klf4.[46–48] In every case, the combination of these factors expressed in more differentiated cell types successfully produced ESC-like colonies. These studies demonstrate that Oct4 and Sox2 are critical for reprogramming cells, while c-Myc and Klf4 expression, though not critical for transformation, does significantly increase reprogramming efficiency. In three cases, iPSCs were generated without the use of an oncogenic factor. In the first study by Thompson and colleagues, they successfully reprogrammed human fetal and neonatal fibroblasts using Lin28 and Nanog in place of Myc and Klf4. The ability to eliminate oncogenic factors like Myc and Klf4 may be attributable to differences in plasticity of reprogramming less and more differentiated cells.[49,50] In the remaining two studies, Oct4 and Sox2 were utilized along with either SV40 large T antigen to reprogram human fibroblasts or with a histone deacetylase inhibitor to reprogram mouse fibroblasts.[49,50]

Genetic integration using either a lenti- or retrovirus has been the most common method for iPSC production. One problem with generating iPSCs using lentiviral integration occurs when the inserted pluripotent genes are not silenced over time, thereby preventing differentiation of the cells for clinical application.[50] For this reason, retroviruses have been employed using a similar strategy. With retroviruses, gene silencing usually occurs a few weeks after host cell integration. However, compared to lentiviruses, retroviruses exhibit lower transduction efficiencies since these viruses specifically target replicating cells and in some cases, silencing does not occur.[51]

One main issue in iPSC induction is avoiding factors which promote tumorigenesis. To circumvent the issue of oncogenic transgene integration into the host genome, several methods are used including adenoviral transduction, transient transfection, piggyBac transposon gene-delivery system, and various chemical reagents including the direct delivery of the reprogramming proteins themselves.[51–55] The minimal set of transcription factors required to induce reprogramming is constantly being refined, as well as the application of chemical inhibitors and signaling molecules. Chemical inhibitors involved in DNA methylation, histone methylation, and acetylation not only improve reprogramming efficiencies and kinetics, but also prevent the use of additional reprogramming factors. For instance, chemical inhibitors such as AZA, valproic acid (VPA), and BIX-01294 (BIX), involved in epigenetic processes have been demonstrated to improve reprogramming when combined with conventional reprogramming factors, such as Oct4 and Nanog.[49,56–60] Other molecules such as Wnt3a, 2i, and A-83-01, have also been employed to target specific pathways which appear to enhance the transition to fully programmed iPSCs.[58–60] Nonetheless, it still remains a challenge to reprogram somatic cells by chemical treatment alone. Another critical issue in iPSC derivation is the safety of small molecules used to generate therapeutically relevant iPSCs. For example, some of these chemicals not only exert known localized changes in cells, but they also promote global modifications which will most likely result in genetic aberrations and/or dysregulation of genes. A specific example of this concept is AZA, which is known to induce DNA damage.[61]

Independent of the iPSC derivation method used, iPSC-like colonies appear to form 1 to 4 weeks after transfection. This time depends, in part, on the differentiated state of the host cells with less differentiated cells requiring a shorter time to form colonies. These colonies are then selected for clonal propagation based on morphology. Pluripotent expression patterns normally take an additional 3 to 4 weeks to develop depending on the cells and methods involved. At this point, colonies should exhibit greater than 70% to 90% $OCT4^+$ cells. Many colonies will never completely transform into pluripotent stem cells so it is very important to select and purify cells based on pluripotent cell surface markers.[62] It is the authors' experience that TRA-1-60 or TRA-1-81 appear to be more effective than SSEA4 for identifying pluripotent human cells.

Another issue in iPSC induction is the low rate of transformation of the transfected host cells. With the use of transgene expression alone 0.001% to 0.1% efficiencies have been reported, but with the addition of other "enhancing" molecules or hypoxic cell culture conditions,[36] this rate is reported to be at most 3% in human cells and 10% in mouse cells.[46,49] It also appears that higher efficiency rates are correlated with cells from earlier developmental tissue. For instance, work from our laboratory has shown efficiencies of ~2% with only the addition of two genes when applying iPSC technology to human PGCs (Figure 2). Pera and

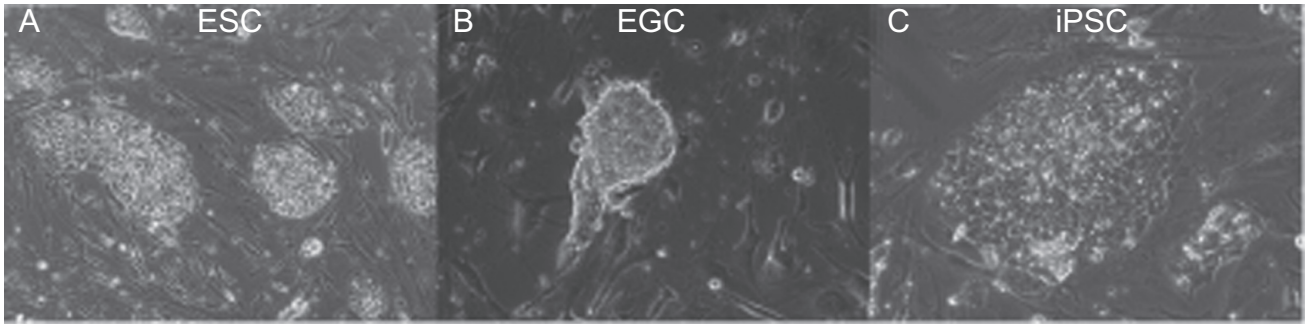

Figure 2 Pluripotent stem cell derivation produce colonies similar in morphology and culturing conditions. **A**) ESC colonies. **B**) EGC and **C**) PGC-derived iPSC colony.
Abbreviations: ESC, embryonic stem cells; EGCs, embryonic germ cells; PGC, primordial germ cells; iPSC, induced pluripotent stem cells.

colleagues also demonstrated that a subpopulation of human adult fibroblasts expressing the pluripotency marker stage specific embryonic antigen 3 (SSEA3) were the source of iPSC colonies after transduction with Oct4, Sox2, Klf4 and cMyc.[63] In this report, the efficiency of iPSC derivation was increased by 8-fold while no colonies were generated from SSEA3 negative cells. This evidence lends support to one of two models recently proposed by Yamanaka to explain the low efficiency and partial nature of iPSC generations. Here, the possibility that only SSEA3+ cells generate colonies supports that only an "elite" subset of cells are competent for reprogramming.[64] Alternatively, Yamanaka also proposes a stochastic model in which most, if not all, differentiated cells have the potential to become iPSCs. His laboratory and others provide evidence for this model by demonstrating that iPSC formation is impaired by general mechanisms involved in regulating senescence including the p53 and p21 pathways.[65,66] Thus, while studies generating iPSCs provide hope for reprogramming adult cells for therapeutic uses, iPSC research also emphasizes the need to continue finding mechanisms that regulate pluripotency and cellular reprogramming. For this purpose, future studies identifying the factors that regulate cellular reprogramming of lineage-restricted cells will be critical.

Lineage reprogramming has been shown to occur naturally in lower vertebrates by several different strategies including dedifferentiation[62] and transdifferentiation.[67] In fact, transdifferentiation in mammalian cells has been recently highlighted by two studies. Graf and colleagues have shown that lineage switching is possible in the hematopoietic system whereby the overexpression of C/EBPα and β is sufficient to reprogram B lymphocytes into macrophages. Melton and colleagues have also demonstrated lineage switching by directly converting mature pancreatic exocrine cells to endocrine β cells in adult mice.[68,69] In this study a genome-wide expression analysis of >1100 transcription factors from the developing pancreas was employed which revealed specific expression in distinct progenitor cells. Using knockout studies to pinpoint genes required for β cell fate specification, they were able to identify just three factors that together reprogrammed adult exocrine cells into β cells. These studies illustrate the significance of elucidating the molecular machinery that underlies reprogramming which can in turn be utilized to develop strategies to reprogram cell fate that do not require the pluripotent state.

Embryonic germ cells

Primordial germ cells are the progenitor cells of the germ cell lineage, which are the sole source of gametes in the adult. During human development, 50 to 100 PGCs are first distinguishable at ~22 days in the endoderm of the dorsal wall of the yolk sac, near the allantois and in the mesenchyme of the stalk. From there, they proceed to migrate through the hindgut during the fourth week and dorsal mesentery in the fifth week to reach the genital ridge.[70–72] By the end of the fifth week or early in the sixth week, ~1000 PGCs begin to actively migrate from the dorsal mesentery to the genital ridge.[73–75] At this time, in the female, premeiotic PGCs begin extensive mitotic expansion until they arrest in prophase of meiosis I beginning around week 10 in gestation.[70] In contrast, at 8 weeks male PGCs begin extensive mitotic expansion and then arrest around 10 weeks gestation.[73,74,76–78] To derive human EGCs, PGCs are isolated from the fetal gonad between 5 and 10 weeks' gestation. This time period coincides with peak PGC proliferation and encompasses the period in which the gonad undergoes sexual dimorphism into either an ovary or testis starting in the 7th week.[79] PGCs are unipotent in that they are lineage-restricted to become germ cells, do not exhibit self renewal and do not survive past one week under standard tissue

culture conditions.[80] The derivation of EGCs from human tissue has been performed by adapting methods based in part from the original EGC derivation in the mouse. Unlike other pluripotent stem cells which are primarily derived on primary mouse embryonic feeder cells (MEF), EGCs have been mainly derived using the transformed mouse embryonic fibroblast line, Sandoz Thioguanine- and Ouabain-resistant mouse fibroblasts (STO). A few reports deriving mouse and recently one involving human PGCs has successfully utilized MEFs for EGC derivation.[81–84]

In most studies, EGC growth media has consisted of serum as first reported for human ESC derivation.[23] However, a recent report suggests that MEFs with serum replacement are more efficient for human EGC derivation.[81] These results highlight that more research in this area is needed to improve EGC derivation. For instance, deriving EGCs is a unique process when compared to deriving other ESCs by the addition of several different factors. Forskolin is one such pharmacological agent which raises intracellular cAMP levels and has been shown to stimulate mitosis in PGC cultures.[80] Forskolin has been uniquely employed in EGC derivation to increase derivation efficiency, however, it is not required. Derivation of human EGCs also relies on leukemia inhibitor factor (LIF). LIF was originally known for its inhibitory role in liver cell differentiation[85] and later employed for the derivation of mouse embryonic stem cells (mESC) where it signals via the LIF receptor (LIFR), gp130 and intracellular stat3b to maintain mESC pluripotency.[86] However, activation of this pathway does not maintain self-renewal of human ESCs, but is required for human and mouse EGC culture. Another growth factor which may be critical for EGC derivation is stem cell factor (SCF), also known as c-kit ligand. Used for mouse EGC derivation, this factor is well known for its role in mPGC proliferation and survival and has been attributable to the reduced ability of MEFs when compared to STO for culturing mPGCs.[85] Although our laboratory has not seen an effect of adding SCF in our human EGC cultures, we have shown a positive correlation in PGC proliferation and survival in cultures with subcloned STO feeder cells expressing increased concentrations of transmembrane SCF.[87] Finally, EGC derivation like all other pluripotent stem cells relies on fibroblast growth factor (FGF2) for proliferation and survival. FGF2 functions as a potent mitogen in many cell types, and was also the determining factor which led to the first EGC derivation from mice.[88–90]

In the first week, most human PGC cultures do not produce visible EGC colonies. Staining for tissue non-specific alkaline phosphatase (TnAP) activity demonstrates the presence of solitary PGCs with either stationary or migratory morphology. After 2 to 3 weeks, large and recognizable EGC colonies develop at approximately 10% to 20% efficiency rates. Compared to other pluripotent stem cells, EGCs are challenging to maintain due to the difficulty in disaggregating colonies. This issue together with problems in obtaining PGCs significantly hampers research in this area.

Germ-line stem cells

Recent studies have shown that pluripotent stem cells can also be produced from germ cells isolated further along in male development.[91–93] These cells, known as germ-line stem cells (GSCs), were first generated from mouse spermatogonia, and last year, the first study was reported deriving GSCs from testicular biopsies of men.[91–93] Spermatogonia stem cells (SSCs) of the male germ line are present at birth. They develop from PGCs in the fetal gonad and consist of multiple subpopulations that either self renew or continue the differentiation process leading to sperm development. Although GSCs derived from the neonate mouse produced teratomas, cells initially reported from the adult testis did not, suggesting that they were multipotent.[94,95] However, Scholer and colleagues using a different method derived GSCs from adult mice which demonstrated teratoma and chimera formation, including germ cell contribution and transmission.[96] Similar experiments have also been performed on human adult testicular biopsies which in one study produced GSCs capable of teratoma formation.[94,97] As with any human germ cell line, these cells cannot be utilized for chimera testing.

In both the mouse and human studies, the ability to generate fully reprogrammed GSCs that can form teratomas and contribute to chimeras appears to be dependent on the method of cell selection and possibly the growth factors employed. For instance, these studies utilized different cell-surface markers including CD49f, CD90, GDNFRα1, and CD133 in combination with various cell culture matrices like collagen and laminin, to select the "appropriate" spermatogonial subpopulation.

The success of deriving GSCs is attributable to a long history of studying SSCs in culture.[98–100] Interesting comparisons can be made between GSC culture conditions and culture conditions used to produce other pluripotent stem cells. For instance, like EGCs, several lines of evidence suggest that GSCs may require LIF for complete reprogramming. This is supported by Conrad and colleagues who demonstrated by testing various conditions with and without LIF, that LIF alone was sufficient to produce viable GSC lines capable of forming teratomas. His study also

noted that the addition of fibroblast growth factor 2 (FGF2) or glial-derived neurotrophic factor (GDNF) did not increase the efficiency rate.[94] In contrast, Kossack and colleagues attempted to generated human GSCs without the use of LIF and were unable to demonstrate teratoma formation.[97] In addition, culturing cells over a longer period of time in the presence of LIF under ESC culture was attributed to the generation of mouse GSCs which not only formed teratomas, but also produced chimeras with germ-line transmission.[96] Interestingly though, unlike EGCs, human GSC generation does not appear to require FGF2 or GDNF which have been shown previously to support SSC survival in culture.

One benefit of GSCs it that they provide an adult source for pluripotent stem cells without the complications of reprogramming. Yet further analysis and functional validation in animal studies are required to evaluate their potential for clinical applications. Two primary concerns about the use of GSCs for clinical applications include their uniparental epigenetic imprints and the potential availability they may have for only male patients.

Parthenogenetic stem cells

Parthenogenesis is the development of a diploid embryo from a female gamete without contributions from a male. This process occurs naturally in some invertebrate and vertebrate species (such as reptiles, fish), but is very rare in mammals. Parthenogenetic activation can also be induced experimentally using chemicals to mimic sperm-induced Ca^{2+} oscillations (such as alcohol, ionomycin, or cycloheximide) or by physical stimulation including mechanical stimulation, cold temperatures and electrical shock.[101–103] After activation, exposure to cytoskeletal inhibitors (such as 6-dimethylaminopurine) prevent the extrusion of the second polar body creating a diploid parthenote. In mammals, parthenogenetic embryos are unable to thrive beyond the early postimplantation stage, largely because of the lack of paternally expressed imprinted genes required for the normal development of extra embryonic tissues.[104,105] In fact, Kono and colleagues have demonstrated the ability to produce the birth of live parthenogenetic mice which were able to produce offspring when the appropriate imprinting of key genes were expressed.[106,107] In humans, this concept is also demonstrated in a single case report of spontaneous parthenogenetic chimerism in which the patient survived with a mixed makeup of normal and parthenogenetic cells.[108]

The first report of the intentional creation of human patient specific pESC lines was published by Pryzhkova and colleagues.[109] These lines possessed all of the typical characteristics of human ESC lines generated from IVF embryos. This includes pluripotent marker expression (summarized in Table 2), the ability to differentiate into cellular derivatives of all three germ layers *in vitro* and the ability to form teratomas in immuno-deficient mice (reviewed in).[110] Although chimeric studies cannot be applied to human pESCs, mouse pESCs have contributed to adult tissue in chimeras including germ-line transmission.[111–113] Human pESCs lines have also been generated by other laboratories.[114,115] In fact, the erroneous report by Hwang and colleagues declaring the first successful derivation of a human somatic cell nuclear transfer (SCNT) ESC-line was later identified by Daley and colleagues to be pESCs which contained genetic material solely from the oocyte donor.[116,117] Together these studies have shown that pESCs can be derived successfully at relatively high efficiency rates, ~10% to 16% when compared with other stem cells.[102,114,115,117,118]

From work in mice, it was originally thought that human pESCs would also be for the most part genetically homozygous.[119] This is critical from a clinical standpoint in terms of minimizing the risk of immunological rejection in patients. However, two landmark reports on this issue demonstrated several human pESC lines that were heterozygous at several loci which resulted from genetic recombination events during oocyte maturation. Importantly, loci included the major histocompatibility complex (MHC) which plays a defining role in autoimmunity.[109,114] This issue was resolved by two groups who demonstrated methods to generate HLA-homozygous pESC lines by pre-selecting an HLA-homozygous egg donor[120] or by generating haploid parthenogenetic embryos.[115,120] These cells are produced by eliminating the cytoskeletal inhibitor step which permits the extrusion of the second polar body after oocyte activation.[120]

Table 2 Pluripotent stem cell markers

Marker	ESC	GSC	EGC	pESC	iPSC	ECC
Tra-1-60, 81	+	+	+	+,+	+	+
SSEA3	+	+	+	+	+	+
SSEA4	+	+	+	+	+	+
SSEA1	–	–	+	–	–	–
TnAP	+	+	+	+	+	+
Telomerase	+	+	+	+	+	+
Oct4	+	+	+	+	+	+
Nanog	+	–	+	+	+	+
Sox2	+	+	low	+	+	+

Abbreviations: ECC, embryonal carcinoma cells; EGC, embryonic germ cells; ESC, embryonic stem cells; pESC, parthenogenic ESC; GSC, germ-line stem cells; iPSC, induced pluripotent stem cells.

In general, pESC derivation mimics those of ESCs in terms of blastocyst isolation and cell culture. Oocytes are collected from donors after hormonal stimulation for *in vitro* fertilization (IVF) purposes and then subjected to electric stimulation or as Pryzhkova and colleagues have shown, chemical induction alone with ionophore, for egg activation followed by kinase inhibitor 6-dimethylaminopurine (6-DMAP) to prevent the extrusion of the second polar body (this step eliminated for homozygous lines).[102,109] After activation, embryos are cultured and the inner cell mass (ICM) is isolated from days 5–6 blastocysts. Like ESCs, derivation requires either human or mouse fibroblasts as a feeder layer. Growth media and serum requirements for pESC derivation are also similar to those used for ESC derivation which include knockout serum replacement (Invitrogen) or human serum. While all studies utilize FGF2 most, but not all, used LIF demonstrating LIF is not critical for deriving pESCs (pers comm, Dr Marina Pryzhkova).[115]

Another possible source for human pESCs has been demonstrated in reproductively incompetent oocytes, including those that are immature, failed or abnormally fertilized from IVF. These cells are normally discarded during IVF and so alleviate some of the ethical concerns that surround using normal embryos. The process of IVF or intracytoplasmic sperm injection (ICSI) can sometimes produce aneuploid embryos with none or multiple pronuclei.[121] In 2004, Suss-Toby and colleagues reported the generation of a human ESC line from a mononuclear zygote following ICSI, which demonstrated normal diploid female karyotype (46,XX) and corresponding ESC characteristics.[121] These authors suggest, that mononuclear zygotes can develop into normal blastocysts after sperm penetration as a result of asynchronous formation of pronuclei. Although, this study did not determine the parthenogenetic origin of the ESC line, others have now shown that mononuclear zygotes discarded from IVF can be an additional source for creating human pESCs. Of importance, these parthenogenetic lines expressed all of the properties of a normal euploid hESC line.[115,118,122]

One important factor in the therapeutic application of pESCs is that currently human pESCs have only been derived from fresh oocytes making this stem cell limited to women who are able to donate eggs. However, if human pESCs could be derived from cryopreserved oocytes this would provide the opportunity to treat women with decreased ovarian reserve and women needing chemotherapy. Two recent studies show great promise in this area. For example, mouse pESC lines that have been derived from cryopreserved ovaries, express ESC-specific markers and differentiate into embryoid bodies *in vitro* and teratomas *in vivo*.[123] Another study has also demonstrated the ability to produce parthenogenetic human blastocysts from cryopreserved oocytes at high efficiency rates.[124] Together these studies provide promise for cryopreserved eggs in the future as a potential source for pESCs.

Unlike work done in ESCs and EGCs, few studies have explored the clinical application of pESCs using transplant models.[125] For instance, one study has shown the stable and functional hematopoietic engraftment of mouse pESCs derived from uniparental genomes.[126] Another study demonstrated the ability of rabbit pESCs to differentiate into myogenic, osteogenic, adipogenic, and endothelial lineages. These cells were injected into a chemically induced injured tibialis muscle of nude mice, where they were able to integrate and form muscle- and bone-like tissues.[127] In addition to these studies, pESCs from nonhuman primates have also been derived that could differentiate into dopamine neurons that restore function in a rat model of Parkinson's disease.[128]

Clinical considerations

Given their properties of unlimited self renewal and differentiating potential, pluripotent stem cells hold the promise of providing sufficient numbers of differentiated cells that could potentially be used to treat a wide variety of human conditions, including heart disease, diabetes, and many neurological disorders. However, it is unknown which source of pluripotent stem cells will provide the best therapy for any given disease or affliction. In fact, it seems more reasonable given the uniqueness of different pluripotent stem cells that there will not be only one given stem cell line or approach that provides the single resolution for the diverse needs across all cell-based therapies. Most importantly, the ideal candidate must be easily and reproducibly cultured and manipulated so that the stem cells possess the necessary characteristics for successful differentiation, transplantation and engraftment. This includes taking steps to prevent unregulated proliferation, unwanted cell migration from the lesion site, incorrect differentiation, and poor functioning of transplanted cells.

Ethical and scientific hurdles remain when using pluripotent stem cells in cell-based therapy. For instance, the ethical issues surrounding the use of embryonic and fetal sources for many of these lines present a challenge. In this respect, iPSCs from adult tissues are less controversial and provide an avenue for producing patient-specific cell lines which would eliminate complications involving allograft rejection.[46,129–131] However, one of the main obstacles for utilizing these cells is the use of oncogenic factors or vectors

which may cause tumorigenesis. In fact, all of the iPSCs reported to date require an oncogenic factor to produce lines from adult tissue at a notable efficiency.[4] Several studies have reported on karyotypic abnormalities which develop in iPSC lines.[50,132] For instance, Cheng and colleagues found that utilizing the SV40 large T antigen (Simian Vacuolating Virus 40 TAg) to increase transduction efficiency, led to the majority of iPSC lines with abnormal karyotypes. This is not surprising given that the large T antigen is an oncogene associated with the transformation in a variety of cell types.[133] Another problem facing iPSC technology is poor efficiency with reported induction rates of only 0.001% to 10% from transfected cells. These rates strongly suggest the involvement of other factors that are critical to regulate reprogramming.

Several reports of the therapeutic use of human pluripotent stem cell-derived cells have been reported across animal models representing a variety of treatable diseases and injuries. First clinical reports were shown in neural-derived cells from human ECCs and EGCs.[130] These studies included the use of EGC-derived neural stem cells in animal models of stroke and motor neuron injury. In a rat model of spinal cord injury, transplanted cells appeared to promote partial recovery of the spine by protecting motor neuron death.[134] However, in a mouse excitotoxic brain damage model, EGC-derived cells migrated away from the lesion sight and toward damaged areas within the striatum, hippocampus, thalamus, and white matter tracts.[135] Models other than neuronal differentiation have also been employed using EGCs-derived cells. For example, EGC-derived cells have also been shown to successfully replace certain bladder defects induced in rats.[136]

Reports using animal models have also shown the therapeutic use of human ESC-derived cells. These have included a gamut of cell types including insulin secreting islets, retinal cells, liver, chondrocytes, cardiomyocytes, and cells of the neural lineage.[129,137–139] Studies have shown that cells derived from human ESCs led to improvements in animal models of osteochondral defects,[140,141] diabetes,[142] heart ischemia,[143–146] Parkinson's,[147,148] spinal cord injury,[134,149–152] stroke,[153–156] liver disease,[157] macular degeneration[158] and multiple sclerosis.[159,160] In fact, work led by Keirstead and colleagues involving ESC-derived glial cells in rat spinal cord injury models almost led to the first FDA-approved human clinical trials involving ESC-derived progenitors in the summer of 2009.[161] This trial sponsored by Geron Corp (Menlo Park, CA, USA), involved injecting human ESC-derived oligodendrocyte progenitors into patients with severe spinal cord injury, but was halted when benign appearing cysts began developing in some of the animal trials. Other clinical trials have also been reported in the near future using ESC-derived retinal pigmented epithelium for macular degeneration and ESC-derived β islet cells for the treatment of diabetes.[158,162]

Despite there being no current reports using human iPSC-derived cells, studies have begun to show the utility of mouse iPSCs in animal transplant models. Several of these models involve neural and cardiac afflictions. For instance, one study has shown that mouse iPSC-derived neurons have the ability to not only integrate themselves into fetal brains, but also improve symptoms of rats with Parkinson's disease.[163] Another study implicated iPSC-derived progenitors in the treatment of acute myocardial infarction.[164] With the exciting promise of iPSC-based therapies studies have begun demonstrating the ability to derive iPSCs from patients with a specific disease. The first report of patient-specific lines were those developed by Cheng and colleagues from patients with sickle cell anemia.[50] Since then, human iPSCs have also been generated from skin biopsies of patients with spinal muscular atrophy, amyotrophic lateral sclerosis and familial dysautonomia which demonstrated the ability to differentiate into motor neurons.[165–167] Likewise, iPSCs from patients with type 1 diabetes have been derived that could differentiate into insulin-producing cells.[168] Proof of iPSC potential for patient-specific treatments comes from a landmark paper describing the treatment of a humanized sickle cell anemia mouse model with iPSCs generated from autologous skin.[169] In this study, Hanna and colleagues derived an iPSC line from a transgenic mouse expressing a human sickle cell gene, corrected the mutant α-globin gene producing this disease and then showed that the differentiated cells from the corrected iPSC line were able to treat the disease when injected back into the knock-in mice. More recently, iPSCs have also been generated from β-thalassemia patients.[170]

Two primary concerns for the use of pluripotent stem cell-derived tissue are host-graft rejection and tumor formation. Graft-versus-host rejection is a critical factor in nonpatient-derived pluripotent stem cells. Therefore, it is critical to have stem cell-derived transplants that are a similar match to the histocompatibility complex of the patient in order to prevent complications associated with long-term immune suppression.[171] This is especially pertinent to stem cell-based therapies where cells are integrated into host tissue and as such cannot be surgically removed. However, one solution is to generate a registry of HLA-typed pluripotent stem cell lines from various ethnic groups. The possibility to create a bank of HLA-homozygous

stem cell lines, which could be MHC, matched for the majority of human population, is dependent on determining a realistic number that would satisfy a sizable population. However, the number of actual ESC lines that would be needed for a perfect tissue match is still under considerable debate, with estimates by different groups ranging from the hundreds to the thousands.[171,172] The considerable range in these studies can be in part contributed to the genetic diversity of the population as well as the criteria set forth for HLA mismatch. In contrast, a few papers have also estimated smaller numbers for pESC lines. These studies estimate that 10 to 70 lines of homozygous human pESC lines would be needed to cover the majority of the Japanese, UK and US populations.[172–174]

Several issues about pluripotent stem cells have raised concerns on their potential for tumor formation in clinical applications. First, all pluripotent stem cell lines have the propensity to become chromosomally abnormal over long-term culture, a characteristic feature of carcinogenesis. In fact, ESCs and iPSCs share similarities with the pluripotent cancer stem cells, ECCs, including abnormalities in chromosomes 12, 17, and X all of which are implemented in generating teratocarcinomas.[47,175–178] Using array-based comparative genomic hybridization (aCGH), a recent report comparing 17 human ESC lines also identified amplification at 20q11.21 and a derivative of chromosome 18.[179] It will remain to be seen if the application of this relatively new technology for stem cell purposes will identify more abnormalities missed by chromosomal banding techniques. Secondly, there is always the possibility that some stem cells remain pluripotent even after long-term culturing conditions which promote differentiation. Thus, efficient differentiation protocols along with rigid cell selection must be available to provide pure populations prior to transplantation.

Nevertheless, there is a risk that less differentiated progenitors derived from pluripotent cells may also generate tumor formation. This can be caused by either the innate properties of the cells themselves or by the host environment. For example, it has been shown that leukemias develop more frequently when hematopoietic stem cells are derived from umbilical cord blood as compared to bone marrow or blood suggesting that immature cells may carry higher risks for malignancy.[180] There are also numerous examples of bone marrow transplantation where donor-derived human bone marrow cells contributed to solid organ cancers.[181–183] Whether these bone-marrow-derived cells are responsible for tumor formation or contributed to a microenvironment that supports tumor growth is not clear.[47,184,185]

Studies have suggested that the frequent presence of fetal-derived cells in the stroma of malignant breast cancer tumors associated with pregnancy and in some cervical cancers may play a role in their cancer progression.[186,187] This issue was further highlighted by the first report of tumor formation from nonmarrow-derived stem/progenitors involving the therapeutic use of human neural stem cells to treat a young patient with inherited ataxia telangiectasia.[188] In this case, the quality of stem cells was not reported, raising concerns of proper quality controls as well as raising an important issue about the age of the patient at the time of therapy. In fact, the authors of that report, who did not perform the therapy, caution the use of any progenitors or stem cells in a young environment which alone may drive oncogenesis in otherwise stable cells. This is also consistent with the last decade of human fetal neural stem cell therapy in older adults for Parkinson's disease, where tumor formation was not demonstrated. Or in the only reported study involving cells derived from a human pluripotent stem cell source. In this clinical study (performed by Layton Bioscience, Inc, Sunnyvale, CA), human ECCs were used to produce postmitotic neurons to treat stroke patients. After almost a decade of follow-up with multiple older patients there are still no reports of tumor formation.[189–192]

Future directions to eliminate possible stem cell-derived tumor formation have been proposed.[47] These include genetic controls and cell targeting to selectively eliminate tumor forming stem cells after transplantation. Although experimental animal models and clinical trials in cancer gene therapy provide support for the utility of these strategies the need in the future will be to test these strategies in pluripotent stem cell derived transplants.

Theoretical considerations

Not only does the study of pluripotent stem cell derivation provide a potential source for patient-derived stem cell sources, but they also provide an excellent experimental model for regenerative medicine. Specifically, processes involved in cellular programming can be elucidated that can then be applied to other adult tissues. For instance, studying the process of derivation of iPSCs and EGCs from their differentiated predecessors can provide critical information regarding pathways involved in dedifferentiation. While iPSCs provide hope for reprogramming adult cells for therapeutic uses, they also stress the necessity in finding mechanisms regulating pluripotency and avoiding those associated with oncogenesis. For this purpose, the factors regulating cellular reprogramming of lineage-restricted cells

like that seen in PGCs and adult germ cells during their derivation into pluripotent stem cells may be helpful.

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