Cell Stem Cell
Perspective

Expanding the Boundaries of Embryonic Stem Cells

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The boundaries of embryonic stem cell (ESC) research have extended considerably in recent years in several important ways. Alongside a deeper understanding of the pluripotent state, ESCs have been successfully integrated into various fields, such as genomics, epigenetics, and disease modeling. Significant progress in cell fate control has pushed directed differentiation and tissue engineering further than ever before and promoted clinical trials. The geographical distribution of research activity has also expanded, especially for human ESCs. This review outlines these developments and future challenges that remain.

Introduction

The isolation of mouse embryonic stem cells (mESCs) from mouse blastocysts three decades ago dramatically advanced the field of mouse genetics, resulting in the groundbreaking technology of gene targeting. The impact of the derivation of human ESCs (hESCs; Thomson et al., 1998) almost two decades later was just as dramatic, placing the study of pluripotent stem cells at the forefront of biomedical research. Indeed, in recent years as the ethical constraints associated with hESC research have become a less prominent topic of debate, the scientific boundaries of this field have expanded considerably. In this Perspective, we cover several aspects of this "expansion" and discuss the major issues that have occupied the field in recent years.

In the past 5 years, "core research" on ESCs, i.e., research into their self-renewal and differentiation capacities, took advantage of state-of-the-art genome-wide technologies to extend our understanding of the pluripotent state. This increasing understanding has allowed the ESC field to reach beyond the boundaries of the laboratory, toward the fulfillment of its promise for regenerative medicine, with increasing numbers of preclinical and, more recently, clinical trials performed with ESC-derived cells. This maturation, in turn, facilitated the entry of new players into the ESC field. A growing number of physicians, regulatory agencies, and industrial companies are joining the academically driven journey of ESCs toward the clinic. During the past few years, ESCs have also gone beyond their traditional role as a tool for studying pluripotency and have become a fundamental player in various domains of molecular biology; more and more studies make use of mESCs and hESCs for answering general questions in genetics, epigenetics, and cell biology and for developing novel technologies whose applications may go beyond pluripotent cells. The boundaries of ESC research have also spread in the literal geographic sense across political borders, with laboratories from all over the world making significant contributions to the field.

The Global Village: ESC Research Worldwide

Recent years have seen increasing interest in ESCs throughout the world. In the past 5 years, laboratories from 50 different countries published papers about ESCs, more than doubling the total output of original research papers in the field, relative to the prior 5 years. This increase is even more impressive when considering research on hESCs: between 2007 and 2011, laboratories from 41 countries published papers using hESCs, compared to only 27 countries between 2002 and 2006 (Figure 1), more than tripling the number of papers on these cells. This expansion can probably be attributed, at least in part, to the establishment of clear guidelines for hESC research in many countries.

Although the increase in the total number of publications and in the number of countries involved is especially remarkable for hESCs, there is a similar trend for nonhuman ESC research, mostly on mESCs, which also expanded considerably over the same time frame (Figure 1). Importantly, this growth cannot be credited merely to the breakthrough of induced pluripotent stem cells (iPSCs), as papers that involve iPSCs were not included in this analysis.

The growing global interest in ESC research is also reflected by the relative contribution coming from various parts of the world. While the United States is still the most prolific country in the field, the relative "share" of papers published by laboratories from Europe and Asia has become much more significant (Figure 1). For example, China has doubled its share in the total publication count, in both human and nonhuman ESC-related research. In summary, the increasing number of articles that come out every year, the number of contributing countries, and the relative contribution of these countries all suggest that ESC research is on the rise.

Inside the Network: Understanding Pluripotency

When examining the pluripotency literature from the last few years, one is overwhelmed by the quantity and quality of genome-wide studies performed in attempts to deconstruct the pluripotent state. It seems that no cutting-edge technology has gone unnoticed by the ESC field, which harnessed these state-of-the-art tools to uncover the global state of pluripotency (e.g., its genome, transcriptome, proteome, methylome, etc.), in what could be aptly described as "the 'Omics' era of ESC research" (Loh et al., 2011). The large-scale genome-wide studies exposed complex and dynamic multilayered regulation involving transcriptional networks, chromatin modifications, and posttranscriptional regulation (Ng and Surani, 2011; Orkin and Hochedlinger, 2011; Young, 2011) (Figure 2).

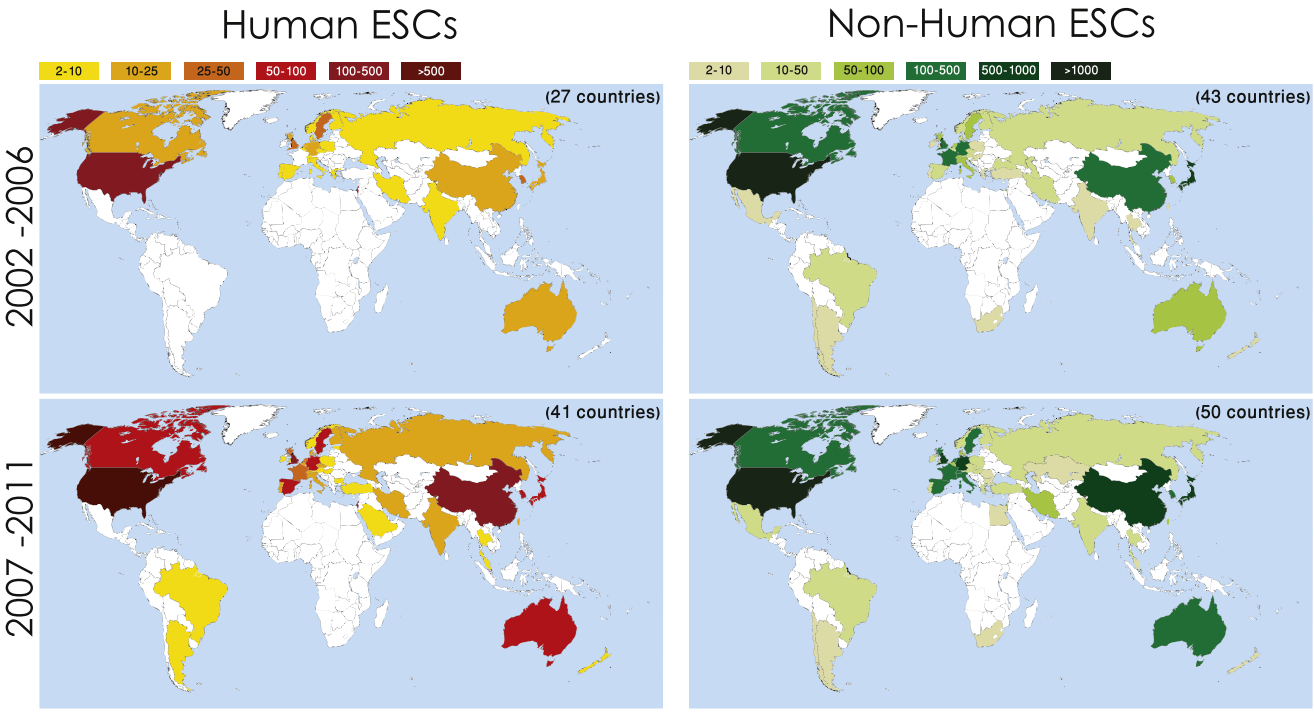

Figure 1. ESC Research Distribution throughout the World

World maps comparing the distribution of stem cell research throughout the world between two 5 year periods: 2002–2006 and 2007–2011. The numbers of publications involving human and nonhuman ESCs were assessed separately and are thus presented in separate maps. Nonhuman ESCs are mostly, but not exclusively, mouse ESCs. The maps are color-coded by the absolute number of articles published by laboratories from each country. The total number of contributing countries during the examined years appears in the upper right side of each map. Articles dealing with iPSCs were removed from the analysis. Quantification of articles was carried out using "ISI Web of Science" (http://apps.isiknowledge.com).

Transcriptional Networks

The core transcriptional regulatory network of pluripotency was investigated in a series of studies using various genome-wide chromatin-IP based technologies (ChIP-on-chip, ChIP-PET, ChiP-seq, and biochip). Whereas the first studies identified the key players of the network and its general architecture (Boyer et al., 2005; Loh et al., 2006), recent analyses refined our understanding of this network, revealing a set of distinct-yet-intimately-connected modules that cooperate and regulate each other (Chen et al., 2008; Kim et al., 2008, 2010). These studies divided the pluripotent network to its two main compartments, the Oct4-centric and the Myc-centric modules; revealed the interactions within and between each of these modules; highlighted the importance of coregulation and autoregulation for the proper function of the network; and integrated novel signaling pathways into it. More recently, the core pluripotency genes were also shown to control germ layer fate choice, extending the original role of the pluripotent network beyond the maintenance of self-renewal (Thomson et al., 2011). Comparison of the mouse and human pluripotent networks revealed, quite surprisingly, that species-specific transposable elements have considerably altered the transcriptional pluripotent circuitry, so that in each species the same core factors bind a distinct set of TF-binding sites and play distinct roles in pluripotency regulation (Kunarso et al., 2010; Wang et al., 2012).

Noncoding RNAs

Another recently identified layer of pluripotency regulation is that of noncoding RNAs: micro RNAs (miRNAs) and large intergenic noncoding RNAs (lincRNAs). The importance of miRNAs in ESCs was demonstrated in several studies. Like their unique characteristic mRNA signature, ESCs exhibit a defined characteristic miRNA signature (Marson et al., 2008). Global loss of miRNAs resulted in defects in both self-renewal and differentiation, whereas specific miRNAs were found to regulate ESC cell cycle, expression of pluripotency factors, and differentiation (Martinez and Gregory, 2010). The miRNAs themselves are, in turn, regulated by pluripotency factors such as Lin28 (Viswanathan et al., 2008), demonstrating the crosstalk between layers of pluripotency regulation. More recently, hundreds of lincRNAs that are involved in the control of the pluripotent state were also discovered (Guttman et al., 2009). Many of them were reported to be bound by Oct4 and Nanog in their promoter regions, directly integrating them into the core pluripotency circuitry (Guttman et al., 2009). Specific lincRNAs were already reported to be essential for pluripotency (Sheik Mohamed et al., 2010), but much is left to be discovered regarding their role.

Proteomics

Proteomic approaches have recently revealed interactions between the core pluripotency proteins, contributing to the growing understanding of pluripotency (Pardo et al., 2010; Wang et al., 2006). Further studies added phosphorylation dynamics as another layer of regulation in ESCs, identifying significant changes in the phosphoproteome of ESCs during their differentiation (Brill et al., 2009; Van Hoof et al., 2009). The study of protein interactions also shed light on the important

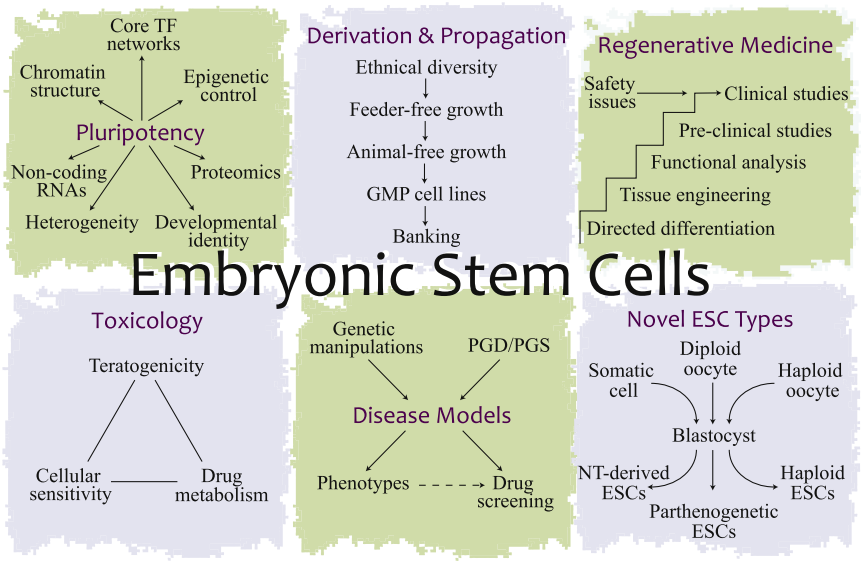

Figure 2. Major Achievements and Challenges of ESC Research
A schematic representation of the main topics in ESC research in recent years. The major advancements in each of these topics, and the challenges that lie ahead, are elaborated in the text.

role of chromatin in pluripotency. Direct and indirect interactions between core transcription factors and chromatin modifiers/remodelers were shown to play a crucial part in maintaining the "open" chromatin state, which is unique for pluripotent cells (Gaspar-Maia et al., 2011). Moreover, there are direct regulatory interactions between these transcription factors and chromatin remodelers (Ang et al., 2011; Gaspar-Maia et al., 2009), further stressing their importance for the pluripotent state.

Epigenetic Control

One of the most remarkable achievements in recent pluripotency research is its intimate involvement in groundbreaking discoveries in epigenetics, elucidating novel layers of regulation in the complex control of the pluripotent state (Meissner, 2010). At the level of DNA methylation, various studies characterized ESC-specific methylation profiles and linked them directly to the core transcriptional networks of ESCs (Fouse et al., 2008; Meissner et al., 2008). A recent study applied Methyl-seq technology to map the ESC methylome at a single-base resolution, revealing a novel class of DNA methylation at non-CpG sites (Lister et al., 2009). These unique non-CpG methylations are enriched in exons of highly expressed genes (Lister et al., 2009) but appear to be dispensable for pluripotency (Ziller et al., 2011), so their role in regulation of gene expression is still unclear. In another major discovery, a novel type of DNA methylcytosine modification was recently discovered in ESCs in which the Tet-family proteins Tet1 and Tet2 transform methylated cytosines into 5′-hydroximethylcytosines (5hmC) (Ito et al., 2010; Koh et al., 2011; Pastor et al., 2011; Tahiliani et al., 2009). This series of studies demonstrated that Tet1 and Tet2 affect self-renewal and differentiation of mESCs.

Another layer of epigenetic regulation that has been studied extensively in ESCs is histone modifications. ESCs are characterized by bivalent domains generated by the co-occupation of the transcription start sites of genes that control cell fate decisions by the activating mark H3K4me3 and the repressive mark H3K27me3 (Bernstein et al., 2006). This phenomenon has drawn much interest in the field, as the bivalent domains are considered to "poise" genes for their rapid activation upon differentiation. Recent studies have improved our understanding of the molecular mechanism that underlies these unique domains, demonstrating the important role of the polycomb group (PcG) proteins PRC1 and PRC2 (Margueron and Reinberg, 2011), and introducing new players that participate in the generation and maintenance of this delicate balance of modifications (Margaritis and Holstege, 2008; Pasini et al., 2010; Peng et al., 2009). Other histone modifications such as H3K9 methylation have also been studied in ESCs (Wen et al., 2009), and together these studies begin to decipher what seems to be an ESC-specific "histone code."

Having discovered elements that participate in regulating pluripotency, the main challenge that lies ahead seems to be the integration of all these "layers," "modules" and "subnetworks" into a consolidated regulatory circuitry. Attempts to describe the crosstalk between different layers of regulation have already been reported, connecting, for example, gene expression with DNA methylation (Bock et al., 2011), transcription with histone modifications and protein levels (Lu et al., 2009), or DNA methylation with histone modification (Viré et al., 2006). With the increasing focus on combinatorial regulation and on crosstalk between network elements, we expect many more exciting "connections" to be revealed in the future.

One but Not the Same: Emerging Variability of the Pluripotent State

The increasing understanding of the molecular mechanisms that govern pluripotency inspired fruitful discussions regarding the nature of the pluripotent state and helped refine the very definition of the term "pluripotency." The similar yet distinct pluripotent states of various types of ESCs were coined "different flavors of pluripotency" (Buecker and Geijsen, 2010), and as our understanding of these pluripotent states is becoming more solid, so does our control of the transitions between them.

Developmental Identity of PSCs

The focus of investigation in this area is the difference between mouse embryonic and epiblast stem cells (ESCs and EpiSCs, respectively), and between mESCs and hESCs. MESCs are derived from the inner cell mass of blastocysts, while mouse EpiSCs are derived from postimplantation epiblasts (Brons et al., 2007; Tesar et al., 2007); consequently, these cell types differ in their morphology, culture requirements, developmental potential, expression profile, and amenability to homologous recombination. These differences led to the emergence of the

concepts "naïve" (or "ground-state") versus "primed" pluripotent cells (Nichols and Smith, 2009). Recent work showed that mouse cells can acquire "metastable" pluripotent states that could be interconverted by endogenous genetic determinants or by exogenous factors (Hanna et al., 2009).

HESCs share many features with mESCs but, intriguingly, they also share some of their characteristics with mouse EpiSCs, suggesting that they may represent the primed ESC state and therefore may not harbor the full developmental potential of naïve ESCs. Recently, several groups reported the derivation of hESCs and hiPSCS with biological properties similar to those of mESCs (Buecker et al., 2010; Hanna et al., 2010; Li et al., 2009; Wang et al., 2011a). These hESCs exhibited morphology, growth properties, expression profiles and signaling dependence that were comparable to those of mESCs, but they were not stable in the absence of genetic manipulations. Whether these naïve hESCs are indeed superior to their primed counterparts in terms of developmental potential remains to be determined; however, the fact that culture conditions are sufficient to interconvert between pluripotent states, both in mESCs and in hESCs, indicates that plasticity in the pluripotent state is more widespread than was previously appreciated.

Heterogeneity of PSCs

The variability between ESCs has received attention from other directions as well; in recent years, ESCs were shown to be more heterogeneous than previously thought. Both intraculture and interculture heterogeneity exist: within undifferentiated cultures of mESCs and hESCs and of mouse EpiSCs, distinct subpopulations were identified, differing in their expression of molecular markers and in their differentiation potential and therefore presumed to correspond to distinct developmental stages (Canham et al., 2010; Han et al., 2010; Martinez Arias and Brickman, 2011; Stewart et al., 2010; Toyooka et al., 2008). Between undifferentiated hESC lines, large-scale comparisons revealed differences in gene expression and in differentiation, suggesting that not all ESCs lines are equally suitable for any given purpose (Adewumi et al., 2007; Bock et al., 2011).

ESC to Every Lab: Advances in ESC Derivation and Propagation

The techniques for deriving, propagating, and banking ESCs have significantly improved in recent years, and these types of advances are key for moving ESCs toward the clinic (Figure 2). Much progress has been made in adapting culture conditions to enable rapid and efficient thawing, passaging, and cryopreservation of hESCs. The most notable discovery in this regard was probably that the Rho-associated kinase (ROCK)-inhibitor Y-27632 permits the survival of dissociated hESCs (Watanabe et al., 2007). Follow-up studies uncovered the molecular mechanism that underlies the high sensitivity of hESCs to dissociation (Chen et al., 2010; Ohgushi et al., 2010) and also utilized this inhibitor for deriving and propagating hESCs in suspension (Amit et al., 2010; Steiner et al., 2010).

Several groups have also directed much effort at determining the components of defined media that would enable feeder-free growth of hESCs (Akopian et al., 2010) and eliminating animal products from such media, thus making it xeno-free (Lei et al., 2007; Valamehr et al., 2011). These efforts culminated in the generation of good manufacturing practice (GMP) clinical-grade hESCs (Unger et al., 2008). In order to standardize the use of hESCs in biomedical research and, eventually, in the clinic, consensus guidelines for banking and supply of hESCs were proposed (International Stem Cell Banking Initiative, 2009).

As human ESC lines are now derived on a weekly basis, the ethnical diversity within the human ESC pool has greatly expanded so that it currently represents dozens of different ethnic backgrounds (Amps et al., 2011). This diversity will be important for the study of ethnically relevant diseases, for the removal of confounding effects due to specific genetic backgrounds, and for the banking of hESCs that would be compatible with as large a population as possible.

Apart from the abovementioned advances in the culture of hESCs, derivation and culture techniques were also developed in recent years for ESCs of various species, expanding the repertoire of pluripotent stem cells available for research. In addition to mouse, monkey, and human ESCs, in the past 5 years ESC lines were derived from multiple species including rabbit, canine, and—most importantly—rat (Martins-Taylor and Xu, 2010). These stem cell types should enhance our understanding of the pluripotent state and, especially in the case of the rat, enable the generation of novel model animals for studying human disease.

Eyes on the Target: ESCs in Regenerative Medicine

Many clinical conditions such as neurodegenerative disorders, diabetes, and some forms of heart and hepatic failure are caused by loss of functionality or insufficient quantity of a particular cell type. The potential of hESCs to differentiate into any cell type of the human body raised the hope for treatment of these clinical conditions and has thus drawn hESCs into the public spotlight. Indeed, exciting recent progress is paving the hESC path into the practice of regenerative medicine (Figure 2).

Directing Differentiation

For hESCs to live up to expectations, it will be essential to control their differentiation course. One of the most efficient strategies designed to control a pluripotent cell fate is the recapitulation of developmental steps through which cells assume a specific fate during normal development (Murry and Keller, 2008). The first step in the differentiation of a pluripotent stem cell is transition into one of the three embryonic germ layers: the ectoderm, mesoderm, and endoderm. Multiple studies that applied knowledge of developmental biology to ESC differentiation demonstrated that despite the known differences between hESCs and mESCs (Nichols and Smith, 2009), the signaling pathways that control primary differentiation are very similar (Cohen and Melton, 2011; Murry and Keller, 2008).

After acquiring their initial lineage identity, application of various growth factors and culture conditions can continue to direct the cells along multiple differentiation paths. Recently, small molecules have become more useful in differentiation protocols, presenting an appealing alternative to recombinant growth factors, especially when considering the potential for mass production of cells for clinical use (Rubin, 2008). Small molecules are less expensive and more stable than recombinant proteins and can contribute to the development of fully defined

media. They also allow for more straightforward minimization of differences between batches of reagents, which can reduce experimental variance and help establish reproducible differentiation protocols. However, in some cases there are no known chemical compounds that modulate the desired signaling pathway. In such cases, unbiased screening offered a potential solution. In a landmark study, thousands of chemical compounds were screened in order to identify molecules that can replace Activin A in the induction of definitive endoderm (DE) (Borowiak et al., 2009). Two such small molecules were uncovered in both mESCs and hESCs. Additional studies used the unbiased screening approach to efficiently produce desired ESC derivatives such as pancreatic progenitor cells and cardiomyocytes (Chen et al., 2009; Takahashi et al., 2003).

Advances in the genetic manipulation of ESCs (Giudice and Trounson, 2008) have enabled the successful generation of several fluorescent reporter hESC lines that are extremely useful for a variety of applications. They can be used to distinguish undifferentiated hESCs from their differentiated derivatives (Eiges et al., 2001) or to visualize the appearance of desired differentiated cells in culture (Lavon et al., 2004; Singh Roy et al., 2005). Moreover, reporter cell lines enable the isolation and analysis of various cell populations using fluorescence-activated cell sorting (FACS) even without previous knowledge of specific cell surface markers or available antibodies. The reporter cell lines are also valuable for microscopy-based high-throughput screening and can therefore assist with the optimization of differentiation protocols. The engraftment, survival and integration of transplanted cells can be tracked more easily using such reporter hESCs. Although they are mostly useful for in vitro analyses and preclinical studies, reporter cell lines may be beneficial for determining the fate and function of transplanted cells in clinical trials as well (Ellis et al., 2010).

The tremendous progress in applying understanding of embryogenesis to differentiation protocols has enabled the generation of diverse cell types in vitro, including highly specified cells such as retinal pigment epithelium (RPE) (Idelson et al., 2009), mechanosensitive hair cells (Oshima et al., 2010), and primordial germ cells (Hayashi et al., 2011). However, there are still many challenges on the way to the ultimate goal of ''cells on demand.'' Differentiation is a stepwise process, passing through several intermediate progenitor cells on the way to a fully-differentiated cell of interest. The first differentiation step into the desired germ layer is usually the most efficient step, and the differentiation efficiency often decreases with each step of the protocol (Cohen and Melton, 2011). As a result, the end product is usually a heterogeneous cell population that contains only low percentage of the specific cell type. A major challenge will therefore be to improve the differentiation efficacy and design reliable methods for isolating the desired cell populations. An appealing alternative can be to propagate intermediate progenitor cells, as was recently demonstrated with definitive endoderm progenitor cells (Cheng et al., 2012). Another related challenge is the reproducibility of differentiation protocols. Different batches of reagents and slight differences in cell culture techniques sometime make it very difficult to recapitulate differentiation protocols successfully and with comparable efficiency rates to those originally reported. The distinct differentiation propensity of different hESC lines (Bock et al., 2011) adds to this complexity, because it compromises the generalization of some differentiation protocols. Of note, most ESC-derived differentiated cells are not fully mature, and their in vitro maturation is another obstacle that awaits a solution (though, in some cases, maturation does take place in vivo after cell transplantation (Hayashi et al., 2011; Kriks et al., 2011; Kroon et al., 2008)).

Generation of Complex Differentiated Cell Types

The main focus of most directed differentiation experiments is to maximize the derivation of one desired cell type for cell replacement therapy. The replacement of complex tissues, however, presents a greater challenge that involves differentiation into several cell types with a three-dimensional (3D) organization. Recently, two different approaches have made progress toward meeting this challenge. The first approach makes use of the potential of ESCs to respond to extrinsic signals and recapitulate developmental cell fate decisions to generate ''tissues in a dish.'' Several recent studies demonstrated that ESCs can not only differentiate to all cell types, but also generate organizer cells that may affect the fate of adjacent cells during embryogenesis (Sharon et al., 2011). Moreover, the cells possess in vitro self-organization capacity and can therefore generate organized and complex 3D tissues, such as cortical structures (Eiraku et al., 2008), the optic cup (Eiraku et al., 2011), adenohypophysis (Suga et al., 2011), and intestinal tissue (Spence et al., 2011). The second approach is the in vivo generation of tissues and organs using chimeric animals. In a remarkable experiment, xenogeneic organ complementation was achieved when rat pluripotent stem cells were injected into mouse blastocysts that lacked the Pdx1 gene. As a result, the rat-mouse chimera's pancreas was composed exclusively of rat cells, demonstrating the feasibility of organ generation through interspecies chimeras (Kobayashi et al., 2010). As the generation of viable chimeras from a nonhuman primate was recently demonstrated (Tachibana et al., 2012), these breakthroughs raise fascinating possibilities regarding organ generation but also raise significant technical, legal, and ethical questions.

Preclinical Evaluation

Before transplantation of differentiated cells into patients, it is essential to conduct pre-clinical trials to demonstrate the integration capacity and functionality of the cells in animal models. Finding an appropriate animal model and analyzing the mechanism that underlies the observed improvements can be a challenging task. Demonstrating the functionality of differentiated cells in an animal model that entirely lacks the relevant cell type is the most stringent and straightforward approach; for example, several groups demonstrated the potential of hESC-derived β cells (Kroon et al., 2008) and RPE cells (Idelson et al., 2009) to functionally replace their in vivo equivalents. Importantly, most in vivo transplantation experiments are currently conducted in murine models, and the scalability of these assays thus remains an open question. Tackling this issue, a recent study demonstrated the in vivo survival, integration, and function of hESC-derived dopaminergic neurons in rat and mouse Parkinson's disease models and went on to show their survival and integration in parkinsonian adult rhesus monkeys (Kriks et al., 2011).

Clinical Studies

The progress in various aspects of ESC technology, discussed above, has established a solid platform for therapeutic implementation. Although some safety issues as well as differentiation and pre-clinical challenges are still unresolved, it seems as though hESCs are starting to fulfill their promise, as reflected by a couple of ongoing clinical trials. In these pioneering clinical trials, differentiated derivatives of hESCs were transplanted into patients suffering from various clinical conditions: spinal cord injury, dry age-related macular degeneration (AMD), and Stargardt's disease (Goldring et al., 2011; Trounson et al., 2011). This encouraging progress was hindered by the surprising decision of Geron, the first company that took ESCs to the clinic, to terminate all ESC-related experiments and ongoing clinical trials. This blow, however, was somewhat balanced by the positive preliminary report of Advanced Cell Technology (ACT), describing the results of their first hESC-based clinical trial in human patients (Schwartz et al., 2012). In this report, hESC-derived RPE cells were transplanted into patients with either AMD or Stargardt's disease. Four months after the implantation, the survival and engraftment of the cells, together with functional visual improvement, were identified, whereas no signs of tumorigenicity or immune rejection were observed (Schwartz et al., 2012). These preliminary clinical results, together with the ever-improving differentiation protocols into highly specified, complex and mature cell types, suggest that a new era of hESC-based therapy might not be very far away.

Better Safe Than Sorry: The Tumorigenicity and Immunogenicity of ESCs

As human ESC products get closer to the bedside, safety issues have become a serious hurdle that must be overcome before ESC-derived cells can be routinely injected into patients (Goldring et al., 2011). The major safety problem the field is currently facing is the potential tumorigenicity of the cells, mainly due to residual undifferentiated cells. Several approaches to selectively remove undifferentiated cells from culture have been suggested in order to solve this problem, including the use of genetic labeling, ablation of teratoma-specific genes, sorting out pluripotent stem cells based on antibodies against pluripotent-specific molecules, and specific cytotoxic antibodies (Ben-David and Benvenisty, 2011). Most recently, biomarkers unique to human pluripotent stem cells were used to eliminate pluripotent stem cells from mixed populations (Tang et al., 2011; Wang et al., 2011b). Concomitantly, efforts are being made to characterize the most likely tumors, i.e., teratomas and teratocarcinomas, in an attempt to prevent their formation (Blum et al., 2009; for a discussion of the appropriate terminology for these tumors, see Damjanov and Andrews, 2007; Lensch and Ince, 2007). Although no tumor formation was reported in the preliminary report of the first clinical trial with ESC-derived cells (Schwartz et al., 2012), the tumorigenicity risk has not been resolved yet and remains a concern that limits the number of cells injected into human patients.

Another concern that may affect the safety of ESC-based treatments is their genomic stability in culture. In recent years, large-scale comparison studies revealed recurrent genomic aberrations in hESCs and began to pinpoint the genes that drive these frequent aberrations (Amps et al., 2011; Baker et al., 2007; Mayshar et al., 2010). Some of these aberrations may be associated with oncogenic transformation, and thus would increase the tumorigenicity of the cells (Baker et al., 2007; Ben-David et al., 2011; Lefort et al., 2008). Until strategies to prevent the accumulation of such genomic alterations in ESC cultures are developed, the genomic integrity of the cells needs to be monitored carefully prior to their clinical application. It is worth noting that the genomic instability of ESCs is deleterious for additional reasons: it may compromise their differentiation propensity, the functionality of the differentiated cells, and their usefulness for disease modeling and drug screening.

The immunogenicity of ESCs and of ESC-derived cells is a third issue related to the safety—and, obviously, to the success—of ESC-based treatments (Kadereit and Trounson, 2011). HLA matching is a major hurdle for hESC-based therapies, especially for treatments of tissues that are not immune privileged. Recent preclinical and clinical trials applied pharmacological immunosuppression to avoid graft rejection (Kriks et al., 2011; Schwartz et al., 2012); however, in one case the immunosuppression was shown to be incomplete (Kriks et al., 2011) and in another the patient did not comply with the immunosuppression regimen (Schwartz et al., 2012), indicating that this important issue is not fully resolved. Although some strategies for tolerance induction have been suggested (Robertson et al., 2007), the main strategy for circumventing this obstacle remains the assembly of ESCs with diverse major histocompatability complex (MHC) haplotypes in the ESC banks that are founded these days throughout the world.

ESCaping Drug Attrition: ESCs in the Service of Toxicology

Assuring the safe use of new drugs requires the analysis of their safety in the developing embryo and in the adult. In recent years, hESCs have begun to play a major role in toxicology assays (Laustriat et al., 2010). The pluripotent capacity of the cells and their ability to differentiate into many cell types make them a valuable pharmacological tool in three main ways (Figure 2). First, hESCs can be used for screening of teratogens, compounds that are selectively detrimental for the embryo or the fetus, based on the ability of hESCs to mimic early stages of human development (Mayshar et al., 2011; West et al., 2010). The second aspect is drug metabolism, which primarily takes place in the liver. The capability of hESCs to differentiate into fairly mature hepatocytes (Agarwal et al., 2008; Basma et al., 2009) may make them a suitable tool for testing the hepatic metabolism of potential drugs. The third aspect is tissue toxicity, which is based on the growing ability to obtain rather pure populations of clinically relevant cells, such as cardiomyocytes and neurons, from hESCs. Using these differentiated cells for cardio- and neurotoxicity screens will allow tissue-specific assessment of drug toxicity (Mandenius et al., 2011). During the past few years, good progress has been made in all three fronts, and it is predicted that in the future hESCs will be routinely used by the pharmacological industry (Wobus and Loser, 2011).

Diseases in a Dish: Modeling Human Genetic Disorders with ESCs

Mouse ESCs are the major tool for generating mouse models of human diseases, and the contribution of transgenic mouse to

understanding human disease has been tremendous. However, in many disorders the mouse models fail to recapitulate the human phenotypes. Therefore, hESCs represent an alternative tool for modeling human diseases, by introducing mutations to normal ESCs (Urbach et al., 2004) (Figure 2).

In the past 5 years, many different methodologies have been used to manipulate the genome of hESCs, including homologous recombination by plasmids and BAC constructs (Song et al., 2010) or by zinc finger and TALE nucleases (Hockemeyer et al., 2009; Hockemeyer et al., 2011; Leavitt and Hamlett, 2011), and the use of RNAi to knock down specific genes (Tulpule et al., 2010). While both the efficiency and the specificity of these methods have improved in recent years, off-target activity remains a concern, as it may introduce "collateral damage" that may jeopardize their applicability for disease modeling. An alternative to induction of mutations in normal hESCs is the isolation of genetically aberrant hESCs from blastocysts carrying genetic diseases. Screening for diseased embryos by the analysis of single blastomeres at the preimplantation stage is becoming a more common methodology. Thus, preimplantation genetic screening (PGS) is used to identify chromosomal aberrations in human embryos, and hESCs can be derived from such aneuploid embryos, generating in-vitro models for chromosomal disorders such as Down syndrome (Biancotti et al., 2010). In addition, preimplantation genetic diagnosis (PGD) is conducted to screen for embryos that carry monogenic disorders, and multiple ESC lines were derived from such mutated blastocysts (reviewed in Ben-Yosef et al., 2008).

Some of the derived disease model hESCs were analyzed to identify disease characteristic phenotypes, either in the undifferentiated state or after their differentiation in culture (Colman and Dreesen, 2009). In developmental disorders, these models also enabled the characterization of phenotypes that are unique to the embryonic stage of the cells. Once defining a phenotype of interest, these powerful models can potentially serve to identify novel drugs that would enable treatment of currently untreatable disorders. The abundance of available cells could be exploited either for testing drugs that target known candidate genes or for performing unbiased high-throughput screens with libraries of varied molecular entities.

It is clear that research on hESCs paved the way to the analysis of human disorders using human iPSCs (Robinton and Daley, 2012). It is also evident that the availability of somatic cells from practically any human disorder has made the generation of such models in human iPSCs very accessible for most labs. There is some indication, however, that iPSC models may be affected by an epigenetic memory from the somatic cells and thus might be inferior to ESCs in reflecting developmental aspects of the disease (Urbach et al., 2010). Nonetheless, iPSCs are clearly becoming the system of choice for disease modeling, and most disease models are already generated using this methodology (Robinton and Daley, 2012).

The New Kids on the Block: Novel Types of ESCs

Traditionally, hESCs are derived from blastocysts of IVF embryos and thus represent the outcome of the natural fertilization process. Recently, however, new types of ESCs were introduced, diversifying the ESC toolbox with ESCs derived from "artificially generated" blastocysts (Figure 2).

Somatic cell nuclear transfer (SCNT) experiments, culminating in the cloning of Dolly the sheep, paved the way for cloning other mammals (Wilmut et al., 1997; Wakayama et al., 1998). In humans, there were various attempts to generate nuclear transfer (NT)-derived hESCs, but the initial successful report was found to be fraudulent (Normile et al., 2006), leading the field to stagnation. Successful SCNT with human cells was finally reported last year, resulting in nuclear transfer (NT)-derived hESCs (Noggle et al., 2011). Although these ESCs were triploid, as the oocyte genome could not be removed, this study has revitalized interest in using SCNT for deriving "personalized" ESCs. Comparing these NT-ESCs to normal ESCs and to iPSCs would eventually be necessary to determine whether important differences exist between these pluripotent cell types.

Another type of ESCs known for a long time in mouse was finally generated in humans as well: human parthenogenetic ESCs derived through parthenogenetic blastocysts (Kim et al., 2007b). Such blastocysts are generated by the activation of unfertilized oocytes, which undergo duplication of their genomic content and thus harbor two copies of the maternal genome. Human parthenogenetic pluripotent stem cells may serve for the generation of MHC-matched cells for transplantation, as was demonstrated in mouse (Kim et al., 2007a), and may also be used for the study of imprinting (Stelzer et al., 2011).

A third striking type of ESCs was recently reported in mouse. Using the same technique of activating haploid oocytes, mESCs were derived from parthenogenetic embryos grown under specific culture conditions, and were than FACS-sorted for low DNA content, resulting in haploid mESC lines (Elling et al., 2011; Leeb and Wutz, 2011). These haploid mESCs may become an invaluable tool for forward and reverse genetics, as was elegantly demonstrated in these two groundbreaking papers. Similar attempts to generate haploid hESCs are currently underway, hopefully to be crowned with success.

The Pluripurpose Cell: Using ESCs beyond Pluripotency Research

One of the most interesting developments in the ESC field in recent years is the way it has been integrated into affiliated research fields and influenced all areas of genetics, epigenetics, and cell biology. Indeed, the boundaries between ESC research and other research fields often become blurry, as studies "with" ESCs, rather than "of" ESCs, are already rapidly accumulating into a significant body of work.

ESCs have been used as a tool for the investigation of basic questions in various areas of biology, and by now these cells are responsible for major advancements "outside" the traditional borders of ESC research. To name just a few examples, the identification of an active DNA demethylation enzyme (Bhutani et al., 2010), lincRNAs (Guttman et al., 2009), and extensive transcription initiation (Guenther et al., 2007), as well as improvements in genomic techniques such as zinc finger nucleases (Collin and Lako, 2011) and high-resolution methylation mapping (Jeddeloh et al., 2008), are all discoveries made in ESCs but with implications that go far beyond the biology or the manipulation of pluripotent cells.

It seems, therefore, that ESCs have recently made great impact on a variety of research arenas, serving as a sort of "pluripurpose cells," a well characterized in vitro system of normal human proliferating cells that might possibly even replace HeLa cells as "default" cells of choice. ESCs have thus gone beyond what is usually perceived as "ESC research" and are extensively used in the biological and biomedical sciences (a fact that is unfortunately overlooked in some public discussions, when the necessity and utility of these cells is drawn into question).

Concluding Remarks: The Next 5 Years

Soon after Yamanaka and Thomson first reported the generation of human iPSCs, President George W. Bush referred to this achievement as a "scientific advancement within ethical boundaries" (Kolata, 2007); however, the expectation that iPSCs would replace embryonic stem cells (ESCs) in the study of pluripotency has proven wrong. On the contrary, the vibrant and flourishing ESC research community received a substantial boost from the induced pluripotency breakthrough, and since 2006 these two pluripotent cell types have complemented and promoted each other (Scott et al., 2011).

The rapid discovery rate in the ESC field, with the surprising twists and turns it sometimes takes, makes it very difficult to predict where ESC research will be 5 years from now. Nonetheless, the achievements of recent years do seem to suggest that ESC research is far from reaching its full capacity and is predicted to continue expanding. Geographically speaking, the gap that still exists between mESCs and hESCs in terms of countries involved suggests that more countries are probably about to get actively engaged in hESC research. From a basic research point of view, there is no doubt that much is left to be discovered regarding the pluripotent state and its control, with an emphasis on combining the complex multilayered regulation into a coherent regulation circuitry. From the perspective of regenerative medicine, data regarding the safety and efficacy of current and future clinical trials will undoubtedly determine when ESC research is mature enough to fulfill its promise in the clinic, and success in this arena is bound to draw more private companies into the field. Novel ESC types and reprogramming methods, possible advancements in the generation of human-animal chimeras, and increasing numbers of disease models are further reasons to believe that exciting years lie before us.

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