Towards the use of human embryonic stem cells in the clinical setting: recent progress

Alessia Deglincerti, Ph.D.
Laboratory of Vertebrate Molecular Embryology, The Rockefeller University, New York City, NY
Email: adeglincer@rockefeller.edu

Abstract
Human embryonic stem cells (hESCs) offer great therapeutic promise for the treatment of human diseases due to their ability to generate all tissues in the body and potentially regenerate or replace damaged tissues in patients. Here, I discuss recent progress in the derivation, maintenance, growth, and differentiation of hESCs that are leading the stem cell field one step closer to the efficient and effective utilization of hESCs in the clinical setting.

Introduction

Human embryonic stem cells (hESCs) are characterized by two unique properties: they can be cultured to remain undifferentiated indefinitely (self-renewal), or they can differentiate and give rise to all cell types in the body (pluripotency). As such, hESCs are a valuable system for understanding the complex mechanisms involved in the early stages of human development. Moreover, hESCs hold a rare potential for basic research, drug discovery, and clinical applications, opening new possibilities in the field of regenerative medicine. However, the signaling mechanisms involved in hESC differentiation to various cell types and the appropriate culturing conditions for the large-scale production of stable and homogeneous cell populations are still being investigated. In this review, I discuss recent advances in hESC derivation and differentiation and how these advancements are paving the road for the use of hESCs in the clinical setting.

Derivation of hESCs

Classic derivation protocols

Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of a blastocyst, an embryo at an early stage in development. Pioneering work in the mouse has led to the establishment of a classic protocol for deriving mouse embryonic stem cells (mESCs) from the mouse blastocyst (Evans et al., 1981; Martin, 1981) (Figure 1). These cells have a normal karyotype and are able to differentiate in vitro and in vivo to give rise to different cell types (teratocarcinomas) (Evans et al., 1981; Martin, 1981).

It was not until almost twenty years later that a very similar protocol was used to successfully generate the first hESC lines from human embryos that had been produced by in vitro fertilization for clinical purposes, almost twenty years after the generation of mESCs (Thomson et al., 1998; Reubinoff et al., 2000). To date, hundreds of different hESC lines have been generated from surplus embryos (the current number of lines in the NIH human embryonic stem cell registry is 234).

Although both mESCs and hESCs are capable of self-renewal and pluripotency, hESCs seem to have been derived from an earlier developmental stage than mESCs, thus raising questions when comparing data between the two species (Brons et al., 2007; Tesar et al., 2007). This might be part of the reason why mESCs and hESCS rely on different signaling pathways - and therefore culturing conditions - for the maintenance of pluripotency: leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) signaling for mESCs (Qi et al., 2004), fibroblast growh factor (FGF) and Nodal/Activin signaling for hESCs (Vallier et al., 2005). Several recent studies have described methods to derive and culture "primed" mESCs derived from the post-implantation epiblast, a

later developmental stage (so-called epiblast stem cells, or EpiSCs), which share a variety of epigenetic and gene expression properties with hESCs (Brons et al., 2007; Tesar et al., 2007). Similarly, "ground state naive" hESCs have been derived in vitro from already established hESC lines that epigenetically and functionally resemble mESCs more closely (Gafni et al., 2013). These cells will offer an optimal platform for comparisons across species.

Induced pluripotent stem cells

One of the greatest challenges in exploiting and harnessing the full clinical promise of hESCs is

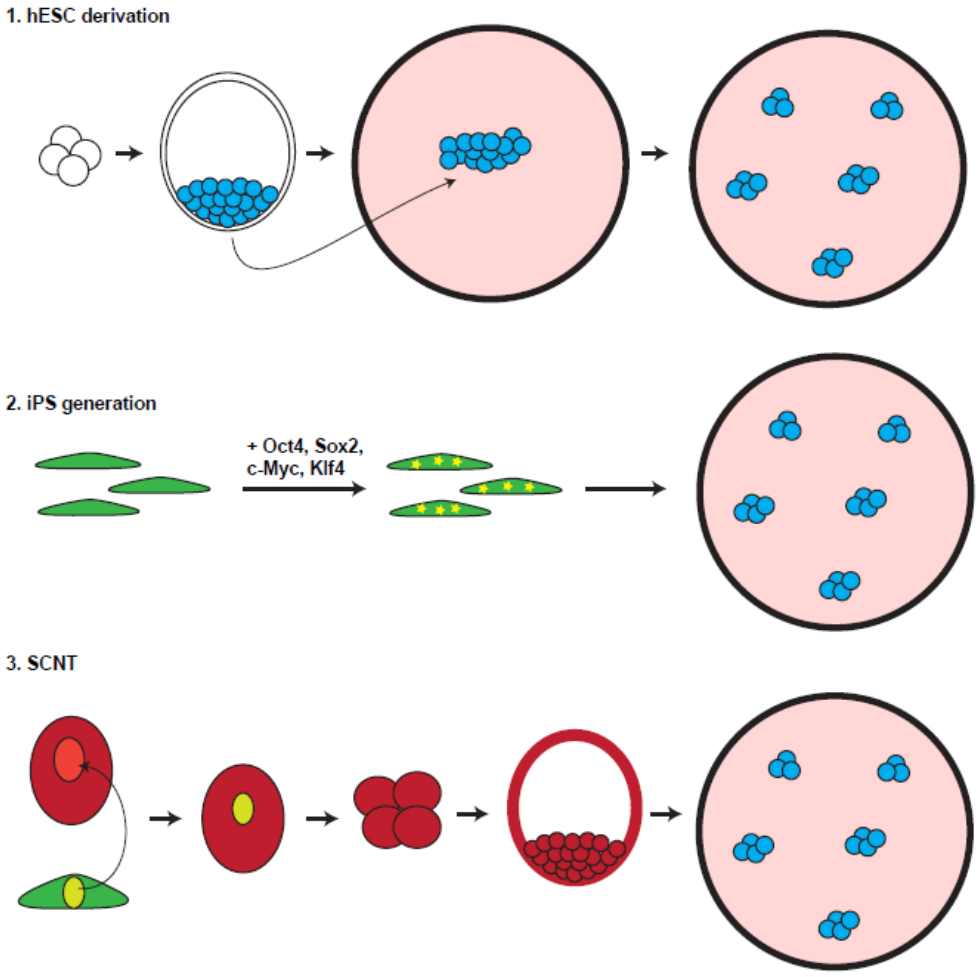

Figure 1. Derivation of hESCs and hES-like cells

1. Classic derivation of hESCs. Cells from the inner cell mass (ICM, blue) are isolated from a blastocyst and put in culture, where they give rise to colonies of hESCs.
2. Induced pluripotent stem cells (iPS cells) are generated in vitro by allowing the expression of the four transcription factors Oct4, Sox2, c-Myc, and Klf4 in a fibroblast (green). These genetically modified fibroblasts in culture are reprogrammed to hES-like cells.
3. hES-like cells can also be generated by somatic cell nuclear transfer (SCNT). In this protocol, a human oocyte (red) is enuclated and the nucleus is replaced with the nucleus from a fibroblast (green). The derived embryo is allowed to divide to reach the stage of blastocyst and the cells from the ICM are isolated and put in culture to give rise to nuclear transfer hESCs (NT-hESCs).

to be able to generate patient-specific hESCs. Because these cells can be converted into any cell type, they provide a system to model a disease in vitro, to study its pahtogenesis, and to build a platform for drug screening, with obvious potential for therapeutic advancements. Moreover, because they come from the patient, these cells could be used in transplantation procedures since they should not be recognized as foreign by the patient's immune system and therefore should not cause immune reactions or transplant rejection. However, patient-specific hESCs cannot be isolated using the classic protocol described above, which requires a blastocyst and leads to the destruction of the embryo.

The breakthrough came in 2006, when Takahasi and Yamanaka described for the first time how a combination of four transcription factors can reprogram a differentiated fibroblast to pluripotent stem cell-like conditions (Takahashi et al., 2006) (Figure 1). These induced pluripotent stem cells (iPS cells) retain the properties of self-renewal and pluripotency characteristic of hESCs, but offer a superior alternative to hESCs for the generation of patient-specific stem cells since the required starting material can be the skin biopsy from a patient. However, the process of iPS generation has proven to be slow and inefficient, with high lab-to-lab variability (Robinton et al., 2012). Moreover, generation of iPS cells requires genetic manipulation of the fibroblasts to induce the expression of the four transcription factors above. This has been traditionally achieved through delivery of viral vectors that integrate in the genome, significantly increasing the risks of associated dangerous mutations or cancer (Robinton et al., 2012). Non-integrating viral vectors as well as direct delivery of proteins and mRNA have been used, but the resulting reprogramming had low efficiency compared to the standard protocols (Robinton et al., 2012). Recently, a novel strategy using small molecule compounds to generate pluripotent stem cells from mouse somatic cells was published, thus opening the possibility of generating iPS-like cells without genetic manipulation of the cell (Zhu et al., 2010; Hou et al., 2013). Future studies and the application of this protocol to human iPS-like generation will be required to understand the feasibility of this approach for regenerative medicine.

Somatic cell nuclear transfer

Despite progress in the generation of iPS cells have made the process more efficient and streamlined, a number of studies have shown genetic and epigenetic differences between hESCs and iPS cells (Robinton et al., 2012). These differences are subtle yet significant, and might affect the potential utilization of iPS cells especially in the clinical setting. Somatic cell nuclear transfer (SCNT) recently emerged as an alternative to iPS cells for the generation of hESCs (Tachibana et al., 2013). In SCNT, an early-stage embryo is formed by replacing the nucleus of an oocyte with the nucleus derived from a fibroblast (Figure 1), the embryo is allowed to undergo a few cell divisions in vitro, and stem cells are isolated from the in vitro generated blastocyst. It isn't clear at this stage how the stem cells derived via nuclear transfer (NT-hESCs) compare to hESCs and iPS cells, and a key test in the future will be to contrast genetic and epigenetic profiles of hESCs, iPS cells, and NT-hESC. However, a potential advantage of NT-hESCs over iPS cells comes from the "uncoupling" of nucleus and cytoplasm, which can originate from different individuals. Because a major component of the cell cytoplasm is mitochondria, SCNT could offer new avenues of research in the study and possibly treatment of mitochondrial diseases.

From feeders to feeder-free to 3D culturing of hESCs

When the first hESC lines were derived, mouse embryonic fibroblasts (MEFs) feeder layers were used to support hESC growth and maintenance of pluripotent conditions (Thomson et al., 1998). However, because of the artificial system represented by the co-colturing of hESCs and MEFs and bearing in

mind the potential clinical applications of hESCs, various research groups have determined the culturing conditions required for hESC growth and maintenance in feeder-free conditions. In the lab, we routinely use matrigel as a coating reagent to allow hESC attachment in feeder-free conditions. hESCs grown on matrigel can be kept pluripotent by culturing them in media conditioned by MEFs and supplemented with basic fibroblast growth factor (bFGF) (Xu et al., 2001). Alternatively, chemically defined media have been introduced to allow feeder-free and serum-free conditions (Xu et al., 2005; Ludwig et al., 2006; Yao et al., 2006; Chiao et al., 2008). One problem with feeders and extracellular matrix proteins such as matrigel is that they only allow for growth and differentiation of hESCs on a two-dimensional (2D) surface. This is quite different from the in vivo conditions where tissues form within a three-dimensional niche during embryonic development. Thus, several lines of research have started pursuing 3D culturing systems to more closely resemble in vivo differentiation (Sasai, 2013). It is not a coincidence that the classic way of differentiating stem cells, embryoid bodies (EBs), involves allowing cells to grow in a 3D environment (Desbaillets et al., 2000; Itskovitz-Eldor et al., 2000). EBs are obtained by growing hESCs in suspension in low-attachment plates or as hanging drops. Within an EB, hESCs spontaneously differentiate into various cell types, although with a random pattern and without a true organization. More recent studies have been able to generate structures in vitro that more accurately resemble real organs, so-called "organoids" (Sasai, 2013), including intestinal (Sato et al., 2009; Sato et al., 2011), gastric (Barker et al., 2010), hepatic (Huch et al., 2013), mammary gland (Dontu et al., 2003), prostate (Lukacs et al., 2010), trachea (Rock et al., 2009), pancreatic (Greggio et al., 2013), and cerebral organoids (Lancaster et al., 2013). Organoids are generated by allowing cells to self-organize in 3D, rather than by forcing the cells to form 3D structures through the use of artificial 3D scaffolds (Sasai, 2013). Because a more "natural" differentiation and organization program underlies their generation, tissue-derived organoids could potentially better integrate, survive, and function within a tissue in vivo if they were transplanted (Sasai, 2013). However, it remains to be seen if these organoids or tissues derived from them can effectively and safely be applied to regenerative medicine, and if the more complex tissues can efficiently be recapitulated in vitro. Nonetheless, these organoids represent an advantage for developmental biology and the study of tissue formation since now the process of tissue growth and organization can be modeled in vitro, and could be used as a platform to study the effects of genetic mutations within the more complex environment of a tissue.

Clinical applications of hESCs

The ultimate goal in applying hESCs to the clinic is to establish clinically relevant therapies that use hESCs in the context of regenerative medicine. To this end, protocols need to be developed that allow the formation of functional 3D tissues and their maintenance in an *in vivo* environment. Although much progress has been achieved at the basic research level with the successful differentiation of hESCs into various cell types *in vitro*, therapeutic application of hESCs is still at a very early stage. Areas of ongoing research include cardiovascular repair (Menasche et al., 2001), hepatic regeneration (Touboul et al., 2010), treatment of diabetes (Van Hoof et al., 2009), and neural regeneration (Lee et al., 2007). However, a major hurdle is the very low efficiency of differentiation from the undifferentiated stem cells, as well as the prolonged time required for terminal differentiation (in the order of several weeks for derivation of neurons, for example)(Chambers et al., 2011). The use of bioreactors, scaffolds, and small molecules has shown some advantages over classical culturing methods (Gerecht-Nir et al., 2004; Gerecht-Nir et al., 2004; Chambers et al., 2012), but more definite

data is needed to assess the applicability of these techniques to the larger scale required for clinical applications.

A more immediate area of hESC use is that of drug screening and toxicology testing. Currently, drug screens are performed on primary cells isolated from biopsies, which require continuous supply of new cells due to cell senescence, or on cancer cell lines, which may be abnormally respond or be irrelevant to the process under study. Thus, both these cell types present significant limitations. However, hESCs could represent an "infinite" source of material that can be differentiated towards the physiologically relevant cell type to perform drug screening studies. Moreover, the use of diseased hESCs (or patient-specific iPS cells or SCNT-hESCs) could allow modeling of specific human diseases in a dish. This, in turn, could provide the opportunity to perform drug screens and to identify novel diagnostic markers and tools.

Conclusion

The field of hESC research has extensively evolved over the past few years and promises to take the center stage over the next decade. hESCs provide a window into early human embryogenesis, and therefore hold the promise of advancing the understanding of our own development. More importantly, the ability to generate various specialized cell types *in vitro* has opened the door for potential clinical and therapeutic applications of hESC research to regenerative medicine. However, several critical questions remain. A major challenge will be to define systems in which to test and validate stem cell therapies. Animal models such as dogs, pigs, and non-human primates may provide an advantage over classical rodent models in evaluating clinical treatments. Furthermore, because stem cells have the intrinsic ability to rapidly proliferate and generate all cell types and therefore have the potential to give rise to tumors, it will be important to assess the safety of stem cell therapeutics before they can be applied to the clinical setting.

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