Chapter 12
Human Embryonic Stem Cells: Hype or Hope?

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Embryonic stem (ES) cells have emerged in recent years as powerful tools for the study of in vitro and in vivo development. The engineering of mouse ES cells has been the basis of major advances in mouse transgenesis and subsequent breakthrough findings pertaining to many aspects of development and disease (31) as well as therapeutic targeting (47). Similarly, the recent derivation of human ES (hES) cells (40,46) has generated significant interest in the scientific as well as the lay community, especially in view of their potential as a universal cell source for cell replacement therapies. These efforts have also kindled complex ethical and political debate centered over the moral implications of the blastocyst derivation of ES cells and the arguable obligations to seek therapies that stand to alleviate human suffering. Current federal policy limits funding of hES cell research to work involving a small group of ES lines derived before August 9, 2001 and listed in a National Institutes of Health registry. Funding of somatic nuclear transfer for the derivation of cloned ES cells is also prohibited. The purpose of this brief review is to present recent advances in human ES cell research with special emphasis on central nervous system (CNS) applications.

Some of the most striking advantages of ES cells as compared with any other cell type are their extensive self-renewal capacity and differentiation potential, access to the earliest stages of neural development, and ease of inducing stable genetic manipulations (43). In fact, ES cells can be propagated almost indefinitely without losing their multipotential capacity and are capable of generating all cell types including germ cells.

Interest in the neural field derives from early successes in directing ES cells towards neural fates in vitro, including CNS progenitors, young neurons, and glia, as well as highly differentiated neuronal subtypes such as dopamine (DA) neurons.

NEURAL DIFFERENTIATION OF HUMAN ES CELLS

ES cells are obtained by dissecting the inner cell mass (immunosurgery) of a 3 to 5-day-old blastocyst. Undifferentiated human ES cells are typically cultured on layers of mouse embryonic fibroblasts. The cells grow into high density clusters that can be propagated multiply in their undifferentiated stage. If injected into the peritoneal cavity, these cells will differentiate randomly to give rise to a teratoma. Neural induction has been achieved in mouse ES cells via several paradigms: Embryoid body formation (54), co-culture in the presence of stromal feeders (22), or “default induction” (39) under minimal conditions necessitating the presence of basic fibroblast growth factor (bFGF) and absence of bone morphogenetic protein signaling.

hES cells can also be readily directed towards a neuroectodermal fate using modifications of the protocols mentioned above. The early studies describing the generation of hES cells demonstrate neural induction and differentiation into glia and neurons, mostly glutamatergic and GABAergic (39,54). More recently, we have described highly efficient neural induction using a coculture system of hES cells (lines H1(WA-01) or H9(WA-09) with a bone marrow stromal feeder cell layer,MS5 (37). This system yields neuroepithelial structures reminiscent of early neural tubes, and named neural rosettes (Fig. 12.1). Cells at this stage express markers compatible with neural plate identity such as Pax6, nestin, NCAM and Sox1. Markers associated with undifferentiated ES cells such as Oct4 and Nanog, are

downregulated, implying cell fate restriction towards the neuroectodermal lineage. After an additional 2 weeks of differentiation in serum-free medium, > 90% of the hES cells on MS5 express neural markers and form large clusters (1--8 mm in diameter) composed of hundreds of individual rosettes with persistence of rare clusters of Oct4-expressing cells. At 4 weeks of differentiation, cells emanating from the borders of individual rosettes are often positive for Tuj1 and exhibit neuronal morphologies. At this point, the “neural rosettes” can be dispersed and passaged multiply in the presence of bFGF to yield large numbers of neural progenitors that display in vitro characteristics similar to those exhibited by primary neural stem cells. At this stage, these “naïve” neural stem cells can acquire a region-specific neural identity such as a midbrain DA neuron or a cholinergic motor neuron following exposure to specific morphogens. Morphogens provide patterning information or specification of the anteroposterior and dorsoventral identity of the neural cell. Comparable results were obtained with stromal feeder cells derived from the AGM (aorto-gonado-mesonephros) region such as the line S2 (3). The neural inducing effects were confirmed in multiple human and nonhuman primate ES lines.

DERIVATION OF DA NEURONS FROM HUMAN ES CELLS

Fate specification of human ES cells in vitro is achieved by mimicking the events occurring during development. In the case of DA neuron fate, the neural precursors emanating from the rosettes require “ventralization” signals as well as exposure to factors that define anterior-posterior patterning before terminal differentiation. Previous work identified sonic hedgehog (SHH) and FGF8 as crucial factors in the specification of midbrain DA neurons (52). These studies in explant culture and subsequent work with mouse ES cells (3,25,30) demonstrated that the effect of SHH and FGF8 on dopaminergic differentiation is limited to distinct developmental windows that correspond to the establishment of the isthmic organizer and events controlling dorsoventral patterning during neural tube closure. Based on the work in mouse ES cells, we predicted that, in hES cells, SHH and FGF8 might act between Days 12 and 20 of differentiation to specify human midbrain DA neuron fate. This time period corresponds to the onset of neurulation until the time of neural tube closure in human development, taking into account the presumptive age of hES cells at the time of isolation (5--7 days) (34). Exposure to SHH and FGF8 from Day 12 to Day 20 of differentiation, followed by differentiation in the presence of ascorbic acid (AA) and BDNF (brain derived neurotrophic factor), resulted in a threefold increase in tyrosine hydroxylase (TH+) DA neurons(37) (Figure 15.2). The overall DA neuron yield from hES cells is enormous compared to any other culture system and completely eliminates concern about adequacy of cell numbers. In a rough approximation, we estimate that a single undifferentiated ES cell will ultimately generate 100 DA neurons and that one million DA neurons can be harvested from a single 6 cm cell culture dish. It is estimated that 100,000 to 200,000 DA neurons per hemisphere are required for successful transplantation in Parkinson’s disease and that the substantia nigra in a normal adult brain contains one million DA neurons. In addition to neural fates, hES cells have been differentiated to date into cardiomyocytes (23,51), hematopoietic progenitors (5), endothelial cells (26,49), insulin-secreting cells (32,38,42), hepatocyte-like cells (13), trophoblast (15) and germ cells (6).

TRANSPLANTATION OF hES CELLS INTO THE CNS

Early reports of neural induction of hES cells included grafting of neural progenitors into the cerebral ventricles of newborn mice (40,46). The cells migrated throughout the parenchyma and differentiated into all three CNS lineages. There were no reports of teratoma or uncontrolled growth. These results were very promising and were similar to data obtained with human fetal CNS cells in terms of widespread migration across the ventricular wall and differentiation (12,13). However, a greater challenge from a therapeutic perspective is the derivation of region-specific neuronal subtypes such as DA or motor neurons, and their ability to integrate into the adult brain. Previous experience in the transplantation field has demonstrated that the adult brain provides a less permissive milieu and

that appropriate regenerative cues may be restricted to the neurogenic zones, i.e., the subventricular zone and the dentate gyrus. In fact, the most successful grafting strategies nearly always involve directing the progenitors towards a specific fate in vitro before grafting into a disease model. The most common disease targets have been neurodegenerative models such as Parkinson's (DA neurons), Huntington's disease (ã-aminobutyric acid-ergic neurons), amyotrophic lateral sclerosis (motor neurons), or trauma models such as spinal cord injury, whereby multiple cellular components may be required, e.g., oligodendrocytes and motor neurons.

At the time of publication, there remains a paucity of studies detailing the behavior and fate of hES cells upon grafting into the mature CNS. Reports of grafting of hES-derived cells into Parkinson's disease models have raised issues of poor cell survival and incomplete correction of behavioral deficits for reasons that remain unclear (36,53). Some investigators have raised concerns about the stability of the DA phenotype in vivo despite in vitro expression of the full complement of transcription factors that characterize a midbrain DA neuron identity, such as Engrailed1, Pax2, Nurr1, and Lmx1b. Others postulate that the in vitro-generated DA neurons exhibit significant vulnerability to early cell death post grafting, a phenomenon also observed in primary human fetal DA neurons whereby a 5% survival rate is commonly observed. The DA neuron survival problem is likely to be resolved in the near future by ongoing studies in multiple laboratories.

A recent publication reports transplantation of hES-derived oligodendrocyte progenitor cells into adult rat spinal cord injuries with good survival and persistence of oligo marker expression but limited migration (33). The authors report enhanced remyelination and improved locomotor ability in the rat group that received the transplant 7 days post injury, but no significant change in a chronic injury model (10 months post lesion).

Of particular interest to our group, was the study of the potential of the hES-derived cells for widespread migration within the adult brain and their ability to respond to environmental cues. To that end, we derived stable green fluorescent protein (GFP)-expressing hES cells from lines H1 (NIH code WA01) and HES3 (NIH code ES03) after transduction with a lentiviral vector expressing GFP under the control of the PGK promoter (44). Green colonies of undifferentiated ES cells were then neurally induced in the presence of a layer of stromal feeders. The cells were expanded for a month in the presence of bFGF and EGF, and were devoid of undifferentiated ES cells and expressed markers of neural precursors (nestin+, A2b5+, Oct4- and SSEA4-); when differentiated in vitro, they generated glia and neurons. The nestin+ GFP+ cells were then transplanted into the striatum of adult rat brains. Interestingly, the hES-derived cells exhibited widespread migration following white matter tracts and were distributed both ipsi- and contralateral to the injection site within the corpus callosum, fornix, fimbria, striatum, and cortex. The hES-derived cells also differentiated into neurons, astrocytes, and oligodendrocytes. When injected into rats with demyelinating lesion, the hES cells contributed to remyelination integrating seamlessly into the repaired lesion along with host cells (Figure 3).

A small percentage of human cells were also noticed in the subventricular zone in the demyelinated and normal brains. We thus performed a series of experiments including serial time-based BrdU analyses following transplantation of the hES-derived precursors in the subventricular zone to investigate whether the human cells would integrate into the adult neural stem cell niche of the subventricular zone (SVZ). Our data demonstrate clearly that the hES-derived neural precursors integrated into the endogenous precursor pool in the subventricular zone, a site of persistent neurogenesis. Like adult neural stem cells, hES-derived precursors traveled along the rostral migratory stream to the olfactory bulb, where they contributed to neurogenesis. The human cells acquired the entire complement of markers that are normally found in the SVZ and its derivatives, in a region-specific manner. This included GFAP, Pax6 and dlx2 at the SVZ and doublecortin and TuJ1 in the rostral migratory stream (Figure 4). BrdU-positive human neurons expressing calretinin were found in the olfactory bulb of all transplanted rats indicating their hES background and the fact that they were born in vivo.

Recent studies have demonstrated that stem cell plasticity can be mimicked by fusion between host and graft cells (1,28). We thus performed genomic in situ hybridization, but could not identify any evidence of rat-human nuclear fusion. Similarly rat-specific cytoplasmic markers were not identified in any human cells and vice versa, thus excluding any significant rate of nuclear or cytoplasmic fusion.

THERAPEUTIC CLONING

Despite the immunologically privileged status of the CNS, most investigators consider immunosuppression to be a prerequisite for adequate graft survival in humans when using allografts (50). The recent placebo-controlled clinical trials of nonmatched fetal DA neuron transplants in Parkinson's disease patients used low-dose (35) or no immunosuppression (14). A close analysis of the data shows initial improvement in clinical scores with subsequent deterioration 4 to 6 months later. Some investigators attributed this response to gradual graft rejection (50). Activated microglia were noted in and around the graft in the few postmortem specimens reported, thus suggesting an immune response against the allograft. Studies of hES cells have demonstrated low levels of expression of MHC-I in undifferentiated hES cells, but a multifold increase upon differentiation (11). Exposure to interferon-ã also induced significant upregulation of MHC-I while MHC-II was not detectable (8). This data is preliminary and limited (10); additional studies encompassing the various differentiation fates acquired by hES cells and in vivo studies are required before a final determination of the antigenic potential of hES cells.

At this time the need for immunosuppression remains a major concern when proposing clinical application using nonmatched allografts. A possible solution may lie in the generation of autologous ES cells derived from the potential ES graft recipient. In collaboration with Teru Wakayama, our group derived autologous mouse ES cells by nuclear transfer using fibroblast or cumulus cell nuclei transferred to enucleated oocytes (48). The oocytes were allowed to develop into blastocysts and ES cells isolated from the inner cell mass. This work resulted in the derivation of multiple nuclear transfer ES (ntES) lines from different genetic backgrounds. The ntES lines were tested extensively and found to maintain their multipotential character as well as their ability to fully differentiate along CNS lineages including functional DA neurons. The resultant ntES cells were genotypically analogous to the somatic cell donor. This process is often referred to as therapeutic cloning. Implanting the nuclear transfer derived blastocysts into the womb of a mouse resulted in the development of cloned mouse progeny. This is referred to as reproductive cloning and was performed as a proof of principle that the ntES cells are truly pluripotential. However, our main interest lies in the therapeutic value of nuclear transfer. When subjected to the neural induction protocols with subsequent patterning and differentiation, the ntES lines generated large numbers of DA neurons in a manner indistinguishable from other ES lines. We are in the process of testing their function and immunogenicity in vivo.

The nuclear transfer process does bypass the normal processes of fertilization of two individual gametes and the resulting transfer nucleus is exposed the reprogramming activity of the oocyte cytoplasm that affects the entire genome. Thus the parent-specific methylation differences between the sperm and egg are erased resulting in erratic regulation of imprinting genes. In contrast, during normal fertilization, the epigenetic differences between the parental genomes are maintained. Improper reprogramming is considered a major barrier in the formation of normal cloned embryos but does not seem to interfere in the proper function of cells derived in vitro from a cloned blastocyst (21). Our recent work in mice does support this theory except for the generation of normal-appearing clones that went on to undergo normal reproduction (48). However, these mice were not examined carefully for subtle pathology or behavioral deficits.

Overall, the outcome of our studies of mouse nuclear transfer ES cells was very encouraging; if translated to humans, nuclear transfer could result in a significant breakthrough in therapeutic terms as it allows the generation of autologous grafts that are matched to a donor patient. The study of "cloned" hES cells from different disease backgrounds will also provide an invaluable wealth of information by expanding our understanding of the genetic and epigenetic factors leading to specific disease processes. Just recently, Woo-Suk Hwang et al. (18,19) have successfully generated multipotent hES lines by transfer of skin cell nuclei from patients with disease or injury into donor oocytes. These cloned hES lines matched the donor patient's DNA and exhibited in vitro characteristics that are identical to those seen in hES cells derived post fertilization. At this time, federal regulations in the United States prohibit the use of NIH funding for the study of these lines, but their derivation has generated significant excitement in the ES cell field. Full characterization of the differentiation potential of these cloned lines is eagerly anticipated and is expected to parallel findings in mouse ntES cells.

POTENTIAL FOR CLINICAL APPLICATION

Considerable progress has been achieved in understanding the basic properties of hES cells and the ability to harness their potential towards clinical application. Current data demonstrate a consistent and highly robust response

to directed differentiation into specific CNS cell subtypes. The hES-derived cells also exhibit an impressive ability to migrate widely in the white matter of the adult brain and to respond to environmental cues across species, namely the integration into the adult stem cell pool.

Nonetheless, additional questions remain to be addressed before the anticipation of clinical application. These include the long-term in vivo survival and phenotypic stability of hES-derived progeny and a demonstration of behavioral improvement in a recognized model of animal disease, a process that is likely to be addressed in the near future. Other safety and regulatory concerns include differentiation into undesirable phenotypes or malignant transformation, and transmission of an infectious non-human pathogen.

We and others have demonstrated the feasibility of eliminating the risk of teratoma formation by highly enriching the hES-derived cultures in neurally committed cells. In vivo differentiation into undesirable phenotypes is dependent on the persistence of Oct4 expressing undifferentiated ES cells which can also be eliminated by positive selection methods.

Some investigators have raised concerns regarding karyotype stability (7,9). The majority of studies demonstrate excellent karyotype stability over extended culture periods (up to 140 passages) (4,41), but there are reports of abnormal karyotypes that may be attributed to the passaging methods used. There are no reports of accumulation of mutations over time or of unstable hES lines despite the presence of cytogenetic abnormalities in some cell subpopulations (16). A more imminent concern is the fact that all hES cells in the NIH registry have been derived in the presence of xenogeneic substrates namely mouse embryonic fibroblast feeders during propagation, and animal sera or protein. This raises the possibility of contamination with a non-human pathogen. A recent study demonstrates that hES cells cultured with animal sera or serum replacement and murine feeders incorporate a non-human sialic acid, against which many humans have circulating antibodies (27). Grafting of such cells is likely to result in rejection. There are now reports of generation of hES lines using human feeder layers (17, 20) or in a feeder-free system using Matrigel as an extracellular matrix substrate (24). However, none of these lines qualify for Federal funds since they were generated after August 9, 2001 and are thus not included in the NIH Registry of hES lines (32).

Studies of hES cells for basic or therapeutic applications have stimulated significant controversy and debate over the ethics of their derivation and use. Opponents of this research voice grave concerns about the destruction of blastocysts inherent to the derivation of ES lines. The main argument is essentially a philosophical belief that life begins at conception or fertilization and that society has an obligation to protect it. The opposite argument is more pragmatic and points out the nature of the blastocyst as only a potential embryo (or multiple embryos) with no chance of developing into a fetus unless implanted into the womb of a willing parent. Supporters of hES research are concerned with depriving actual patients from a highly promising technology that can bring relief to many agonizing disease conditions. Several hundred thousand “excess’ embryos are created each year in the United States in the process of in vitro fertilization. The fate of these embryos is usually destruction or permanent cryogenic storage in fertility clinics. There have been calls to alter regulation to allow donation of these excess embryos by informed parents for research purposes. This suggestion is tampered by calls for adoption of the embryos in storage by “volunteer parents” giving rise to the so-called “snow flake babies” movement. The question of somatic nuclear transfer met with further controversy in view of concerns over extrapolation of such research down a slippery slope into reproductive cloning. In Europe, regulatory agencies have prohibited reproductive cloning while allowing somatic nuclear transfer, a proposition that remains highly contested in the United States. Some scientists believe that nuclear transfer may actually represent a good compromise by allowing the generation of blastocysts via an “unnatural” route that does not involve fertilization or intent to create an embryo. Furthermore, current understanding of human development suggests that there are essentially insurmountable biological barriers related to the dysregulation of imprinting that are inherent to the nuclear transfer process and that will prevent successful reproductive cloning (21). Thus a blastocyst obtained by somatic nuclear transfer is not likely to potentially generate a live healthy human being. Nonetheless, the President’s Council on Bioethics does not recommend revision of the current federal regulations that limit funding to a list of lines generated before August 9, 2001, and has advised against approval of somatic nuclear transfer. More recently, the Council has proposed an alteration to nuclear transfer that includes the introduction into donor cells of a mutation gene (e.g., CDX2) known to result in death at the blastocyst stage in mice. These embryos can still give rise to mouse embryonic stem cells. A similar mutation in human cells would result in an ethically uncontroversial source of hES cells, according to the Council (45). This

proposition was met with significant scientific skepticism owing to the lack of data in human systems and the significant efforts required to properly investigate and achieve the technical manipulations inherent to this suggestion (29).

It is likely that the ethical debate over hES cells will persist and evolve as our knowledge of hES cell biology develops further, but it is important to note that, in the United States, this debate only impacts the use of government funds to support hES research. Private companies are free to pursue this research and are doing so eagerly. This dichotomy will generate another type of debate, this time over licensing and patent rights to hES cells. It effectively excludes academic institutions from active involvement in this research in view of their dependence on Federal funds, and subjects advances in the field to the laws of marketing and commerce. Many scientists and patient advocates find this compromise to be more problematic, as it further complicates the ethical challenge.

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Fig. 12.1 Neural rosette derived from hES cells and immunostained for nestin, a neural stem cell marker (green) and TuJ1, a neuronal marker (red)(original magnification, x200).

Fig. 12.2 Human DA neurons derived from hES cells. Immunostaining for Tyrosine Hydroxylase (green) and neurofilament (red) (original magnification, x200).

Fig. 12.3 Transplantation of hES-derived neural precursors into the striatum of the young adult rodent brain. Camera lucida drawing of a representative coronal section (G) through the brain of a lesioned animal illustrating the distribution of hNA+ cells 11 weeks post grafting. (A) GFP+/NeuN+ neurons in the graft core (green outline in G), (B) Migrating hNA+/Nestin+ cells in the contralateral corpus callosum, (C) z-stack confocal image of a GFP+/MBP+ cell located in the remyelinated cingulum following lysolecithin lesion. (h) Diagram of a sagittal brain section illustrating the location of hNA+/Dlx2+ cells (d) and hNA+/GFAP+ (D, inset) in the subventricular zone. (E) hNA+/doublecortin+ neuronal precursors in the RMS. (F) z-stack confocal image of hNA+/Calretinin+ neuron in the olfactory bulb. DAPI nuclear counterstains are in blue. Scale bars, 10μm.

Fig. 12.4 At 42 days after transplantation, hES cell-derived precursors persisted in the subventricular zone and were aligned in human NCAM+ chains (green) (original magnification, x400).