Human embryonic stem cells for brain repair?

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Cell therapy has been perceived as the main or ultimate goal of human embryonic stem (ES) cell research. Where are we now and how are we going to get there? There has been rapid success in devising *in vitro* protocols for differentiating human ES cells to neuroepithelial cells. Progress has also been made to guide these neural precursors further to more specialized neural cells such as spinal motor neurons and dopamine-producing neurons. However, some of the *in vitro* produced neuronal types such as dopamine neurons do not possess all the phenotypes of their *in vivo* counterparts, which may contribute to the limited success of these cells in repairing injured or diseased brain and spinal cord in animal models. Hence, efficient generation of neural subtypes with correct phenotypes remains a challenge, although major hurdles still lie ahead in applying the human ES cell-derived neural cells clinically. We propose that careful studies on neural differentiation from human ES cells may provide more immediate answers to clinically relevant problems, such as drug discovery, mechanisms of disease and stimulation of endogenous stem cells.

Keywords: embryonic stem cells; neuroectoderm; neural patterning; neural transplantation; cell therapy

1. INTRODUCTION

During early embryonic development, the inner cell mass of a blastocyst-stage embryo differentiates into three major embryonic germ layers: endoderm, mesoderm and ectoderm. These cells go on to form all the tissue types of the body in a strictly temporal and spatial order. The inner cell mass cells of the preimplantation embryo can be artificially maintained as embryonic stem (ES) cells *in vitro* for years in a genetically stable, pluripotent state capable of giving rise to all three germ layer cell types. Establishment of human ES cells (Thomson *et al.* 1998) has attracted particular attention because these cells offer an unprecedented source of human cells for multiple applications including unravelling early human development, discovering function of novel genes, screening toxins and pharmaceuticals, and producing specialized cells such as neural cells for potential cell therapy. Since our previous overview (Zhang 2003), significant progress has been made, especially in generation of neural subtypes and examination of *in vitro* produced neural cells in animal models. In this brief review, we will focus on the potential of human ES cells in relation to brain repair. A large body of literature on mouse ES cell neural differentiation can be found elsewhere (Du & Zhang 2004; Lang *et al.* 2004; Keller 2005; Sonntag *et al.* 2005).

2. CERTAIN NEURAL SUBTYPES CAN BE EFFICIENTLY DIFFERENTIATED FROM HUMAN ES CELLS

The pluripotent nature of ES cells precludes the direct therapeutic application of undifferentiated stem cells to

patients. The first step towards the use of human ES cells is therefore the production of enriched or purified populations of functional target cells. Two main strategies, directed differentiation and purification (enrichment) of target cells, have been employed in a similar manner to that used for mouse ES cells in the past decade (reviewed by Du & Zhang 2004).

(a) Directing neuroepithelial differentiation

Neuroepithelial or neuroectoderm cells, which form the neural plate and neural tube during early embryo development, are ancestors of all neuronal and glial types in the brain and spinal cord. From a developmental biology perspective, a method to direct human ES cells to neuroepithelial cells that faithfully reflects neuroectodermal development *in vivo* will provide a tool to dissect out how the neuroectoderm is initially induced and how neuronal and glial cells are subsequently specified. From an application standpoint, neuroepithelial cells or more restricted neural progenitors, rather than differentiated neurons, are more amenable for cell preparation and transplantation. The production of neuronal and glial subtypes requires the specification of region- and/or transmitter-specific neuroepithelial or progenitor cells. Hence, the generation of neuroepithelial cells from human ES cells bears both scientific and clinical significance.

The general procedure for neural progenitor differentiation from human ES cells includes removal of self-renewing factors, aggregation of ES cells and addition of morphogens/mitogens or stromal cell-derived factors to promote specification and/or selective amplification of the neuroepithelial population (Du & Zhang 2004). Detailed accounts and comparison of methods for neuroepithelial differentiation from human ES cells have been discussed elsewhere (Zhang 2005). Ideally,

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the neural differentiation method should be simple, efficient, chemically defined, scalable and reproducible, and the end product should be an enriched or purified homogeneous neural progenitor population.

The first step towards differentiation is the removal of self-renewal supporting factors. ES cells grown on feeder cells need to be separated from each other by aggregating the ES cell colonies. Human ES cells grown on matrigel without feeder cells can be directly differentiated into neural progenitors by removing feeder cell-conditioned medium and application of neural induction medium (Gerrard *et al.* 2005; Yao *et al.* 2006). A similar approach has been used for mouse ES cell differentiation (Ying *et al.* 2003). Aggregation of ES cells creates an environment that mimics the one occurring during early embryo development in which cells interact with each other and 'sense' their individual positions in a three-dimensional sphere. Such cell-cell interactions are crucial in triggering the normal, intrinsic cellular programme. We therefore favour the ES cell aggregation approach. Continued suspension culture of human ES cell aggregates often leads to random differentiation to derivatives of the three major germ layers with formation of an inner cyst (Itskovitz-Eldor *et al.* 2000), referred to as an embryoid body. However, ectoderm and neuroectoderm are specified during gastrulation which takes place between the second and the third weeks of human gestation. Therefore, the ES cell aggregates need to be induced by neutralizing factors before the aggregates form embryoid bodies in order to achieve an efficient neural differentiation.

The seemingly random differentiation of ES cell aggregates can be steered to the neural lineage by subsequent application of factors. Retinoic acid (RA) is a strong differentiation-inducing factor and perhaps the most frequently used factor in ES cell differentiation. RA promotes multilineage differentiation including neural cells (Keller 2005). RA needs to be combined with other conditions such as serum-free environment and/or low-density culture in order to increase the neural proportion from human ES cells (Carpenter *et al.* 2001). RA is also a strong morphogen and induces caudally fated neuroepithelial cells (Li *et al.* 2005). Thus, it is ideal to use RA to produce caudally fated neuroepithelial cells such as spinal cord cells. However, it should be avoided or its concentration adjusted in order to achieve differentiation of fore- or midbrain cells, such as dopamine neurons.

One theory on how neural tissue is induced is the so-called 'default' pathway, which states that the embryonic ectoderm will become neuroectoderm when transforming growth factor- β (TGF- β) family signalling is inhibited (reviewed by Wilson & Edlund 2001). By applying noggin, a bone morphogenic protein (BMP) antagonist, several groups have shown an increase in the number of neuroepithelia-like cells formed from human ES cells (Pera *et al.* 2004). While it is clear that adding exogenous noggin can enhance neural differentiation in some culture conditions, it has not been demonstrated that noggin alone can efficiently direct all differentiating ES cells to a neural fate. Additional work in animal embryology (Wilson & Edlund 2001) as well as studies of neural differentiation

of mouse ES cells (Ying *et al.* 2003) have lead to a more nuanced picture of neural induction that includes, but is not limited solely to BMP antagonism.

The neural induction process may also involve active induction by factors such as the fibroblast growth factor (FGF) family (Wilson & Edlund 2001). Using a single growth factor, FGF2, in a chemically defined culture condition, we are able to induce human ES cells to efficiently differentiate into neuroepithelial cells, with 70–90% efficiency among the total differentiated progenies (Zhang *et al.* 2001; Zhang 2005). Importantly, the neuroepithelial cells organize into a neural tube-like rosette structure at a time window that is equivalent to the human embryonic stage when the neural plate begins to form the neural tube. Thus, our differentiation system appears to mimic the normal neuroectodermal development taking place in a human embryo. The neuroepithelial cells express known neural markers, such as Pax6, Sox1 and nestin, but not mature neuronal or glial cell markers, indicating a synchronized population of neural precursors. This population of neuroepithelial cells is distinct from neural stem/progenitors isolated from foetal brain tissues in that the human ES cell-derived neuroepithelial cells can be readily specified to region-specific neuronal subtypes (see below). Thus, they provide a unique intermediate source for production of more specialized neurons and glia.

Co-culture with stromal cells, such as HepG2 (Schulz *et al.* 2003), PA6 (Mizuseki *et al.* 2003; Buytaert-Hoefen *et al.* 2004) and MS5 (Perrier *et al.* 2004) can also promote neuroepithelial differentiation from human ES cells. Interestingly, a single ES cell colony can generate a colony comprising nearly a 100 neural tube-like rosettes (Perrier *et al.* 2004), suggesting a powerful mitogenic activity of the stromal signals. Our own experience indicates that some human ES cells retain the stem cell state for a considerably long period, whereas others readily differentiate into neuroepithelial cells on the MS5 feeder cells, thus creating developmentally heterogeneous populations of neural precursor cells. The co-culture method also appears to be particularly effective in inducing certain neuronal subtype differentiation (see §2*b*(i)). It is thus attractive at the present for certain applications. However, the neural induction process often requires direct contact of human ES cells with the stromal cells, which introduces unknown and potentially risky factors as most of these cell lines are tumorigenic. Identification of the stromal factors that promote the differentiation and/or proliferation of neural precursors and that induce dopaminergic neuronal differentiation will be useful.

Neuroepithelial cells produced from human ES cells using various methods, as outlined above, may appear similar in morphology and expression of certain neural precursor markers. However, they in fact differ significantly from each other depending upon the culture conditions. RA treatment almost always generates neural cells that are fated to hindbrain and spinal cord identities. Many stromal cells, such as PA6 and MS5, appear to favour the differentiation of neural progenitors with mid/hindbrain characters. The chemically defined neural differentiation system established

by Zhang (2001) and refined by his colleagues represents a simple and robust differentiation system that produces a developmentally synchronized neuro-epithelial population for further induction of neuronal and glial subtypes with forebrain, midbrain and spinal cord identities.

(b) *Making the right types of neurons*

Every neuron has a unique position in the brain and spinal cord and possesses a special set of neurotransmitter(s). Both of these characteristics are crucial for forming the appropriate connections and correct chemical communications with targets. In the CNS, neurons with the same transmitter identity often aggregate in a specific location, suggesting the close relationship between the position and the neurotransmitter specificity. Both appear to be endowed during early development, although transmitter phenotypes may be altered in later life (e.g. Black *et al.* 1984; Schotzinger & Landis 1990). At present, little is known about how neuronal transmitter identity is specified during development. Hence, specification of position-specific neural progenitors provides a rational approach to producing functional neuronal subtypes with a desired transmitter phenotype.

At present, major attention is paid to a few 'high-value' neuronal subtypes such as midbrain dopamine neurons that are degenerated in Parkinson's disease patients and motor neurons that are lost in amyotrophic lateral sclerosis (ALS) patients. These are large projection neurons and are born at early embryonic stages. Molecular pathways that lead to the specification of both neuronal types are relatively well understood from animal studies, although some crucial steps involved in midbrain dopaminergic neuron specification remain to be elucidated. Success or failure in producing these neuronal subtypes will undoubtedly provide us with insights into the principle of neuronal specification and lessons for future modification of protocols.

(i) *Dopamine neurons*

Among the ten papers on dopaminergic differentiation from primate ES cells coming within the past few years (many more are expected to come shortly), eight reports employ co-culture with stromal cells, including PA6 (Kawasaki *et al.* 2002; Buytaert-Hoefen *et al.* 2004; Zeng *et al.* 2004; Takagi *et al.* 2005; Park *et al.* 2005), MS5 (Perrier *et al.* 2004; Sanchez-Pernaute *et al.* 2005) and HepGII (Schulz *et al.* 2004). As discussed in §2b, the key to the generation of neuronal subtypes is the specification of region-specific progenitors. Signals from PA6 and MS5 cells seem ideal to promote the differentiation of neural progenitors with a mid/hindbrain character. In most studies, additional signalling molecules, including FGF8 and sonic hedgehog (SHH), have been added to promote specification of midbrain neural progenitors, although it is not clear whether these growth factors are necessary in this culture system.

It is clear that neurons expressing tyrosine hydroxylase (TH) and releasing dopamine can be generated from both human and non-human primate ES cells. All the human ES cell lines reported can produce dopamine neurons; however, the proportion of TH+ neurons

varies widely, from less than 1 to 75% of the neuronal population. This is partly due to the methods used for quantitation. In some reports, only neurons in the peripheral growth area are counted and cells in the densely packed progenitor cluster area are excluded, thus artificially increasing the dopamine neuron production efficiency. Most of the analyses are limited to the demonstration of TH expression and dopamine release. Expression of midbrain dopamine neuron phenotypes is generally performed using reverse transcriptase-polymerase chain reaction (RT-PCR). Since neural progenitors with various regional specificities are present in most of the differentiation culture conditions, the mere expression of midbrain neural transcription factor mRNA in bulk cultures is not sufficient to demonstrate that the TH+ neurons are the midbrain type of dopamine neurons. Co-localization of TH with nuclear midbrain transcription factors will be necessary to confirm the midbrain dopamine neuron identity. Studer and colleagues (Perrier *et al.* 2004) have demonstrated that the TH+ neurons, generated through co-culture with MS5 stromal cells, co-express engrailed 1 (En-1), a transcription factor necessary for midbrain development and is present in the midbrain dopamine neurons. They have also demonstrated the presence of enzymes relating to dopamine metabolism such as aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter 2 (VMAT2) in TH+ neurons.

This stromal cell co-culture approach has the advantage of simplicity and efficiency. In particular, dopamine neurons generated in this way appear to bear some midbrain phenotypes (Perrier *et al.* 2004; Takagi *et al.* 2005). The disadvantage of this approach is the contamination of target neural cells with stromal cells. We have co-cultured human embryonic stem cells (hESCs) with irradiated green fluorescent protein (GFP)-labelled PA6 or MS5 cells for dopamine neuron differentiation. It becomes obvious that these GFP+ stromal cells will be carried over for at least three to four weeks (S.-C. Zhang 2005, unpublished observation). The contamination of these supporting cells has ramifications. From the application standpoint, these cells must be removed, e.g. by fluorescence-activated cell sorter (FACS), before the human ES cell progenies may be considered for use in patients. Even in a preclinical experimental setting, the contamination of these supporting cells may skew the outcome. For example, human ES cell-derived dopamine neurons have failed to survive or maintain their dopaminergic phenotypes following transplantation into the striatum of Parkinsonian animals. The presence of these supportive stromal cells may enhance the survival of the *in vitro* produced dopamine neurons, which may otherwise fail after the stromal cells are removed. Hence, it is now important to identify the molecular makeup that is essential for dopaminergic specification and/or differentiation. This will allow researchers to create a chemically defined system for the production of dopamine neurons. In addition, some human ES cells retain the stem cell state when co-cultured with the stromal cells. Thus, an additional step in removing the undifferentiated cells will be necessary.

We have developed a chemically defined system for neuronal subtype differentiation. This is based on our

Figure 1. Stereotypic neuronal specification from human ES cells. (a) Schematic procedures for inducing dopamine neuron (DA) and motor neuron (MN) differentiation. ES cells were differentiated to primitive neuroepithelial cells (NE) and then neuroepithelial cells that exhibit neural tube-like rosettes in two weeks of differentiation in a chemically defined neural medium (Zhang *et al.* 2001) without the presence of morphogens. (b) Treatment with FGF8 and SHH after neuroepithelial cells have formed resulted in differentiation of dopamine neurons that possess forebrain features (upper row), whereas the same set of morphogens at the primitive NE stage caused differentiation of dopamine neurons with midbrain characters including the expression of En-1 (lower row; Yan *et al.* 2005). (c) Similarly, neuroepithelial cells, after treatment with RA and SHH, differentiated to neurons that expressed Isl1, but not the motor neuron-specific transcription factor HB9+ (upper row). In contrast, treatment of primitive neuroepithelial cells with the same set of morphogens resulted in the production of motor neurons that express HB9+ (Li *et al.* 2005). Blue indicates Hoechst stained nuclei. Bar = 50 μ m. (b) and (c) are reproduced from Yan *et al.* (2005) and Li *et al.* (2005), respectively, with permission.

well-defined neuroepithelial differentiation system (see §2a) and the principles of midbrain development. Neuroepithelial cells, in the form of neural rosettes, are first enriched by removing the surrounding non-neural cells. This step becomes extremely useful as it not only removes non-neural cells that may interfere with subsequent dopamine neuron induction process, but also more importantly removes any possible undifferentiated ES cell. Contamination of ES cells in the differentiated progenies is one of the major concerns for ES cell-based therapy. The enriched neuroepithelial cells, in response to FGF8, a morphogen organizing the mid/hindbrain region, and SHH, a ventralizing morphogen, differentiate robustly into neurons, among which approximately 50–60% express TH and other enzymes that are necessary for dopamine synthesis and metabolism (Yan *et al.* 2005). Importantly, we have discovered that neuroepithelial cells at different developmental stages differentiate into dopaminergic neurons with different characteristics, in response to the same set of morphogens. Early or primitive neuroepithelial cells, treated with FGF8 and SHH, differentiate into TH+ dopaminergic neurons which exhibit characteristics of large projection neurons and co-express the midbrain transcription factor En-1 in cell nuclei. Late or definitive neuroepithelial cells

differentiate into a similar proportion of TH+ dopaminergic neurons but these neurons are generally bipolar cells and rarely co-express En-1 in the nuclei. In addition, many of the dopamine neurons, produced by the late neuroepithelial cells, co-express gamma aminobutyric acid (GABA), suggesting that they are likely forebrain (e.g. olfactory bulb) dopamine neurons. We have confirmed that many of these dopamine neurons express a forebrain transcription factor BF-1 (figure 1). This study illustrates the similarity between *in vitro* and *in vivo* neural lineage specification and the importance of applying developmental insights into the production of correct types of neurons.

(ii) Motor neurons

Motor neurons are one of the largest projection neuronal types in the brain and spinal cord. They are the target of many neurological disorders including the devastating ALS. Hence, generation of motor neurons not only provides a potential source for therapeutic application but also offers a tool for research purposes. Thanks to the well-defined molecular pathways underlying the birth of spinal cord motor neurons in vertebrate animals (Jessell 2000; Wichterle *et al.* 2002), we have established a defined system for

efficient generation of spinal motoneurons from human ES cells (Li *et al.* 2005). Similar to what we have found in the dopaminergic neuron differentiation from human ES cells, neuroepithelial cells need to be 'instructed' by RA at the primitive stage for them to adopt the spinal cord motor neuron fate. Neuroepithelial cells at a later stage, even though they can be caudalized by RA and ventralized by SHH to some degree, do not readily produce motor neurons (figure 1). This may explain why neural stem cells isolated from the spinal cord do not usually produce motor neurons. It requires the coupling of the intrinsic programme of the precursors with extrinsic morphogen actions. Furthermore, molecular analyses reveal that a simple caudalization and ventralization by morphogens are not sufficient. Rather, the generation of motor neurons requires activation of a set of transcription factors and at the same time inhibition of another set (Li *et al.* 2005). The sequential activation/inhibition of transcription factors may also be crucial for producing neurons with appropriate functions (see §§3 and 4). The *in vitro* generated motor neurons exhibit morphological features of large projection neurons and express HB9, HoxC8, choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT).

(iii) Other neuronal subtypes

Although dopamine and motor neurons are at present the main target cells for differentiation from human ES cells, other neuronal subtypes are frequently found in these culture systems. Since dopamine and serotonin neurons are located adjacent to each other in the mid- and hindbrain, respectively, their specification and growth requirement are very similar. Therefore, serotonin neurons are frequently observed in cultures designed for dopamine neuron differentiation (Salli *et al.* 2004; Perrier *et al.* 2004). Non-human primate ES cells produce a particularly high proportion of serotonin neurons when the neuroepithelial cells are treated with FGF8 and SHH (Salli *et al.* 2004, S.-C. Zhang 2004, unpublished observation). These observations suggest that specification of hindbrain serotonin neurons in primates may be different from that in rodents. Generation of serotonin neurons from mouse ES cells requires the presence of FGF4 in addition to FGF8 and SHH (Goridis & Rohrer 2002; Barberi *et al.* 2003).

Neurons carrying the inhibitory transmitter GABA are most frequently observed, especially after the neuroepithelial cells are expanded with FGF2 (Zhang *et al.* 2001). GABA neurons are also frequent by-products of the above dopaminergic differentiation cultures, as specification of GABA neurons in the forebrain (olfactory and striatum) and midbrain also depends on the signalling of SHH and FGF8 (Hynes & Rosenthal 1999; Goridis & Rohrer 2002). In order for neuroepithelial cells to make a decision between dopaminergic, serotonergic and GABAergic neuronal fates, the neuroepithelial cells have to be able to 'sense' the nuances within the set of morphogens (e.g. amount, sequence, isoforms, etc.) and/or additional signals. Treatment with FGF8 at an early developmental stage biases neuroepithelial cells towards a dopaminergic fate, whereas at a later stage the same set of morphogens

facilitate GABAergic neuronal differentiation and the dopaminergic neurons often carry a GABAergic phenotype (Yan *et al.* 2005). Hence, developmental principles and nuances need to be integrated in order to achieve directed differentiation of neural subtypes from primate ES cells.

These non-targeted observations reassure us of the potential of human ES cell-derived neuroepithelial cells to produce a wide spectrum of neuronal subtypes. However, the frequently generated neuronal subtypes, as summarized above, are mostly located in the mid/hindbrain and spinal cord. Neuronal subtypes from the forebrain are under-reported. This is possibly due to a better understanding of signalling pathways governing neuronal subtype specification in the hindbrain and spinal cord than in the forebrain. It may also be attributed to the use of common morphogens and mitogens, such as FGFs and RA, which ultimately caudalize ES cell-derived neural cells. We have observed glutaminergic neurons, presumably forebrain neurons, in our culture system (Zhang *et al.* 2001; Yan *et al.* 2005). Carefully designed strategies will be needed in order to direct ES cells to the vast array of neuronal subtypes that are harboured in the primate forebrain.

(c) Producing myelinating glial cells

During embryonic development, glial cells, including astrocytes and myelinating oligodendrocytes, are generated following the birth of major neuronal cells. *In vitro*, human ES cell-derived neuroepithelial cells produce neurons first, followed by astrocytes and oligodendrocytes. In our chemically defined culture system, human ES cells generate neuroepithelial cells in 2–3 weeks. These neuroepithelial cells differentiate predominantly into β III-tubulin+ neurons within three weeks (or 5–6 weeks of ES cell differentiation). Glial fibrillary acidic protein-positive (GFAP+) astrocytes appear thereafter, 6–9 weeks after the differentiation of human ES cells. O4+ oligodendrocytes arise in a much longer period of differentiation (Zhang *et al.* 2001). This temporal sequence of neuronal and glial differentiation is the same as occurs *in vivo*, and the time line corresponds to the observations made from limited samples of foetal tissues (Sidman & Rakic 1982). Thus, the intrinsic programme governing neuronal and glial lineage development is retained *in vitro*. This timing issue points to potentially challenging issues associated with directing human ES cells to the late-born oligodendrocytes (Zhang 2003, 2005).

There appears to be a significant progress in producing oligodendrocytes from human ES cells. Keirstead and colleagues have recently reported the generation of pure cultures of oligodendrocytes using a rather simple protocol (Nistor *et al.* 2005). Human ES cells are cultured in a 'glial-restricted medium', similar to the Sato medium (Bottenstein & Sato 1979), with the presence of 2–4 ng ml⁻¹ of FGF2 and 20 ng ml⁻¹ of epidermal growth factor (EGF) for 6 weeks. The culture is exposed to RA (10 μ M) for 8 days after ES cells are differentiated for 2 days, which results in the formation of 'yellow spheres'. Upon removal of EGF, these cells become multipolar with branched processes, possessing some aspects of the morphological features of oligodendrocytes. These cells can be stained with a

series of antibodies against common oligodendrocyte antigens (Nistor *et al.* 2005).

The rationale behind the choice of this culture system for oligodendrocyte differentiation is not clear. FGF2 and RA may play a role in inducing a neural fate as outlined above, although early treatment with RA (within 6 days) often results in non-neural lineage differentiation (M. T. Pankratz & S.-C. Zhang 2006, unpublished observation). Oligodendrocytes are mostly generated from the ventral side of the neural tube. Hence, generation of oligodendrocytes will presumably require the action of SHH. The production of pure population of oligodendrocytes in the absence of SHH is somewhat surprising, although it is possible that endogenous SHH, potentially induced by RA, may play a role. A striking observation made in this study is that cells in the yellow sphere appear to be a rather pure population of oligodendrocyte progenitors by day 10 of the '42-day differentiation protocol' as most of them express oligodendrocyte-related transcription factors, such as Olig2, Olig1, Sox10, NG2 proteoglycan and platelet-derived growth factor receptor alpha (PDGFR α ; Nistor *et al.* 2005). This is at odds with the findings so far made in the field (Carpenter *et al.* 2001; Zhang *et al.* 2001; Perrier *et al.* 2004; Li *et al.* 2005; Yan *et al.* 2005). Nevertheless, most neural transcription factors are present in the cytoplasm in addition to cell nuclei and membrane antigens being distributed throughout the cells. This is accompanied by the atypical morphology of oligodendrocytes *in vitro*. The morphological transformation and the corresponding antigenic appearance in the oligodendrocyte lineage are stereotypic along *in vitro* differentiation (Zhang 2001; Rowitch 2004). The production of rather mature oligodendrocytes within 7 weeks of ES cell differentiation is relatively short for the development of oligodendrocytes in a normal human embryo. Mature oligodendrocytes do not appear in the human CNS until three months of gestation (Sidman & Rakic 1982). The birth of oligodendrocyte progenitors in humans is not very clear but peaks at four to five months of gestation, which also makes it easy for isolation (Grever *et al.* 1999; Windrem *et al.* 2004). Nevertheless, the early appearance of oligodendrocytes could be attributed to the exceedingly high concentration of RA (10 μ M). It will be important for other laboratories to produce similar pure populations of oligodendrocytes using this simple protocol.

3. *IN VITRO* PRODUCED HUMAN NEURONS EXHIBIT TYPICAL ELECTROCHEMICAL PROPERTIES

The single most important functional property of a neuron is its ability to transduce electrochemical signals through synapses. Neurons produced from human ES cells in a Petri dish have been shown to possess basic electrophysiological properties such as maintenance of a resting membrane potential, ability to fire a Na⁺ gated action potential and transduction of synaptic currents (Carpenter *et al.* 2001; Li *et al.* 2005), similar to neurons that are grown in the brain. The *in vitro* generated neurons can also synthesize, store and release neurotransmitters such as dopamine and serotonin, and the

release is generally activity dependent (Perrier *et al.* 2004; Yan *et al.* 2005). Depolarization of neurons in cultures elicits an increase in transmitter release (figure 2). Some of the properties may require environmental cues or specific connections. We have shown that the *in vitro* generated human neurons can form functional synapses in culture. These synapses include both excitatory and inhibitory currents, and the synaptic currents can be blocked by specific pharmacological transmitter blockers (Li *et al.* 2005; figure 2). Furthermore, ES cell-derived human motor neurons can communicate with their target muscle cells by inducing acetylcholine receptor clustering in cultured muscles and elicit muscle contractions (Li *et al.* 2005; figure 2). Thus, human neurons, produced in a Petri dish, can be functional.

The appearance of the basic electrophysiological properties of human neurons generated from ES cells using various protocols suggests that these properties are inherently associated with the neuronal development programme. Since these functional properties are developed in a Petri dish, i.e. outside the brain, the ES cell differentiation system offers a unique tool to study how these functional features are acquired by a neuronal cell. By reconstructing a neural circuitry, e.g. by co-culturing with target cells, one may dissect out how additional functional features are gained by a subtype of neurons. On top of this circuitry, genetic and pharmacological interventions can be applied to create a condition that mimics certain aspects of neurological processes. Such a system will provide a dynamic and controllable model to dissect certain developmental and pathological processes involved in human neurological disorders. It can also be a simple functional tool for screening pharmaceuticals that may affect these processes (Krencik & Zhang 2006). Both applications can lead to the ultimate goal of brain repair. Yet, owing to overwhelming focus on generating specialized cells for cell therapy and owing to scientific bias against the use of an *in vitro* system for developmental and pathological studies, such a simple and functional human model system has been astonishingly overlooked.

4. HUMAN ES CELL-DERIVED NEURAL CELLS MATURE IN THE BRAIN AND CONTRIBUTE TO FUNCTION IN SOME CASES

Human ES cell-derived neural cells have been transplanted into normal and injured animal brains in an attempt to assess changes in function. Taking advantage of the easy accessibility to the developing brain and spinal cord at a very early stage in chick embryos, Benvenisty and colleagues injected human ES cells directly into the somites at the stage of neuroectodermal development. The injected ES cells differentiate robustly into neuroepithelial cells that form neural tube-like rosettes (Goldstein *et al.* 2002), similar to the structure formed in our *in vitro* neural differentiation cultures (Zhang *et al.* 2001). This observation confirms the *in vitro* finding that human ES cells can respond to extrinsic factors for efficient neural differentiation. It also reinforces the notion that human ES cells retain the intrinsic capability to form

such structures as neural tube-like rosettes both in *in vitro* and in ectopic *in vivo* regions.

From a therapeutic perspective, differentiated neural progenies instead of ES cells themselves will be used as donors. Injection of ES cell-derived neuroepithelial cells into the ventricles of a neonatal mouse brain results in widespread distribution of the grafted human cells. These human cells, identified by human-specific markers, such as human nuclear proteins, differentiate into neurons in neurogenic areas and glial cells in white matter areas (Reubinoff *et al.* 2001; Zhang *et al.* 2001). In a long-term study (up to nine months) when the neural precursors were initially transplanted into the ventricles of neonatal SCID mice, we have found that the grafted human neuroepithelial cells become region-specific neurons and form synapses with host cells. In neurogenic areas, such as the subventricular zone and the rostral migratory stream, grafted human cells retain an immature state (Guillaume *et al.* 2006), again suggesting the ability of *in vitro* produced human neural precursors to respond to local environmental signals for fate determination. Transplantation of hES cell-derived neural precursors to the mouse brain at an even earlier stage (E14) results in robust differentiation of neurons that resemble host neurons in the same region (Muotri *et al.* 2005). More importantly, electrophysiological analysis based on GFP-labelled cells indicated that these graft-derived neurons are active and form functional synapses with the surrounding neurons. Hence, *in vitro* produced human neurons can become functional in a conducive environment. Transplantation of similar neuroepithelial cells into the adult rodent brain results in a similar migration pattern, but mainly along the rostral migratory stream for neuronal differentiation and in white matter for glial differentiation (Tabar *et al.* 2005). This ability to differentiate into versatile neuronal subtypes in a neurogenic environment places human ES cell-derived neuroepithelial cells as a useful source of cells for neural replacement therapy in multiple neurological disorders. The restricted migration and differentiation in adult brains suggest that in non-neurogenic adult brain regions, it may require further specification of the neuroepithelial cells to a more restricted fate in order to achieve targeted differentiation. Alternatively, modification of the non-neurogenic environment will be necessary if uncommitted neuroepithelial cells are transplanted. In all these studies, no teratomas have been observed, suggesting the effectiveness of neural differentiation from the pluripotent human ES cells and the plastic response of the *in vitro* produced cells to environmental signals.

Transplantation of differentiated neurons such as dopamine neurons to adult or diseased non-neurogenic regions such as the striatum has yielded less promising outcomes. Several research groups including ourselves have found that human ES cell-differentiated dopamine neurons survive poorly following transplantation into the 6-hydroxydopamine (6-OHDA)-lesioned rat striatum (Ben Hur *et al.* 2004; Zeng *et al.* 2004; Park *et al.* 2005; Sanchez-Pernaute *et al.* 2005; Takagi *et al.* 2005). It is presently not clear why the high percentage of TH+ cells evident *in vitro* does not persist following transplantation. One possibility is that the *in vitro*

produced dopamine neurons are not *bona fide* dopamine neurons. In virtually every protocol outlined above for dopamine neuron differentiation, ascorbic acid and cAMP are included. These two chemicals can activate TH gene expression in many different cells (Lim *et al.* 2000; Yan *et al.* 2001). Hence, the expression of TH alone does not necessarily indicate the identity of a dopamine neuron. The second possibility is that the dopamine neurons may not be the correct type of dopamine neurons. As it has already been shown, a slight shift in the application sequence of morphogens can lead to the production of dopamine neurons with very different phenotypes (Yan *et al.* 2005). The midbrain dopamine neurons that synapse with striatal neurons are located in the substantia nigra. Dopamine neurons in other parts of the mid- and forebrain areas do not form direct connections with striatal neurons. These dopamine neurons also possess distinct molecular profiles (Chung *et al.* 2005). Thus, if the dopamine neurons do not possess the nigra dopamine neuron characteristics, they may fail to form stable synapses with striatal neurons, which may explain why the functional contribution is short-lived. Other cell types in the donor mixture may also contribute to the survival of dopamine neurons. As discussed above, serum-free dopaminergic differentiation cultures contain few or no astrocytes when dopamine neurons are first born. *In vivo*, astrocytes (and radial glia) are crucial in specifying and maintaining the phenotypes of midbrain dopamine neurons (Hall *et al.* 2003). Therefore, multiple strategies may well be needed to achieve a better survival and/or phenotypic maintenance of the transplanted dopamine neurons and subsequent functional contribution.

The transplant study performed by Takagi *et al.* (2005) appears more successful. This group has generated dopamine neurons from monkey ES cells using the PA6 stromal cell co-culture system. Initial yield of dopamine neurons was low by the simple co-culture, approximately 10% of neuronal cells. However, treatment with FGF2 and FGF20 increased the yield to 24%. Transplantation of the differentiated cultures into the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated Parkinsonian monkey brain has resulted in the survival of TH+ and dopamine transporter + dopamine neurons in the graft with concomitant partial recovery of the monkey behavioural deficit. It is not clear why this transplant experiment works. As mentioned above, stromal cells, such as PA6 and MS5, can promote mid/hindbrain neural progenitor specification. Dopamine neurons generated by co-culturing with PA6 cells, especially together with a treatment with FGF20, may generate some dopamine neurons with the nigra identity. The presence of astrocytes in this differentiation culture may also contribute to the better survival of dopamine neurons. In addition, trophic support from the graft may also be partly involved. This can be seen in the early functional recovery within 4 weeks of transplantation. This may be particularly useful in primates since TH+ dopamine neurons are present in the basal ganglia. Finally, species matching might play a role in the success of this transplant work,

Figure 2. Functional properties of human ES cell-derived midbrain DA neuron and spinal motor neurons. (a) Electrophysiological recording indicated that action potentials were evoked by depolarization current steps in TH+ dopamine neurons. Immunostaining showed that the recorded neuron (positive for biocytin, from the recording electrode) was TH+. (b) High performance liquid chromatography measurement indicated that dopamine was released from dopamine neuronal cultures spontaneously and the release was increased by depolarization (56 mM KCl in HBSS). (c) Electrophysiological analyses indicated that hES cell-derived motor neurons received both inhibitory and excitatory synaptic currents from neighbouring neurons (i). The outward inhibitory currents were blocked by bicuculline (20 μ M) and strychnine (5 μ M) (ii). Subsequent application of D(-)-2-amino-5-phosphonopentanoic acid (AP-5; 40 μ M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μ M) blocked the remaining the inward excitatory currents (iii). (d) Co-culture of human ES-derived motor neurons with C2C12 myoblasts resulted in aggregation of acetylcholine receptors on myotubes as shown by α -bungarotoxin (BTX) staining in areas where synapsin+ or ChAT+ fibres landed. Bar (a) = 50 μ m and (d) = 30 μ m. (a + b) and (c + d) are reproduced from Yan *et al.* (2005) and Li *et al.* (2005), respectively, with permission.

whereas other transplants involved xenotransplants of human ES-derived cells to lesioned rats or mice.

Human ES cell-produced neuroepithelial cells can also differentiate into glial cells, such as astrocytes and oligodendrocytes following transplantation into the white matter regions of the brain (Reubinoff *et al.* 2001; Zhang *et al.* 2001; Tabar *et al.* 2005). Keirstead and colleagues transplanted the oligodendrocyte progenitors, generated using their protocol outlined above, into the spinal cord of shiverer mice which exhibit dysmyelination due to mutations in the myelin basic protein (MBP) gene. The grafted human cells appear to differentiate into oligodendrocytes and produce MBP+ myelin sheaths in approximately 6 weeks (Nistor *et al.* 2005). A similar transplant study in the injured rat spinal cord results in increased myelination and locomotive function recovery compared with control animals that received fibroblasts or medium only. These results are promising as human ES cell-derived oligodendroglial progenitors may be of therapeutic value for myelin disorders and traumatic brain and spinal cord injuries. An interesting observation made in the spinal cord injury model is that this transplant therapy will be effective only when these cells

are grafted shortly after injury (7 days), indicating a crucial window for cell therapy (Keirstead *et al.* 2005). Functional recovery is seen within 4 weeks post-transplantation. As discussed below, it takes a much longer period of time for human oligodendroglial progenitors to mature and produce myelin sheaths (Windrem *et al.* 2004). These phenomena suggest that the functional improvement seen in the transplant experiment may be at least partly attributed to trophic support that prevents further tissue destruction following spinal cord injury.

5. HUMAN ES CELLS FOR BRAIN REPAIR: CHALLENGES AHEAD

(a) Efficient neural induction and/or enrichment remains a major focus in the near future

Despite significant progress in an efficient differentiation of neuroepithelial cells (70–90%) and a few neural subtypes, such as dopamine neurons (approx. 30%), spinal motor neurons (approx. 20%) and oligodendrocytes (more than 90%), protocols for generating many other neuronal subtypes need to be established. Even for the extensively invested studies on

dopamine neuron differentiation, most laboratories use undefined protocols such as the stromal cell co-culture system. This type of technique has merits in its simplicity and the differentiated progenies appear to resemble more closely those born *in vivo*. It is now important to identify the stromal signals that are responsible for the induction of dopamine neurons. This will be a crucial step moving towards the application of human ES cell progenies in clinics, like the series of efforts in removing undefined components in ES cell derivation and maintenance (Xu *et al.* 2005).

Formation of regionally specified neurons with unique transmitter profiles in the developing nervous system is a highly regulated process in spatial and temporal scales involving activation (or inactivation) of sets of transcription factors. Applying principles of developmental biology will be essential in devising and optimizing neural differentiation protocols aimed at mimicking these processes *in vivo* as well as characterizing the neural cells that are derived from these protocols. Activation of different sets of neurogenic transcription factors by RA and SHH at different developmental stages of neuroepithelial cells results in different classes of neurons (Li *et al.* 2005). This study also demonstrates that the production of a class of neurons like motor neurons requires sequential activation of a complete set of transcription factors. Incomplete activation of molecular pathways may result in the birth of a cell type with a deficit in phenotypes and/or function. The dopamine neurons produced using the reported procedures may fall into this category, which possibly explains why cells that express TH *in vitro* do not survive and integrate following transplantation into the brain. Even though they express enzymes necessary for dopamine metabolism and release dopamine in an activity-dependent manner *in vitro*, most *in vitro* generated dopamine neurons do not possess transcription factors, such as Nurr1 and Ptx3 in cell nuclei, which are crucial for their survival and function. Activation of both the Nurr1-TH and Ptx3-TH pathways is necessary for the generation of a correct type of midbrain dopamine neurons. Thus, a defined procedure for producing the correct type of midbrain dopamine neurons from human ES cells remains, at least partially, unrealized.

Differentiation studies thus far suggest that human ES cells retain the intrinsic programme to produce different classes of neurons and glia *in vitro*. We have found that neuroepithelial cells, in response to RA and SHH, produce Olig2-expressing neural progenitors after 4 weeks of human ES cell differentiation. These Olig2+ progenitors first produce post-mitotic motor neurons (Li *et al.* 2005). However, they do not produce oligodendrocytes until after a significant lag, at least 4–5 weeks after the generation of motor neurons (X. J. Li & S.-C. Zhang 2005, unpublished observation). This observation is in line with the time line observed in human development (Sidman & Rakic 1982; Grever *et al.* 1997; Grever *et al.* 1999; Windrem *et al.* 2004). Oligodendrocytes differentiated in this way exhibit the same morphology as those isolated from brain tissues (Zhang *et al.* 2000; Windrem *et al.* 2004). Based on this lengthy developmental time line and the transition between neurogenesis and gliogenesis that human

precursors would have to undergo, we initially predicted that generation of an enriched population of oligodendrocytes from human ES cells using an epigenetic approach would be challenging (Zhang 2003, 2005). Our pessimistic prediction is now shaken by the production of a near-pure population of oligodendrocytes (Nistor *et al.* 2005) and we are eager to see these results replicated in additional laboratories. It is fascinating that a high percentage of oligodendrocytes can be produced with a simple culture condition in such a short developmental window. In essentially all of the other work outlined above involving the derivation of specific neuronal subtypes, the differentiation time *in vitro* has roughly matched the projected developmental time of primate neuronal development. Presumably, during the formation of oligodendrocytes *in vitro* either the whole process is accelerated or some of the steps are skipped. It is certainly easier and faster if we can skip certain steps that occur in the 'long-term' human embryo development. However, cells that have not received the stepwise 'training' that normally takes place in an intact embryo may have difficulty adapting to the new environment in which they need to survive and function for cell therapy. Alternatively, these cells may be smart enough to learn all the tricks when they are placed in the brain.

Owing to the long-term induction procedure for human neuronal and glial subtypes, it would be ideal to be able to expand the neural progenitor populations. We have expanded the ES-derived human neuroepithelial cells in the presence of FGF2 for several months (up to 35 passages) and found that these neural precursors can still produce a high proportion of β III-tubulin-positive neurons. However, the major neuronal subtype is GABAergic (S.-C. Zhang 2005, unpublished observation). This phenomenon is in line with the need for a large number of neurons to be generated over a long period of development in order to reach the size of a human brain. The predominant GABAergic neuronal population also coincides with the developmental programme, although the use of FGF2 may also bias the system. Identification of culture conditions that can maintain and/or expand specified subtype neuronal progenitors will significantly facilitate practical applications.

Preliminary transplant experiments using differentiated and enriched neural progenitor cells suggest that they may in fact be rather safe with a low incidence of tumour formation (Reubinoff *et al.* 2001; Zhang *et al.* 2001; Tabar *et al.* 2005). Nevertheless, purification or enrichment of the target neural population is another crucial step towards the application of ES cell derivatives. This step will aid in quality control and safety. However, this requires the identification of surface molecules that are expressed by stage-specific subtype neural cells, which are not as rich as the blood system. An alternative approach is to label neural subtypes with genetic means (e.g. Zwaka & Thomson 2003; Singh *et al.* 2005), followed by cell sorting to isolated the target population. However, this approach may not be ideal for clinical applications. FACS isolation of dissociated neural cells may not always be desirable as dissociated neural cells survive poorly.

Additionally, appropriate maturation and function of a special class of neurons often requires neighbours, including interneurons and glial cells, that would be lost in the sorting process. Genetic labelling and sorting of specific neural populations do, however, hold promise as a means for discovering specific cell surface molecules.

(b) Demonstration of functional integration of human neural cells in the brain is awaited

Transplant studies have demonstrated the ability of human ES cell-derived neural progenitors, and in some cases differentiated neural cells, to survive, migrate and produce neurons and glial cells. It is now important to demonstrate that the grafted neurons and glial cells integrate in a functional manner. The ultimate demonstration will be behavioural improvement. Owing to potential protective or modulatory role of stem cell transplants, it is crucial to establish the link between behavioural outcome and cell transplant. Human nuclear proteins or other human-specific markers can be effective. However, marking living cells for functional analyses (e.g. electrophysiological recording) remains a technical challenge. Significant efforts have been made to label human ES cells or their derivatives with various vectors carrying fluorescent reporters (Ma *et al.* 2003; Vallier *et al.* 2004; Costa *et al.* 2005). However, these reporters, while successful at labelling ES cells and immature neural cells, are eventually downregulated in terminally differentiated neurons almost without exception. Development of a reliable approach to trace functional human neurons live *in vivo* will significantly move the field forward.

Given the intrinsic maturation process of human neural cells, transplanted human cells likely mature slowly in the brain. Transplantation of human foetal midbrain tissue, for example, results in functional improvement several weeks following transplantation, corresponding to the maturation and potential connection with endogenous neurons, of dopamine neurons in the graft (Stromberg *et al.* 1995; Annett *et al.* 1997). Oligodendrocyte progenitors enriched from developing brain tissues (20-week gestation human brain) will produce myelin sheaths in the dysmyelinating shiverer brain in two to three months, whereas similar cells isolated from adult human brain tissues produce myelin sheaths in only one month (Windrem *et al.* 2004). Hence, the early functional recovery seen in animals with human ES cell-derived dopamine neurons and oligodendrocytes (Keirstead *et al.* 2005; Takagi *et al.* 2005) could be partially attributed to a protective role of the graft. Long-lasting functional improvement requires incorporation of the grafted neurons into the endogenous neural circuitry. This similarly requires long-term observation in grafted animals. Development of animal models under the immune-deficient background will ease the huge technical challenges facing long-term immunosuppression in the xenotransplant paradigm.

(c) Genetic engineering to regulate transmitter release may broaden the horizon of human ES cells for brain repair

Major neural circuits are built during early development and are functionally modified over a long period.

It is thus hard to imagine that a single part of the adult neural circuitry can be readily replaced by cell transplants as simply as a new part can be used to repair an automobile. Under most circumstances, neural transplant acts as a localized chemical replacement, a typical example being neural transplantation in Parkinson's patients. Since grafted cells are unlikely to be integrated into the existing neural circuitry in a short period, the release of neurotransmitters is uncontrolled. This may be one of the reasons that some patients who receive foetal tissue transplants suffer from dyskinesia (Freed *et al.* 2001; Olanow *et al.* 2003). Ability to regulate transmitter release following transplantation into the brain would overcome this problem. This will be particularly useful at early stages, before the grafted neurons integrate into the circuitry, and in ectopic transplantation such as in the case of Parkinson's where some of the feedback regulation may be permanently lost. ES cells are amenable for genetic manipulation and a precursor cell with a regulatory transmitter system could shape the future of stem cell therapy for neurological disorders.

(d) Immunological rejection may be overcome by somatic nuclear transfer

Despite a general concern over immune rejection, cells transplanted into the brain and spinal cord are less susceptible to immune rejection than peripheral tissue transplant. Parkinson's patients who received foetal brain tissue transplant retain the graft for years in the absence of immune suppression (Piccini *et al.* 1999). Limited reports suggest that human ES-derived neural progenitors express a low level of major histocompatibility complex (Drukker *et al.* 2002). However, it is likely that the expression level will change along further differentiation *in vitro* and *in vivo*.

There are ways to minimize or overcome immunological barriers in stem cell transplant therapy, such as immunosuppression and induction of tolerance (Kaufman & Thomson 2002; Zhang 2003). The most direct way is to produce ES cells that contain a patient's own DNA through nuclear transfer or reprogramming of the patient's own somatic cells. This technique needs optimization. Another potential approach to avoiding issues of immune rejection would be the creation of a human ES cell bank that would encompass a wide enough range of cells with different immune backgrounds. If and when a cell transplant therapy became available, an individual patient could then be immunomatched to a cell line to avoid rejection of differentiated cells.

(e) Multiple measures should be taken to ensure safety

It is a general perception that ES cell derivatives are less safe than 'adult' tissue stem cells and that differentiated target cells must be purified in order to achieve safety. Human ES cells are exceedingly stable even with current technology. They maintain chromosomal stability for at least 200 doubling times (Draper *et al.* 2004), whereas most somatic stem/progenitor cells will likely alter their genetic background due to excessive amplification beyond the normal cell cycle numbers accomplished with a large amount of growth factors.

That being said, safety issues need to be taken seriously given the inherent ability of ES cells to produce all cell types in our body. This can be achieved at multiple stages. As discussed above, significant progress has been made in directing ES cells to several neural lineages. Many of these procedures do, however, need refinement by using low-density cultures in chemically defined systems without the presence of stromal cells. This will essentially remove the chances for ES cell survival and also facilitate a synchronized neural differentiation (Zhang *et al.* 2001; Li *et al.* 2005; Yan *et al.* 2005). With the development of strategies for directed differentiation and/or enrichment of target cells, the risk of teratoma formation by grafted human ES cell derivatives may well be less of a concern. The site-specific homing and differentiation of human ES cell-derived neuroepithelial cells, both in the developing and in the adult brain environments (Zhang *et al.* 2001; Tabar *et al.* 2005; Guillaume *et al.* 2006), also make them less likely to retain the undifferentiated state. Finally, enrichment of lineage-committed neural progenitors or immature neurons (Singh *et al.* 2005) will not only eliminate any contaminating ES cells and non-neural lineages but also avoid other neuronal lineages that may cause unwanted effects.

(f) Beyond ES cells: pathfinding and reinnervation by human ES cell-derived neurons are roadblocks to successful cell therapy

Restoration of brain function requires the grafted neurons to be able to find their targets and reinnervate them in a functional manner. The transplanted neurons also need to be innervated in a correct circuitry. For example, if the ES cell-derived motor neurons can survive transplantation into the spinal cord, the axons need to overcome the barrier in the CNS–PNS boundary. Successful axons will then be guided to the denervated muscles over a long journey. Whether denervated targets will still be able to receive innervation from the newly grafted, *in vitro* produced neurons remains an open question. To complicate matters, all these steps are now taking place in the adult non-neurogenic, and most likely hostile pathological, environment. Therefore, expectation for quick stem cell replacement therapy is unrealistic, at least for most neurological disorders.

6. PERSPECTIVES

While this overview focuses on the potential of human ES cells for brain repair, efforts from a number of laboratories including ourselves are to delineate the molecular pathways that lead to the birth of individual neural subtypes from human ES cells. These efforts are by no means separated from the ultimate objective of brain repair. These developmental studies will be essential, at least at present, for developing simple and scalable production of therapeutically relevant target neural cell types. Understanding the mechanism of neural specification using the human ES cell model may also enable us to instigate regeneration from endogenous stem cells.

Human ES cell-generated neural cells provide an unprecedented tool for screening pharmaceuticals that

may have therapeutic values in neurological disorders. As outlined above, some neural subtypes have already been efficiently generated from self-renewing human ES cells, which provide a simple and standardized population of human cells for drug discovery and toxicity screening. The availability of human ES cells with natural diseases (Verlinsky *et al.* 2005), via somatic nuclear transfer, or through genetic alterations in laboratories, will provide not only disease-specific neural cells for drug screening but also a tool to unveil some fundamental pathological processes underlying individual neurological disorders.

Application of human ES cell-derived neurons for functional replacement in patients may take some time. It not only requires continued effort in producing clean and functional subtypes of neuronal cells, but also more importantly depends on our ability to guide the stem cell-derived neurons and promote them to make functional connections with the therapeutic targets in the diseased environment. That being said, some aspects of stem cell behaviours, such as provision of a substrate for neuronal function and supplement of trophic factors to limit destruction may well be employed in the more near future to treat some devastating neurological injuries and diseases such as spinal cord injury and ALS.

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