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Human embryonic stem cells: challenges and opportunities

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Abstract. Human and non-human primate embryonic stem (ES) cells are invaluable resources for developmental studies, pharmaceutical research and a better understanding of human disease and replacement therapies. In 1998, subsequent to the establishment of the first monkey ES cell line in 1995, the first human ES cell line was developed. Later, three of the National Institute of Health (NIH) lines (BG01, BG02 and BG03) were derived from embryos that would have been discarded because of their poor quality. A major challenge to research in this area is maintaining the unique characteristics and a normal karyotype in the NIH-registered human ES cell lines. A normal karyotype can be maintained under certain culture conditions. In addition, a major goal in stem cell research is to direct ES cells towards a limited cell fate, with research progressing towards the derivation of a variety of cell types. We and others have built on findings in vertebrate (frog, chicken and mouse) neural development and from mouse ES cell research to derive neural stem cells from human ES cells. We have directed these derived human neural stem cells to differentiate into motoneurons using a combination of developmental cues (growth factors) that are spatially and temporally defined. These and other human ES cell derivatives will be used to screen new compounds and develop innovative cell therapies for degenerative diseases.

Extra keywords: culture, karyotype, neural differentiation.

Human and non-human primate embryonic stem cells

In this review, we will describe some of the unique qualities of non-human primate and human embryonic stem cells, the challenges in maintaining these unique phenotypes and the opportunities associated with differentiation towards neural stem cells and specific phenotypes such as motoneurons. The advances are based on previous discoveries in developmental biology.

Stem cell derivation

The isolation of the first human embryonic stem (hES) cells (Thomson *et al*. 1998), as well as primate embryonic stem cells from rhesus monkey (Thomson *et al*. 1995) and marmoset (Thomson *et al*. 1996), was remarkably similar to the mouse embryonic stem (mES) cell protocol/media used 17 years previously. Within the same year that hES cells were isolated, another research group derived pluripotent human embryonic germ (hEG) cell lines from fetal gonads 5–9 weeks post fertilisation (Shamblott *et al*. 1998).

Regardless of species, ES cell isolation requires the selection away from, or removal of, the trophectoderm (external cell layer surrounding the inner cell mass (ICM) that gives rise to a portion of the placenta). The ICM is often plated on a mouse embryonic fibroblast feeder cell layer (mEFs) and allowed to grow and expand. This culturing system is maintained by means of continuous passaging and subculture. The doubling time for a hES cell line is between 35 and 40 h (Rosler *et al*. 2004).

The human embryos used to start ES cell derivation were donated by *in vitro* fertilisation (IVF) clinics to the research community, and were either in excess of what was needed for implantation or were not of sufficient quality. However, one can further delineate these excess embryos into two more categories (Fig. 1). The first subgroup is the excess embryos that had formed adequate cell numbers in both the trophoblast and ICM. These higher quality embryos are usually frozen to be thawed later for transfer into the reproductive tract, donated to infertile couples or donated for research. The second subgroup contains excess embryos determined not to be viable enough to either initiate a pregnancy or to freeze for later transfer. This second subgroup of non-viable embryos is usually discarded. We were able to use these embryos to derive three of the most highly investigated embryonic stem cells in the NIH registry (BG01, BG02 and BG03; Mitalipova *et al*. 2003). No publications to date have shown that these hES cell lines differ in their usefulness in regenerative therapies when compared with other hES

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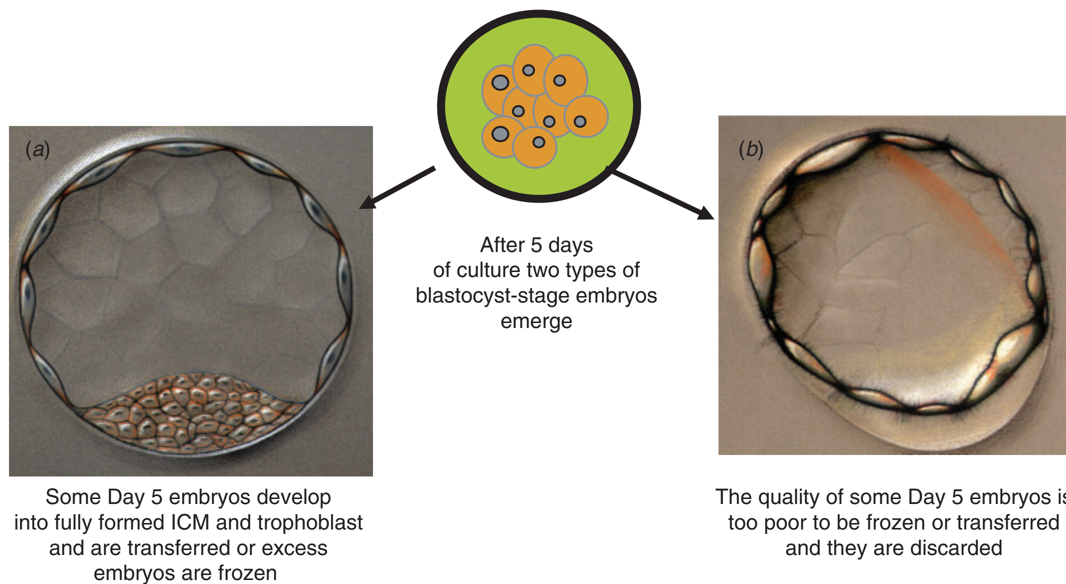

Fig. 1. (*a*) Type of human embryos used to derive embryonic stem cells. (*b*) Poor-quality embryos that are discarded are available and were used to make three of the 21 human embryonic stem cell lines on the National Institute of Health registry. ICM, inner cell mass.

cell lines. For the list of NIH (National Institute of Health)-approved ES cell lines, see the NIH stem cell registry website (http://stemcells.nih.gov/research/registry/eligibilityCriteria.asp, verified September 2006).

Human embryonic stem cell chromosomal stability and gene expression

Variations in chromosomal number could lead to alterations in the genetic expression patterns of certain hES cell lines. Karyotyping most often involves a procedure called G-banding to count chromosomal number and/or chromosomal rearrangements within a cell. In terms of the established hES cell lines, it has been reported that lines can maintain a normal karyotype in continuous culture under certain conditions. For example, hES1–6 cell lines have a normal karyotype (46 chromosomes, XY) during 24–140 passages of continuous culture (Buzzard *et al*. 2004), and H1, H7 and H9 cell lines during passage number 60–100 (Rosler *et al*. 2004). However, reports of abnormal karyotypes similar to those found in embryonic carcinoma (EC) cell lines of trisomy 17 and 21 have recently surfaced in the BG01 and BG02 NIH-registered hES cell lines after bulk hES cell passaging compromised the genetic integrity of cells as quickly as 23–25 passages after manual passaging techniques had ceased (Mitalipova *et al*. 2005).

Aneuploidy has been associated with abnormal gene expression. Theoretically, abnormal gene expression may be a risk factor when used in cell transplant studies. Therefore, abnormal karyotype may up-regulate genes associated with teratoma formation. Our laboratory found that manually passaged cells could maintain a normal karyotype, but when using enzymatic or even non-enzymatic techniques over 20 passages, trisomies at 12, 17, 14, 20 and X in BG01 and BG02 lines eventually developed. Short-term bulk passaging, typical of methods used in other stem cell types including mES cells, was successful in maintaining a normal karyotype from 13 passages and 15 passages for the non-enzymatic method using cell dissociation buffer and collagenase/trypsin, respectively (Mitalipova *et al*. 2005). By quantitative real-time (RT) polymerase chain reaction (PCR), the majority of over 80 genes associated with pluripotency and early differentiation were significantly up-regulated in aneuploid cells (Mitalipova *et al*. 2005). Brimble and colleagues (2004) stated that abnormal karyotypes, especially trisomy 17 and 21, can be acquired through various single-cell disaggregating passage techniques by positively selecting for aneuploidy. They confirmed that manually passaged BG01 and BG02 hES cell lines maintained a long-term normal karyotype (tested only up to 52 passages for a period of 6 months) (Brimble *et al*. 2004). Together with our study, these reports suggest that bulk hES cell passaging will compromise the genetic integrity of cells as quickly as 23–25 passages after manual passaging techniques have ceased.

With all the above issues surrounding each hES stem cell line, a standard protocol for their derivation, culture, maintenance and characterisation is needed to ensure the validity of all lines and their uses. An international initiative named The International Stem Cell Forum (http://www.stemcellforum.org.uk, verified September 2006) was founded in January 2003 comprising 15 countries with the intention of establishing a set of standards for the characterisation and culturing of all the reported hES cell lines.

Table 1. Phenotype marker expression for among-model embryonic stem (ES)-cell lines
Modified table from Pera *et al.* 2000. EC, embryonic carinoma; EG, embryonic germ; ES, embryonic stem; hTERT, telomerase reverse transcriptase; N/A, antibodies do not react with mouse cells. It is unknown whether this is due to lack of expression or species specificity of the antibody; N/D, data not found; SSEA, stage-specific embryonic antigen; TRA-1-60, TRA-1-81 and GCTM-2, extracellular antigenic epitopes

Marker	Human ES cells	Human EG cells	Human EC cells	Primate ES cells	Mouse ES, EG and EC cells
SSEA-1	−	+	−	−	+
SSEA-3	+	+	+	+	−
SSEA-4	+	+	+	+	−
TRA-1-60	+	+	+	+	N/A
TRA-1-81	+	+	+	+	N/A
GCTM-2	+	N/D	+	+	N/A
Alkaline phosphatase	+	+	+	+	+
Nanog	+	+	N/D	N/D	N/D; + (ES)
Oct-4	+	+	+	+	+
hTERT	+	N/D	N/D	N/D	N/D; + (ES)

Laboratories in 11 of the 15 participating countries contributed hES cells (totalling 75 different lines) for the purpose of flow cytometric analysis of 17 surface antigens, quantitative RT PCR and microarray analysis of 100 selected gene transcripts, and for epigenetic studies (Andrews *et al*. 2005). To date (January 2006), there is not a specific gene or surface marker that can unequivocally define ES cell status, but rather only a series of trends in gene expression levels as an ES cell line begins to differentiate.

Morphology and marker expression

Morphological distinctions between mES, hES and non-human primate ES cells exist in terms of phenotype and marker expression. Owing to their evolutionary closeness, hES and non-human primate ES cells share similar phenotypic properties of flat monolayer colony growth with distinct cell borders in culture, whereas mES cells have a more rounded, clumped appearance with indistinguishable cell borders. Beyond phenotypical differences between species ES cells, gene-marker expression also differs among the model lines as illustrated in Table 1. Several studies report that cell-surface markers specific to the ICM lineage of the blastocyst have been maintained during prolonged hES cultures. It should be noted that the markers shown in Table 1 are not completely stage specific and have been shown to be expressed in other tissue types.

Expression marker description

Cell origins are often defined by one or more cell surface and or intracellular epitopes unique to that particular cell type. Stage-specific embryonic antigen (SSEA) markers are used to distinguish early stages of cell development, denoting pluripotency. These markers are globo-series glycolipids and are recognised by monoclonal antibodies. The SSEA-4 epitope is the globo-series glycolipid GL7. It is produced by the addition of sialic acid to the globo-series glycolipid Gb5, which carries the SSEA-3 epitope (Kannagi *et al*. 1983*a*, 1983*b*). It has been demonstrated that GL7 can react with antibodies to both SSEA-3 and SSEA-4 (Kannagi *et al*. 1983*a*, 1983*b*). Human and non-human primate ES cells will express SSEA-3 and -4 during pluripotency and only SSEA-1 upon differentiation (Andrews *et al*. 1996; Thomson and Marshall 1998; Thomson *et al*. 1998; Reubinoff *et al*. 2000). Interestingly, mES cells have reversed SSEA marker expression patterns compared with primates (Kannagi *et al*. 1983*a*).

Other cell-surface antigens are coupled to the pericellular matrix, more specifically a series of related keratin sulfate proteoglycans within the matrix. The TRA-1-60 epitope adheres to a particular epitope of the proteoglycan and is sialidase sensitive, whereas antibody TRA-1-81 reacts with another unknown epitope of the same core proteoglycan molecule. Both antibodies recognise proteoglycans found on all human and non-human primate stem cell types. The reason is unclear why these antibodies do not label mES or EC cells, but it is postulated that either mouse cells lack that particular antigen or the antibodies are species specific. The antibody GCTM2 has been reported to recognise an epitope associated with a cell-surface keratin sulfate, but its relative closeness to TRA-1-60 and TRA-1-81 has not been ascertained (Cooper *et al*. 1992).

All ES, EC and EG cells express alkaline phosphatase activity. In humans, there are four isoforms of the enzyme and it is not clear which isoform hES cells express. Human EC cells express the non-specific tissue form, along with a form (antibody detectable) that will cross-react with the germ cell or placental form (Pera *et al*. 2000).

Nanog is a NK-2-type homeodomain gene thought to encode a transcription factor that is critically involved in the self-renewal of stem cells. Thus, it may possibly act to repress genes necessary for differentiation and activate

those involved in self-renewal. Lin and colleagues (2005) demonstrated that the tumour suppressor p53 binds to the promoter of Nanog, suppressing its expression after DNA damage induced by UV irradiation or chemical treatment in mES cells. Therefore, p53 can stimulate differentiation of embryonic stem cells into alternate cell types that undergo p53-dependent cell-cycle arrest and apoptosis when genetic integrity is not preserved.

Oct-4, a POU-domain transcription factor, is highly expressed in ES cells (Thomson *et al.* 1998; Reubinoff *et al.* 2000; Niwa 2001) and has been shown to be essential for maintaining pluripotency in mES cells (Niwa 2001). It has been reported that Oct-4 transcripts are nearly exclusively found in pluripotent cells *in vivo* and within culture. Oct-4 down-regulation is observed in differentiating cells (Rosner *et al.* 1990). Not only is Oct-4 necessary for the maintenance of pluripotency, but its expression level governs three cell fates once differentiation occurs (Niwa *et al.* 2000; Hay *et al.* 2004). Niwa *et al.* (2000) showed a 2-fold increase promoted mES cell differentiation into embryonic and extraembryonic cell types typically produced upon withdrawal of the cytokine leukemia inhibitory factor (LIF), whereas a reduction in the level of Oct-4 to less than 50% triggered dedifferentiation of these cells into the trophoblast. These authors hypothesise possible roles for Oct-4 as a master regulator for initiation, maintenance and differentiation of pluripotent cells along with preventing respecification and dedifferentiation into the extraembryonic ectoderm (Niwa *et al.* 2000; Niwa 2001). Several candidate genes have been reported as targets of Oct-4 based on stem cell expression patterns and immuno-precipitation, but few have been conclusively verified. In the ongoing search for the identification of pluripotent markers, Xu and colleagues have reported that the catalytic component of telomerase, telomerase reverse transcriptase or hTERT, is expressed in undifferentiated cells and down-regulated upon differentiation (Xu *et al.* 2001).

Neural stem cells derived from human embryonic stem cells

A greater understanding of mammalian cellular differentiation and cell-fate specification has, and continues to be, of intense interest to developmental biologists. With the advent of hES cells, human developmental biology has progressed in recent years. Research on cell fate-specification in the central nervous system (CNS) is of enormous interest given the therapeutic potential in neuronal repair strategies. The mammalian central nervous system is developed from the neural tube. The early neural tube is composed of a single layer of pseudo-stratified columnar epithelium of neural epithelial progenitor cells. These neural progenitor cells are induced to form specified neural phenotypes such as motoneurons (Sanai *et al.* 2005). Below we will discuss the stepwise differentiation of hES cells to neural progenitors and further differentiation to motoneurons.

Table 2. Phenotype profile of mouse and human neural progenitor (NP) stem cells

Mouse data from Liu *et al.* 2002, human data from Shin *et al.* 2006

Antigen	Mouse NP cells	Human NP cells
Nestin	+	+
Sox2	+	+
A2B5/4D4	–	–
GFAP/CD44	–	–
RC1/S100/Vimentin	–	–
Sox10/NG2/PDGFRα	–	?
O4/GALC	–	–
Oct-4	–	–

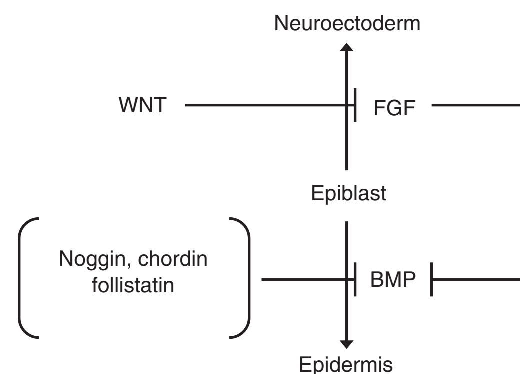

Fig. 2. Signalling pathways in neural induction.

Neural epithelial progenitors, or neural progenitor (NP) cells, are self-renewing cells that can differentiate into neurons, oligodendrocytes and astrocytes. According to Liu, who examined immunohistochemistry patterns in mouse tissue sections, the phenotype of NP cells can be characterised by several specific markers (Table 2) (Liu *et al.* 2002). Likewise, it has been shown that human NP cells have a phenotype characterisation similar to that found in mouse (Mayer-Proschel 2002). These cells expressed Nestin and Sox2, but did not express any other late stage neuronal or glial lineage markers. Neural progenitor cells give rise to all the cells that comprise the mammalian central nervous system, including various types of neurons and glial cells. However, it is not clear whether they multiply by symmetric or asymmetric cell division.

During early human development, NP cells form the neural tube during the third and fourth weeks of gestation. To acquire this NP cell induction from the epiblast, several molecules are required. Major signalling pathways involved in this induction are bone morphogenic protein (BMP), fibroblast growth factor (FGF) and Wnts (Fig. 2). Bone morphogenic protein signals block neural fate and promote epidermal fate and are excluded from prospective neural cells (see Wilson and Edlund 2001). Noggin, chordin and follistatin are known BMP inhibitors. Fibroblast growth factor has the dual role of repression of BMP expression and

promotion of a neural pathway that is independent of the BMP pathway repression, whereas Wnts block some of FGF's effect on BMP repression. Serum contains numerous undefined proteins including growth factors and molecules that could stimulate or prevent differentiation towards a particular lineage. Tropepe *et al*. (2001) proposed neural induction as a default choice of cell fate. When mES cells were dissociated and cultured in serum- and feeder-deprived conditions, colony-forming primitive neural stem cell populations could be obtained. Thus, defined culture will be beneficial not only for directed differentiation but also for the elimination of mesodermal and endodermal differentiation.

Strategies used to isolate human neural progenitor cells

Different strategies have been used to induce neural differentiation in hES cells. The most routine method of generating differentiated cell types has been through the three-dimensional structure of the embryoid body (EB). Embryonic stem cells in this agglomerate start spontaneous differentiation to form a sphere in suspension culture. Differentiated EBs contain neural stem cells, the proportions of which increase with retinoic acid exposure. Reubinoff and colleagues cultured hES cells until spontaneous differentiation occurred, then isolated a subpopulation to make neurospheres (Reubinoff *et al*. 2001). Pera *et al*. (2004) introduced a BMP inhibitor of noggin into this prolonged culture system. However, EB culture has disadvantages compared with adherent culture in that phenotype observation within the sphere is not possible with standard microscopy. In addition, stochastic differentiation yielded multiple cell lineages and limited the overall yield of the desired cells.

Ying and colleagues developed a monolayer differentiation method to obtain efficient neural induction of mES cells. When differentiation was triggered by the withdrawal of LIF, the mES cell monolayer chose a neural fate in serum-deprived medium (Ying *et al*. 2003). This efficient neural determination did not occur in medium containing serum. Though it was uncertain whether hES cells would behave similarly to their mouse counterparts, adherent differentiation in defined culture is an attractive strategy. We developed an adherent hES cell differentiation in defined culture conditions (Shin *et al*. 2006). The differentiation process was followed and examined immunohistochemically, suggesting a gradual but uniform differentiation to NP cells when serum was removed and FGF2 and LIF were added (Table 2). Derived NP cells were characterised both genetically and by their differentiation capacity. Subsequently, NPs were propagated using defined medium for 6 months while maintaining their differentiation potential.

Neural patterning in development of motoneurons

Several mature neuronal cell phenotypes have been derived from hES cells when exposed to growth factors first determined in neural patterning studies. As an example, we will describe motoneuron patterning and how it relates to deriving this phenotype *in vitro*. Cell fate determination occurs during and following neural-tube closure. Among several molecules, sonic hedgehog (Shh), retinoic acid (RA) and FGFs have been well defined molecules involved in dorsal–ventral and anterior–posterior axis formation. Here we review the role and interaction of these three factors in motoneuron development. Based on this prior understanding, we were able to derive a motoneuron phenotype from hES cells in 2005 (Shin *et al*. 2005).

Roles of sonic hedgehog

Dorsoventral axis formation involves the action of two opposing signalling pathways. Sonic hedgehog (Shh) originates ventrally from the notochord and later from the floor plate, whereas BMP diffuses dorsally from the boundary of neural and non-neural ectoderm and later from the roof plate. The notochord is the source of signals involved in the specification of the floor plate and secondarily to the formation of motoneurons and ventral interneurons with Shh being the major regulator of this signal. Loss- and gain-of-function studies of Shh have suggested that it is both necessary and sufficient to induce the floor plate (for review see Wilson and Maden 2005). Briefly, Shh-neutralising antibodies inhibited induction of ventral types in the developing notochord. Also, Shh-knockout mice lack floor plate and motoneuron development, although four other classes of ventral neurons still develop. In contrast, when the explant was exposed to Shh, an ectopic floor plate and motoneurons developed. Sonic hedgehog has a membrane-bound and non-membrane-bound form. It is presumed that the bound-protein form is involved in floor-plate induction and the soluble secreted form is in charge of motoneuron specification. Soluble secreted Shh has been shown to also act as a morphogen, eliciting different cell fates at varying concentration thresholds. Graded Shh activity directs neural identity through a set of homeodomain proteins that exhibit mutual cross repressive interactions.

Within the ventral spinal cord, five progenitor domains can be identified by unique combinations of transcription factors. The patterns of gene regulation in the progenitor domains are established by the high-ventral to low-dorsal gradient of Shh. Sonic hedgehog can either induce or repress the expression of the transcription factors within progenitor cells. These graded responses (either positive or negative) to Shh lead to the patterned expression of unique combinations of factors in each progenitor cell domain. A second level of transcriptional regulation is also in place involving homeodomain (HD) transcription factors, termed class I (Pax7, Pax6, Dbx1/2, and Irx3) and class II (the Nkx6 and Nkx2) proteins. Sonic hedgehog signalling regulates the expression of these HD class I and class II factors that have an opposing cross-repressive interaction (for review see Wilson and Maden 2005).

Roles of retinoic acid

Retinoic acid (RA) is the biologically active derivative of vitamin A and it induces a variety of embryonic carcinoma and neuroblastoma cell lines to differentiate into neurons. Retinoic acid acts through at least two sets of receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which can interact with multiple putative coactivators and corepressors to yield a complex molecular pathway with a variety of pleiotropic effects. Retinoids are thought to function as morphogens during anterior–posterior patterning *in vivo*. In fact, endogenous retinoid expression has been documented at very low levels in the forebrain and midbrain. However, the hindbrain contains localised gradients of RA, with high expression observed in the spinal cord (Horton and Maden 1995). Ectopic expression of RA within whole *Xenopus* embryos led to increased expression in the hindbrain and spinal cord with a corresponding decrease in the forebrain. Retinoic acid affects the development of the head structure in a concentration-dependent manner while possessing the ability to alter tail structures as well. Within the hindbrain, gradually increased RA concentrations generate a stepwise gene activation effect in sequentially more posterior segments. In addition, RA is implicated in establishing regional identity within the spinal cord itself. It appears to act at sequential developmental stages to impose different rostrocaudal positional values.

Roles of fibroblast growth factor

Although FGF has been shown to play a major role as a repressor of differentiation, it has recently been shown to contribute to neural patterning. Fibroblast growth factor (including -2, -4, and -8) inhibits differentiation of adjacent neural tissue that opposes the effect of RA. Fibroblast growth factors 2, 4 and 8 are secreted by presomitic mesoderm and caudal cells, inhibiting neural tissue from differentiation and maintaining the caudal region as a stem zone (for review see Wilson and Maden 2005). They also affect patterning of the ventral spinal cord by differential inhibitory action on basic helix-loop-helix (bHLH) (e.g. Olig2) and homeobox transcription factors. Novitch and colleagues demonstrated that forced expression of FGF within *in vivo* neural cells resulted in marked repression of class I proteins and limited repression of class II proteins (Novitch *et al.* 2003). They also observed a joint action between FGF and RA causing an induction of Olig2 expression in the absence of Shh. This suggests that there is an Shh-independent pathway in ventral neural pattern formation. The independent role of FGF was also observed in oligodendrocyte progenitor induction from neocortical precursors in culture (Kessaris *et al.* 2004). This FGF activity was not affected by cyclopamine, a Shh pathway inhibitor. In contrast, PD173074, an inhibitor of FGF receptor (FGFR), blocked Shh activity. It has been demonstrated that constitutive activity of FGFR maintains a basal level of phosphorylated mitogen activated protein kinase (MAPK), and that Shh depends on MAPK for Olig2 induction.

Human neural progenitor to motoneuron differentiation using FGF2, RA and Shh

Recently, we demonstrated that human NP cells could form a motoneuron phenotype (Shin *et al.* 2005). The effect of inductive signals from FGF2, RA and Shh were examined. Gene expression changes in motoneuron progenitors (Olig2 expressing) of the motoneuron specific gene (*HB9*) were monitored using quantitative RT PCR. We found that all three factors had an additive effect, but individually, FGF2 was the most potent inducer of motoneuron-associated gene and marker expression. In the end, when these inducing factors were used on our progenitors, 10–20% of the cells in these cultures contained a motoneuron phenotype.

Future research to further enrich motoneuron phenotypes will require additional spatial and temporal cues. Motoneurons are derived from the same lineage as oligodendrocytes and it is possible that motoneuron progenitors could follow the same pathway. Also, motoneurons may require neurotrophic support, so although more progenitors are produced, the mature motoneurons might not survive. In the developing vertebrate, *in vivo* motoneuron survival depends on neurotrophic support. The predominant signalling molecules required include glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and cardiotrophin-1 (CT-1) (Li *et al.* 2005). In this same report, optimal motoneuron development was obtained when early-stage NP cells from hES cells were induced using Shh and RA.

Conclusions

Previous studies have indicated that neuronal differentiation can be achieved using mouse and human ES cells. However, research towards uniform directed differentiation and isolation of desired cell types remains an ongoing effort in many laboratories. It has been shown that defined medium prevents mesodermal differentiation of mES cells and adherent differentiation was introduced for efficient homogeneous differentiation. In addition, inductive signals during *in vivo* development have been shown to induce motoneuron differentiation in mouse and non-human primate ES cells.

In this review, we have described progress towards culturing stable euploid hES cells that can be maintained and propagated. These hES cells could theoretically then be used in an adherent differentiation with defined medium process to establish homogeneous populations of NP cells. Finally, morphogens can be used to direct differentiation towards a specific phenotype. We have described the roles of FGF2, RA and Shh in the differentiation of a hES cell into a motoneuron.

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