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Genetic Modification of Human Embryonic Stem Cells

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Introduction

Embryonic stem (ES) cells, originated from the inner cell mass (ICM) of a blastocyst embryo, are self-renewable (Evans and Kaufman, 1981; Martin, 1981). The pluoripotent nature of ES cells endows them as a great tool for dissecting cell lineage development in mammals and they can be used as a limitless source for producing specialized cells for potential cell therapy. Application of gene targeting technology in mouse ES cells has allowed the cloning of mice with modified genomes (Frohman and Martin, 1989; Koller *et al*., 1989), thus significantly widening the use of mice as a vertebrate experimental model in biological research today. Forced overexpression or knockout of critical genes by genetic modification has become a routine technique in mouse ES cells to create transgenic mice for analyzing gene function and genetic pathways in the context of intact animals and in cell lines derived from such animals. Gene targeting in mouse ES cells also allows the creation of mouse models of human diseases (Bedell *et al*., 1997a; Bedell *et al*., 1997b), which offers insights into the genetic, biochemical and pathological basis of the diseases and may help developing treatments.

Like their murine counterparts, human ES cells (Reubinoff *et al*., 2000; Thomson *et al*., 1998) offer invaluable and perhaps the only system for directly studying early human development as manipulation of human embryos is prohibited and information gained from model organisms (Anderson and Ingham, 2003) does not always reflect

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Abbreviations: Ad: adenovirus, bFGF: basic fibroblast growth factor, BMP: bone morphogenetic protein, CNS: central nervous system, ES: embryonic stem, FACS: fluorescence activated cell sorting, HIV: human immunodeficiency virus, ICM: inner cell mass, LIF: leukemia inhibitory factor, MEF: mouse embryonic fibroblast, RNAi: RNA interference, SCNT: somatic cell nucleus transfer, UTR: untranslated region.

what is occurring in humans. The ability of human ES cells to produce almost any cell types in our body brings hopes to many incurable diseases affecting the central nervous system (CNS) (Dunnett *et al*., 2001; Silani *et al*., 2004), pancreas (Bonner-Weir and Weir, 2005), and heart (Srivastava and Ivey, 2006), etc. The potential use of human ES cells can be further enhanced by genetic modification (Drukker, 2005; Kobayashi *et al*., 2005). Application of currently available genetic modification techniques in human ES cells will provide researchers with even more versatile tools to study early human development and to expand the potential use in medicine. Basic mechanisms underlying early human development can be dissected through interfering with specific signaling pathways by forced gene overexperssion or silencing. Genetic defects may be corrected in human ES cells by site directed gene modifications, which may lead to the development of treatment for many inheritable diseases (Chang *et al*., 2006; Rideout *et al*., 2002). By labeling cells with tissue specific markers, genetic modification may help us obtain pure functional cells of desired types after differentiation, which can be used as more effective transplant medicine.

Unlike their murine counterparts, genetic modification in human ES cells has been technically challenging. Standard chemical or mechanical methods for gene delivery exhibit very low efficiency in human ES cells, with the most effective approaches yielding only approximately one stable transfectant per 10^5 transfected cells (Eiges *et al*., 2001; Zwaka and Thomson, 2003). After transfection, transgene expression appears to be suppressed more severely in human ES cells than in mouse ES cells (Xia, 2006). Thus successful examples are still rare. In this review, we will summarize the current methodologies for genetic modification of human ES cells from a very limited literature and propose future directions to overcome the present problems in genetic manipulation of human ES cells.

Techniques for genetic modification of human ES cells

TRANSFECTION

(i) Electroporation

Electroporation is the most commonly used mechanical method for introducing DNA into a wide range of cell types. This technique allows the passage of foreign DNA through pores formed transiently in the cell membrane caused by electric pulses. While the method has been proven very efficient for mouse ES cells (Thomas and Capecchi, 1987), early trials have shown that human ES cells did not survive traditional electroporation conditions well, with a cell survival rate of only about 1% (Eiges *et al*., 2001; Zwaka and Thomson, 2003). This was improved to some extent by using modified electroporation parameters combined with isotonic protein-rich electroporation solution (Zwaka and Thomson, 2003). However, the survival rate remained low. We have observed a survival rate of about 10% using the same improved conditions (unpublished observations). A systematic study of the electroporation parameters for human ES cell transfection indicated that by optimizing the voltage, pulse duration, and the number of pulses, high cell viability (>60%) and relatively high percentage of transfected cells (14%) can be achieved (Mohr *et al*., 2006). While

it is not proven whether transfected cells retain their ES cell state, the study indicated that the low transfection efficiency and survival may not be inherent to human ES cells. Technical improvement may ultimately lead to efficient transgene delivery to human ES cells.

Despite the high cell death rate and relatively low transfection efficiency, electroporation is so far the only method that has been reported to achieve homologous recombination in human ES cells (Zwaka and Thomson, 2003). Considering the essentiality of homologous recombination for site specific gene modification, electroporation will remain to be indispensable until more effective novel methods are devised.

(ii) Chemical reagents

In the first attempts of human ES cell transfection, three commonly used chemical transfection reagents (LipofectAmine plus,

FuGene and ExGen 500) were tested by Eiges and coworkers . Although the survival rate for human ES cell was not reported, we observed very little cell death (unpublished observations) following the protocol described by them. Among the different chemical reagents Eiges *et al*. tested, ExGen 500 showed significantly higher transient transfection rate than others. The stable transfection rate for ExGen 500, as reported by the authors, was about 10^{-5}. Zwaka *et al*. (Zwaka and Thomson, 2003) have re-evaluated two of the chemical reagents (FuGene and ExGen 500) and reported that the stable transfection rate for FuGene was about twice that of ExGen 500, both in the magnitude of 10^{-5}.

Taken together, the efficiency of standard transfection methods (electorporation and chemical reagents) in human ES cells is much lower, as compared to other cells, both in transient and stable transfection. We have also observed that most successfully transfected cells tend to locate on the edge of the colony, and usually appear flat and show early signs of differentiation, as shown in *Figure 1*. This may result in overestimation of transfection efficiency in human ES cells. Therefore it is important to validate whether the transfected cells retain the expression of ES cell markers such as SSEA1 and Oct 4.

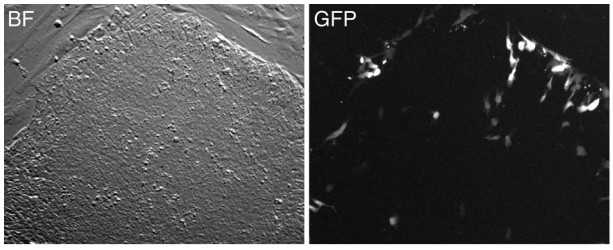

Figure 1. Location of transfected cells in a hES colony. Cells were transfected with a GFP construct using FuGene following manufacturer's instruction. Most of the transfected green cells were located on the edge of the colony.

VIRAL TRANSDUCTION

Viral transduction generally is more efficient and results in less damage to many types of cells than transfection. Several types of virus have been tested, to enable transient or stable expression of exogenous genes in human ES cells. For transient expression, adenovirus (Ad) has been shown to be efficient in mouse ES cells when appropriate promoters are used (Kawabata *et al*., 2006; Kawabata *et al*., 2005). Ad5 based adenoviral vectors have been proven to be capable of mediating transient expression in human ES cells under both undifferentiated and differentiated states (Smith-Arica *et al*., 2003). Because adenoviral vectors do not insert into the host genome and remain epichromosomal in the host cells (Russell, 2000), they are ideal for therapeutic applications in which no interruption of the endogenous genes by the transfected genes is desired.

Another type of virus, lentivirus, is derived from human immunodeficiency virus (HIV), which has the property of being able to stably infect both nondividing and dividing cells (Naldini *et al*., 1996), including human ES cells (Gropp *et al*., 2003; Ma *et al*., 2003; Xiong *et al*., 2005). It is probably the most promising gene delivery vehicle for stable expression of transgenes in human ES cells for the following reasons. (1) High transduction efficiency. Up to 70% of transduction efficiency has been achieved in human ES cells using a high titer of self-inactivating lentiviruses (Xiong *et al*., 2005). (2) The transgenes are integrated permanently into the host genome so that gene expression is stable and inheritable (Cockrell and Kafri, 2003). (3) Less vector-associated immunogenicity was observed for lentivirus mediated transduction than other viral vectors (Kordower *et al*., 2000). Lentiviral and adenoviral vectors can be used in genetic modifications of human ES cells to complement each other, depending on the purpose of modification.

Besides safety concerns, several other disadvantages should be considered when using viruses for genetic modification of human ES cells. First, viral integrations are random and unpredictable, which makes it unsuitable for site-directed genetic modification. Secondly, virus delivered transgenes usually undergo gene silencing in the host cells, meaning that expression of the genes will be gradually turned off during propagation and/or differentiation (Cherry *et al*., 2000; Laker *et al*., 1998). The extent of silencing differs among various promoters. In general, β-actin based promoters are less silenced during propagation and differentiation (Costa *et al*., 2005; Muotri *et al*., 2005), whereas many other commonly used promoters are eventually turned off completely, such as following terminal neuronal differentiation (Guillaume, 2006). Thirdly, besides gene silencing, lentivirus delivered transgenes may be suppressed immediately after transduction; therefore the transgenes are never expressed in the host cells (Xia, 2006). This gene suppression phenomenon is also highly promoter dependent. Unfortunately, promoters that are relatively exempt from gene suppression (like EF1α and PGK promoters) usually suffer from severe gene silencing during later differentiation, whereas the afore-mentioned β-actin based promoters (e.g., CAG) are seriously suppressed in human ES cells. Therefore, caution should be taken in choosing the appropriate promoter based on the particular experimental purpose (Xia, 2006).

NUCLEOFECTION

Nucleofection is a mechanical method for DNA delivery developed by AMAXA Biosystems (AMAXA Biosystems, Gaitherburg, MD). The technique uses combinations of electric current and solutions tailored for specific cell types and enables introduction of plasmid DNA directly into the nucleus, giving rise to high gene delivery efficiency even in those hard-to-transfect cells such as non-dividing primary cells (Gresch *et al*., 2004). This technique has been recently applied to human ES cells and shown to be superior to other transfection methods (Lakshmipathy *et al*., 2004; Siemen *et al*., 2005). Compared to traditional non-viral methods like electroporation, the authors showed that nucleofection appeared to cause significantly reduced cell death (<30%) and increased efficiency (66%). The technique has not been widely used in human ES cells and the transfection efficiency remains to be confirmed by more research groups. Although the transfected cells have been shown to retain the ES cell specific markers including Tra-1-60, Tra-1-81 and Oct4, it is not shown whether the transfected cells retain the same potential for terminal differentiation.

As a summary, the methodologies used for genetic modification of human ES cells are listed in *Table 1*.

Table 1. Methodologies for genetic modification of human ES cells

Method	Cell survival%	Transient transfection %	Stable transfection %
Electroporation	~5%(Lakshmipathy *et al*., 2004) >60% (Mohr *et al*., 2006)	14%	10^{-5}
LipofectAMINE plus	Low cell death	<10%	N/A
FuGene	Low cell death	<10%	10^{-5}
ExGen 500	Low cell death	<10%	10^{-5}
Adenovirus	Low cell death	~ 30%	N/A
Lentivirus	Low cell death	N/A	>70%
Nucleofection	>70%	66%	N/A

Applications of genetic modification in human ES cell research

Genetic modification of human ES cells is still at its early stage. Successful establishment of stable transgenic human ES cell lines are at present limited to a couple of laboratories. Judging from the impact of genetically modified mouse ES cells and resultant animals, we predict that genetically modified human ES cells will have a major impact on our ability to elucidate the mystery of early human development and pathogenesis of genetic disorders, to identify therapeutic targets, as well as to develop technology for more efficient stem cell maintenance and directed differentiation.

TRANSGENIC CELL LINES FOR ELUCIDATING PLUORIPOTENCY AND FOR ES CELL MAINTENANCE

One question central to stem cell biology is how the pluoripotency of ES cells is maintained. The answer to this question is instrumental to developing the appropriate technology for effectively maintaining ES cells. Studies from mouse ES cells have shown that a network of transcription factors including Oct4 , Sox2, Nanog and others are essential to the maintenance of stemness (Boiani and Scholer, 2005; Ivanova *et al*., 2006; Takahashi and Yamanaka, 2006). This transcription factor network in mouse ES cells can be maintained by a relatively simple factor, leukemia inhibitory factor (LIF). However, LIF cannot support self renewal of human ES cells (Thomson *et al*., 1998). Instead, high concentrations of basic fibroblast growth factor (bFGF) and suppression of bone morphogenetic protein (BMP) signaling by noggin have been shown to sustain undifferentiated proliferation of human ES cells (Levenstein *et al*., 2006; Xu *et al*., 2005), suggesting a possible discrepancy in the regulatory networks maintaining the stemness of human and mouse ES cells. Using a genetic modification approach, Darr and coworkersn have established a human ES cell line overexpressing one of the transcription factors important for mouse ES cell pluripotency, Nanog, and showed that the cell line can be expanded under a feeder-free system in an undifferentiated state, indicating that common transcription factors are involved in the pluripotency of both mouse and human ES cells, even though they may be regulated by different sets of extracellular signals (Darr *et al*., 2006). Establishment of such human ES cell lines not only enables us to elucidate the mechanism underlying pluripotency, but also may help us to ultimately develop chemically defined systems for maintaining human ES cells, which will be a significant step toward potential clinical use of human ES cells.

Genetically marked undifferentiated human ES cells can be separated from differentiated cells, which can facilitate mechanistic analyses. Using the murine Rex1 promoter to drive the EGFP gene, a mouse ES cell line has been established, in which GFP is expressed at high levels in undifferentiated ES cells but at dramatically lower levels when cells are differentiated (Eiges *et al*., 2001). This allows the sorting and analyzing of a pure population of undifferentiated ES cells. In a more sophisticated manner, a cassette containing an internal ribosomal entry site (IRES) and an EGFP gene was knocked into the 3' untranslated region (UTR) of the Oct4-encoding gene POU5F1 in human ES cells, which is also expressed exclusively in the pluripotent cells (Zwaka and Thomson, 2003). These fluorescently marked stem cells will greatly facilitate our understanding of stem cell pluripotency and developing technology for stem cell maintenance in chemically defined conditions.

TRANSGENIC CELL LINES TO PROMOTE DIRECTED DIFFERENTIATION

Directed differentiation of human ES cells toward a specific fate is a key to potential therapeutic applications. Protocols have been devised for differentiating and/or enriching specific cell types from differentiated human ES cells (Kaufman *et al*., 2001; Kehat *et al*., 2001; Levenberg *et al*., 2002; Reubinoff *et al*., 2001; Xu *et al*., 2002; Zhang *et al*., 2001). Many other cell types, such as pancreatic cells, have not been as successfully differentiated from ES cells, partly because the particular

extracellular signaling pathways that lead to pancreatic specification are not known. Genetic alteration (forced expression or silencing) in genes that determine specific cell fates can aid in the generation of specialized cells and potentially help identifying extrinsic factors that facilitate specification of a particular cell lineage. Forced expression of Pdx1 and Foxa2, transcription factors involved in pancreas development, in human ES cells indicates that Pdx1 enhances the expression of pancreatic enriched genes (Lavon *et al*., 2006), although additional signals are required for differentiation of cells that are capable of insulin induction. This preliminary study has indicated the potential of using genetically modified human ES cells to identify extracellular signals that promote lineage differentiation.

Although not yet reported, interference with signaling pathways during differentiation using similar genetic manipulation will also likely help identify conditions for directing the commitment of cells towards specific lineages.

TISSUE SPECIFIC FLUORESCENT CELL LINES TO FACILITATE PURIFICATION

Differentiation of ES cells usually results in a mixed population of cells. For example, the presently most effective protocols for differentiating human ES cells to spinal motor neurons or dopaminergic neurons yield about 30% of the target cells among the total differentiated progenies (Li *et al*., 2005; Perrier *et al*., 2004; Yan *et al*., 2005). For mechanistic analyses or potential future clinical applications, a pure or highly enriched population of differentiated cells is desirable. By transiently transfecting with a GFP construct driven by the motor neuron specific Hb9 homeobox gene promoter (Arber *et al*., 1999; Nakano *et al*., 2005) in human ES cells that are differentiated toward spinal motor neurons, Singh *et al* show that differentiated motor neurons turn on GFP. The fluorescent cells were then sorted out by fluorescence activated cell sorting (FACS), which led to more than 10-fold enrichment of motor neurons (Singh Roy *et al*., 2005).

Stable cell lines have also been established to trace specific cell types (Lavon *et al*., 2004; 2006). The reporter EGFP gene driven by the albumin promoter, which is relatively specifically expressed in the hepatic lineage, has permitted detection of a small fraction (6±2%) of hepatic-like cells in human ES differentiated cells. Human ES cells stably transfected with EGFP driven by the pancreatic-specific Pdx1 gene promoter has similarly allowed researchers to trace a small fraction (5±2%) of pancreatic β-like cells from human ES differentiated cells. These stable cell lines eases transfection burdens considerably. Considering the difficulty in efficiently directing human ES cells to such lineages as pancreatic β cells and hepatic cells, these fluorescently marked cells under lineage-specific promoters will be extremely useful in isolating sufficient cells through sorting. The fluorescent marker is also a great readout for identifying or optimizing conditions for differentiating human ES cells to these particular lineages.

Future directions

The most successful application of mouse ES cells is perhaps the generation of knockout mice, by using mouse ES cells with ablated genes. The technique to achieve

gene ablation in mouse ES cells, i.e. homologous recombination, has been brought to human ES cells with success using modified transfection methods (Zwaka and Thomson, 2003). However the *in vitro* success can not be extended to *in vivo* for obvious reasons. As a result, complete gene knockout can not be easily accomplished in human research unless the gene is located in one of the sex chromosomes, since homozygotes are typically generated by animal breeding in non-human research. For this reason homologous recombination may not be as valuable for gene knockout in human ES cells, although it is still a crucial technique for other site-specific genetic modification. An alternative way to achieve gene silencing is through RNA interference (RNAi) (Hannon, 2002). RNAi bypasses the need for homozygote since only one copy of small interference RNA transcribing gene is required. Gene knockdown by RNAi appears technically feasible in human ES cells (Liu *et al*., 2005; Zaehres *et al*., 2005). However, development of stable RNAi human ES cell lines has been hampered by technical hurdles described above, mainly the low gene delivery efficiency. So far no human ES cell line with stable RNAi knockdown of endogeneous gene has been reported yet. Another issue is whether the RNA polymerase III promoters (such as U6 and H1) commonly used to drive the transcription of small hairpin RNA will be silenced during the differentiation of human ES cells, as many of the RNA polymerase II promoters are. Although there are still many unsolved issues, RNAi is expected to be used broadly in human ES cells in the near future. A database of human ES cell lines with specific genes silenced will be a great asset to the research community, as its counterpart of knockout mice.

Cell lines which allow inducible gene overexpression or silencing are probably even more valuable than those constitutively overexpressing or silencing genes. Using the tet-on technique (Gossen *et al*., 1995), Kyba and coworkers have established a founder mouse ES cell line which has greatly facilitated the construction of inducible mouse ES cell lines (Kyba *et al*., 2002). Recently the tet technique was further successfully applied to primate ES cells (Adachi *et al*., 2006). All these progresses suggest that the extension of this technique to human ES cells is merely a matter of time.

The conditional knockout technique developed in the 1990s has further expanded the scope of the traditional knockout technique (Gu *et al*., 1993). Using tissue specific promoters and Cre/loxp recombination, a specific gene can be selectively knocked out in certain types of tissues, which will enable researchers to determine gene function in different tissues. The conditional knockout technique requires generation of a homozygous floxed exon, which as we discussed above, is not applicable to human research in most cases. However, a combination of RNAi, Cre/loxp recombination and tissue specific promoter still allows tissue specific gene silencing in human ES cells and derivatives (*Figure 2*). The strategy employs a tissue specific promoter to drive the expression of cre recombinase. As a result, the floxed stop codon is removed in the particular tissue through cre/loxp recombination so that the full length hairpin RNA is transcribed, which leads to silencing of the target gene. The first step is the establishment of founder human ES cell lines expressing the cre recombinase under a tissue specific promoter. Many founder mouse lines expressing the cre recombinase under various tissue specific promoters have been created and are made available through non-profit institutions like the Jackson Laboratory. Similar human ES cell lines expressing the cre recombinase under tissue specific promoters will undoubtedly change our research in human biology.

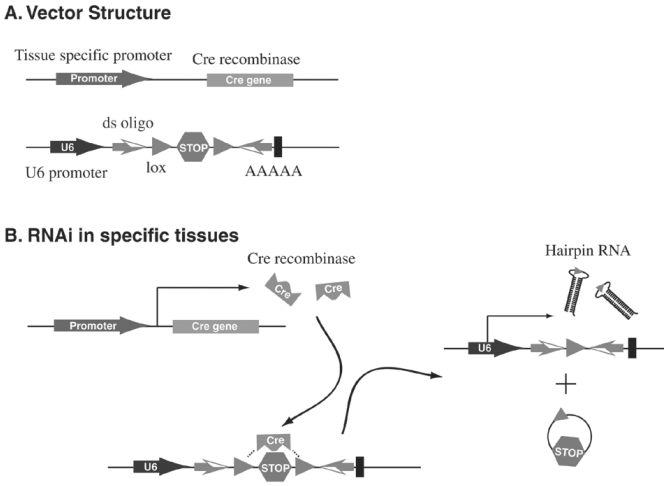

Figure 2. Strategy to achieve tissue specific gene slicing in hES cells and derivatives. **A.** structures of the two constructs used in the strategy. One construct drives the expression of cre recombinase under a tissue specific promoter. The other construct uses U6 promoter to drive the expression of small hairpin RNA. The two reverse complementary double strand oligos were separated by a floxed stop codon. **B.** In the specific tissue where the promoter is active, cre recombinase is expressed so that the floxed stop codon is removed through cre/loxp recombination. This enables the transcription of the full self-complementary oligo so that small hairpin RNAs are generated which lead to knockdown of the target gene.

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