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Neural Development in Human Embryonic Stem Cells—Applications of Lentiviral Vectors

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ABSTRACT

The derivation of neural lineages from human embryonic stem cells (hESCs) in vitro is based largely on exposure of hESCs to exogenous signals and substrates, designed to mimic conditions in the developing embryo. However, selection of specific lineages and the discovery of gene function in human neural development may be enhanced by the ability to intrinsically regulate gene expression. Recombinant lentiviral vectors provide an efficient method to stably introduce genes into hESC and their differentiating derivatives. Here we review the methods used to derive neural cells from hESCs, transduction of these cells with lentiviral vectors, and improvements that have been made to the vectors to enhance viral integration and transgene expression. Finally, we explore prospects for future uses of lentiviral vectors in hESC research, including their applications in library screening for drug development, zinc finger nucleases for gene editing and optogenetics to interrogate cellular pathways and function. J. Cell. Biochem. 112: 1955–1962, 2011.

KEY WORDS: HUMAN EMBRYONIC STEM CELLS; NEURONAL DEVELOPMENT; LENTIVIRAL GENE TRANSFER; ZINC FINGER NUCLEASES; OPTOGENETICS

Human embryonic stem cells (hESCs) were first isolated in 1998 [Thomson et al., 1998] based on developments in in vitro fertilization (IVF) embryo culture including the isolation of the inner cell mass (ICM) from blastocysts in 1994 as well as advances in non-human primate stem cell culture [Bongso et al., 1994; Thomson et al., 1995]. hESCs possess the dual characteristics of self-renewal and pluripotency. Self-renewal refers to the ability of hESCs to maintain a population of uncommitted or undifferentiated cells, allowing nearly unlimited production and maintenance over many years and laboratories worldwide. hESCs are also pluripotent; that is they possess the ability to differentiate into derivatives of the three germ lineages; ectoderm, mesoderm, and endoderm. POU5F1 (Oct4) is commonly used as a marker of pluripotent cells [Niwa et al., 2000], but other markers such as Nanog [Chambers et al., 2003], SOX2 [Boyer et al., 2005], Germ Cell Tumor Marker-2 (GCTM-2) [Andrews et al., 1996], TG30, TRA-1-60, SSEA3, and SSEA4 [Adewumi et al., 2007] are also used. A useful test of pluripotency in hESCs is to transplant them into an immune-compromised mouse. If the transplanted cells are pluripotent, a benign tumor, or teratoma, consisting of derivatives of all three germ lineages will develop [Stevens, 1962].

The applications of hESCs are numerous, including therapeutic potential for regenerating tissue and for understanding developmental processes. Lineage selection in hESCs has traditionally been mediated by varying substrates and/or addition of growth factors or cytokines, based primarily on developmental studies in rodents and other model organisms. Additionally, knockout studies have led to the identification of key genes in lineage regulation. The ability to selectively regulate such genes in hESCs or during neural differentiation may allow more defined lineage selection for regeneration applications and an increased understanding of gene function in human neural development. Here we review the use of recombinant lentiviral vectors to genetically modify hESCs, discuss directed derivation of specific neural cell types by lentiviral-mediated gene manipulation and suggest avenues for further protocol optimization and future prospects.

NEURAL DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

Many of the protocols used to direct lineage fate in hESCs have been informed by embryonic development. Neural development

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can be divided into three main stages: neural induction, neural specification, and neural maturation. Each of the stages in the developing embryo can be modeled in vitro in hESCs. As our understanding of each stage increases so do the opportunities to manipulate and selectively enhance specific neuronal cell fates.

Both indirect and direct methods have been used to induce hESCs to differentiate into neuroectodermal-like cells. The indirect approach is to maintain hESC in culture beyond one week without passaging, allowing their spontaneous differentiation into all three germ lineages. Differentiation toward neuroepithelial-like cells can be easily identified by a rosette-like morphology. These neural rosettes contain columnar epithelial cells, organized radially [Elkabetz et al., 2008]. Neural rosettes express neuroectodermal markers, such as PAX6 and SOX1, during the neural induction phase [Davidson et al., 2007]. They can then be mechanically isolated and cultured in suspension to form aggregates, referred to as "neurospheres" [Dottori and Pera, 2008]. An adaptation to ES neural induction by spontaneous differentiation uses a feeder-free suspension culture of ES cells. These clusters of cells, known as embryoid bodies (EB), can also differentiate toward any of the three germ lineages (Fig. 1). For this reason the EB technique is commonly used as an in vitro test to determine pluripotency [Itskovitz-Eldor et al., 2000]. The cell culture media used in EB formation may be standard or may contain factors that promote neural induction.

A more directed approach for hESC neural induction involves culturing cells on a feeder layer of mouse stromal cells such as PA-6 or MS-5 cell lines [Barberi et al., 2003]. It appears that these stromal feeder layers secrete factors that promote neural induction. Recent publications have moved away from a feeder layer system and now culture hESC directly on laminin substrates in media containing neural inducing factors [Denham et al., 2010]. Directed systems are favorable due to the fact that hESC differentiation is directed toward the neural lineage and not mesoderm or endoderm. A non-feeder system also allows higher efficiency of lentiviral transduction in hESC progenitors (see below).

Most hESC neural induction methods involve antagonizing of bone morphogenic protein (BMP) signaling. Pera et al. [2004] showed that addition of the BMP inhibitor protein, noggin, to hESC cultures for 14 days resulted in a high proportion of the cells converting to a neural stem cell phenotype (Fig. 1), as evidenced by positive staining for SOX2, PAX6, and NESTIN, and lack of mesoderm and endoderm lineage markers [Pera et al., 2004]. BMPs belong to the Transforming Growth Factor-beta superfamily (TGF-β) of receptors which mediate their signals through the class of SMAD transcription factor proteins. Inhibition of different SMAD signaling pathways, using small molecules in combination with noggin, resulted in even greater hESC neural induction efficiency [Chambers et al., 2009]. This suggests that the mechanism of hESC neural induction operates by inhibiting specific SMAD signaling pathways.

Following neural induction, hESC-derived neural progenitors are cultured as clusters in suspension, to promote neurosphere formation. hESC-derived neurospheres frequently display rosettes within their structure, possibly indicating a niche of proliferating neuroepithelial-like cells residing within the sphere itself [Davidson et al., 2007; Dottori and Pera, 2008]. Within the neurosphere microenvironment, neural progenitors begin to express early embryonic markers of anterior/posterior and dorsal/ventral neural cell types of the developing nervous system [Davidson et al., 2007; Denham et al., 2010]. The timing and expression of neural patterning genes depends upon the method of neural induction used and exposure of exogenous factors during this stage. For example, noggin-treated hESC colonies show high expression of PAX6; however, this expression is down-regulated coinciding with up-regulation of PAX7 when the cells are transferred to neurosphere forming conditions [Davidson et al., 2007]. In contrast, hESC neural induction using the PA6 co-culture system results in earlier onset of

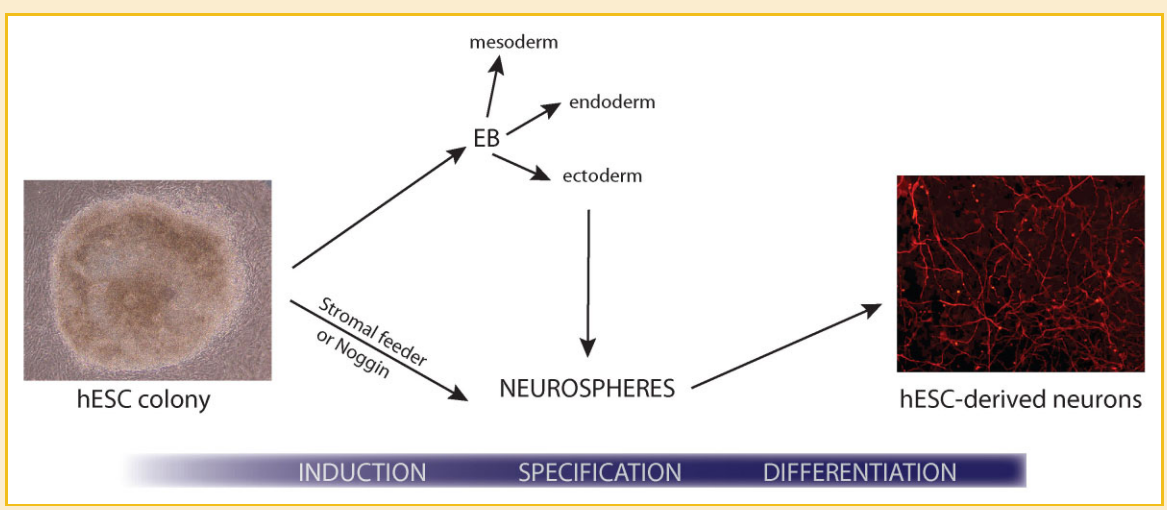

Fig. 1. Methods of hESC neural differentiation. hESC undergo neural induction by either co-culturing with stromal feeder cells, noggin treatment and/or embryoid body (EB) formation. Neural progenitors are then harvested and propagated as neurospheres. Specification and commitment of progenitors to lineages also occurs during this stage of development. Neurospheres can then be plated onto substrates for their differentiation to mature neuronal or glial cell types. Scale bar 200 μm.

PAX7 expression [Denham et al., 2010]. It has also been shown that treatment of hESC with sonic hedgehog during the neural induction phase results in higher proportion of ventral neural cell types [Denham et al., 2010]. This demonstrates that neural specification occurs at early stages of hESC neural differentiation, which is very important to consider when developing methods to direct hESC differentiation to specific neuronal lineages.

Exposing hESC and their derivatives to exogenous signals is not necessarily always effective for obtaining specific cell types. Studies from our laboratory have shown that treatment of hESC with high concentrations of sonic hedgehog during neural induction was ineffective in biasing their differentiation toward cell types of the embryonic ventral floorplate [Denham et al., 2010]. This was overcome using a forced intrinsic approach using lentiviruses. Thus, intrinsic differentiation of hESC and neural progenitors can sometimes be difficult to override despite culture conditions.

The third stage of hESC neural development, neural differentiation, is the maturation of specified progenitors to neuronal or glial lineages. Neuronal differentiation is usually achieved by plating neurospheres onto laminin substrates in the absence of mitogenic factors [Dottori and Pera, 2008]. Fibronectin substrates are used for glial differentiation and mitogenic factors are maintained for a specific period [Dottori and Pera, 2008]. Neurons and glial cells can be identified by their morphology and expression of specific neuronal (e.g., β-III tubulin, MAP2; Fig. 1) and glial markers (S100β and GFAP), respectively [Dottori and Pera, 2008]. At this stage specific mature neuronal cell types can be identified, and the efficiency of the neuronal differentiation protocol can be determined.

In summary, the methods used for hESC neural differentiation have essentially relied on exposing cells to exogenous signals within the media, and culture conditions throughout the differentiation protocol. Whilst this system of differentiation is useful for obtaining neurons and/or glial cells in general, we are still at the mercy of intrinsic cellular signals, which can be challenging to override and inevitably result in a heterogenous population of cell types. For this reason, genetic modification of hESC and their progeny to intrinsically drive their differentiation toward specific lineages is sometimes the best option.

LENTIVIRUSES AS GENE TRANSFER VECTORS

Lentiviral vectors are vital tools for hESC research. The earliest studies on hESC viral transduction used retroviral vectors due to their capacity to target dividing cells and stably integrate into the host genome. However, retroviral-delivered transgenes are often silenced due to methylation of the long-terminal repeats (LTRs) [Pfeifer et al., 2002]. The development of lentiviral vectors [Naldini et al., 1996] has mirrored the development of hESC methods and these vectors are now widely exploited in hESC studies.

The lentiviruses, including human, simian, equine and feline immunodeficiency viruses are a genus of the *Retroviridae* family. In contrast to the *gammaretroviruses*, which require breakdown of the nuclear membrane during cell division for genome integration, lentiviruses can target the nucleus of both dividing and non-dividing cells. In addition, lentiviral LTRs are less prone to methylation and gene silencing, therefore, are able to maintain long-term control over viral transcription [Pfeifer et al., 2002]. The lentiviral genome is a positive-sense single-stranded RNA encoding core *gag* (capsid), *pol* (protease, reverse transcriptase, and integrase) and *env* (envelope) genes as well as a number of accessory genes important for viral virulence and pathogenesis (Fig. 2A). The accessory genes, which vary depending on lentiviral type, are dispensable for the generation of recombinant vector systems; however, *gag* and *pol*, and an envelope usually from another virus, are retained for recombinant viral packaging. Packaging of virus is achieved by triple (2nd generation) or quadruple (3rd generation) transfection (Fig. 2B) of human embryonic kidney cells (HEK293). *Gag* and *pol* genes from HIV, and the envelope, most commonly the vesicular stomatitis virus glycoprotein (VSV-G), are provided in *trans* and these proteins packaged into recombinant virions. The recombinant viral genome contains the desired transgenes and promoter elements together with a packaging signal and modified LTRs (Fig. 2). The 5'LTR in the third generation systems contains CMV or RSV elements providing Tat-independent transcription during packaging, thereby further reducing lentiviral genes in the system. Most recombinant lentiviral genomes also contain a deletion in the 3'LTR, which, during integration into host cells, is replicated at the 5' end. This renders the virus self-inactivating (SIN) as it no longer has the capacity to generate full length genomes from the integrated provirus, providing an additional safety mechanism against the generation of replication-competent virus [Iwakuma et al., 1999] and promoter activity from the LTR. Several other enhancements have been made including the reintroduction of the central polypurine tract (cPPT) to enhance nuclear import of the lentiviral genome [Follenzi et al., 2000], and the woodchuck hepatitis virus enhancer (WPRE; Zufferey et al. [1998]) to increase transcript termination and viral titer [Higashimoto et al., 2007]. Recombinant lentiviral particles therefore contain minimal viral RNA (less than 40%), transgene/s and regulators (promoters and/or enhancers) packaged together with protease, reverse transcriptase and integrase proteins; in a lentiviral capsid surrounded by a lipid bilayer studded with envelope glycoprotein. Viral particles harvested from the packaging cell media can be used directly for transduction, or concentrated by ultracentrifugation prior to transduction of hESCs or neural derivatives. Viral integration provides stable, long-term expression of the transgene/s in all progeny of the transduced cells (Fig. 2C).

LENTIVIRAL VECTORS AND hESCs

Methods to stably transduce hESC cells or neural progeny using lentiviral vectors have been widely reported. hESCs are either seeded on matrigel in mouse embryonic fibroblast (MEF)-conditioned or defined media prior to transduction [Xiong et al., 2005], transduced in suspension prior to replating on MEFs [Clements et al., 2006], or transduced directly on irradiated MEF feeder cells [Zaehres et al., 2005]. The latter method also results in transduction of MEFs; however, these cells are eventually lost through successive hESC passaging.

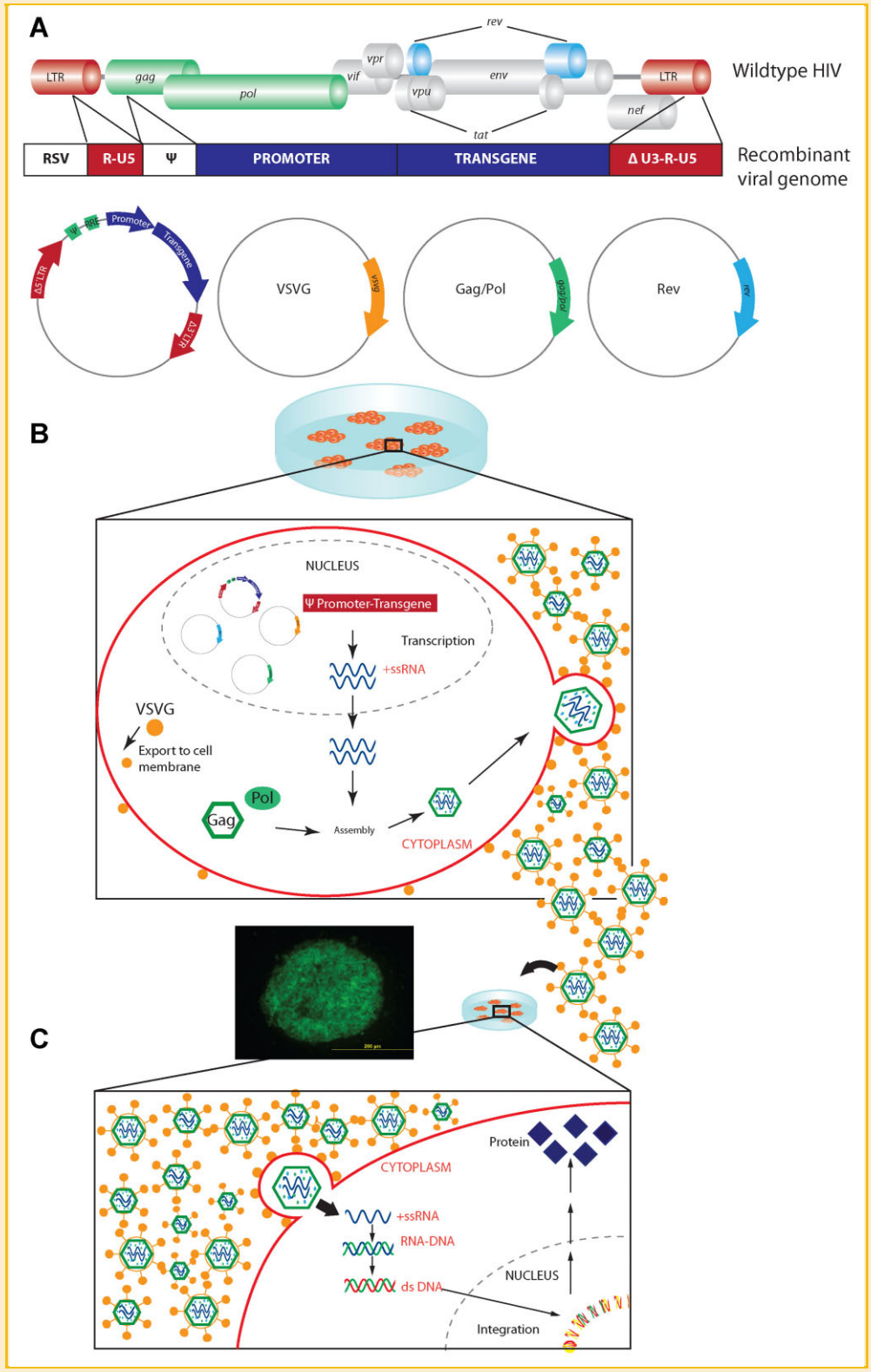

Fig. 2. The generation of recombinant HIV-derived lentiviral vectors. (A): The genome of the recombinant lentiviral vector is derived from minimal sequences of wildtype HIV. The long-terminal repeats (LTR) are modified by deletion of part of the 3'LTR and introduction of a chimeric 5'LTR to provide self-inactivation and tat-independent replication, respectively. The recombinant lentiviral genome encodes the transgene/s of interest under the regulation of an internal promoter. Part of the *gag* gene (Ψ) is retained to allow packaging of this RNA within the viral particle. Further components required for construction of a viral particle including *gag/pol* and *rev* genes and an envelope are provided *in trans*. (B): Pol proteins together with the recombinant RNA genome are packaged into a capsid (gag). Lentiviral particles bud from the VSV-G containing packaging cell membrane into the cell culture media. (C): Transduction of hESCs and expression of transgenes. Photo shows a hESC-derived neurosphere transduced with LV-EF1α-GFP. Scale bar 200 μm.

The efficacy of lentiviral-mediated gene transfer to any cell type depends on a number of aspects of the vector and cellular target. Uptake of the viral particle into a cell is dependent on the affinity of the viral envelope for target cell surface receptors, endocytosis, and transfer, integration and expression of the viral genome within the target cell. Most studies of lentiviral transduction of hESCs have measured efficacy based solely on transgene expression. Further improvements may be possible to allow enhanced efficiency at lower multiplicity of infection (MOI) by assessing and optimizing earlier steps in transduction such as viral binding and endocytosis. For example, inhibition of proteosome function with MG132 during transduction has been shown to enhance lentiviral efficiency [Santoni de Sio et al., 2008]. Changing the envelope glycoprotein can be used to alter viral tropism to selectively transduce hESCs or neural derivatives. While VSV-G is the most commonly used envelope, the RD114 glycoprotein from endogenous feline leukemia virus and the GALV glycoprotein from gibbon ape leukemia virus have also been tested in hESCs. These envelopes show selective tropism for hESCs whilst not transducing MEF feeder cells [Jang et al., 2006]. Lentiviruses pseudotyped with rabies or LCMV glycoproteins show tropism for neurons [Mazarakis et al., 2001] and astrocytes [Cannon et al., 2011], respectively, in murine cells. However, to the best of our knowledge, these pseudotypes have not yet been tested in hESC or hESC-derived neural cells.

Whilst hESCs and their neural progeny are efficiently transduced by lentiviral vectors compared to other viral and non-viral methods, the stability throughout neural differentiation is dependent on the chromatin structure of the integration site/s and the promoter used to drive transgene expression. As is true for the retroviral LTRs, internal promoters can also be subject to epigenetic modification; this is commonly seen with the CMV promoter in various cell types including hESCs [Suter et al., 2006; Kim et al., 2007; Xia et al., 2007]. The genome of a differentiating stem cell is subject to significant epigenetic modification [Bartova et al., 2008], which can lead to silencing of transgenes. Transgene expression can be enhanced by introduction of insulator sequences that aid in protecting the promoter from local chromatin changes [Ma et al., 2003]. The constitutive human elongation factor 1 alpha (EF1α) promoter has been widely used in hESCs (Fig. 2C) and is generally found to be stable even after multiple passages [Ma et al., 2003; Xiong et al., 2005; Kim et al., 2007] although expression can be down-regulated during differentiation [Xia et al., 2007] especially at high MOI [Clements et al., 2006].

Although there is still potential for improvements, lentiviral delivery remains the most efficient method to introduce exogenous factors into hESC and their applications in stem cell biology are continuing to expand.

BEYOND TRANSDUCTION ASSAYS—RECENT AND POTENTIAL APPLICATIONS OF LENTIVIRAL VECTORS IN hESCs

Now that lentiviral-mediated gene transfer to hESC and neural progeny has become routine in many labs, research has moved toward exploiting the technology to understand gene function during early human development and to provide new tools to develop therapies and disease models. Although it is possible to test gene function in vivo in mice and other species, there are already several examples where genes do not always play the same role in humans as in mice, such as the involvement of Leukemia inhibitory factor in ES cell maintenance [Okita and Yamanaka, 2006]. Another example is that of the role of PAX6, a pan-neuroepithelial marker and SOX1 in neural induction: in mESCs, Sox1 is the first factor to be expressed in the presumptive neuroectoderm [Pevny et al., 1998]; but in hESCs, the neural determinant is PAX6 [Zhang et al., 2010].

Recent research has focused on the role of neural specification and differentiation genes in human development and to isolate subpopulations of cells from differentiating hESC cultures. Cell type specific promoters driving expression of a reporter gene such as GFP can be used to isolate specific subpopulations of cells from differentiating hESCs. This has already been effectively used in hESCs to isolate hESC-derived myocardial cells [Coppola et al., 2010], and via homologous recombination to introduce GFP into the OLIG2 [Xue et al., 2009] or NKX2.1 [Goulburn et al., 2011] loci to identify and isolate of neural progenitors or basal forebrain progenitors, respectively. Although the later two examples used traditional non-viral methods to introduce the reporters, these may well be accomplished with greater efficiency using a lentiviral approach based on a non-integrating lentiviral vector (described later).

Drug-inducible systems such as the Tet-system [Zhou et al., 2006; Fu et al., 2008] allow the generation of hESC clones in which constitutive transgene expression would prevent maintenance of hESCs. Drug-inducible systems can also be used to study the effects of a gene at different stages of differentiation by knocking down or over-expressing genes during neural induction, specification or differentiation. This was recently illustrated in mouse ES cells by Wang and colleagues who demonstrated that the transcriptional regulator Fezf2 plays a role in early anterior posterior forebrain patterning prior to its previously recognized function in cortical differentiation [Wang et al., 2011]. Expression of Gli1 specifically in hESC-derived neural progenitors was also shown to specify their fate to floorplate-like cells [Denham et al., 2010]. Not only are these types of studies vital to understanding gene function, they may also allow the development of new methods to purify specific populations for testing drug treatments or eventually as a source for cell replacement therapies.

A natural extension of the ability to generate large numbers of purified cell types from a renewable cell source such as hESCs is their use in library screens. Library screens provide a high-throughput/high-content method to interrogate genes and biological pathway functions and to identify new drug targets. Several lentiviral RNAi libraries covering the human genome [Moffat et al., 2006], or selected biological pathways [Duan et al., 2010] have been developed, and it is likely that this technology will soon be applied to hESC and their neural derivatives.

Another potential application of lentiviral vectors in hESC is generation of models of genetic disease or for gene knockout to explore gene function. Genes can be selectively edited, deleted, or added either by traditional homologous recombination strategies, or via engineered zinc finger nucleases [Urnov et al., 2005], which

display significantly increased efficiency [Hockemeyer et al., 2009]. Zinc finger nucleases combine two target sequence-specific zinc finger-binding proteins fused to a *FokI* nuclease. Binding of the zinc fingers to adjacent target sequences and *FokI* dimerization generates a double-stranded break between the zinc finger-targeted sequences. The double stranded break can either be repaired by non-homologous end rejoining, often resulting in errors; or by homology-directed repair from a donor sequence containing homologous sequences (Fig. 3A). For this system to work in a lentiviral context, the viruses are packaged in the absence of functional viral integrase (integrase-deficient lentiviral vectors). This allows episomal expression of both the zinc finger nucleases targeting a specific locus, and a donor containing the edited gene or insert for homology-directed repair (Fig. 3A). Expression from this system is transient; however, sufficient to induce permanent editing of the host genome. Proof-of-principle studies have already demonstrated efficient targeting of the *IL2RG* locus, the gene mutated in X-linked SCID, by lentiviral zinc finger nucleases [Lombardo et al., 2007] and the *OCT4* and *AAVS1* loci in hESCs [Hockemeyer et al., 2009]. The demonstration of effective gene editing in hESCs, paves the way for the development of both new disease models and the generation of cell lines expressing reporter genes at defined loci. This site-selective technology therefore allows the purification of specific cell lineages for cell replacement without potential for insertional mutagenesis associated with traditional lentiviruses.

The developing field of optogenetics (the manipulation of cell function with light) is rapidly becoming the gold standard for the

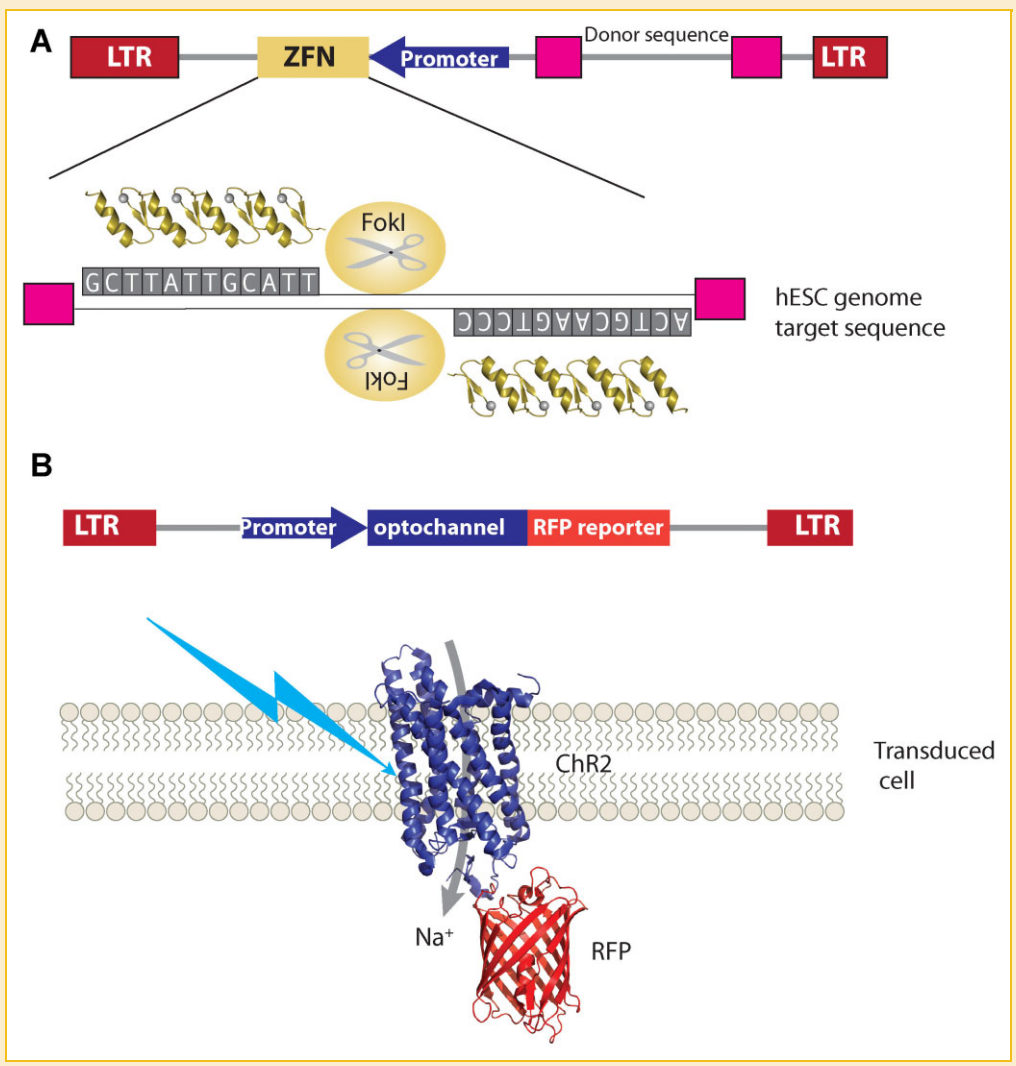

Fig. 3. Applications of lentiviral vectors in hESCs. (A): Lentiviral-mediated gene editing. A zinc finger nuclease (ZFN) contains two zinc finger DNA binding domains with specificity for a genomic target each fused to a *FokI* nuclease domain. Binding of the ZFNs to a genome target induces FokI dimerization and double strand cleavage. Breaks may be repaired using the donor sequence from the lentiviral DNA (pink homologous sequence) to introduce point mutations, insertions, deletions or reporter genes. (B): Lentiviral-mediated optogenetics. A chimeric optogenetic probe containing a red fluorescent protein reporter (RFP) and a rhodopsin-like membrane channel. Activation by light of a specific wavelength induces conformational changes in the channel and changes in cellular activity (in this case cell depolarization by Na^+ influx). Expression via a cell-type specific promoter in hESC can limit expression to subsets of differentiating cells.

analysis of neural circuits and in the normal and diseased brain [Miller, 2006; Stuber, 2010]. Optogentic constructs delivered by lentiviral vectors offer enormous potential in the hESC field. The original optogenetic constructs were engineered light-activated ion channels from bacterial species. These provide either silencing of electrical activity (hyperpolarize target cells) or depolarize and activate target cells (Fig. 3B) depending on the form of channel and light frequency used. Additional optogenetic constructs have been designed to modulate G-protein coupled receptors, allowing interrogation of intracellular signaling cascades [Moglich and Moffat, 2010]. The potential exists to use this technology to modify any pathway or enzyme within any cell type including hESCs and derivatives with precise spatial and temporal control [Toettcher et al., 2011].

CONCLUSION

Lentiviruses remain the most effective way to stably introduce exogenous factors into hESCs and their applications in stem cell biology remain at the forefront. The continuing development of lentiviral vectors and the increasing range of potential applications in hESC research provides exciting new opportunities to understand developmental processes and move toward clinical applications.

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