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Genetic Manipulation of Human Embryonic Stem Cells

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Abstract

One of the great advantages of embryonic stem (ES) cells over other cell types is their accessibility to genetic manipulation. They can easily undergo genetic modifications while remaining pluripotent, and can be selectively propagated, allowing the clonal expansion of genetically altered cells in culture. Since the first isolation of ES cells in mice, many effective techniques have been developed for gene delivery and manipulation of ES cells. These include transfection, electroporation, and infection protocols, as well as different approaches for inserting, deleting, or changing the expression of genes. These methods proved to be extremely useful in mouse ES cells, for monitoring and directing differentiation, discovering unknown genes, and studying their function, and are now being extensively implemented in human ES cells (HESCs). This chapter describes the different approaches and methodologies that have been applied for the genetic manipulation of HESCs and their applications. Detailed protocols for generating clones of genetically modified HESCs by transfection, electroporation, and infection will be described, with special emphasis on the important technical details that are required for this purpose. All protocols are equally effective in human-induced pluripotent stem (iPS) cells.

Keywords: Human ES cells, Genetic manipulation, Transfection, Electroporation, Infection

1 Introduction

1.1 Genetic Modification Approaches and Their Potential Applications

There are basically two types of strategies that can be applied for inducing permanent changes in the DNA of HESCs. One approach depends on random integration of foreign DNA sequences into the genome while the other approach relies on targeted mutagenesis.

1.1.1 Random Integration of Foreign Sequences into the Genome

Random integration of foreign sequences into the genome is typically applied for overexpression of genes, or for the downregulation of endogenous genes in *trans* (knock-down). Overexpression is usually useful for constitutive or facultative expression of either cellular or foreign genes. It may also be applied for the introduction of reporter or selection genes, under the regulation of tissue-specific promoters. These procedures allow to label and track specific cell lineages following induced differentiation of human embryonic stem cells (HESCs) in culture. Moreover, they can be employed for the isolation of pure populations of specific cell types, by the use of selectable markers. The marker gene may either be a selectable reporter, such as green fluorescent protein (GFP),

resulting in the production of green glowing cells which can be selected for by fluorescent activated cell sorter (FACS), or a drug resistance gene (1–8). The ability to isolate pure populations of specific cell types and eliminate undifferentiated cells prior to transplantation has great importance in cell-based therapy; this is because transplantation of undifferentiated cells may lead to teratoma formation. Overexpression experiments may also be employed for directing the cell fate of differentiating ES cells in culture. This can be achieved by introducing master genes that play a dominant role in cell commitment, forcing the cells to differentiate into specific lineages that otherwise are rarely obtained among many other cell types in culture (9–12). Random integration of promoter-driven transgenes may also be employed for the generation of cell-based delivery systems by producing therapeutic agents at the site of damaged tissue. The use of ES-derived cells as therapeutic vectors has been previously shown to be feasible in mice, where grafting of ES-derived insulin secreting cells normalized glycemia in streptozotocin-induced diabetic mice (13).

Apart from tagging, selecting, and directing the differentiation of specific cell types, it is possible to inactivate endogenous genes to study their function. This can be achieved by downregulating the activity of particular genes in *trans* by overexpressing specific short hairpin RNA (shRNA) molecules. ShRNAs are short sequences of RNA that by forming hairpins silence target gene expression via RNA interference (RNAi) pathway. They are processed into small interfering RNAs (siRNAs) by the enzyme Dicer, and then paired with the target mRNA as they are incorporated into an RNA-induced silencing complex (RISC), leading to the degradation of the target mRNA. The great advantage of this system is that it provides a specific, long-lasting, gene silencing effect. This is why it is being considered as one of the most applicable tools for gene silencing in living organisms. Furthermore, since shRNAs operate in *trans* and are not involved in the modification of the targeted gene, it is relatively simple to apply and particularly efficient in achieving transient or conditional gene silencing effects. Expression of shRNA in HESCs is typically accomplished by transfection or through viral infection. Applications of this loss-of-function approach are now widely used not only to study developmental roles of specific genes in human, but also for their utility in modulating HESC differentiation in vitro (14, 15).

An additional use for the random integration approach can be the search of unknown genes whose pattern of expression suggests that they might have developmental importance. The identification of such genes is performed by the gene trap method, which is based on the random disruption of endogenous genes (reviewed by (16)). As opposed to targeted mutagenesis (*see* below), it involves the random insertion of a reporter gene that lacks essential regulatory elements into the genome. Because the expression of the reporter

gene is conditioned by the presence of an active endogenous regulatory element, it may serve to identify only transcribed sequences. Using this method, a large-scale gene disruption assay is possible, allowing the discovery of new genes and the creation of wide variety of mutations (17).

1.1.2 Targeted Mutagenesis

Targeted mutagenesis, or site-directed mutagenesis, is a procedure which involves the replacement of a specific sequence in the genome by a mutated copy through homologous recombination with a targeting vector. The targeting vector that contains the desired mutation and a selectable marker, flanked by sequences that are interchangeable with the genomic target, pairs with the wild-type chromosomal sequence and replaces it through homologous recombination. Targeted mutagenesis is most widely used technique for inactivating genes in ES cells. By targeting both alleles, using distinct selection markers, it is possible to create "loss-of-function" or so-called knockout phenotypes in ES cells that can be used for functional studies of specific genes. This technology has been well practiced in mice for gene function studies, in which genetically altered cells are introduced into wild-type embryos, resulting in the creation of germ-line transmitting chimeras (18). The genetically manipulated animals can be further mutated to generate animals that are homozygous for the desired mutation. The creation of HESCs with a null genotype for specific genes may have great importance for modeling human diseases, and for the study of crucial developmental genes that in their absence are embryonic lethal (19). Thus, these cells should be valuable for basic research studies, but more importantly for exploration of new gene therapy-based treatments and drug discovery.

A very similar approach that relies on targeted mutagenesis involves the insertion of foreign sequences into the genome at desired loci. This strategy, termed knock-in, is commonly used to study the regulatory function of specific elements for example, by positioning a reporter gene under the regulation of a native gene. Therefore, it can be applied to follow the expression of a target gene in situ during ES cell differentiation and monitoring the expression of the endogenous genes, enabling to identify HESCs differentiated cell derivatives (20, 21).

It should be emphasized that both gene targeting approaches, knock-out and knock-in, depend on homologous recombination events however, the efficiencies of homologous recombination is extremely low (ranging from 1 in 10^6 to 1 in 10^7), limiting the routine use of these techniques in HESC manipulation until recently. Yet, as double strand breaks dramatically improve the rate of homologous recombination, it was hypothesized that by targeting double strand DNA breaks to specific sites in the genome one may significantly improve the efficiencies of targeted mutagenesis. Indeed, due to the recent advancements in the field of artificially

engineered nucleases, it has been possible to insert, replace, or remove specific DNA sequences from the genome of HESCs/iPS in a fairly uncomplicated procedure. This technology, termed genome editing, depends on the direction of unspecific DNA nucleases to desired sites in the genome, where they induce double strand DNA breaks and by that significantly enhance the rate of homologous recombination. There are by now three different types of engineered nucleases that can be applied for this purpose; zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases (RGENs). All result in the elevation of gene targeting events through homologous recombination by at least 2–3 orders of magnitude relative to the conventional method by transient expression (22–27). Detailed description related to their composition, targetable sites specificities, off-target mutational rates, and complexity in design and preparation, which are beyond of the scope of this chapter, can be found in other excellent reviews (28, 29).

1.2 Methods for Genetic Manipulation

Several gene transfer techniques are now available for manipulating gene expression in HESCs. The latter include chemical-based (transfection), physical (electroporation), and viral-mediated (infection) techniques. No single transfection method will work for all HESC lines, and even within a lab, the method of choice may vary.

1.2.1 Transfection

Transfection is probably the most commonly used method for introducing transgenes into HESCs. It is straightforward, relatively easy to calibrate, provides a sufficient number of cells for clonal expansion, can be performed on adherent cell cultures, and allows the insertions of constructs of virtually unlimited size. This system is based on the use of carrier molecules that bind to foreign nucleic acids and introduce them into the cells through the plasma membrane. In general, the uptake of exogenous nucleic acids by the cell is thought to occur through endocytosis, or in the case of lipid-based reagents, through fusion of lipid vesicles to the plasma membrane. There are many factors that may influence transfection efficiency: phase of cell growth, number of passages, size and source of the transgene, vector type and size, and selection system. However, the most important factor is the transfection method. The first study to describe stable transfection in HESCs was based on the use of a commercially available reagent, ExGen 500, which is a linear polyethylenimine (PEI) molecule that has a high cationic charge density (1, 13). The unique property of this molecule is due to its ability to act as a "proton sponge," which buffers the endosomal pH, leading to endosome rupture and DNA release. This method routinely produces transient transfection rates of approx 10–20 % and stable transfection efficiencies of $1{:}10^{-5}$ to 10^{-6} (1). Since then, other chemical-based transfection methods have been found

to be equally effective. For example, Fugene6 (Roche) and Lipofectamine (Life Technologies) are commonly used by many labs. Both reagents are based on the presence of a positively charged cationic lipid compound that forms small unilamellar liposomes and are useful in obtaining transient and stable transfections in HESCs as well (15, 30). Usually, the cells are plated to 50–70 % confluence at the time of transfection. The plasmid DNA and lipid reagent are mixed in a tube, and only then administered to the cells as a DNA-lipid complex.

1.2.2 Electroporation

Electroporation is a method that employs the administration of short electrical impulses that create transient pores in the cell membrane, allowing foreign DNA to enter into the cells. Although efficient and most popular in mouse ES cells, this procedure gave poor results in HESCs, both in transient and stable transfection experiments. This is most probably due to the low survival rates of HESCs after the voltage shock. Zwaka and Thomson reported a protocol to increase the yield of electroporation 100-fold, thereby achieving an integration rate of approx $1{:}10^{-5}$ (21). This was performed by carrying out the procedure on cell clumps rather than on single cell suspension. In addition, electroporation was performed in standard cell culture media, which is a protein-rich solution, instead of PBS and altering the parameters of the protocol used in mouse ES cells. Using this method, 3–40 % homologous recombination events among resistant clones were reported, subject to vector properties (14). A substantial number of HESC clones obtained by homologous recombination have been created thus far using different constructs, demonstrating the feasibility of this technique for site-directed mutagenesis in HESCs.

1.2.3 Infection

Unlike in all nonviral-mediated methods (transfection and electroporation), gene manipulation by viral infection can produce a very high percentage of modified cells. To date, genetic manipulation of HESCs by viral infection has been reported by several groups using adeno- as well as Baculovirus and lenti-viral vectors (26, 31–33). Infection studies with RNA and DNA viruses have demonstrated that these viral vectors have two distinct advantages over other systems: high efficiency of DNA transfer and single-copy integrations. However, integration occurs randomly and cannot be targeted to a specific site in the genome. Yet, because of its high efficiency, this method could prove useful for bypassing the need for selection and time consuming clonal expansion, as well as for experiments that aim for random insertion mutagenesis or gene trap.

Lentiviral-based vectors offer an attractive system for efficient gene delivery into HESCs. Lentiviral vectors (LVVs) can transduce both dividing and nondividing cells and were shown to drive gene expression efficiently in various types of ‘stem’ cells. Gene delivery into HESCs by vectors derived from lentiviruses has the following

advantages: (1) lentiviral vectors efficiently transduce HESCs; (2) they integrate into the host-cell genome, thus promoting stable transgene expression; (3) transgene expression is not significantly silenced in undifferentiated HESCs as well as following differentiation; and (4) transduced HESCs retain their self-renewal and pluripotent potential. To improve vector biosafety and performance, all pathogenic coding sequences were deleted, resulting in a replication-defective vector. In addition, the proteins necessary for the early steps of viral infection (entering into the host cell, reverse transcription, and integration) were provided in *trans* by two additional plasmids: a packaging plasmid expressing the gag, pol, and rev genes, and an envelope plasmid expressing a heterologous envelope glycoprotein of the vesicular stomatitis virus (VSV-G). Third, a large deletion was introduced to abolish the viral promoter/enhancer activity. These steps resulted in a vector that could only undergo one round of infection and integration, a process termed transduction. Moreover, they minimized the risk of generation of wild-type HIV-1 by recombination.

Random chromosome integration of viral vectors poses the risk of insertional mutagenesis, oncogene activation, and cellular transformation. In addition, lentiviral vectors may not be suitable for transient transgene expression. Viral vectors derived from adenovirus and adeno-associated virus (AAV) have a much lower risk of insertional mutagenesis and have been tested in HESCs, but their transduction efficiencies were less satisfactory (26). The insect baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors have also been introduced as a type of delivery vehicle for transgene expression in mammalian cells (34). The virus can enter mammalian cells but does not replicate, and it is unable to recombine with preexisting viral genetic materials in mammalian cells. One significant advantage of using baculovirus AcMNPV as a gene delivery vector is the large cloning capacity to accommodate up to 30 kilobases (kb) of DNA insert, which can be used to deliver a large functional gene or multiple genes from a single vector.

1.2.4 Short- vs. Long-Term Expression

Gene transfer experiments can be subdivided into short-term (transient) and long-term (stable) expression systems. In transient expression, the foreign DNA is introduced into the cells and its expression is examined within 1–2 days. The advantage of this assay is its simplicity and rapidity. Furthermore, because the foreign DNA remains episomal, there are no problems associated with site of integration and the copy number of the transgene. Yet, it does not allow conducting experiments over long periods. Moreover, transfection efficiency usually does not exceed 20 %. For short-term induction, efficient transient expression can be

achieved through the insertion of supercoiled plasmid DNA rather than the linear form. Transient expression in HESCs usually peaks roughly 48 h after transfection, and frequently results in high expression levels attributed to the high copy number of plasmid DNA molecules that occupy the cell. During long-term assays, one isolates a clone of HESCs that has stably integrated the foreign DNA into its chromosomal genome. The major advantage of this method is the ability to isolate stable ES cell lines that have been genetically modified and can be grown indefinitely in culture. In this type of experiment, it is important to linearize the vector, leading to greater integration and targeting efficiencies. When the target gene is nonselectable, one must introduce also a positive selection marker under the regulation of a strong constitutive promoter. This can be performed either by cotransfecting the selectable marker on a separate vector, or as is frequently done, by fusing the selectable marker to the targeting vector. Selection should not be carried out immediately after transfection but at least 24 h later, giving the cells time to recover, integrate the foreign DNA and express the resistance conferring gene.

2 Materials

2.1 Tissue Culture (See Notes 1 and 2)

1. Knockout DMEM-optimized Dulbecco's modified Eagle's medium for ES cells (Life Technologies; cat. no. 10829-018).
2. DMEM 4.5 g/L glucose (Sigma, Dorset, UK; cat. no. D5796).
3. 1 *M* β-mercaptoethanol (Sigma; cat. no. M7522).
4. Nonessential amino acids 100× stock (Biological Industries, Kibutz Beit-Haemek, Israel; cat. no. 01-340-1B).
5. Insulin-transferrin-selenium 100× (Life Technologies; cat. no. 41400-045).
6. Bovine serum albumin (Sigma; cat. no. A-4919).
7. Mitomycin C (Sigma; cat. no. M-0503).
8. 0.1 % gelatin (Sigma; cat. no. G-1890).
9. Collagenase type V (Life Technologies; cat. no. 17104-019).
10. Hygromycin B (Sigma; cat. no. H-3274).
11. 6-thioguanine (Sigma; cat. no. A-4660).
12. Opti-MEM I (Life Technologies; cat. no. 31985-047)
13. TransIT-LT1 transfection reagent (Mirus).
14. TrypLE Select (Life Technologies, cat. no. 12563-011)
15. KnockOut SR-serum-free formulation (Life Technologies; cat. no. 10828-028).
16. Fetal calf serum (Biological Industries).

17. L-glutamine 100× stock (200 m*M*/L, Biological Industries; cat. no. 03-020-1).
18. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) 100× stock (Biological Industries; cat. no. 03 031-1B).
19. Human basic fibroblast growth factor (bFGF) stock solution (2 ng/μL) (human recombinant; Life Technologies; cat. no. 13256029).
20. Trypsin-EDTA: 0.25 % trypsin and 0.05 % EDTA (Biological Industries; cat. no. 03-052-1).
21. G418 (Geneticin; Sigma; cat. no. G-9516).
22. Hexadimethrine Bromide (polybrene) (Sigma H9268-5G).
23. Puromycin (Sigma; cat. no. P8833).
24. ROCK inhibitor Y-27632 (ATCC; cat. no. ACS3030)
25. Dimethylsulfoxide (DMSO; Sigma; cat. no. D-2650).
26. 10 m*M* β-mercaptoethanol: dilute 1:100 in PBS, filter, sterilize, and store at 4 °C.
27. 50× Mitomycin-C: dissolve 2 mg in 4 mL MEF medium, store in 4 °C.
28. bFGF solution: add 10 μg of bFGF solution to 5 mL of filter-sterilized 0.1 % bovine serum albumin dissolved in 1× PBS (with Ca^{2+}/Mg^{2+}), to give a final concentration of 2 μg/mL, store 1-mL aliquots in −20 °C.
29. 0.1 % gelatin solution: add 0.1 g of gelatin into a bottle containing 100-mL distilled water and autoclave immediately. The gelatin is dissolved while boiling in the autoclave, store at 4 °C.
30. MEF media: add to a 500-mL bottle of DMEM (high glucose and L-glutamine) 50-mL fetal calf serum, 2.5 mL penicillin/streptomycin, 5 mL Glutamine.
31. HESC medium: add to a 500-mL bottle of Knockout DMEM: 75 mL KnockOut SR, 6 mL nonessential amino acids, 6 mL glutamine (2 m*M*), 3 mL insulin-transferrin- selenium, 60 μL β-mercaptoethanol (0.1 m*M*), 3 mL penicillin/streptomycin, and 1 mL bFGF. ES media should be protected from light (*see* **Note 3**), and stored in 4 °C up to 1 month.
32. Collagenase solution: dissolve 10 mg of Collagenase type V in 5 mL serum-free DMEM (2 μM/1 mL working solution) and filter through a 0.2 μm filter under sterile conditions. Prepare fresh once a week. Store in 4 °C.
33. Freezing medium: add 1 mL of DMSO and 1 μL of ROCK inhibitor Y-27632 (10 μM stock) to 9 mL of appropriate HESC media. Media should be prepared fresh.
34. Leishman's stain (BDH, Poole, England) in 100 % methanol.

35. 293T cells medium: add to a 500-mL bottle of DMEM (high glucose and L-glutamine) 50-mL fetal calf serum, 2.5 mL penicillin/streptomycin, 5 mL Glutamine.

2.1.1 Equipment and Supplies for Tissue Culture

1. Laminar flow hood.
2. Humidified incubator set at 37 °C and 5 % CO_2.
3. Phase contrast microscope (objective range from 10× to 40×).
4. Liquid nitrogen storage tank.
5. Refrigerator (4 °C) and freezers (−20 °C, −70 °C).
6. 37 °C water bath.
7. Electroporator (Biorad, Gene Pulser II System).
8. Swing-out centrifuge for conical tubes (15- and 50-mL).
9. Cell counter.
10. Gene pulser cuvette 0.4 cm electrode gap (Bio-rad cat# 165-2088).
11. Pipetmen (2, 10, 20, 200, and 1,000 μL) designated for tissue culture use only.
12. Sterile forceps and scissors for dissecting mouse embryos.
13. Falcon tissue culture plates (100 × 20 mm) and 6-, 12-, and 24-multiwell trays (Falcon, Bedford, MA; cat. no. 353047, 353047, 353043, 353046).
14. Falcon 15-mL and 50-mL (Falcon; cat. no. 352097, 352098) polypropylene conical tubes.
15. Cryo vials (1.8-mL CryTube; Nunc, Roskilde, Denmark; cat. no. 363401).
16. Plastic pipets (1-, 2-, 5-, and 10-mL).
17. Tips for 2-, 10-, 20-, 200-, and 1,000-μL pipetmen.
18. Eppendorf tubes (1.5-mL).
19. Disposable filter unit FP 30/0.45 CA-S, 0.45 μm and 0.2 μm, cellulose acetate sterile (Whatman cat. no. 10462100 and 10462200, respectively).
20. Syringes sterile 20 mL.

2.2 Transfection

1. TransIT-LT1 Transfection reagent (Mirus).
2. Humidified incubator set at 37 °C, 5 % CO_2.
3. Tips for 2-, 10-, 20-, 200-, and 1,000-μL pipetmen.
4. 15-mL Falcon tubes.
5. Sterile eppendorf tubes (1.5-mL).
6. Opti-MEM I Reduced-Serum Medium (Life Technologies).

2.3 Infection

1. DMEM growth medium with 10 % FCS, and Glutamine (1 mg/mL), without penicillin/streptomycin.
2. 27 μL of TransIT-LT1 (Mirus).
3. Hexadimethrine Bromide (polybrene) 5 μL (8 mg/mL).
4. Humidified incubator set at 34 °C, 3 % CO_2.
5. Tips for 2-, 10-, 20-, 200-, and 1,000-μL pipettes.
6. 15-mL tubes.
7. Eppendorf tubes (1.5-mL).
8. Tissue culture plates

2.4 Colony Picking

1. HESC medium (*see* Section 2.1, **item 26**).
2. G418 (200 μg/mL).
3. Puromycin (0.5–1 μg/mL).
4. Hygromycin (100 μg/mL).
5. 6-Thioguanine (1 μg/mL).
6. 6-, 12-, and 24-well Falcon tissue culture plates (*see* Section 2.1.1, **item 11**).
7. Mouth apparatus consisting of an aspirator mouthpiece, tubing, and Pasteur pipette pulled on flame for collecting single colonies (*see* **Note 4**).

3 Methods

3.1 Tissue Culture (See Notes 5 and 6)

3.1.1 MEFs

The special growth conditions that are required for supporting undifferentiated growth of HESCs in culture rely mostly on the presence of inactivated fibroblasts, serving as a feeder layer. The feeder layer sustains undifferentiated growth by secreting unknown growth factors, and by serving as a growth matrix that allows the cells to adhere and grow as monolayer culture. So far, primary mouse embryonic fibroblasts (MEFs) were the most commonly used in the propagation and derivation of HESCs. However, STO cells (34), fetal muscle (35), foreskin fibroblasts (36, 37), and marrow cells (38) were also reported to be equally effective in supporting undifferentiated growth. The feeders are prepared only from early passage MEFs (up to passage 5). Their mitotic inactivation is carried out by the treatment with mitomycin-C (39), but can also be achieved through irradiation (40). Normally we prepare MEFs from 13.5-days-old ion cyclotron resonance (ICR) embryos. However, inactivated primary fibroblasts are required not only for routine maintenance of ES cells in culture, but also for stable transfection experiments, where drug selection is applied. Therefore, it is a prerequisite that feeder cells be resistant to the drug employed. For this purpose,

one must separately prepare MEFs from different strains of mice that bear resistance to the desired drug or alternatively, use feeders that carry multidrug-resistant genes by intercrossing between different strains. For instance, the transgenic strain of mice DR-4, expresses four different drug-selected genes and can be used for the production of MEFs, which confer resistance to G418, puromycin, Hygromycin, and 6-thioguanine drugs (41). The DR-4 strain, therefore, represents a suitable and an economical donor for the production of drug-resistant MEFs, and is especially advantageous for gene targeting experiments, which normally involve sequential selection for multidrug-resistant markers. There may be a significant variability between various batches of MEFs, with respect to their capacity for supporting undifferentiated proliferation of HESCs. To overcome this problem, the competence of different batches of MEFs to support undifferentiated growth can be assessed by testing their ability to maintain undifferentiated proliferation of mouse or primate ES cell lines before their use.

Isolation of MEFS

1. Coat plates with 0.1 % gelatin by incubation for 10 min at room temperature.
2. Collect 13.5-days-old fetuses from pregnant mice using sterile equipment: sacrifice pregnant mice and dissect the embryos by removing the uterus and transferring it into a sterile PBS-containing Petri dish.
3. Rinse twice in PBS and relocate all work to laminar flow hood.
4. Using sterile tweezers and scissors, remove the fetuses from the uterus, separate them from extraembryonic tissues (amniotic and yolk sacs) and transfer them to a clean Petri dish with PBS.
5. Count the number of collected fetuses and prepare, for later use, 1 × 10-cm gelatin-coated tissue culture dish for every three fetuses.
6. Remove head and internal parts (liver, heart, kidney, lung, and intestine) with sterile tweezers under a stereomicroscope.
7. Cut the remaining tissues into small pieces in a minimal volume of PBS (1–2 mL) and transfer into a sterile 50-mL Falcon tube.
8. Disaggregate the cell clumps obtained by passing them through a 5-mL syringe with an 18-gauge needle, no more than 10 times.
9. Add MEF media to reach 10 mL per three embryos, distribute cell suspension evenly into 10-cm tissue culture dishes and incubate.
10. Change media the following day. When plates are confluent (2–3 days after dissection) split 1:3 by trypsinization.

11. Change media (10 mL) every 2 days. When cell density reaches confluence, trypsinize the cells and freeze each 10-cm plate in one cryovial, store in liquid nitrogen.

Mitomycin-C Inactivation of MEFs

1. Thaw contents of one cryotube into 3× 10-cm culture dishes.
2. Grow the cells to confluence by changing the media every other day.
3. Further propagate the cells by splitting them twice at a 1:3 dilution (sums to 27 plates).
4. To inactivate the cells, add 40 μL of mitomycin-C stock solution (1 mg/mL) to 5 mL culture media (final concentration of 8 μg/mL) and incubate at 37 °C, 5 % CO_2, for 3 h.
5. Aspirate the mitomycin C-containing medium and wash the plates twice with 6 mL PBS.
6. Tripsinize cells by adding 1 mL of trypsin-EDTA and incubate at 37 °C, 5 % CO_2, for 5 min.
7. Add 5 mL medium and suspend the cells by vigorous pipetting.
8. Collect cell suspension into a 50-mL Falcon tube.
9. Centrifuge mitomycin-treated cell pool at 1,000 × *g* for 5 min.
10. Aspirate supernatant and add fresh medium to reach a final cell concentration of 4×10^6 cells/10-cm dish. Feeder plates can be stored in the incubator for 3–4 days, but should be examined under the microscope before use.
11. It is possible to freeze mitomycin-C treated MEFs and keep them for later use. For this purpose freeze 1.5–7 × 10^6 cells in each cryotube and later thaw and plate to give 1–5 × 10-cm dishes, respectively.

3.1.2 Maintenance of HESCs and Genetically Modified Clones

The maintenance of HESCs in culture relies on the continuous and selective propagation of undifferentiated cells. Controlling culture conditions and minimizing the effect of spontaneous differentiation, which constantly occurs, can achieve this. When passing the cells, care must be taken so that the cell number will not drop below a certain density, because this increases their tendency to differentiate, possibly from a lack of autocrine signaling. The differentiation status of the cultures should be followed daily by observation through a phase-contrast microscope. Undifferentiated colonies are easily recognized by their typical appearance, which includes small and equal-sized cells that are defined by a discrete border, pronounced nucleus and clear cellular boundaries. As differentiation begins, the cells at the periphery of the colonies lose their typical morphology. At that stage, splitting must be performed.

Subculture of HESCs

1. Aspirate medium from plate and rinse with PBS.
2. Replace with 1 mL serum-free DMEM containing collagenase type IV (2 mg/mL) per well.
3. Incubate at 37 °C in a 5 % CO_2 atmosphere for 40–60 min.
4. Add 1 mL growth medium and suspend the cells by gently pipetting.
5. Using a 2 mL or a 5 mL pipette, collect cell suspension from plate into a conical tube making sure to break up cell clumps by pipetting (colonies should be reduced to approximately 5–20 cells) (*see* **Note 7**).
6. Let cell clumps sink to the bottom of the tube for 10–15 min.
7. Remove medium with collagenase carefully, and resuspend with fresh media by splitting 1:2–1:3.
8. Plate on mitotically inactivated feeders prepared the previous day.
9. After 48 h, replace medium with fresh hESC medium.

Freezing HESCs

1. Collect HESCs and pellet them, as described in Section " Subculture of HESCs", **steps 1–4**.
2. Resuspend cells in an appropriate amount of growth media supplemented with 10 % DMSO and

   1 μL/1 mL ROCK inhibitor (10 μM stock) (*see* **Note 8**).
3. Mix the cells are gently by pipetting up and down and place in a properly marked cryotube.
4. Store at −70 °C in a low temperature vial container filled with isopropanol for at least 1 day.
5. For long-term storage, vials must be kept in liquid nitrogen.

Thawing HESCS (*See* **Note 9**)

1. Incubate the frozen cryovial in a 37 °C water bath until it is completely thawed.
2. Transfer and resuspend the cells with 5 mL growth media in a conical tube.
3. Pellet the cells by centrifugation at 1,000 × *g* for 5 min.
4. Resuspend again in an appropriate amount of fresh media with 1 μL/1 mL of ROCK inhibitor (10 μM stock) (*see* **Note 8**).
5. Plate cells and incubate overnight.

Mouse ES Cells Clonal Assay to Test Competence and Quality of KO-Serum Batch

Batch-to-batch variability in the competence of the KO-serum replacer to support undifferentiated proliferation may be remarkable. Clonal assays with mouse ES cells may be used to test the quality of the serum substitute batch before its use. An established culture of mouse ES cells is used as previously described (42) and all medium components should be those that will be used to culture the HESCs (*see* **Note 9**).

1. Trypsinize mouse ES cells and plate individual cells in pre-gelatinized 6-cm Petri culture dishes at a low density (1,000 cells per plate).
2. Culture either with the medium that was in current use or the new tested medium at 37 °C in a 5 % CO_2 atmosphere (*see* **Note 10**).
3. Change medium once on the fifth day after plating.
4. On the seventh day, rinse the cultures with PBS and stain for 5 min with 0.15 % Leishman's fix and stain.
5. Wash the stained cultures thoroughly with water and let them air-dry.
6. Compare the number of colonies per plate as well as the size and degree of differentiation and select the batch of serum with the best performance compared with the batch in use.

3.2 Transfection (See Table 1 and Fig. 1)

3.2.1 DNA Preparation for Transfection

1. Prepare DNA vector by any commonly used technique to obtain OD280/OD260 absorption ratio value of 1.8 or greater (*see* **Note 11**).
2. To linearize the vector by digesting it with the appropriate restriction enzyme.
3. Assess the completion of the restriction digest by electrophoresis of a small aliquot on a 1 % gel agarose.
4. Ethanol precipitates the DNA and resuspend in a small volume (20–50 μL) of TE or sterile water. Adjust concentration to 1 μg/μL.

Table 1
Transfection protocol timetable

Days	
1	Plate MEF-resistant cells
2	Split/thaw a vial of HESC to high density
4	Transfect HESCs (high density cultures of 8–32 cells/colony)
5	Begin selection
6–10	Change selection media every day
11–15	Change selection media every other day
16–18	Screen for resistant colonies Pick up selected colonies and plate them on MEF-resistant feeder in 1× 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in 1× 12-well twice Freeze and/or screen/further propagate in 1× 6-well trays

MEF mouse embryonic fibroblasts, *HESC* human embryonic stem cell

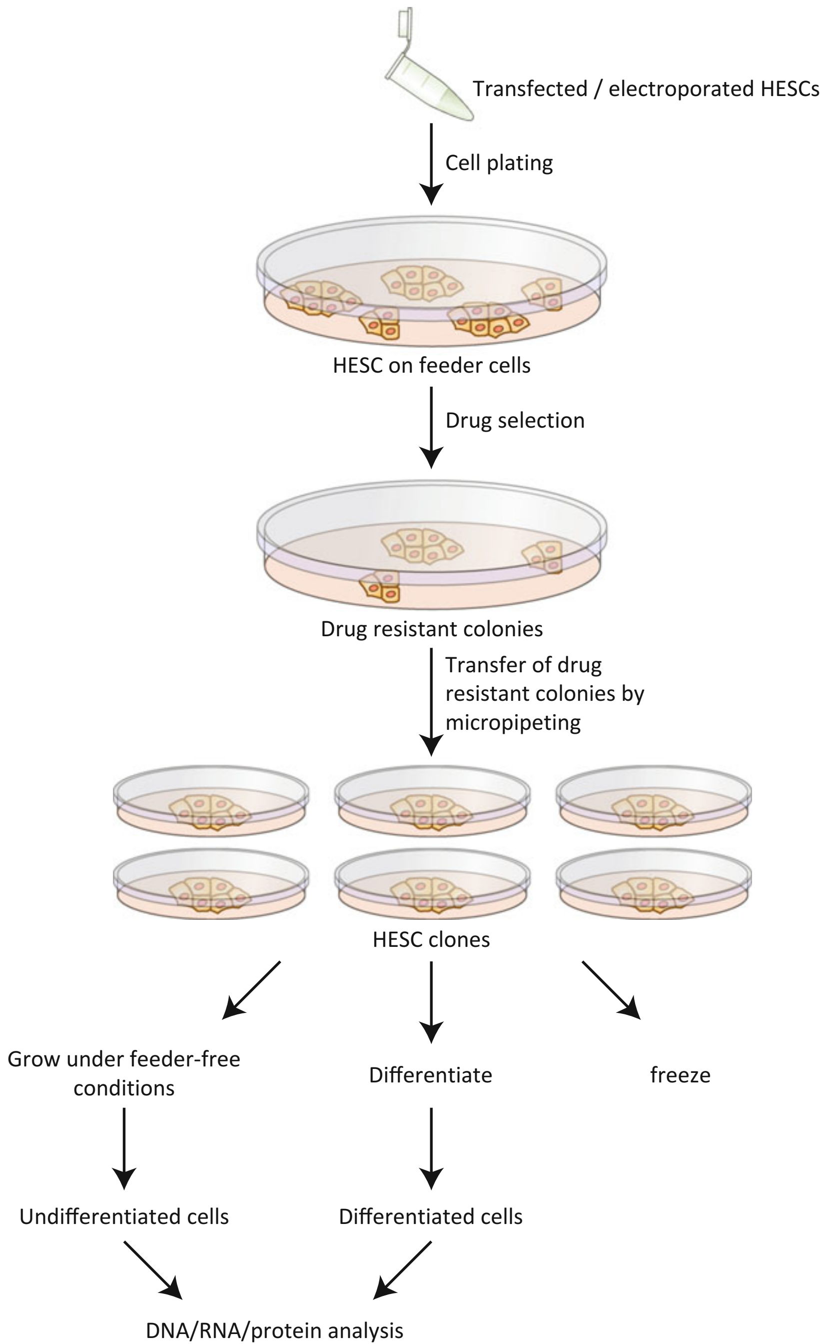

Fig. 1 Schematic illustration describing the methods for generating genetically modified HESCs by transfection

3.2.2 Preparing HESCs for Transfection

1. Grow healthy and undifferentiated cells and split (1:2 or 1:3) 2 days before transfection with Collagenase (*see* **Note 12**).
2. Collect HESC culture (70–80 % confluence) by Collagenase treatment into a 15 mL Falcon tube (*see* **Note 7**).

3. Let cell clumps sink to the bottom of the tube for 10–15 min.
4. Aspirate supernatant and gently rinse with PBS.
5. Centrifuge cells at 600 × *g* for 5 min and aspirate supernatant to obtain a cell pellet.

Transfection with Mirus (TransIT-LT1) Transfection Reagent (*See* **Note 14**)

1. Warm TransIT-LT1 reagent to room temperature and vortex gently before use.
2. For each well of a six-well tissue culture tray prepare a sterile tube containing 250 μL of Opti-MEM I.
3. Add 2.5 μL of DNA (1 μg/μL stock). Pipette gently to mix.
4. Add 7.5 μL TransIT-LT1 reagent to the diluted DNA mixture. Pipette gently to mix.
5. Incubate TransIT-LT1:DNA complex at room temperature for 15–30 min.
6. Add TransIT-LT1:DNA complex on cell pellet.
7. Resuspend cells with the transfection complex with fresh growth media without Pen-Strep (*see* **Notes 13–15**).
8. Plate on drug-resistant MEFs following a 1:3 split, and incubate for 24–48 h.
9. Change to fresh media with Pen-Strep and appropriate selection drug.
10. Change drug containing HESC media once a day (5 days) and then every other day for a period of approximately 10 days, until resistant HESCs colonies begin to appear.

Electroporation (Essentially According to Zwaka and Thomson)

1. Grow healthy and undifferentiated cells in a 6-well tray until they reach cell density greater than 70 % confluence (*see* **Note 12**).
2. Trypsinize cells to collect clumps of undifferentiated HESC by adding 0.5 mL per well of TrypLE for 5 min (*see* **Note 16**).
3. Add 1 mL HESC growth medium to each well.
4. Collect cell suspension into a 15 mL Falcon tube.
5. Centrifuge cells at 600 × *g* for 5 min.
6. Aspirate supernatant and gently resuspend in 0.8 mL of HESC fresh media, containing 20–30 μg linearized DNA vector, to reach a final cell concentration of $1–3 \times 10^7$/0.8 mL.
7. Transfer cell/DNA mix into precooled 0.4 cm cuvettes.
8. Electroporate cells using the following parameters: 320 V, 250 μF. The time constant should be between 9.0 and 13.0 (*see* **Note 17**).
9. Immediately after electroporation, allow cells to recover by standing in the cuvette on ice for 10 min
10. Transfer contents, using 1 mL glass pipette, into 15 mL tube containing 2 mL of prewarmed HESC media.

11. Pellet cells by centrifugation of 600 × *g* for 5 min.
12. Aspirate supernatant and gently resuspend pellet in 10 mL HESC media in the presence of 1 μL/1 mL of ROCK inhibitor (10 μM stock).
13. Plate cells on to two 10 cm culture dishes pre-seeded with 2.5×10^6 inactivated MEF feeders and return to incubator.
14. The following day remove cell debris by washing twice with PBS and then add fresh HESC media.
15. Apply selection the following day (day 2 post electroporation).
16. Change drug containing HESC media once a day (5 days) and then every other day.

3.3 Infection (See Table 2 and Fig. 2)

3.3.1 Retrovirus/Lentivirus Production

1. Plate 293T cells in 10 mm tissue culture dish (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, glutamine, Pen-Strep) 24 h before transfection so that they are 80 % confluent for transfection.
2. Cotransfected 293T cells with 3 μg retroviral/lentiviral vector, 2 μg packaging plasmid, 1 μg VSV-G expression vector and 18 μL TransIT-LT1 (Mirus) per plate according to the suppliers conditions. Transfection of the cells has to be done in medium without antibiotics.
3. After 24 h change medium to full medium (with antibiotics).

Table 2
Infection protocol timetable

Days	
1	Plate 293T cells 2×10^6 cells per plate
2	Transfect the 293T cells with the viral vectors (TransIT-LT1) Split/thaw a vial of HESC to high density
3 4	Change the medium of the 293T cells Filter the viral supernatant (48 h) and infect the HESCs Add new medium to the 293T cells
5	Filter the viral supernatant (72 h) and infect the HESCs
6–10	Change selection media every day
11–15	Change selection media every other day
16–18	Screen for resistant colonies Pick up selected colonies and plate them on MEF-resistant feeder in 1× 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in 1× 12-well twice Freeze and/or screen/further propagate in 1× 6-well trays

MEF mouse embryonic fibroblasts, *HESC* human embryonic stem cell

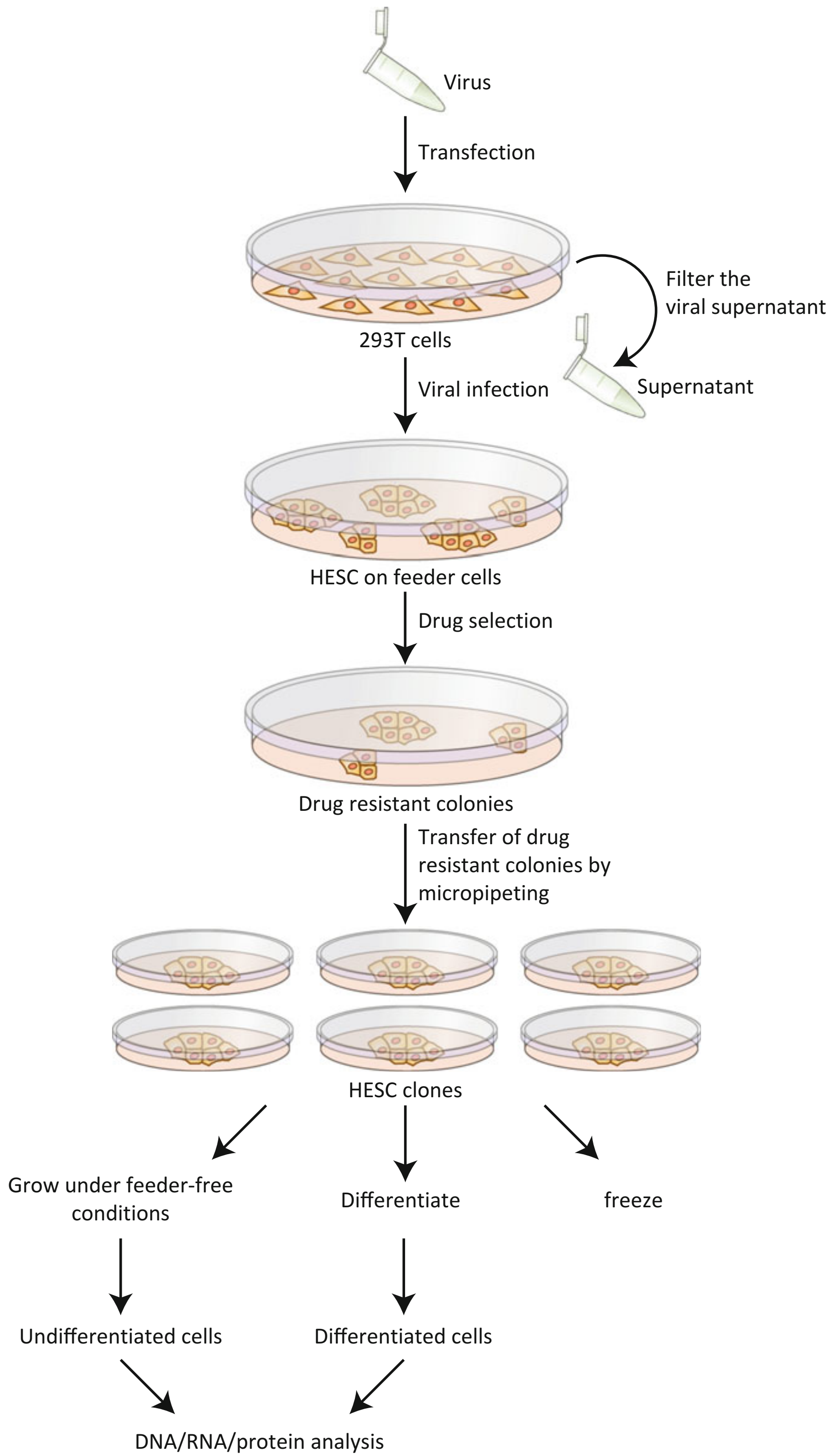

Fig. 2 Schematic illustration describing the methods for generating genetically modified HESCs by infection

4. Collect virus supernatant from all plates 48 h and 72 h after transfection with plastic pipettes and filter supernatant through a 0.45 μm filter.

3.3.2 Retroviral and Lentiviral Gene Transfer into Human ES Cells

1. Cultivate HESCs cultures on mouse embryo fibroblast feeder cells (MEF) or on matrigel in basic fibroblast growth factor (bFGF) supplemented MEF conditioned medium.
2. Plate 1×10^5 HESCs on a tissue culture plate pretreated with Matrigel or Gelatin and MEF attached cells. In the case of Matrigel add MEF conditioned medium supplemented with bFGF (4 ng/mL) to keep the HESCs undifferentiated.
3. Collected and filtered the viral supernatant, after 48 h of cells transfection, together with 6 μg/mL Hexadimethrine Bromide (polybrene).
4. Culture the cells with the virus for 24 h, wash three times with PBS, and then add fresh media or the 72 h viral supernatant, for another 24 h in order to increase the infection efficiency.
5. On day 3 after infection, measure for transgene activity and continue the culture on MEFs or matrigel.

3.4 Colony Picking and Expansion

After 10–12 days in selection media, individual HESC-resistant clones become visible and are big enough to be isolated for expansion.

1. Screen transfected culture plates using an inverted microscope for the presence of resistant clones and mark their location at the bottom of the dish.
2. Manually pick selected HESC colonies (*see* **Note 18**).
3. Disconnect the cell colony from the feeders by dissociating it into small cell pieces using the sharp edge of the glass micropipette while collecting them by aspiration into the tip of the pipette.
4. Plate the small cell clumps on fresh drug-resistant feeder layer, in a single well of a 24-well culture tray and return to incubator for further growth. The replated cell clumps, which have originated from a single cell clone, give rise to round flat colonies with well-defined borders in 3–5 days, while changing the selection media as necessary (*see* **Notes 18–20**).
5. Scale up the clone population by splitting 1:2 with trypsin, twice.
6. When the wells (2×12-well) are approaching confluence, freeze each well in individual cryovial. The remaining cells can be either further expanded (Fig. 3c), by splitting 1:4 or directly used for DNA, RNA, or protein extraction (*see* **Note 18**) (Table 1).

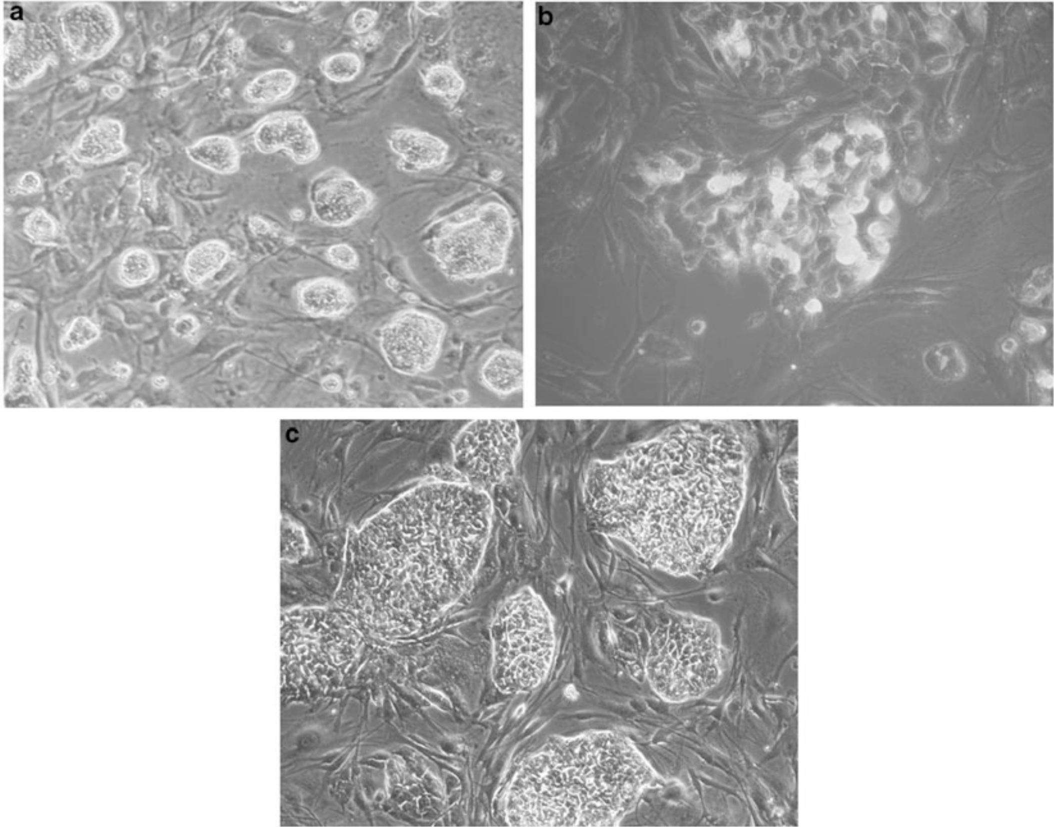

Fig. 3 (**a**) Human embryonic stem (HESC) cell culture on day of transfection. The culture should be composed of many small (8–32 cells) colonies. (**b**) Transient expression of CMV-EGFP in HESCs after 48 h to transfection. (**c**) Established cell line of HESCs after transfection, selection, and clonal expansion of genetically modified cells

4 Notes

1. Section 2.1, **items 1–14** are stored at 4 °C, **items 15–24** at −20 °C, and **item 25** at room temperature. As a rule, all tissue culture protocols must be performed under sterile conditions, in a laminar flow hood, using sterile disposable plastics and clean, detergent-free, glassware.
2. Media should be stored in 4 °C and can be used for up to 1 month.
3. Serum replacement is sensitive to light. Protect supplemented HESC media by covering it with aluminum foil.
4. The mouth-controlled device is the same as the one that is commonly used for handling oocytes and preimplantation embryos in mice. The mouthpiece is available as a part of an aspiration tube assembly from Drummond (model no. 2-000-0001). Sterile glass Pasteur pipettes are pulled on a flame to

create long tubing with a narrow opening. Soften the glass tubing by rotating it in a fine flame until the glass becomes soft. Then, withdraw the glass quickly from the heat and pull both ends smoothly to produce a tube with an internal diameter of about 200 μm. Neatly break the tube and fire polish its tip by quickly touching the flame.

5. All tissue culture procedures are performed under sterile conditions, using prewarmed media and gelatin-precoated plates.
6. As in other cell lines growing in vitro, chromosomal aberrations may occur. Working with cells of low passage number can minimize this. Thus, it is advisable to monitor the karyotype of the cells following prolonged growth in culture and subsequent to stable transfection.
7. It is essential that the HESCs remain as small cell clumps (5–10 cells). Avoid dissociation of the HESCs to single cells when splitting.
8. ROCK Inhibitor Y27632 is a selective inhibitor of the Rho-associated kinase p160ROCK. Treatment with ROCK Inhibitor Y27632 prevents dissociation-induced apoptosis of human embryonic stem cells (HESCs), increasing the survival rate and maintaining pluripotency during freezing and thawing of HESCs.
9. Cell thawing must be performed as quickly as possible.
10. The culture medium is supplemented with 10 % of the tested batch of knockout serum substitute (instead of 15 %) and mouse recombinant LIF at 1,000 U/mL.
11. The purity of the DNA is very critical for successful transfection.
12. The cells should be transfected during the lag phase of cell division. The transfection rate is most efficient when the cell density reaches 50–70 % and the colonies are small (8–32 cells per colony) (Fig. 3a). The colonies should have discrete borders and be composed of similar sized cells, with a pronounced nucleus.
13. Antibiotics will inhibit transfection complex formation and therefore should be excluded from the HESC growth media until the following day.
14. Alternatively, transfection complexes can be added directly to the cells as they grow in culture. However, this may reduce transfection efficiency.
15. In parallel to the experiment, one may consider to carrying out transient transfection on a small number of cells with a construct carrying a constitutive expressed reporter gene, such as CMV-EGFP, to assess transfection efficiency before applying selection (Fig. 3b).

16. For electroporations, it is necessary to dissociate cells to single cells suspension. Therefore, it is essential to trypsinize the cells with TrypLE and then resuspend them with media supplemented with ROCK inhibitor (1 μL/1 mL of ROCK inhibitor from a 10 μM stock) to prevent from cell death associated with colony dissociation.
17. There are various apparatuses that can be applied for electroporation in HESCs. Therefore, electroporation parameters may change and must be adjusted accordingly.
18. The colonies are picked up by the aid of a mouth apparatus connected to a sterile pulled and fire polished Pasteur pipet, as is commonly used for handling oocytes and preimplantation embryos (*see* **Note 4**).
19. We find this pickup method more suitable and efficient for isolating single HESC colonies than the method applied in mouse, where individual ES colonies are collected with a disposable tip, trypsinized, and then plated.
20. In some cases, it is crucial that no feeders will be present during the screen. For this purpose, cells must be propagated in feeder-free culture conditions, for at least one passage. Under such conditions the cells must be grown on vitronectin or matrigel-coated plates, preventing from differentiation and consequently culture loss.

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