Chapter 21

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 initiated in human ES (HESC) cells. 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.

Key words: Human ES cells, genetic manipulation, transfection, electroporation, infection, overexpression, targeted mutagenesis, homologous recombination, knock-down.

1. Introduction

1.1. Genetic Modification Approaches and Their Potential Applications

There are basically four types of strategies that can be applied for genetic engineering of HESCs: overexpression, knockout, knock-in, and knock-down experiments.

1.1.1. Overexpression

Overexpression of genes is usually based on random integration of an exogenous DNA sequence into the genome. It can be applied for constitutive or facultative expression of either cellular or foreign genes. It may also be used for the introduction of reporter or

K. Turksen (ed.), *Human Embryonic Stem Cell Protocols,* Methods in Molecular Biology 584,
DOI 10.1007/978-1-60761-369-5_21,

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), which can be selected for by fluorescent-activated cell sorter (FACS), or a drug-resistance gene (1, 2). Indeed, transgenic fluorescent reporters driven by tissue-specific promoters have been successfully employed to identify various HESCs-derived cell types including neurons (3), cardiomyocytes (4) as well as hepatic and pancreatic committed cells (5, 6). Likewise, the introduction of selectable reporters under the regulation of an inner cell mass-specific promoter may allow the selection for or against undifferentiated cells in culture. This has been previously demonstrated by introduction of EGFP (enhanced GFP) into HESCs under the regulation of both *OCT4* (7) and *Rex1* (8), resulting in the production of green glowing cells only when they are in an undifferentiated state (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 (9–11). In fact, it was possible to show that ectopic expression of HOXB4, a master regulator in blood cell differentiation, can drive HESCs to commit into hematopoietic lineages as they differentiate in vitro (*12*).

Random integration of promoter-driven transgenes may 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 either by disrupting both copies of the gene or by downregulating its activity *in trans*.

1.1.2. Knockout

The most widely used technique for inactivating genes in ES cells is site-directed mutagenesis. This procedure 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. 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 (14). 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. One example for generating a HESC-based disease model by homologous recombination is the targeting of the X-linked gene hypoxanthine phosphoribosyl transferase 1 *(HPRT1)*, which when mutated is responsible for the development of Lesch-Nyhan syndrome. This was performed by introducing a large deletion at the *HPRT1* locus in HESCs of an XY karyotype. The resulting cell lines recapitulate the major biochemical defect that characterizes Lesch-Nyhan affected individuals, which involves the accumulation of uric acid (*15)*. Thus, these cells should be valuable for basic research, but more importantly for exploration of new gene therapy-based treatments and drug discovery.

1.1.3. Knock-In

Similar to the knockout strategy, it is possible to generate clones of HESCs in which the gene of interest is deleted by inserting a promoterless reporter gene through homologous recombination. The method, termed knock-in, allows the positioning of a reporter gene under the regulation of a native gene. Therefore, it can be applied to monitor the expression of a target gene in situ during ES cell differentiation. Accordingly, Zwaka and Thomson have created human knock-in ES cell lines that express either GFP or a neomycin-resistance gene under the regulation of the endogenous OCT4 promoter (16). The *OCT4* gene encodes for a transcription factor that is specifically expressed by pluripotent stem cells. Thus, by replacing *OCT4* with such reporters, the authors were able to monitor and select for undifferentiated HESCs in culture. Likewise, GFP cDNA was inserted into the locus of *MIXL1*, which is a developmentally regulated gene that is transiently expressed in the primitive streak during embryogenesis (*17)*. In this case, the reporter knock-in reflected the expression of the endogeneous *MIXL1*, enabling to identify primitive streak-like cells and isolation of primitive hematopoietic precursors from differentiating HESCs.

The relative ease by which ES cells can be genetically manipulated has made them particularly useful for 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 (18)). As opposed to targeted mutagenesis, 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 a wide variety of mutations.

Finally, the recent identification of the human homolog of the mouse *Rosa26* locus has facilitated in the generation of *Cre*-mediated HESC lines that can be utilized for inserting transgenes into a broadly expressed locus. The importance of this system is that it overcomes problems of gene silencing as well as gene disruption through insertional mutagenesis. Moreover, it does not require drug selection and thus may be useful for therapeutic applications in cases where drug-resistance cassettes are not desirable.

1.1.4. Knock-Down

Downregulation of particular genes can also be achieved by overexpressing specific RNA molecules that inhibit the activity of a given gene through the generation of small interfering RNA molecules (siRNAs). Because siRNAs operate *in trans* and are not involved in the modification of the targeted gene, it is relatively simple to achieve transient or conditional gene silencing using this method. The use of RNA interference (RNAi) was demonstrated to be feasible in mouse ES cells to inactivate genes and shown to be equally effective as the knockout models in the generation of null mutant embryos (*19*). Downregulation by RNAi in HESCs was demonstrated for the *HPRT*, *β2-microglobulin*, *OCT4*, *SOX2*, and other genes (20, 21). Applications of this loss-of-function approach will have widespread use, not only to study developmental roles of specific genes in human, but also for their utility in modulating HESC differentiation in vitro.

1.2. Methods for Genetic Manipulation

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 the selection system. However, the most important factor is the transfection method. 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.

1.2.1. Transfection

Transfection is probably the most commonly used method for introducing transgenes into HESCs (**Fig. 21.1**). 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. The first study to describe stable

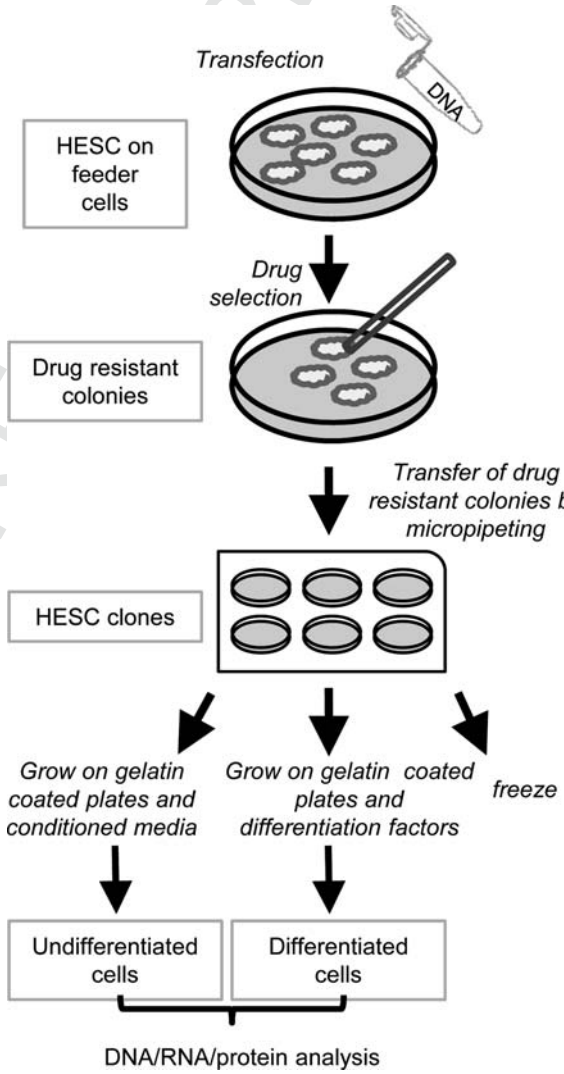

Fig. 21.1. Schematic illustration describing the methods for generating genetically modified HESCs by transfection.

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 (8). 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 $1{:}10^{-5}$–10^{-6} (*8*). Since then, other chemical-based transfection methods have been found to be equally effective. The calcium phosphate precipitation method is a widely used method for transfecting many different cell types. It is also based on negatively charged molecules that interact with DNA to form precipitates that are incorporated by the cells. The calcium phosphate transfection system seems to be slightly more efficient in gene delivery in comparison to ExGen 500. Lipofectamine 2000 reagent is a positively charged cationic lipid compound that forms small unilamellar liposomes and was recently shown to be useful in obtaining transient and stable transfections in HESCs as well (*20, 21*).

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 approximately $1{:}10^{-5}$ (*16*). 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 (22). 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 (**Fig. 21.2**). 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 (23–26). 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

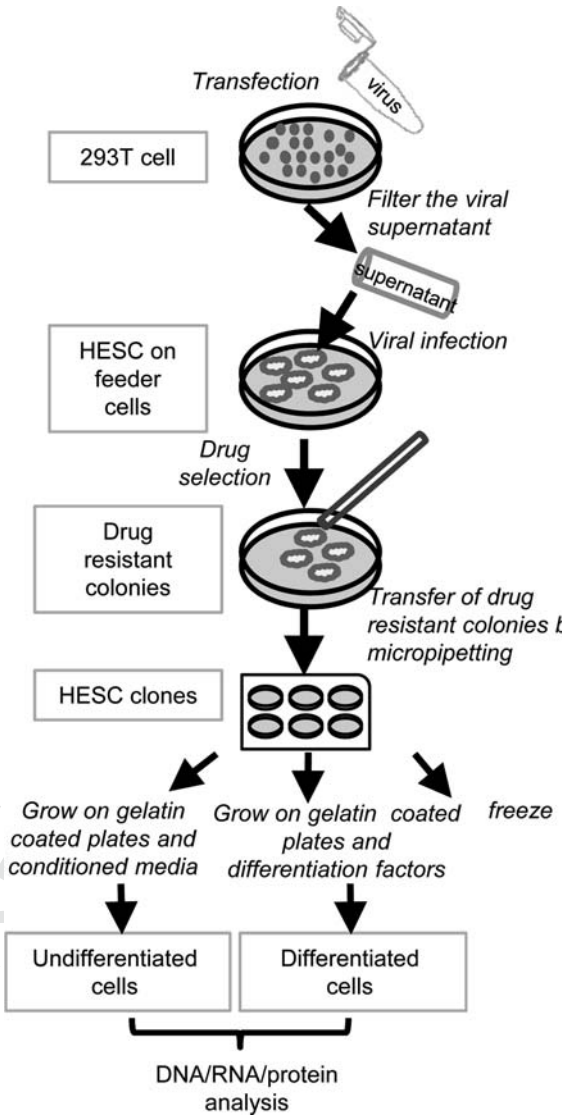

Fig. 21.2. Schematic illustration describing the methods for generating genetically modified HESCs by infection.

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. These vectors are derived from lentiviruses, a group of complex retroviruses that cause slow chronic immunodeficiency diseases in humans and animals. 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.

In order to improve vector biosafety and performance, first all pathogenic coding sequences were deleted from the vector, resulting in a replication-defective vector containing only the transgene and several essential regulatory viral sequences, such as the encapsidation signal and the viral LTR. Second, 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 into the U3 region of the viral LTR abolishing the viral promoter/enhancer activity. The self-inactivating (SIN) vector that was generated contained a heterologous internal promoter driving the expression of the transgene (27, 28). 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.

Human immunodeficiency virus (HIV)-1-based lentiviral vectors were the first viral vectors used to genetically engineer HESCs. However, because of the severe pathogenic effects of HIV-1 replication in humans, the potential emergence of replication-competent retrovirus from HIV-1-based vectors raises concerns over their use in clinical settings. In addition, random chromosome integration of lentiviral 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 adenoassociated virus (AAV) have a much lower risk of insertional mutagenesis and have been tested in HESCs, but their transduction efficiencies were less satisfactory (25).

The insect baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors have recently been introduced as a new type of delivery vehicle for transgene expression in mammalian cells (29). The virus can enter mammalian cells but does not replicate, and it is unable to recombine with pre-existing 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 (Gibco BRL, Carlsbad, CA, USA; 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 100X stock (Biological Industries, Kibutz Beit-Haemek, Israel; Cat. No. 01-340-1B).
5. Insulin-transferrin-selenium 100X (Gibco BRL; 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. Hygromycin B (Sigma; Cat. No. H-3274).
10. 6-Thioguanine (Sigma; Cat. No. A-4660).
11. Knockout SR – serum-free formulation (Gibco BRL; Cat. No. 10828-028).
12. Fetal calf serum (Biological Industries).
13. l-Glutamine 100X stock (200 mM/L, Biological Industries; Cat. No. 03-020-1).
14. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) 100X stock (Biological Industries; Cat. No. 03 031-1B).
15. Human basic fibroblast growth factor (bFGF) stock solution (2 ng/μL) (human recombinant; Gibco BRL; Cat. No. 13256029).
16. Trypsin-EDTA: 0.25% trypsin and 0.05% EDTA (Biological Industries; Cat. No. 03-052-1).
17. G418 (Geneticin; Sigma; Cat. No. G-9516).
18. Puromycin (Sigma; Cat. No. P8833).
19. Dimethylsulfoxide (DMSO; Sigma; Cat. No. D-2650).
20. 1X phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}. For 1 L, mix 3.58 g sodium phosphate ($Na_2HPO_4 \bullet 12H_2O$), 0.24 g potassium phosphate monobasic (KH_2PO_4), 8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), in a final volume of 1 L of double-distilled water (ddH_2O). Aliquot 200 mL solution per bottle and autoclave; store at room temperature.
21. 10 mM β-mercaptoethanol: dilute 1:100 in PBS, filter, sterilize, and store at 4°C.
22. 50X Mitomycin-C: dissolve 2 mg in 4 mL MEF medium and store at 4°C.
23. bFGF solution: add 10 μg of bFGF solution to 5 mL of filter-sterilized 0.1% bovine serum albumin dissolved in 1X PBS (with Ca^{2+}/Mg^{2+}), to give a final concentration of 2 μg/mL; store 1-mL aliquots at –20°C.
24. 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.
25. 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.
26. HESC medium: add to a 500-mL bottle of knockout DMEM, 75 mL knockout SR, 6 mL nonessential amino acids, 6 mL glutamine (2 mM), 3 mL insulin-transferrin-selenium, 60 μL

β-mercaptoethanol (0.1 mM), 3 mL penicillin/streptomycin, and 1 mL bFGF. ES media should be protected from light (*see* **Note 3**) and stored at 4°C up to 1 month.

27. Freezing medium: add 1 mL of DMSO to 9 mL of appropriate media (either hES or MEF media). Media should be prepared fresh.
28. Leishman's stain (BDH, Poole, England) in 100% methanol.
29. 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.
30. FuGENE 6 (Roche 11-988-387).
31. Hexadimethrine bromide (polybrene) (Sigma H9268-5G).
32. Laminar flow hood.
33. Humidified incubator set at 37°C and 5% CO_2.
34. Phase-contrast microscope (objective range from ′10 to ′40).
35. Liquid nitrogen storage tank.
36. Refrigerator (4°C) and freezers (–20°C, –70°C).
37. 37°C water bath.
38. Electroporator (Biorad, Gene Pulser II System).
39. Swing-out centrifuge for conical tubes (15- and 50-mL).
40. Cell counter.
41. Gene pulser cuvette 0.4-cm electrode gap (Bio-rad Cat. No. 165-2088).
42. Pipetmen (2, 10, 20, 200, and 1000 μL) designated for tissue culture use only.
43. Sterile forceps and scissors for dissecting mouse embryos.
44. Falcon tissue-culture plates (100 .′20 mm) and 6-, 12-, and 24-multiwell trays (Falcon, Bedford, MA; Cat. Nos 353047, 353047, 353043, 353046).
45. Falcon 15-mL and 50-mL (Falcon; Cat. Nos 352097, 352098) polypropylene conical tubes.
46. Cryo vials (1.8-mL CryTube; Nunc, Roskilde, Denmark; Cat. No. 363401).
47. Plastic pipettes (1, 2, 5, and 10 mL).
48. Tips for 2-, 10-, 20-, 200- and 1000-μL pipetmen.
49. Eppendorf tubes (1.5 mL).
50. Disposable filter unit FP 30/0.45 CA-S, white rim 0.45 μm, cellulose acetate sterile (whatman Ref No. 1046200).
51. Syringes sterile 20 ml.

2.2. Transfection

1. 2X HBS: 50 mM HEPES and 280 mM NaCl; dissolve 1.57 g NaCl and 1.19 g HEPES in approx 80 mL sterile ddH_2O. Adjust pH to 6.8 and bring to a final volume of 100 mL with ddH_2O. Filter-sterilize and store in 15-mL aliquots at 20°C.
2. 70 mM Na_2HPO_4: dissolve 2.5 g of $Na_2HPO_4 \bullet 12H_2O$ in 100 mL of ddH_2O. Filter-sterilize and store in 15-mL aliquots at –20°C.
3. Transfection buffer: mix 485 µL of 2X HBS with 15 µL of 70 mM Na_2HPO_4.
4. 2 *M* $CaCl_2$: dissolve 27.75 g $CaCl_2$ in ddH_2O to a final volume of 100 mL. Filter-sterilize and store 15-mL aliquots at −20°C.
5. Humidified incubator set at 34°C, 3% CO_2.
6. Tips for 2-, 10-, 20-, 200- and 1000-µL pipetmen.
7. 10-mL tubes.
8. Eppendorf tubes (1.5-mL).
9. ExGen 500 (Fermentas, Hanover, MD, USA; Cat. No. R0511).
10. Vortex.
11. Swing out centrifuge for microplates.

2.3. Infection

1. DMEM growth medium with 10% FCS and glutamine (1 mg/mL), without penicillin/streptomycin.
2. FuGENE 6 27µl (Roche).
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 1000-µL pipettes.
6. 10-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)

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 a monolayer culture. So far, primary mouse embryonic fibroblasts (MEFs) were the most commonly used in the propagation and derivation of HESCs. However, STO cells (30), fetal muscle (31), foreskin fibroblasts (32, 33), and marrow cells (34) 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 (35), but can also be achieved through irradiation (36). Normally, we prepare MEFs from 13.5-day-old ion cyclotron resonance-derived 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 (37). 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.

3.1.1. Isolation of MEFS

1. Coat plates with 0.1% gelatin by incubation for 1 h at room temperature.
2. Collect 13.5-day-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, 1X 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.

3.1.2. Mitomycin-C Inactivation of MEFS

1. Thaw contents of one cryotube into 3X 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-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 1000*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 $1.5–7 \times 10^6$ cells in each cryotube and later thaw and plate to give 1–5X 10-cm dishes, respectively.

3.1.3. 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 (*see* **Note 7**).

3.1.4. Subculture of HESCs

1. Remove culture media and rinse with 6 mL PBS.
2. Add 1 mL of trypsin-EDTA and incubate for 5 min.
3. Add 5 mL growth medium and suspend the cells by vigorous pipetting.
4. Collect suspension into a conical tube and pellet by centrifugation 1000*g* for 5 min.
5. Resuspend with fresh media and plate on mitotically inactivated feeders prepared the previous day.

3.1.5. Freezing HESCs

1. Trypsinize HESCs and pellet them, as described in **Section 3.1.2.1**, **Steps 1–4**.
2. Resuspend cells in an appropriate amount of growth media supplemented with 10% DMSO.
3. Mix the cells 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.

3.1.6. Thawing HESCs (see Note 8)

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 1000*g* for 5 min.
4. Resuspend again in an appropriate amount of fresh media.
5. Plate cells and incubate overnight.

3.1.7. 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 (38) and all medium components should be those that will be used to culture the HESCs (*see* **Note 9**).

1. Trypsinize mouse ES cells (38) and plate individual cells in pregelatinized 6-cm petri culture dishes at a low density (1000 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.
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

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 10**) (*see* **Fig. 21.1** and **Table 21.1**).
2. 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.

3.2.2. Growing HESCs for Transfection

1. Split (1:2 or 1:3) a morphologically undifferentiated and confluent HESC cell culture 2 days before transfection (*see* **Note 11**).

Table 21.1
Transfection protocol timetable

Days	
1	Plate MEF resistant cells
2	Split/thaw a vial of HESCs 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 1X 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in 1X 12-well twice Freeze and/or screen/further propagate in 1X 6-well trays

MEF, mouse embryonic fibroblasts; HESC, human embryonic stem cell.

3.2.3. Transfection by Calcium Phosphate

1. Harvest HESCs and split 1:4 into 10-cm culture dishes containing MEFs that were plated the previous day (*see* **Note 12**).
2. Prepare for each 10-cm plate transfection buffer and DNA in separate tubes. Dilute 10–20 μg of DNA in 240 mM $CaCl_2$ by bringing the DNA to a final volume of 0.5 mL with DDW and then slowly adding 60 μL of 2 M $CaCl_2$ (and not the reverse order).
3. Add very slowly the DNA solution (one to two drops/s) to the transfection buffer, while gently mixing by generating small air bubbles with a sterile disposable tip.
4. Incubate for 10 min at room temperature (*see* **Note 13**).
5. Add the 1-mL solution dropwise onto the cells without swirling or rotating the dish.
6. Incubate at 34°C, 3% CO_2, for 4 h and then change the growth media by aspirating it and washing twice with PBS. Add fresh media and return to the incubator.
7. Apply selection the following day by adding the appropriate drug to the growth media.
8. Refeed the cells with selection media when the medium starts to turn yellow, usually every day during the first 5 days and then every other day. By days 10–12 of selection, colonies should visible and large enough to be picked for further expansion and analysis.

3.2.4. Transfection by Exgen 500 (see Note 14)

1. Two days before transfection by Exgene 500, harvest and split HESCs into 6-well trays containing inactivated and drug-resistant MEFs.
2. About 1 h before transfection, change the growth media by rinsing the cells with PBS and adding 1 mL of fresh media to each well.
3. For each well of a 6-well tissue culture tray, prepare a tube containing 2 μg of DNA to a final volume of 50 μL of 150 mM NaCl and vortex.
4. In a separate tube, mix 10 μL ExGen 500 to 40 vL of 150 mM NaCl and vortex.
5. Mix DNA and transfecting agent by rapidly adding diluted ExGen 500 to DNA (not the reverse order). Vortex-mix the solution immediately for 10 s and then incubate for 10 min at room temperature.
6. Add 100 μL of ExGen/DNA mixture to each well.
7. Gently rock the plate back and forth to equally distribute the complexes on the cells.
8. Centrifuge culture trays immediately for 5 min at 280*g*.
9. Incubate at 37°C, 5% CO_2, for 30 min.
10. Wash twice with PBS and return to incubator (*see* **Note 15**).

3.2.5. Electroporation (Essentially according to Zwaka and Thomson)

1. Grow healthy and undifferentiated cells in a 10-cm culture dish until they reach cell density greater than 70% confluence.
2. Trypsinize cells to collect clumps of undifferentiated HESCs by adding 1 ml of trypsin-EDTA for 3–5 min.
3. Add 10 ml HESC growth medium.
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 hES 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 pre-cooled 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.
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 a 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.

13. Plate cells onto two 10-cm culture dishes preseeded 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 postelectroporation).
16. Change drug-containing HESC media once a day (5 days) and then every other day.

3.3. Infection

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, PenStrep) 24 h before transfection so that they are 80% confluent for transfection (*see* **Fig. 21.2** and **Table 21.2**).

Table 21.2
Infection protocol timetable

Days	
1	Plate 293T cells 2×10^6 cells per plate
2	Transfect the 293T cells with the viral vectors (FUGENE 6) Split/thaw a vial of HESCs to high density
3	Change the medium of the 293T cells
4	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 1X 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF-resistant feeder in 1X 12-well twice Freeze and/or screen/further propagate in 1X 6-well trays

MEF, mouse embryonic fibroblasts; HESC, human embryonic stem cell.

2. Cotransfect 293T cells with 3 μg retroviral/lentiviral vector, 2 μg packaging plasmid, 1 μg VSV-G expression vector, and 18 μl FuGENE 6 (Roche) 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).
4. Collect virus supernatant from all plates 48 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 16**).
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* **Note 17**).
5. Scale up the clone population by splitting 1:2 with trypsin, twice.
6. When the wells ($2 \times$ 12-well) are approaching confluence, freeze each well in individual cryovials. The remaining cells can either be further expanded (**Fig. 21.3c**), by splitting 1:4, or directly used for DNA, RNA, or protein extraction (*see* **Note 18**) (**Table 21.1**).

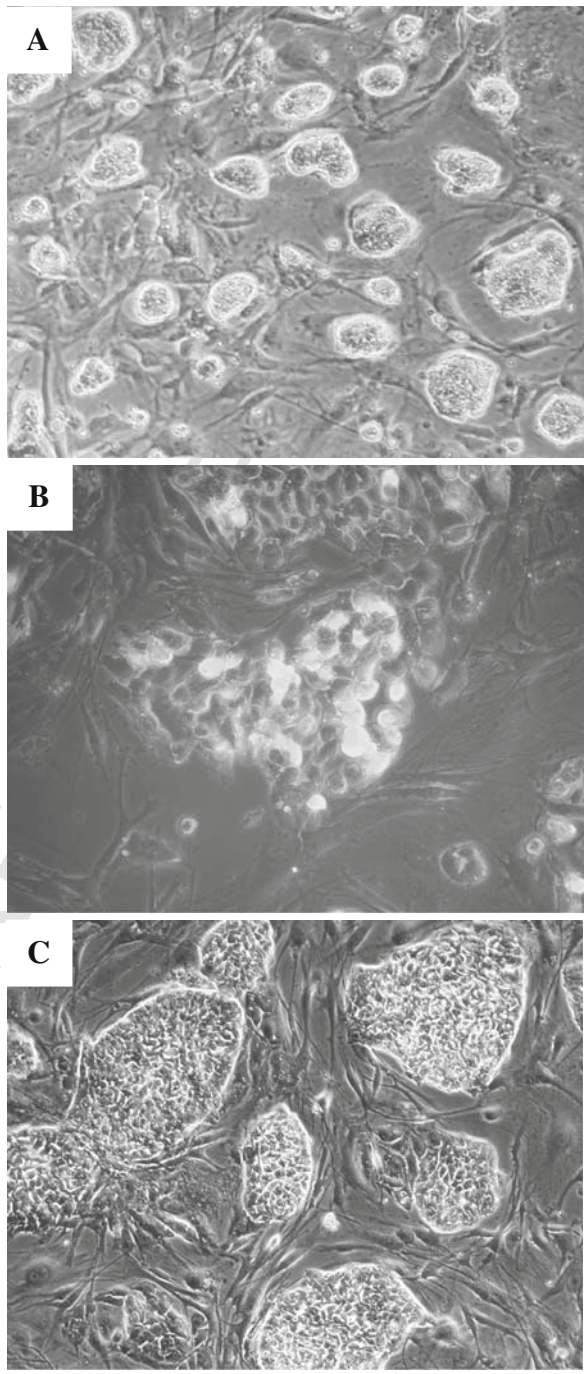

Fig. 21.3. **(a)** Human embryonic stem (HESC) cell culture on day of transfection. The culture should be composed of many small (8–32 cells) colonies. **(b)** Transient expression of CMV-EGFP in HESCs after 48 h to transfection. **(c)** Established cell line of HESCs after transfection, selection, and clonal expansion of genetically modified cells.

4. Notes

1. **Section 2.1, Items 1–10** are stored at 4°C, **Items 11–18** at –20°C, and **Item 19** 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 at 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. Protocols for cell freezing, thawing, and splitting are basically the same for all cell types (feeders and HESCs).
7. 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.
8. Cell thawing must be performed as quickly as possible.
9. The culture medium is supplemented with 10% of the tested batch of knockout serum substitute (instead of 15%) and mouse recombinant LIF at 1000 U/mL.
10. The purity of the DNA is very critical for successful transfection.
11. 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. 21.3a**). The colonies should have discrete borders and be composed of similar sized cells, with a pronounced nucleus.

12. The calcium phosphate ($CaPO_4$) transfection protocol is basically similar to the protocols used for other cell types.
13. At this time, fine DNA-calcium phosphate precipitates should be formed without agitation.
14. ExGen 500 (polyethylenimine, PEI) is a cationic polymer, which is capable of transfecting a wide range of cell types at relatively high efficiency. It interacts with the negatively charged DNA molecules by forming small, stable, and highly diffusible particles, which settle on the cell surface by gravity and absorb into the cell by endocytosis.
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. 21.3b**).
16. The colonies are picked up by the aid of a mouth apparatus connected to a sterile pulled and fire polished Pasteur pipette, as is commonly used for handling oocytes and preimplantation embryos (*see* **Note 4**).
17. 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.
18. 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 gelatinized plates, for at least one passage. Under such conditions, the cells must be grown in conditioned media (CM, HESC cell media conditioned by MEFs for 24 h), preventing from differentiation and consequently culture loss.

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Chapter 21

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