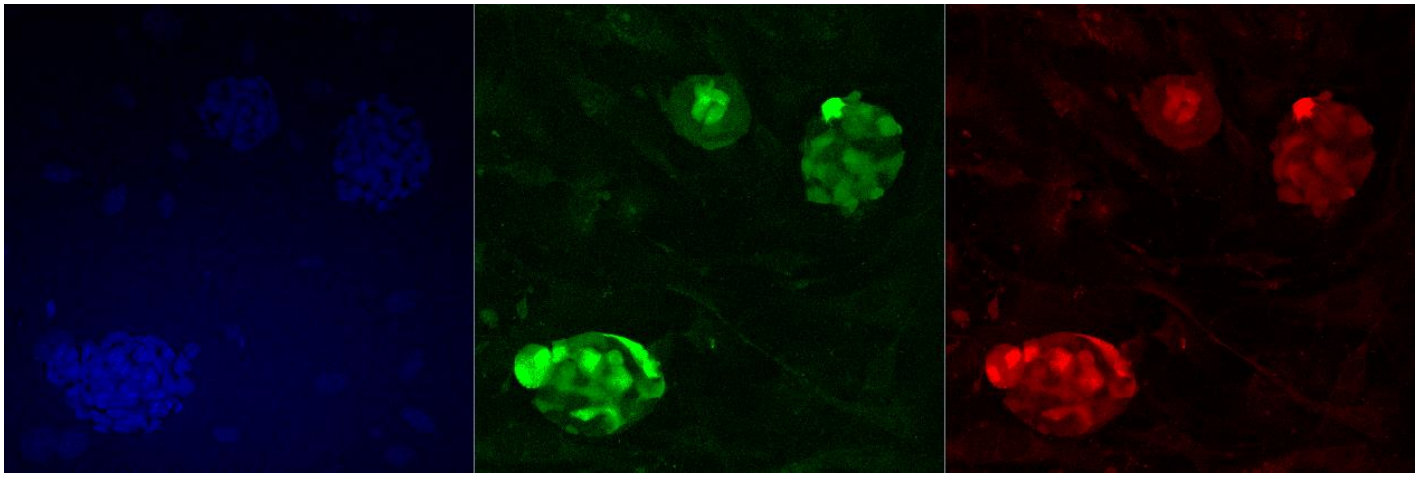
Optimization of mouse embryonic stem cell transfection for the new mutagenesis methods

Bachelor's Degree Thesis



Universitat de Girona

AARHUS UNIVERSITET
INSTITUT FOR MOLEKYLÆRBIOLOGI OG GENETIK

Victor Badillo Lisakowski
B.Sc. in Biotechnology
Universitat de Girona
Erasmus+ Aarhus University
Tutor: Maria Vilanova Brugués
Co-tutor: Ernst-Martin Füchtbauer
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ABSTRACT

Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts, an early-stage of pre-implantation embryo, and are capable of unlimited, undifferentiated proliferation *in vitro*. They are pluripotent, meaning they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. ES cells are key tools for genetic engineering, development of stem cell-based therapies and basic research on pluripotency and early lineage commitment. The use of stem cells as therapeutics to treat genetic defects depends on how efficient are the approaches to manipulate their genome. Traditional non-viral strategies are generally less efficient in delivering DNA and initiating gene expression, but they are safer, cheaper, producible easily in large quantities and have higher genetic material carrying capacity.

Therefore, FuGENE® HD (Promega), SuperFect® (Qiagen), Lipofectamine® 2000 (Invitrogen) and also electroporation were used to transiently transfect fluorescently labelled expression vectors into an mES cell line in order to optimize a reliable and efficient protocol that could be applied for some of the new mutagenesis methods. In most of the new site-directed mutagenesis techniques, more than one plasmid has to be introduced into the cell. For this reason, co-transfection and single transfection efficiencies of plasmids encoding the mCherry fluorescent protein and the EGFP were simultaneously determined.

Transfection and co-transfection efficiencies of 52-83% were found for FuGENE® HD transfection reagent, which was shown to be most efficient and reliable. In addition, in 90-93% of the co-transfected colonies both fluorescent proteins were co-expressed. This optimized transfection protocol was followed to successfully assess a new recombinase mediated cassette exchange (RMCE) system in an mES RMCE-in cell line as a more attractive alternative to the commercial Flp-in cell lines.

RESUM

Les cèl·lules mare embrionàries es deriven de la massa cel·lular interna de blastòcits, un estadi primerenc d'embrions preimplantatoris, i tenen la capacitat de proliferar de manera il·limitada i indiferenciada *in vitro*. Són pluripotents, el que significa que poden diferenciar-se en tots els derivats de les tres capes germinals primàries: ectoderma, endoderma i mesoderma. Les cèl·lules mare embrionàries són eines clau per a l'enginyeria genètica, el desenvolupament de teràpies basades en cèl·lules mare i la investigació bàsica sobre pluripotència i compromís de llinatge. L'ús de cèl·lules mare com a agents terapèutics per al tractament de defectes genètics depèn de com eficients siguin els mètodes per manipular el seu genoma. Les estratègies tradicionals no basades en virus són generalment menys eficients en la introducció d'ADN i en la iniciació de l'expressió gènica, però són més segures i econòmiques, fàcilment produïbles en grans quantitats i tenen una major capacitat de transport de material genètic.

Per tant, els reactius de transfecció FuGENE® HD (Promega), SuperFect® (Qiagen), Lipofectamine® 2000 (Invitrogen) i també electroporació van ser utilitzats per transfectar transitòriament vectors d'expressió marcats amb proteïnes fluorescents en cèl·lules mare de ratolí per tal d'optimitzar un protocol fiable i eficient per ser posteriorment aplicat per alguns dels nous mètodes de mutagènesi. En la majoria de les noves tècniques de mutagènesi dirigida, més d'un plasmidi ha de ser introduït en la cèl·lula. Per aquesta raó, es van determinar simultàniament les eficiències de transfecció i co-transfecció dels plasmidis codificants per les proteïnes fluorescents mCherry i EGFP.

Els resultats mostren eficiències de transfecció i co-transfecció entre 52-83% amb el reactiu de transfecció FuGENE® HD, que ha resultat ser el més eficaç i fiable. A més, en el 90-93% de les colònies co-transfectades ambdues proteïnes fluorescents van ser co-expressades. Aquest protocol optimitzat de transfecció va ser utilitzat posteriorment per testar amb èxit un nou mètode d'intercanvi de casset recombinasa intervinguda (RMCE en anglès) en cèl·lules mares embrionàries de ratolí RMCE-in, com una alternativa més atractiva a les línies cel·lulars comercials Flp-in.

RESUMEN

Las células madre embrionarias se derivan de la masa celular interna de blastocitos, un estadio temprano de embriones preimplantatorios, y son capaces de proliferar de manera ilimitada e indiferenciada *in vitro*. Son pluripotentes, lo que significa que son capaces de diferenciarse en todos los derivados de las tres capas germinales primarias: ectodermo, endodermo y mesodermo. Las células madre embrionarias son herramientas clave para la ingeniería genética, el desarrollo de terapias basadas en células madre y la investigación básica sobre la pluripotencia y compromiso de linaje. El uso de células madre como agentes terapéuticos para el tratamiento de defectos genéticos depende de cuan eficientes sean los métodos para manipular su genoma. Las estrategias tradicionales no basadas en virus son generalmente menos eficientes en la introducción de ADN y en la iniciación de la expresión génica, pero son más seguras y económicas, fácilmente producibles en grandes cantidades y tienen una mayor capacidad de transporte de material genético.

Por lo tanto, los reactivos de transfección FuGENE® HD (Promega), SuperFect® (Qiagen), Lipofectamine® 2000 (Invitrogen) y también electroporación fueron utilizados para transfectar transitoriamente vectores de expresión marcados con proteínas fluorescentes en células madre de ratón con el fin de optimizar un protocolo fiable y eficiente para ser posteriormente aplicado para algunos de los nuevos métodos de mutagénesis. En la mayoría de las nuevas técnicas de mutagénesis dirigida, más de un plásmido tiene que ser introducido en la célula. Por esta razón, se determinaron simultáneamente las eficiencias de transfección y co-transfección de los plásmidos codificantes para las proteínas fluorescentes mCherry y EGFP.

Los resultados muestran eficiencias de transfección y co-transfección entre 52-83% con el reactivo de transfección FuGENE® HD, que ha mostrado ser el más eficaz y fiable. Además, en el 90-93% de las colonias co-transfectadas ambas proteínas fluorescentes fueron co-expresadas. Este protocolo optimizado de transfección fue utilizado para testar con éxito un nuevo método de intercambio de cassette recombinasa mediada (RMCE en inglés) en células madres embrionarias de ratón RMCE-in, como una alternativa más atractiva a las líneas celulares comerciales Flp-in.

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1. Introduction

The process of introducing nucleic acids into eukaryotic cells by non-viral methods is defined as transfection. Different chemical, lipid or physical methods have been developed as gene transfer tools to study gene function and protein expression. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology together with the availability of transfection reagents, provides the foundation to study mammalian promoter and enhancer sequences, trans-acting proteins such as transcription factors, mRNA processing, protein-protein interactions, translation and recombination events (Groskreutz et al., 1997).

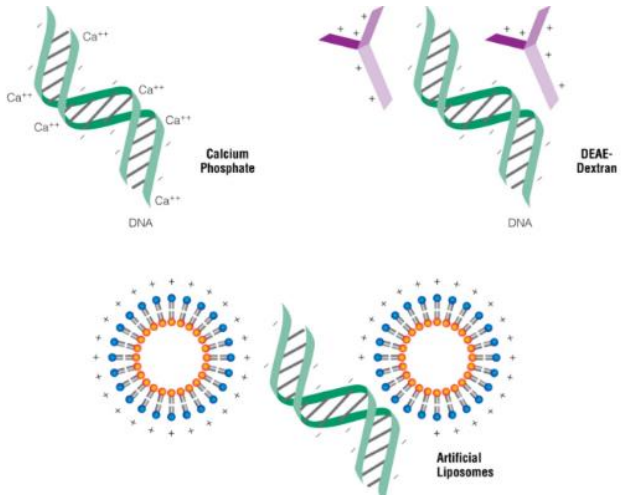

Figure 1. Representation of various transfection technologies and how the negatively charged DNA is neutralized. Lipid-based reagents can coat nucleic acids in addition to forming micelles and associating with DNA by attraction (Promega, Protocols & Applications

The common characteristic of transfection approaches is to help negatively charged molecules to by-pass the anionic cellular membrane (e.g., phosphates of the DNA and RNA backbones). Calcium phosphate, DEAE-dextran or cationic lipid-based reagents, coat the nucleic acids, neutralizing or even creating an overall positive charge around them (*Figure 1*). This enables the complex (nucleic acid: transfection reagent) to cross through the cellular membrane. On the other hand, physical systems like microinjection or electroporation punch through the membrane and introduce directly the DNA into the cytoplasm.

These gene delivery approaches can be applied to stem cell research, a very promising and quickly expanding field of contemporary biology. Stem cells have the potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as an internal repair system, dividing essentially without limit to replenish other cells. When an unspecialized stem cell divides through mitosis, each new cell has the potential either to remain a stem cell or to start cellular differentiation, which can be induced by physiologic or experimental conditions.

In mammals, there are two broad types of stem cells: embryonic stem cells and somatic (or adult) stem cells. The first ones are isolated from the inner cell mass of blastocysts, an early-stage of pre-implantation embryo that is formed 4-5 or 3.5 days post fertilization in humans and in mice, respectively. They are pluripotent stem cells that can differentiate to generate primitive ectoderm, which later differentiates during gastrulation into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. On the contrary, somatic stem cells are multipotent and can produce only a limited number of cell types.

Mouse embryonic stem (mES) cells were first derived and cultured more than 30 years ago (Evans et al., 1981) and are key tools for genetic engineering, development of stem cell–based therapies and basic research on pluripotency and early lineage commitment. The use of stem cells as therapeutics to treat genetic defects depends on how efficient are the approaches to manipulate their genome.

There is a wide range of gene delivery strategies into mES cells, and the most appropriate often depends on several parameters. First, the duration of the expression has to be considered. The delivered gene can remain either separated from the host cell chromosome (transient transfection) or it can be integrated (stable transfection). For the transient transfection, expression of the

transgene usually dissipates within several days, because the expression vector is either degraded or expelled from the host cell. On the contrary, for stable integration the expression of the transferred gene is prolonged, because the vector is integrated into the cell genome (Somia et al., 2000). Parameters like immunogenicity, cytotoxicity, expertise of the researcher, time needed and cost of the equipment should also be taken into account. A summary of these specifications in the context of some of the most common mES cell transfection methods is shown in *Table 1*.

The main gene delivery strategies in ES cells can be divided into two main categories: viral-based methods (gene delivery through lentivirus and adeno-associated virus), and non-viral transfection approaches. The use of virus as vectors to transfect ES cells, like any stable integration method, can involve several drawbacks such as loss of genomic integrity because of the random integration in their genome. In addition, they present other issues such as limited size of DNA, cytopathic effects and safety concerns (Gardlik et al., 2005). They can be used for almost all cell types but are preferentially selected for terminally differentiated cells like neurons and cardiomyocytes (Kaestner et al., 2015), because lentivirus-based transfection is the only efficient method to stably transfect post-mitotic cells. Whereas, traditional non-viral vectors are generally less efficient in delivering DNA and initiating gene expression, but they are safer, cheaper, producible easily in large quantities and have higher genetic material carrying capacity (Luo et al., 2000).

Table 1. Comparison of different gene delivery strategies into ES cells considering different parameters (Lui et al., 2003).

TABLE 1. Summary of the Possible Gene Delivery Strategies

Methods	Cellular context	Length of expression	Relative efficiency	Type of delivered molecule	Suspension vs. adherent	Toxicity	Expertise required	Time required	Cost
Lentivirus	In vitro In vivo	Stable	High	RNA	Adherent and suspension	Low	High	1–3 weeks	$1000
Adenovirus	In vitro In vivo	Transient	High	DNA	Adherent and suspension	Low	High	1–3 weeks	$500–$1000
Adeno-associated virus	In vitro In vivo	Stable	High	DNA	Adherent and suspension	Low	High	1–3 weeks	$500–$1000
Cationic lipids	In vitro In vivo	Transient or stable	Low–moderate	Plasmid Oligos mRNA siRNA Protein	Adherent and suspension	Moderate	Low	1–3 days	$100–$500
Electroporation	In vitro In vivo	Transient or stable	Low–moderate	Plasmid Oligos mRNA siRNA	Suspension	High	Moderate	1–3 days	>$1000
Nucleofection	In vitro In vivo	Transient or stable	High	Plasmid siRNA	Suspension	Moderate	Moderate	1–3 days	>$1000
Microinjection	In vitro In vivo	Transient or stable	High	Plasmid Oligos mRNA siRNA	Adherent	Moderate	Moderate	1–3 days	>$1000
Nuclear transfer	In vitro In vivo	Stable	Moderate	Nuclei	Adherent	Moderate	High	1–3 weeks	>$1000

In general, transfection efficiency of vectors depends on cell type, the kind of DNA and the medium conditions. Other influencing factors that should be considered are the health status of the cells, cell confluence, the number of passages of the cell line and the DNA quantity and quality. During this project, different non-viral transfection strategies were tested and optimized into mES cells: FuGENE® HD (Promega), Lipofectamine® 2000 (Invitrogen) and SuperFect® (Qiagen) transfections, together with electroporation.

FuGENE® HD transfection

Very few is known about the composition of the FuGENE® HD Transfection Reagent (Promega), which is a non-liposomal formulation containing a blend of lipids that can be used to transfect different cell lines with low cytotoxicity. It does require neither serum (even up to 100% serum can remain) or culture medium removal nor washing or changing medium after introducing the reagent-DNA complex (Promega, 2013). Previous studies with C3H10T1/2 pluripotent mouse stem cells have shown high transfection efficiencies with two different plasmid DNAs encoding luciferase or β-

galactosidase using FuGENE® HD (Promega). In addition, highest efficiency for both plasmids was obtained when transfection occurred in the absence of serum (Yamano et al., 2010).

Lipofectamine® 2000 transfection

Lipofectamine® 2000 (Invitrogen) is a cationic liposome formulation for the transfection of nucleic acids into a range of mammalian cell types by lipofection (*Figure 1*), one of the most commonly used gene transfer methods for primary cells. It is a 3:1 (w/w) formulation of the polycationic lipid 2,g3-dioleoyloxy-N-(2(sperminecarboxamino)ethyl)-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) (*Figure 2*), a neutral co-lipid (helper lipid) that allows the entrapped DNA to escape the endosomes. The cationic lipids have three segments: a DNA-interacting head group with a net positive charge in physiological conditions or at lower pH (such as found in the endosome environment); a hydrophobic lipid anchor group such as cholesterol or fatty acid chains of various lengths and unsaturation states; and a linker group that binds the polar group to the lipid moiety (Kaestner et al., 2015). The uni-lamellar DNA-containing liposomes (with positive charge on their surfaces) are called lipoplexes. They can fuse with the negatively charged plasma membrane of living cells, allowing nucleic acid to cross into the cytoplasm by endocytosis (Fraley et al., 1980) and be available to the cell for replication or expression.

In order for a cell to express this transgene, the nucleic acid must reach the nucleus of the cell to begin transcription. This process involves some risks. First, the transfected genetic material may never reach the nucleus, instead it can be degraded somewhere along the delivery process. Second, in dividing cells, the material may reach the nucleus but can be trapped in the reassembling nuclear envelope following mitosis. However, in non-dividing cells, it has been shown that Lipofectamine® 2000 improves the efficiency of transfection, which suggests that it additionally helps the transfected genetic material penetrate the intact nuclear envelope. (Dalby et al., 2004). It can be added directly to cells in culture medium, in presence or absence of serum, and the removal of the complexes or changing/adding medium after transfection is not required (Invitrogen, 2012). Previous studies have shown that ES cells are difficult to lipofect, and most commercially available lipid-based transfection reagents have either low transfection efficiency or high toxicity (Ma et al., 2004).

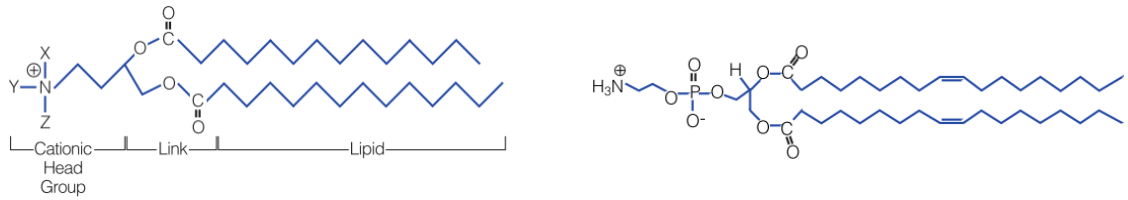

Figure 2. ***Left****: General structure of a synthetic cationic lipid. X, Y and Z represent a number of possible chemical moieties, which can differ, depending on the specific lipid.* ***Right****: Structure of the neutral lipid DOPE. (Promega, Protocols and Applications).*

SuperFect® transfection

SuperFect® Reagent (Qiagen) consists of activated-dendrimer molecules with a defined spherical architecture (*Figure 3*). Branches radiate from a central core and terminate at charged amino groups which can then interact with negatively charged phosphate groups of nucleic acids. It assembles DNA into compact structures that bind to the cell surface and are taken into the cell by nonspecific endocytosis. The reagent buffers the pH of the endosome, leading to pH inhibition of endosomal nucleases, which ensures stability of SuperFect-DNA complexes. This transfection reagent is suitable for a broad range of cell lines and to transfect in the presence of serum (Qiagen, 2002).

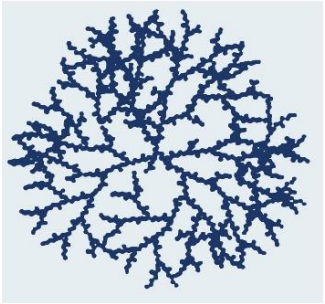

Figure 3. Activated-dendrimer structure of the SuperFect® molecules (Qiagen).

Electroporation

The use of short, high-voltage pulses to increase the permeability of the cell membrane is referred as electroporation. By applying an external electric field that exceeds the capacitance of the cell membrane, transient and reversible breakdown of the membrane can be induced (*Figure 4*). This transient, permeabilized state can be used to load cells with a variety of different molecules, either through simple diffusion in the case of small molecules, like ions and small drugs, or through electrophoretically-driven processes allowing passage through the membrane, as for DNA or protein transfer.

The principal disadvantage of this technique is the extremely low mammalian cell survival rate. In addition, efficient gene delivery into human ES cells by electroporation has been difficult to prove. The optimization of the electroporation efficiency and the cell survival rate depend on the use of correct pulse duration and amplitude, and other parameters like cell size, temperature, post-pulse manipulation and composition of electrodes and pulsing medium, especially on its salt content (Rols et al., 1990). However, when high transfection efficiency is not the objective, electroporation is a useful tool. For example, for the generation of transgenic mice it is better to avoid the delivery of multiple copies of the plasmid containing the sequence of interest into the mES cells.

This approach requires specific equipment including electroporators, purpose-built appliances which create electrostatic field in the cell solution; and glass or plastic electroporation cuvettes containing two aluminium electrodes on both sides.

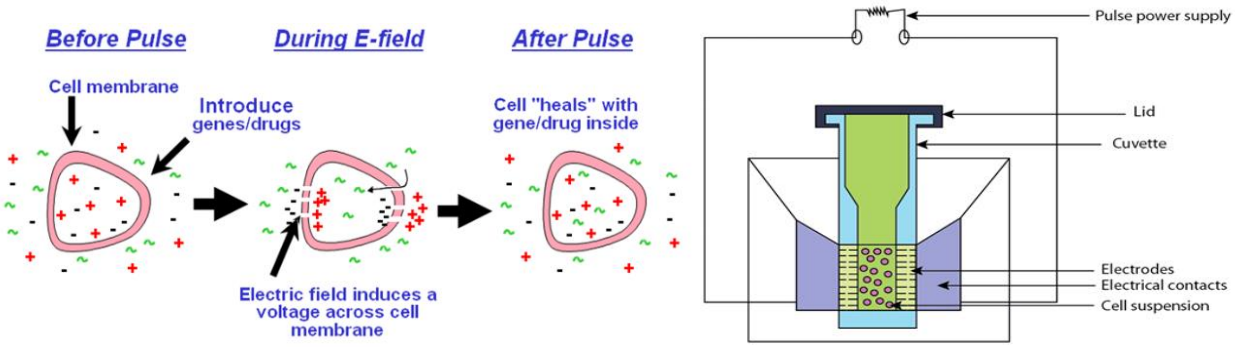

Figure 4. ***Left****: Exogenous molecule uptake by temporary pore formation induced during the application of an electric field (BTX Instrument Division, Harvard Apparatus, Inc.).* ***Right****: Representation of the electroporation settings (NPTEL, Government of India).*

SITE-DIRECTED MUTAGENESIS USING SPECIFIC NUCLEASES

TALEN

Transcription activator-like effector nucleases (TALENs) are artificial restriction enzymes generated by the fusion of a TAL effector DNA-binding domain and a DNA cleavage domain. TAL effectors were discovered in *Xanthomonas sp*., a plant pathogen that injects these effectors into the cells to activate gene transcription by targeting effector-specific gene promoters once they are imported into the plant cell nuclei, a mechanism that may contribute to bacterial colonization (Kay et al., 2007). The effector proteins contain N- and C-termini for localization and activation and a central domain for specific DNA binding (*Figure 5A)*. The central domain is composed of a variable number of tandem monomer repeats, varying from 5 to over 30 with an average of 17.5. Each repeat contains 34 amino acids that recognize one target nucleotide. The amino acid sequence of the repeats is highly conserved with its main variation in the residues at position 12 and 13, a pair of residues known as repeat variable di-residues (RVDs) that determine the nucleotide binding specificity of each TALE

repeat (Boch et al., 2010; Miller et al., 2011). The DNA cleavage domain corresponds to the bacterial restriction endonuclease *FokI*. The cleavage domain is at the C-terminal of the protein, and since it only works in a heterodimeric way, a pair of TALENs is needed to make a cut at a particular site of the genome (*Figure 5B*). This targeted cut in the spacer DNA region, known as double-strand DNA break (DSB), stimulates the cellular DNA repair mechanisms, including error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Wyman et al., 2006). DSBs are generally repaired by NHEJ, often generating small insertions and/or deletions (indels), which is crucial for editing the genome at a specific site. The TALEN-induced indels are often variable in length, generally leading to a frame-shift when it occurs in a coding region of the genome. On the other hand, HDR DNA pathway is possible in the presence of homologous donor DNA, enabling a site-specific insertion of an exogenous DNA sequence (Zu et al., 2013), or a precise replacement of an endogenous nucleotide sequence with a desired or corrected nucleotide sequence in place (Sun et al., 2012) (*Figure 5C*).

Some applications derived from the TALEN-based genetic manipulation are feasible and reliable introduction of exogenous sequences (like tagging FRT/loxP sequences), *in vivo* tagging of genes of interest with fluorescent proteins, easier generation of transgenic animals with conditional expression of a given gene, controlled large genomic rearrangements (particularly important for non-coding RNA genes studies) and building of human disease models in iPS (induced pluripotent stem) cells, among others (Wei et al., 2013). Many of these site-specific nucleases-based approaches have been extended to progenitor cell types, including embryonic stem (ES) cells (Lombardo et al., 2007).

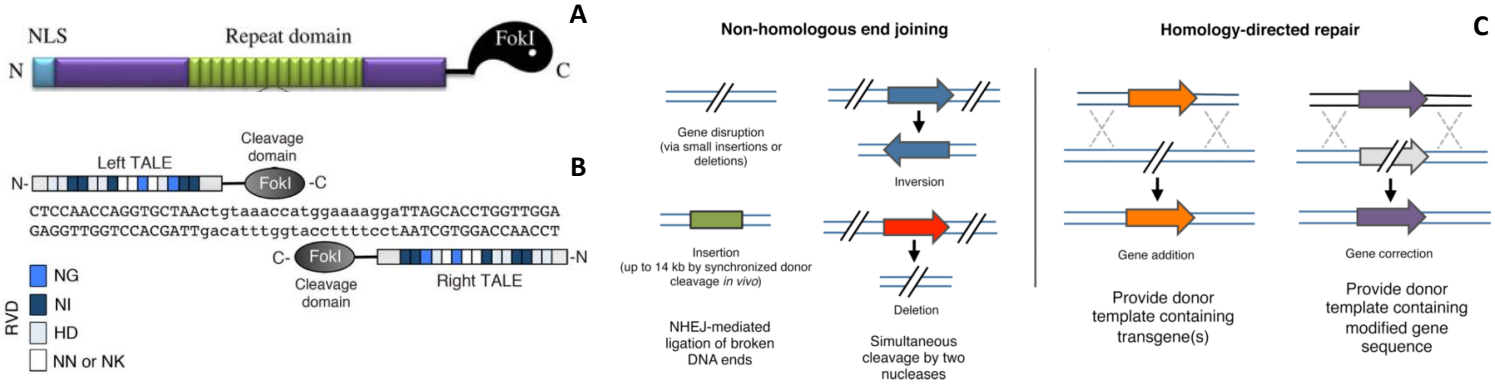

Figure 5. ***A****: A single TALEN, including a nuclear localization site (NLS), a central domain of tandem TALE repeats and a C-terminal domain of the functional endonuclease FokI (Wei et al., 2013).* ***B****: TALEN dimer bound to DNA. The target sites consist of two TALE binding sites separated by a spacer sequence (12-20 bp). TALEs can be engineered to recognize unique left and right half-sites. RVD composition is indicated by the TALE Code, where each di-aminoacid (NG, NI, HD, NN or NK) corresponds to a nucleotide bond (T, A, C, G/A; respectively).* ***C****: Possible genome editing outcomes using site-specific nucleases. DSBs can be repaired by NHEJ pathway, leading to the generation of indels (small insertions/deletions); or by HR DNA pathway, enabling a site-specific insertion of an exogenous DNA sequence (gene addition) or a replacement of the endogenous nucleotide with a desired nucleotide in place (gene correction). Figures B and C were obtained from Thomas Gaj, The Scripps Research Institute, La Jolla, California.*

RMCE

Recombinase-Mediated Cassette Exchange (RMCE) is an integration strategy of increasing relevance in the field of reverse genetics based on site-specific recombination processes, which permits the systematic and repeated modification of higher eukaryotic genomes by targeted integration (Schlake et al., 1994).

The donor and target sequences are each flanked by flippase (Flp) recombinase target (FRT) sites, and crossover events occurring on both sides of the donor and target sequences result in a clean exchange of the target sequence cassette, containing the genetic element of interest. The outcome of the exchange depends on the relative orientation of the participating FRTs, leading to the

inversion of a sequence when it is flanked by two identical but inversely oriented FRT sites; the deletion of a sequence that is flanked by two equally oriented identical FRTs or the integration (addition) of an extra piece of DNA flanked by heterospecific FRT sites (*Figure 6*). One of the most important points of RMCE is that it ensures the integration of the donor cassette excluding its plasmid backbone, which usually contains antibiotic resistance genes and bacterial sequences. In addition, it has the potential to integrate sequences that do not produce a phenotype on their own, and the success of the exchange can be detected simply by the loss of a marker carried by the target cassette (Bateman et al., 2006). This technique has been widely used in ES cells for transgene expression studies and to generate mutated mice (Tchorz et al., 2012), (Minorikawa et al., 2011), Sandhu et al., 2011). A cell line that presents the target cassette is referred as RMCE-in cell line.

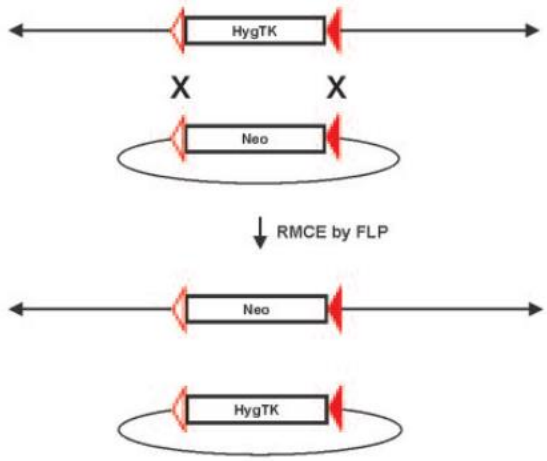

Figure 6. Principle of DNA integration by RMCE. Due to recombination (indicated by X) by a transiently expressed flippase (Flp), an hygromycin/thymidine kinase fusion protein (HygTK) exchangeable cassette, present in a particular locus and flanked by heterospecific FRT sites (filled and open triangles), is exchanged by a neomycin resistance gene (Neo) replacement cassette, present in a circular plasmid and also flanked by the same two heterospecific FRT sites. By application of positive (G418) and/or negative (ganciclovir) selection, clones resulting from exchange between the HygTK cassette and the Neo cassette can be selected (Bateman et al., 2006).

In contrast, the commercial Flp-in cell lines contain a single FRT site for Flp recombination, and the single integration is referred to as a docking site. A genetic element of interest (GEI) can be placed in a plasmid also containing an FRT site and be targeted and integrated into the docking site through Flp recombination. This mutagenesis system presents some issues such as integration of the initial selection marker and of prokaryotic elements from plasmid backbones, which can affect the regulation of GEI. In addition, any random integration in the Flp-in system besides the target integration gives rise to GEI expression (Jakobsen et al., 2010). The recombinase mediated cassette exchange (RMCE) system solves all the problems mentioned above

2. Objectives

The main goal of this project is to test and compare different gene delivery strategies into mouse embryonic stem (mES) cells and try to optimize them in order to find an efficient transfection protocol. FuGENE® HD Transfection Reagent (Promega), Lipofectamine® 2000 Transfection Reagent (Invitrogen), SuperFect Transfection Reagent (Qiagen) and also electroporation were used for this purpose.

The second objective was to test two different genome editing procedures using the best transfection protocol found in objective 1. The genome editing procedures in mES cells proposed were:

1. A new RMCE-in (Recombinase Mediated Cassette Exchange) system created by J.E. Jakobsen (Department of Biomedicine, Aarhus University), as an alternative to the commercial Flp-in cell lines.
2. TALEN-mediated (Transcription Activator-Like Effector Nucleases) knock-in method.

3. Materials and methods

3.1. Cells

For all the transfection tests I used two types of adherent cells, Mouse Embryonic Fibroblasts (MEFs) as non-dividing feeder cells and CJ7 mouse Embryonic Stem (mES) cells.

3.1.1. Feeder cells

Primary MEFs (ATCC® SCRC-1040) mitotically inactivated with Mitomycin C were used as a feeder layer to support the growth of embryonic stem (ES) cells and for the maintenance of ES cells in the undifferentiated state.

3.1.2. mES cells

The CJ7 mES cell line (ATCC® SCRC-1021™) was first derived by Swiatek PJ and Gridley T (Swiatek et al., 1993) from normal and implanted delayed blastocysts from agouti 129/Sv mice cultured on feeder layers of Mitomycin C-treated primary embryonic fibroblasts cells in media containing 1000 U/ml of recombinant leukemia inhibitory factor (LIF) (ESGRO; GIBCO) to maintain the pluripotent stem cells undifferentiated. CJ7 mES cells from passage 11 to 16 were used for the transfection tests.

3.2. Cell culture media

The mES cell medium used for their maintenance and growing contained:

800 ml DMEM 1X (Gibco #41965) (+ 4.5 g/l D-glucose, + L-Glutamine, - Pyruvate)
150 ml FCS (PAN #2602 P250915) heat inactivated
10 ml Glutamine 100X 200 mM (Gibco #25030)
10 ml Non-essential amino acids (Sigma #M7145)
10 ml β-mercaptoetanol (Sigma #M7522) 100X 10 mM in D-PBS
10 ml Nucleoside stock 100X (Sigma #A4036)*
100 µl LIF (Leukaemia Inhibitory Factor) ESGRO™ $5x10^6$ U/ml (Gibco)

*To prepare 100 ml of 100 X Nucleoside stock (Sigma), mix 80 mg Adenosine, 73 mg Cytidine, 85 mg Guanosine, 24 mg Thymidine and 73 mg Uridine.

For the selection of the cells that integrated the resistance gene to geneticin antibiotic, 1ml of G-418® 100X (Roche #04727894001) (potency: 789 µg/ml) was added for every 100 ml of mES cell medium. G-418® (Roche) is an analog of neomycin sulphate that interferes with the function of 80S ribosomes and protein synthesis in eukaryotic cells. When the hygromycin resistance gene was integrated, Hygromycin B® (Invitrogen) at a final concentration of 125 µg/ml was added to the mES cell medium.

The MEFs medium contains DMEM 1X (Gibco #41965) without sodium pyruvate with 10% FCS (PAN #2602 P250915), 1% 100X 200 mM (Sigma) and optional 1% Penicillin (5000 U/ml)/Streptomycin (5000 µg/ml) (Sigma #15140).

3.2.1. Freezing medium

Two different freezing media were used:

1) 2x freezing medium for cells harvested from 6-well or 6cm dishes: 60% DMEM (Gibco #41965), 20% FCS (PAN #2602 P250915), 20% DMSO (Dimethylsulfoxid, Sigma #D2650)
2) 2x HEPES ES cell freezing medium for cells frozen in 96well plates: 60% DMEM HEPES (Sigma #H0887 , 20% FCS (PAN #2602 P250915), 20% DMSO (Sigma #D2650)

3.2.2. Other materials

- Trypsin-EDTA 0.05% (Gibco)
- D-PBS 1X (Gibco)
- Opti-MEM® 1X (Gibco #31985)
- Gelatine 2% (Sigma #G1393) in H2O
- Gene Pulser® Cuvettes (Bio-Rad)
- Gene Pulser Electroporator (Bio-Rad #1652088)
- LB media
- Kanamycin (Sigma) 784 µg/mg
- PFA 4%
- Hoechst stain (#33258) 6.25 µg/µl to a final concentration of 1 µg/ml Gelvatol (Sigma)
- Ampli Taq® DNA Polymerase (Life Technologies)
- 360 GC Enhancer ® (Life Technologies)
- CMF-PBS 1X
- Proteinase K (<600 U/ml, Thermo Scientific #EO0492)

3.3. Culture procedures

3.3.1. Gelatine coating of tissue culture dishes to prepare feeder layer:

For gelatinization, 0.01% gelatine (Sigma #G1393) was poured into a dish or well and left for 5 min. The gelatine was aspired, and the dishes were air dried. Then, either the feeder cells were plated or the gelatinized dishes were stored at 4 °C.

3.3.2. Feeder cell density for dishes or wells (3 - 4 x 10^4 cells/cm^2):

Table of cell densities:

Table 2. Primary MEF (feeder cell) densities required depending on the diameter of the culture dish/well.

Dish/well (ø)	Growth area (cm2)	No. of cells per well/plate
60 mm	28	$1x10^6$
96 well	0.3	$1x10^4$/$1x10^6$
6 well	9.3	$4x10^5$/$2.4x10^6$

Cells will attach to the bottom of the dish within one hour and will give a monolayer after 12 hours.

3.3.3. ES cell culture

Feeder cells were plated on gelatinized dishes or in wells with cover slips at least 6 hours before plating of the ES cells, with a density of 1-3 x 10^6 ES cells per 60 mm dish or 2-5 x 10^6 ES cells per 100 mm dish. Every 24 hours the mES cell medium was carefully aspirated and replaced by fresh medium. Then, the mES cells were splitted 1:4 or used for transfection experiments.

For transient expression of the integrated expression vector, the cells were cultured in a 6-well plate for approximately 40 hours to ensure a maximum protein expression. However, for studying the stable integration of the expression vector, the CJ7 mES cells were first cultured in a 6 cm dish for 40 hours with mES cell medium after the transfection, and then the selection treatment was initiated for a 7 days period, either with G-418® (Roche #04727894001) or Hygromycin B® (Invitrogen).

3.3.4. Freezing

In order to preserve the cells for a long time period, they need to be frozen at extremely low temperatures. First, they were trypsinized and the total cell number was calculated in a haemocytometer. After a centrifugation step, the cell pellet (2-5x10^6 cells/ml/cryotube) was resuspended in 0.5 ml mES cell. Then 0.5 ml of freezing medium was added. The solution was gently pipetted and 1 ml aliquots were transferred into cryotubes, which were then placed in a freezing container, cooled overnight in a -80ºC freezer before finally depositing them into liquid nitrogen.

For a 96-well Nuncon™ Surface plate, the mES cell medium was removed, washed twice with CMF-PBS and then trypsinized with 30 µl of trypsin-EDTA per well and incubated two times 5 minutes at 37ºC. Later, 120 µl of bicarbonate-free freezing medium mix containing 45 µl DMEM/10 mM Hepes mES cell medium and 75 µl mES cell 2X HEPES mES cell freezing medium was added to each well. The content of each well was mixed carefully up and down using a multichannel pipette. The plate was sealed and moved at -80ºC in a styrofoam box.

3.3.5. Thawing

A cryotube that had been stored at -140ºC was taken and cells were rapidly thawed in a 37ºC waterbath until ice crystals disappeared. After being resuspended in a 15 ml Falcon tube containing 5 ml of mES cell medium without LIF to dilute the DMSO, the tube was centrifuged for 5 minutes at 1000 rpm. Once the supernatant was removed, the cells were resuspended in 3 ml of mES cell medium and seeded into a 6 cm dish with feeder cells.

3.4. Transfection experiments

3.4.1. Plasmids

For the mES cell transfection experiments two plasmids encoding the enhanced green fluorescence protein (EGFP) and a red fluorescence protein (mCherry), respectively, were used to follow the transfection success. In both vectors, EGFP and mCherry genes are under the control of the cytomegalovirus (CMV) promoter, whereas the neomycin resistance cassette is under the control of the SV40 early promoter, which can be used for G418® (Roche) selection in mammalian cells. For the TALEN-mediated (Transcription Activator-Like Effector Nucleases) knock-in method test, 3 DNA plasmids encoding the left TALEN DNA binding domain, the right TALEN DNA binding domain and the neomycin resistance gene as the sequence to be knocked-in were used.

Table 3. Resumed description of the different plasmids used for the transfection and co-transfection optimization experiments (mCherry and EGFP) and for a TALEN-based knock-in test.

Vector	Description
pmCherry-N1 (Clontech)	4722 bp, CMV promoter for the **mCherry protein** (CDS) and SV40 promoter for the Kan/Neo resistance (CDS). SV40 PolyA signal 1513-1303.
pEGFP-C1 (Clontech)	4731 bp, CMV promoter for the **EGFP** (CDS) and SV40 promoter for the Kan/Neo resistance (CDS). SV40 PolyA signal 1513-1563.
pROSA-26-RKR	Linearized plasmid (by PvuI) that contains the DNA sequence to be knocked-in (neomycin resistance gene)
Rosa26-TALEN L	Left TALEN DNA binding domain
Rosa26-TALEN R	Right TALEN DNA binding domain

The DNA concentration was calculated using the NanoDrop Lite Spectrophotometer (Thermo Scientific) and adjusted to 1 µg/µl in TE buffer.

3.4.2. Transfections

For the different transfection approaches that were tested, a common setup was designed to be followed in all the experiments in order to have more solid and reproducible results. First of all, cryopreserved mES cells were thawed and plated onto a gelanitinized 6cm dish with feeder cells. Then, the mES cell medium was replaced by fresh medium and the mES cells were splitted until an enough total number of cells was achieved for the following experiment, taking into account that after transfection $2x10^5$ cells/well were needed for an optimal analysis at the fluorescence microscope. The different transfection mixes (transfection reagent:DNA complex) were prepared while the mES cells was being trypsinized, since a 5-20 minutes incubation period is needed for the

formation of the complex. Next, the trypsinized mES cells were resuspended in mES cell medium containing serum and mixed with the transfection reagent:DNA complex. Then, 1 ml of this mix (with $2x10^5$ mES cells) was plated onto a 6-well well, and so on for each well of the plate. 1 ml of mES cell medium was added right after to avoid the drying of the cells.

Since single plasmid transfection and co-transfection were studied and compared, a concrete plating organization was followed to dispose of transfected and co-transfected mES cells from the same original cell mix. This organization consisted in plating transfected mES cells with the mCherry plasmid in the first column of the 6-well plate, the mES cells transfected with the EGFP plasmid in the second and the co-transfected cells in the third column, seeding duplicates of each transfection in the two wells of each column (*Fig. 7, left*). Finally, after 36-48 hours of incubation the transient expression of the fluorescent protein in the transfected mES cells is analysed at the fluorescence microscope.

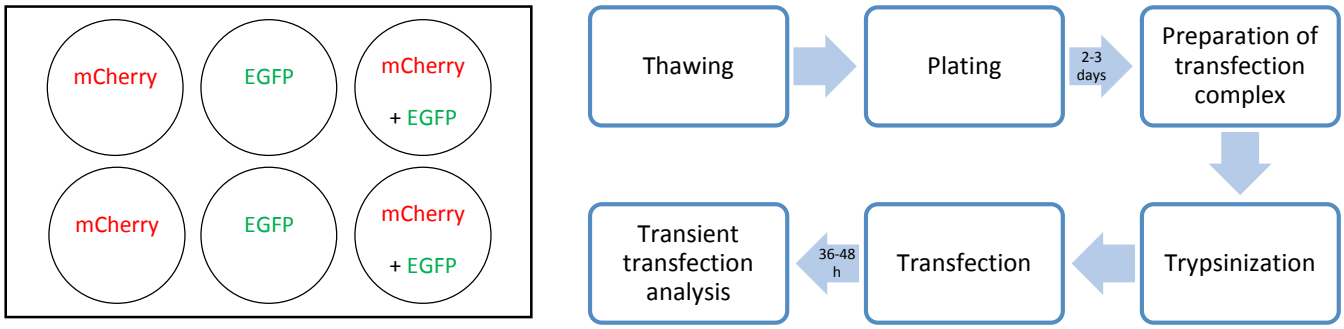

Figure 7. ***Left****: Representation of the common plating organization that was followed for the transfection optimization experiments in a 6-well plate. The single plasmid (mCherry or EGFP) transfected mES cells were plated in the first and second column of the plate, respectively; while the co-transfected (mCherry+EGFP) cells were plated in the third column.* ***Right****: Common schematic planning of a transfection experiment with mES cells, from the thawing of the cryopreserved cells to their final transient transfection efficiency analysis at the fluorescence microscope.*

3.4.2.1. FuGENE® HD transfection mix

While the mES cells are being trypsinized, a tube with 50 µl of Opti-MEM® (Gibco #31985), 1 µg of well mixed DNA and 3 µl of FuGENE® HD Transfection Reagent (Promega #E2311) was prepared for each 6-well well, mixed properly and incubated for 15 minutes at room temperature. Then, the mES cells in mES cell medium were resuspended at a final concentration of $2x10^5$ mES cells/ml.

3.4.2.2. Lipofectamine® 2000 transfection mix

For each 6-well well, 1 vial with 5 µl of Lipofectamine® 2000 (Invitrogen #52887) diluted in 125 µl of Opti-MEM 1X (Gibco #31985) was prepared together with another vial containing 1 µg of DNA diluted in 125 µl of Opti-MEM® 1X (Gibco #31985). The content of both vials was mixed and incubated for 5 minutes at room temperature. Then 1 ml of mES cell suspension ($2x10^5$ mES cells/ml) was added to each vial and mixed gently.

3.4.2.3. SuperFect® transfection mix

While the mES cells were being trypsinized, a tube with 100 µl DMEM 1X (Gibco #41965), 1 µg DNA and 2 µl SuperFect® (Qiagen #301305) was prepared. After mixing properly, the tubes were incubated at room temperature for 20 minutes. Then, the mES cells in mES cell medium were resuspended at a final concentration of $2x10^5$ mES cells/ml.

3.4.2.4 Electroporation

First, the total number of cells to be transfected were washed with 3 ml of D-PBS and centrifuged for 5 minutes at 1000 rpm. Then, the DNA solution containing 10 µl (1 µg DNA/µl) of the DNA to be transfected dissolved in 790 µl of D-PBS was prepared and afterwards mixed with the cell pellet. This 800 µl were transferred into an electroporation cuvette (Bio-Rad #1652088), which was then carefully placed into the electroporator without touching the metal sides. The electroporation conditions were set to 0.240 kV and 500 µF, and the time constant (τ) should be in the range of 6-7 msec. After electroporation, the cuvettes were placed for 20 minutes at 4ºC and then transferred into a 15 ml Falcon tube containing 5 ml of mES cell medium without LIF. Finally, the tubes were centrifuged, the supernatant was removed, the cell pellet was resuspended in mES cell medium with LIF and the cells were divided into the different wells or dishes. For this approach, a final concentration of at least $6x10^5$ mES cells/ml was required for an optimal fluorescence microscope analysis.

3.5. Fixing and embedding

Once the fluorescence protein was expressed, the cells were fixed and embedded to be analysed at the fluorescence microscope. The cells were first washed twice with CMF-PBS and then 2 ml 4% PFA solution was added in each 6-well well with a cover slip. After 30 minutes fixation at room temperature in the dark, the cells were washed twice with CMF-PBS to be finally incubated with 2 ml of CMF-PBS per well also 30 minutes at room temperature and without light. The cover slips were then dipped in double-distilled water and embedded onto microscope slides with a drop of Hoechst + Gelvatol mix. After a couple of hours, the Hoechst stain had completely entered into the mES cell colonies and the fluorescent cells were ready to be observed.

3.7. DNA preparation and PCR analysis

3.7.1. Transformation

In order to dispose of enough DNA for the transfection experiments, a step of transformation of the plasmids was required. First, 20 µl of 5X KCM buffer, 1 µl of DNA and H_2O were mixed to a total volume of 100 µl and placed on ice for 2 minutes. After this period, 100 µl of competent *E. coli* DHα5 cells were added and thawed on ice for 20 minutes. Then, they were incubated at room temperature for 10 minutes, and 800 µl of LB media without antibiotics were added. After 1 hour at 37ºC, 20 µl of the transformed cells were plated on LB-Agar plates supplemented with 30 µl/mg kanamycin. The next day, a single colony was picked with a toothpick and transferred into a 1L conical flask containing 100 ml of LB media with 150 µl of kanamycin. This flask was incubated for 16-18 hours at 37ºC with shaking.

3.7.2. Midi-preparation

After the transformation step, a MIDI prep of the DNA was done according to the protocol of the Qiagen® Plasmid Midi Kit (cat. nos. 12143 and 12145).

3.7.3. Boiling preparation

A boiling prep was performed in order to check whether the colony that was picked up during the DNA transformation had integrated the plasmid with the kanamycin resistance (and then, also the fluorescent protein) or if it was a surviving colony without the plasmid of interest. The first step was to spin 1.5 ml of the cell culture in a 2 ml Eppendorf® tube 1 minute at 12.000 rpm and discard the supernatant. Then, the pellet was resuspended in 200 µl STET solution and mixed with a vortex step. Afterwards, the content of the tube was boiled for 2 minutes at 95ºC, centrifuged for 10 minutes at

12.000 rpm and the pellet was removed with a toothpick. After adding 2 ml of isopropanol, the vial was spun down for5 minutes at 12.000 rpm, washed with 500 µl of 70% EtOH and centrifuged again for 5 minutes. The pellet was air dried for a couple of minutes and resuspended in 50 µl RNAse-TE.

STET solution	RNAse-TE buffer
0.5 ml of 0.5% (v/v) Triton X-100 10 ml of 50 mM EDTA (0.5 M) 5 ml of 50 mM TrisCl (pH 8.0, 1.0 M) H_2O to a final volume of 100 ml	5 ml of 10 mM TrisCl 1 M 1 ml of 1 mM EDTA pH 8 0.5M RnaseA to a final concentration of 100 µg/ml

Separately, the Test Digest solution was prepared by mixing 10 µl of the DNA mini prep, 2 µL Fast Digest® Green Buffer 10X (Fermentas), 0.5 µl restriction enzyme 1 (BamHI), 0.5 µl restriction enzyme 2 (NdeI) and 7 µl H2O. The Test Digest mix was incubated for at least 30 minutes to 1 hour at 37ºC and the 20 µl volume were run on a 1% agarose gel. The restriction enzymes BamHI and NdeI (Life Technologies) were chosen because they cut at separated places in the DNA sequence of the plasmid that is being tested (in order to avoid having very small fragments), and because they are not sensitive to methylation.

3.7.4. Agarose gel electrophoresis

1 gram of UltraPure™ Agarose (Invitrogen) was measured in a 250 ml flask. Then, 100 ml of TAE (Tris-Acetate-EDTA) buffer were added and the flask was microwaved for 2 minutes. After, 5 µl of SYBR® Safe DNA Gel Stain (Life Technologies) were added, mixed well and cooled for 5-10 minutes at room temperature. The liquid agarose was poured onto a casting tray with well combs and tape on the borders. Once it solidified, the tray was sank into the gel box with TAE buffer, the well combs were taken out and the molecular-weight size marker (GeneRuler™ 1kb 0.5 µg/µl, Fermentas) and the different samples together with 10% Fast Digest® Green Buffer 10X (Fermentas) were added. The gel box was closed so that the electrodes were in contact to allow the DNA migration for approximately 40 minutes with an electrical potential of 120V and 3 mA.

3.7.5. Picking up colonies

The morning of picking colonies, the medium of growing mES cells was changed. After 3-4 hours, 50-70 µl Trypsin-EDTA per well of a 96-well U-bottomed plate were added using a multichannel pipette. The plate containing mES cell colonies was washed twice with D-PBS and approximately 1 ml D-PBS was added for every 60 mm plate just to cover it. Individual colonies were picked with a mouth pipette using disposable sterile long tips (Costar) and the colonies were transferred into the Trypsin-EDTA solution in the 96-well U-bottomed plate. After colonies were picked, the 96-well plate was placed in the incubator for 10 minutes. Then, the trypsinized colonies were retrieved from the incubator and 2 volumes of mES cell medium per well (100-140 µl) were added to inactivate the trypsin using the multichannel pipette. Finally, the colonies were disaggregated by pipetting up and down a few times, and then the cells were transferred to the prepared 48-well or 96-well plate with feeder cells.

3.7.6. DNA preparation

The mES cell medium was aspired and the cells were washed with CMF-PBS. Then, 1 ml of DNA Lysis Buffer with recombinant Proteinase K (<600 U/ml, Thermo Scientific #EO0492) was added to a 1 mg/ml final concentration. The cells were incubated with shaking overnight at 58ºC and afterwards, 100 µl of 1.5 M NaCl were added. Finally, 1 ml of 96% ethanol was added and the DNA precipitate was fished after mixing thoroughly. The DNA Lysis Buffer consists in Tris 10 mM pH 7.5, EDTA 10 mM, NaCl 10 mM and 0.5% sarcosyl.

3.7.7. PCR

A PCR amplification (PIK024 ThermoCycler, Thermo Scientific) was performed to determine the integration of the DNA sequence of interest into mES cells through a TALEN-mediated knock-in system in a surviving colony after Hygromycin B® (Invitrogen) selection treatment. The primers used for this amplification were ROSAcaagDEST_61 and ROSA-DEST-CAAG_1355.

Table 4. Primers used for PCR detection of the TALEN-based knock-in test.

Oligo name	Sequence (5' -> 3')
ROSAcaagDEST_61 _forward (Sigma)	CTAGGTAGGGGATCGGGACT
ROSA-DEST-CAAG 1355_reverse (Sigma)	GGAAAGTCCCTATTGGCGTT

Table 5. PCR master mix volumes and cycling conditions.

Volumes
6 µL H_2O
10 µl Ampli Taq®
1 µl GC-enhancer
1 µl reverse primer
1 µl forward primer
1 µl DNA

PCR settings			
Step	Temperature	Time	Cycles
Initial denaturation	94ºC	10 min	1X
Denaturation	94ºC	0:30 min	35X
Annealing	58ºC	0:45 min	
Elongation	72ºC	3 min	
Final elongation	72ºC	10 min	1X
Cooling	4ºC	∞	

3.8. Observation

3.8.1. Fluorescence microscope

The cells were then observed at a Leica (DMS) fluorescence microscope with different light sources (*Table 6*), and with a Zeiss Confocal Laser Scanning Microscope 780 (LSM). The transfection efficiency is measured by randomly counting at least 100 colonies laying on the coverslip and checking whether they are transfected or not.

$$Transfection\ efficiency\ (\%) = \frac{Colonies\ transfected\ (fluorescent\ signal)}{Total\ number\ of\ colonies}\ x100$$

Table 6. Fluorescence microscope light sources used to analyse the expression of the fluorescent proteins encoded in the transfected plasmids.

Excitation (nm)	Colour	Suppression filter (nm)	Emission colour
510-560	Green	590	Red
340-380	UV	425	Blue
450-490	Blue	515	Green

3.8.2. FACS

The Fluorescence Activated Cell Sorting used was BD LSRFortessa™ cell analyser (BD Biosciences) from the Biomedicine Department (Aarhus University). For each experiment, 50.000 fresh cells were analysed and a double negative sample for both the mCherry and EGFP plasmids was taken as a negative control in order to gate the autofluorescence that mES cells have; whereas a co-transfected sampled with FuGENE® HD Transfection Reagent (Promega #E2311) was used as a positive control.

4. Results and discussion

The main objective of this project has been the testing, optimization and comparison of different transfection and co-transfection strategies in mES cells: FuGENE® HD transfection, Lipofectamine® 2000 transfection, SuperFect® transfection and electroporation. The transfection efficiency was determined by delivering plasmids encoding fluorescent proteins into the pluripotent cells and comparing the number of colonies expressing the mCherry and/or EGFP fluorescent proteins 36 to 48 hours after transfection from the total number of colonies that were observed at the fluorescence microscope. In order to have statistically acceptable results, duplicates of each transfection event were plated and at least 100 colonies were counted for each cover slip.

For the transfection optimization experiments, the mean transfection efficiency result and the standard deviation of both duplicates of each transfection event are represented, and five categories are shown in each chart: transfection of the plasmid encoding the mCherry protein, transfection of the plasmid encoding the EGFP and mCherry+EGFP co-transfection. In the two first cases only two possibilities were possible: either the mES cell colony had at least one cell expressing the fluorescent protein or no cell of the colony was transfected. For the co-transfection approach, the cells of a colony could transiently express both proteins, one of them (if despite adding both plasmids to the transfection mix, just one type of plasmid was delivered into the cell) and none. For this reason, the last three categories of the transfection efficiency charts represent the percentage of colonies expressing either both proteins, just the red or just the green, respectively, when both type of plasmids are added into the transfection mix.

In addition, the statistical proportion of the co-transfected and single plasmid transfected colonies (only red or green fluorescence) when both plasmids were added in the transfection mix are also being shown for these experiments. It is particularly interesting to determine if any transfection method shows a higher uptake and expression of both transfected plasmids, because of the general need for delivering more than one plasmid into the cell in the modern mutagenesis methods.

4.1. FuGENE® HD transfection

The efficiencies of five consecutive FuGENE® HD transection experiments is shown on *Fig. 8*. As for all the transfection efficiencies charts, the mean and standard deviation that are shown correspond to duplicate events from the same original transfection mix. The mean transfection results show 63.95% (±10.45%) efficiency for single mCherry plasmid transfection, 67.43% (±15.57%) for EGFP plasmid transfection and 63.56% (±16.72%) for both plasmids. In addition, 8.09% (±5.51%) of the colonies showed only red fluorescence when both plasmids were added, and 1.99% (±1.43%) had green fluorescence. For the fifth experiment only co-transfection was tested and therefore no single plasmid transfection results are available.

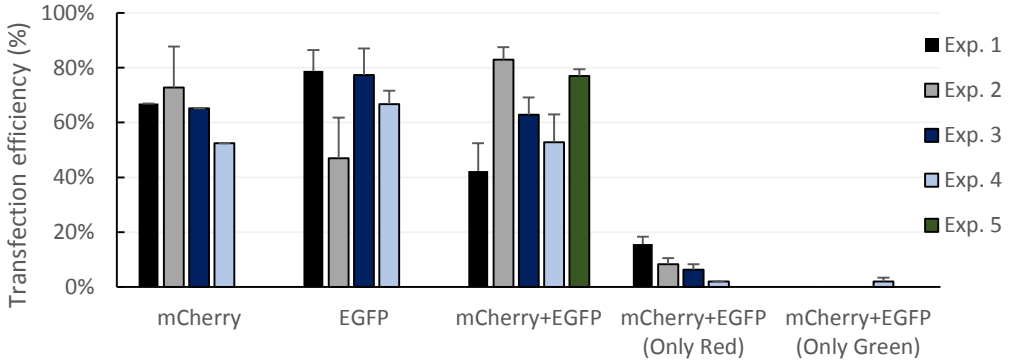

Figure 8. FuGENE HD transfection (mCherry or EGFP) and co-transfection (mCherry+EGFP) efficiency. Data from this results is based on 5 consecutive transfection experiments, represented by different colours.

For the co-transfection experiments, 89.56% (± 9.58%) of the transfected colonies expressed both fluorescent proteins, while 10.44% of the transfected colonies showed single plasmid (red or green) uptake. In *Fig. 9*, the results of each of the duplicates of the 5 co-transfection experiments are presented. For 4A and 4B experiments, no single fluorescent protein expression was found in the transfected colonies, thus 100% of the detected transfected colonies were co-transfected.

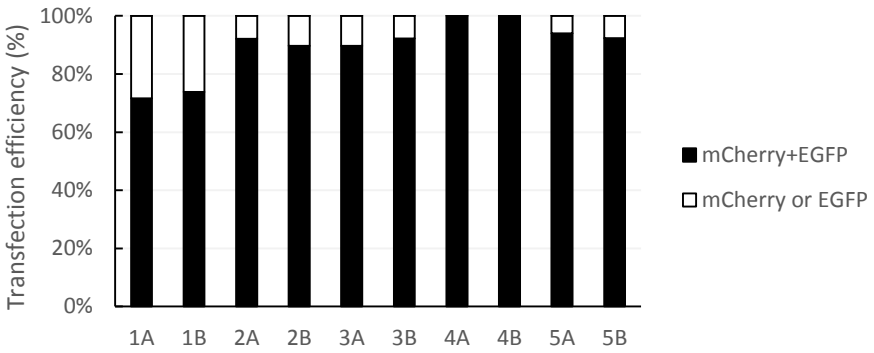

Figure 9. Percentage of expression of both fluorescent proteins (black) and single protein uptake (white) of the transfected cells in the presence of the mCherry and EGFP plasmids in the transfection mix.

Some images were taken using confocal and fluorescence microscopes (*Fig. 10*), which show a sequence of the emissions of the Hoechst stain (for nucleus and mitochondrial DNA), mCherry protein, EGFP protein and the merged picture. The yellowish colour of most of the transfected cells in the merged picture demonstrates the high percentage of co-transfected cells when both plasmids are added in the transfection mix together with FuGENE® HD.

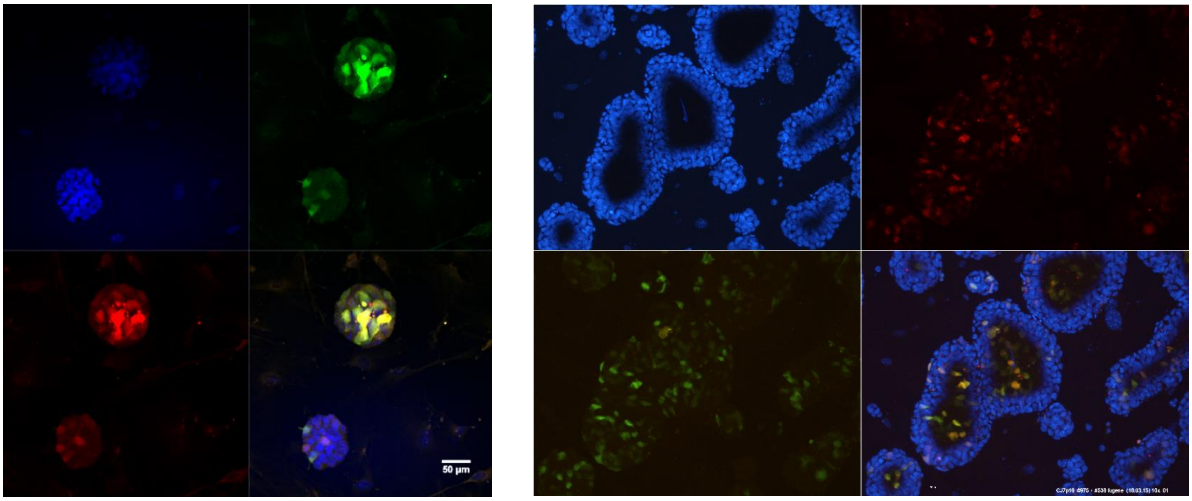

Figure 10. ***Left****: Confocal microscope images (40X magnification) of two co-transfected mES cell colonies laying onto feeder cells.* ***Right****: Fluorescence microscope images (10X magnification) of several co-transfected colonies. The centre of the biggest colonies is not stained because of the high amount of cells and the high viscosity of the Hoechst+gelvatol stain.*

4.2. Lipofectamine® 2000 transfection

Three duplicated transfection experiments were conducted using the Lipofectamine® 2000 transfection reagent. The resulting mean efficiencies were 63.23% (±8.02%) for single mCherry plasmid transfection, 73.02% (± 1.96%) for the EGFP plasmid transfection and 55.75% (±12.9%) for the co-transfection tests. Merely 1.73% (±1.42%) of the colonies showed only red fluorescence when both plasmids were added into the transfection mix, and 3.07% (±3.56%) of the colonies expressed only the EGFP. The efficiencies of each experiment are represented in *Fig. 11* (*left*). In the third experiment only co-transfection of both plasmids was tested.

For the co-transfection events, 92.67% (±3.04%) of the transfected cells in the presence of both plasmids were co-transfected, and 7.33% expressed only the red or the green fluorescent protein (*Fig. 11*, *right*).

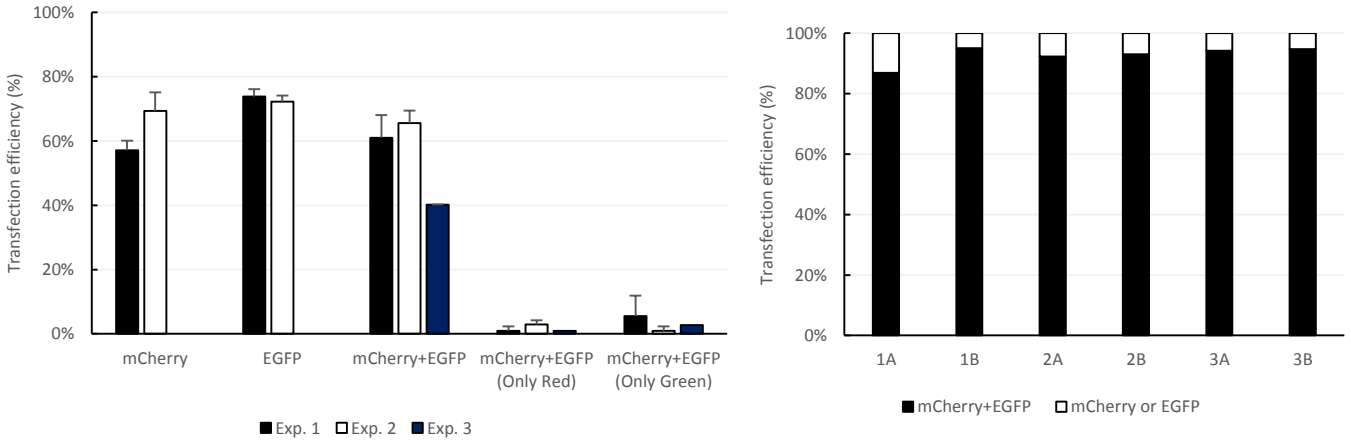

Figure 11. ***Left****: Lipofectamine 2000 transfection (mCherry or EGFP) and co-transfection (mCherry+EGFP) efficiency. Data from this results is based on 3 different transfection experiments, represented by different colours.* ***Right****: Percentage of expression of both fluorescent proteins (black) and single protein uptake (white) of the transfected cells in the presence of the mCherry and EGFP plasmids in the transfection mix. The data corresponds to the duplicates of three different co-transfection experiments.*

In the Lipofectamine® 2000 transfection protocol provided by Invitrogen it is recommended to use 5-12.5 µl of the transfection reagent for each well of a 6-well plate, depending on the cell type and passage number. Since in previous experiments conducted by our group (A.C. Füchtbauer) always 2 µl per well were used to transfect mES cells, we decided to test different volumes (from 2 to 9 µl of Lipofectamine® 2000 per well) in order to find the optimal. The results shown in *Fig. 12* (*left*) seem to indicate no significant differences when taking the standard deviations into account. All the volumes that were tested showed considerably high mean transfection efficiencies (67-74%).

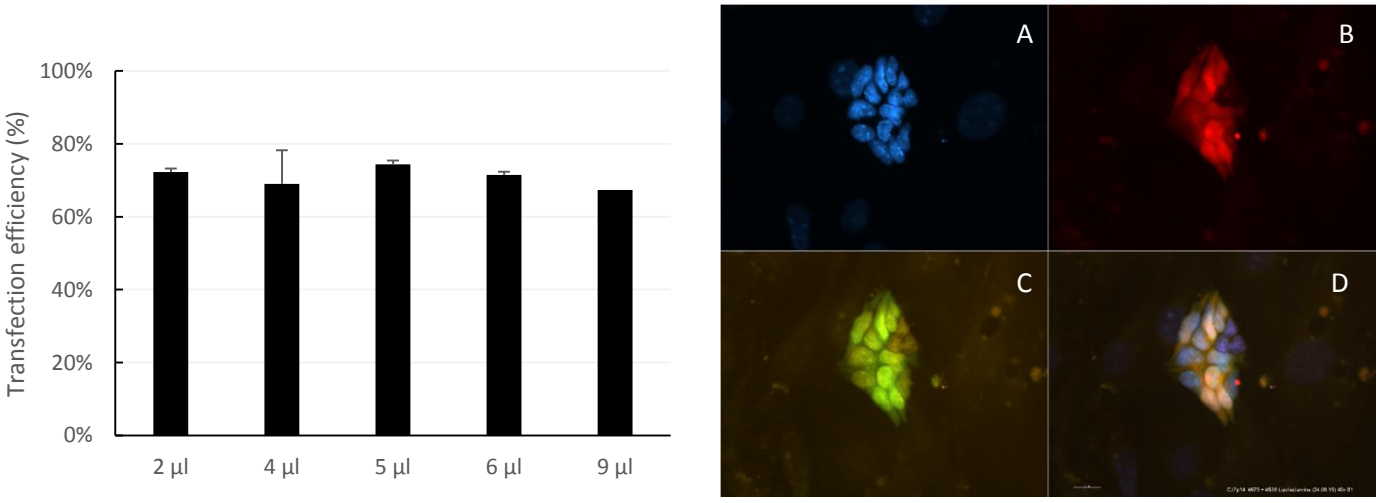

Figure 12. ***Left****: Comparison of the transfection efficiency using different volumes (2 to 9 µl) of Lipofectamine 2000 transfection reagent.* ***Right****: Fluorescence microscope image (40X magnification) of a co-transfected mES cell colony (A) expressing mCherry (B) and EGFP (C) fluorescent proteins. A sequence of Hoechst staining (A), mCherry fluorescence (B), EGFP fluorescence (C) and the merged picture (D) is shown.*

4.3. SuperFect® transfection

The transfection efficiency obtained with SuperFect® transfection reagent following the protocol described in point 3.4 was always in the range of 0-1% for the three different experiments conducted in mES cells. The mean efficiency from three separated tests is shown in *Fig. 13*, *left*. It is recommended to use 5 µl of the transfection reagent for primary cells according to manufacturer's instructions (Qiagen), but only 2 µl were used in some pre-experiments of our group (A.C. Füchtbauer). For this reason a range of DNA:SuperFect ratios (1:2, 1:3, 1:5 and 1:8) were tested to find out the best plasmid uptake efficiency (*Fig. 13*, *right)*. When adding 8 µl of SuperFect reagent (1:8 ratio) in the same cell concentration as for the rest of the tested transfection strategies, a maximum efficiency of 3.24% (±0.61%) was reached. It seems to be a positive correlation between the SuperFect volume used and the final transfection efficiency, but still, an extremely low efficiency was achieved even by using more reagent than suggested by the manufacturer company.

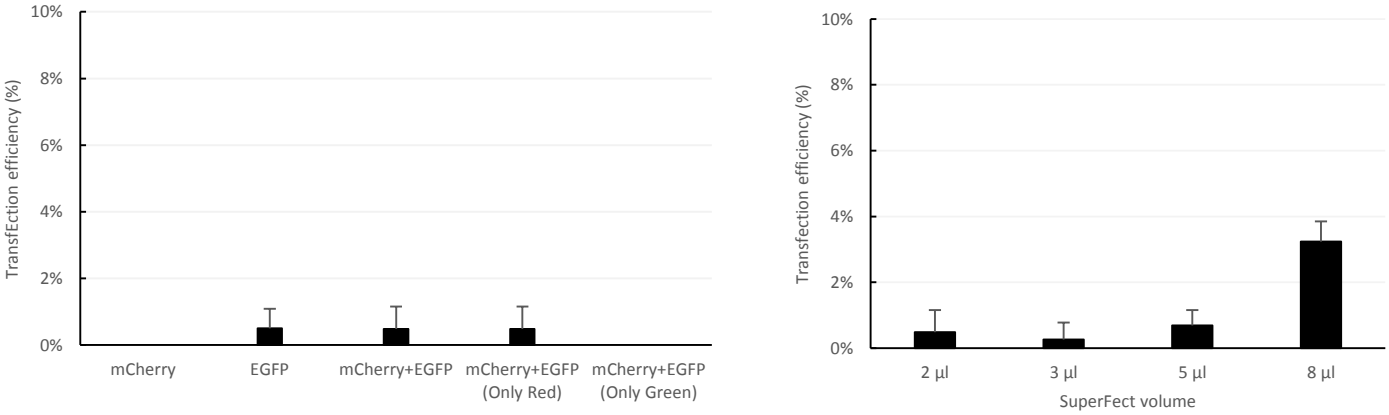

Figure 13. ***Left****: SuperFect transfection (mCherry or EGFP) and co-transfection (mCherry+EGFP) mean efficiency. Data from these results are based on 3 different transfection experiments.* ***Right****: Comparison of the transfection efficiency using different volumes (2 to 8 µl) of SuperFect transfection reagent.*

In *Fig. 14*, an example of a single EGFP plasmid mES cell transfected colony is shown. It was not possible to take a picture of a co-transfected colony strongly expressing both fluorescent proteins, since most of the positive co-transfected colonies (0.48% transfection efficiency when both plasmids were added to be transfected) showed a weak fluorescence emission.

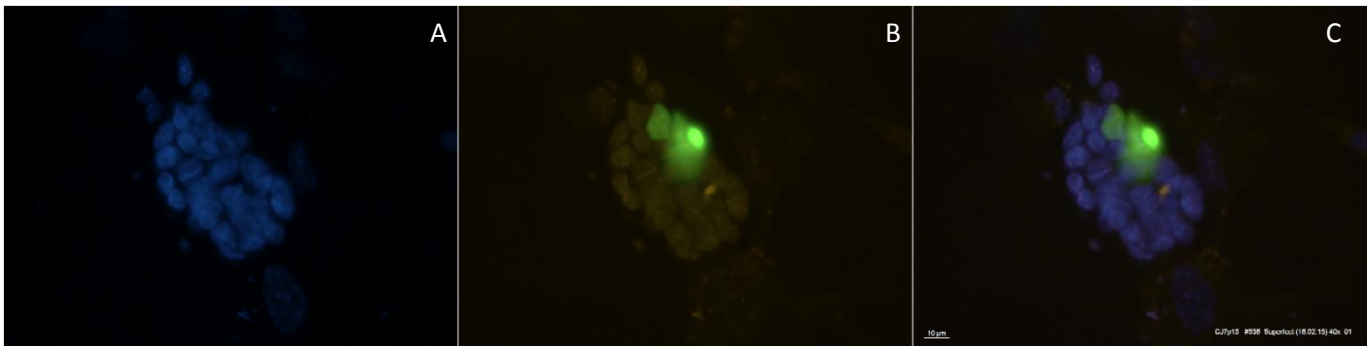

Figure 14. Fluorescence microscope images (40X magnification) of a transfected colony with the EGFP plasmid by using SuperFect transfection reagent. A sequence of Hoechst staining (A), EGFP signal (B) and the merged picture (C) is shown.

4.4. Electroporation

The mean efficiency achieved in three different transfection tests was always in the range of 2-5% for single plasmid transfection, whereas for co-transfection (mCherry+EGFP) no particular efficiency tendency seems to be followed (*Fig. 15*, *left*). In one of these three electroporation experiments no co-transfected cells were detected at the fluorescence microscope, and from the remaining two data sets, co-expression (mCherry+EGFP) and single plasmid expression (mCherry or EGFP) efficiency also shows a high divergence (*Fig. 15*, *right*).

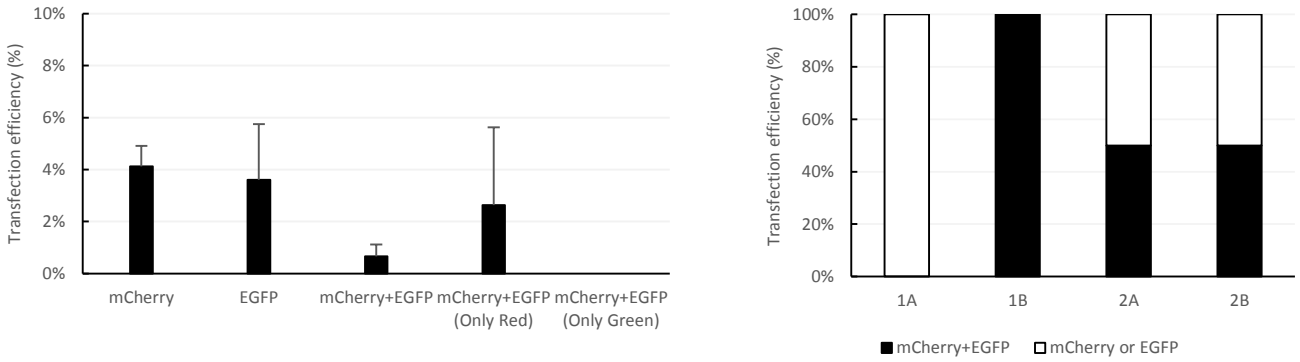

Figure 15. ***Left****: Single plasmid electroporation (mCherry or EGFP) and co-transfection (mCherry+EGFP) mean efficiency. Data from these results is based on 3 different transfection experiments. When both plasmids were added into the transfection no colonies with only green fluorescence were detected.* ***Right****: Percentage of expression of both fluorescent proteins (black) and single protein uptake (white) of the transfected cells in the presence of the mCherry and EGFP plasmids in the transfection mix. The data corresponds to the duplicates of two different co-transfection experiments.*

It is very likely that these results are not as reliable as for FuGENE® HD or Lipofectamine® 2000 transfections for several reasons. First, the fluorescence intensity of the single transfected cells was low, and sometimes the co-transfected cells were barely distinguishable from the autofluorescence background of the non-transfected cells (*Fig. 16*). In addition, due to the very low transfection efficiency, only a few colonies from the whole cover slip showed mCherry or EGFP expression. For this reason, high efficiency percentage differences of duplicate experiments like 1A and 1B (*Fig. 15*, *right*) can be dependent of insignificant variations of very low intensity fluorescent colonies.

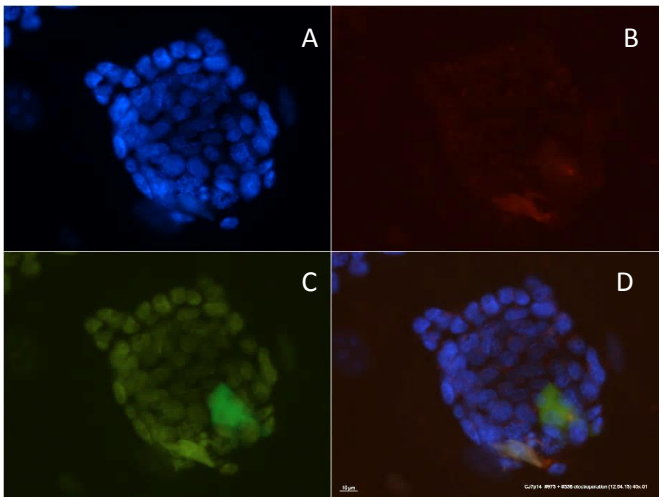

Figure 16. Fluorescence microscope image (40X magnification) of a co-transfected (mCherry+EGFP) mES cell colony by electroporation. Most of the co-transfected cells showed a very low fluorescence intensity, and could not be easily differentiated from the autofluorescence background of the non-electroporated mES cells. A sequence of Hoechst staining (A), mCherry fluorescence (B), EGFP fluorescence (C) and the merged picture (D) is shown.

4.5. Comparison of transfection strategies

Once all individual results for each transfection strategy have been shown, an overview comparison of the mean efficiencies is represented in *Fig. 17* (*left*). A first observation is that two of the studied methods, FuGENE® HD and Lipofectamine® 2000, achieve relatively high mean transfection and co-transfection efficiencies. Since the results obtained with these two reagents are highly similar, and more experiments were taken into account for the FuGENE® HD data (5 experiments for FuGENE® HD and 3 for Lipofectamine® 2000), this last reagent can be considered as the most optimal and reliable for the transfection of mES cell line used .

The low transfection efficiency of electroporation was expected since previous gene delivery into mES cells for the generation of transgenic mice has been carried out by our group (E.M. Füchtbauer). Transient protein expression detection at the fluorescence microscope was usually difficult due to the low fluorescent emission intensity of the transfected cells. Thus, electroporation seems to be more advisable for stable integration into the cell's genome of an expression vector containing selectable markers such as antibiotic resistance genes.

The SuperFect® transfection experiments showed that the reagent using the procedure described here works with an extremely low efficiency. The cause of these bad efficiency results is still unknown for us. For every transfection experiment, including SuperFect® transfections, the same mES cell mix, DNA and materials were used to test at least two approaches at the same time (e.g. FuGENE+Superfect, LipoFectamine+Superfect, etc.), so that the results could be comparable.

Taking only the co-transfection tests into consideration, 90% and 93% of the transiently transfected colonies with FuGENE® HD and Lipofectamine® 2000, respectively, expressed both fluorescent proteins (*Fig. 17, right*). For electroporation this percentage drops till 50%, which means that when a colony is electroporated, only half of the times both plasmids were taken into the cell. For the SuperFect® transfections, not enough co-transfection positives events were found to set some minimal reliable data.

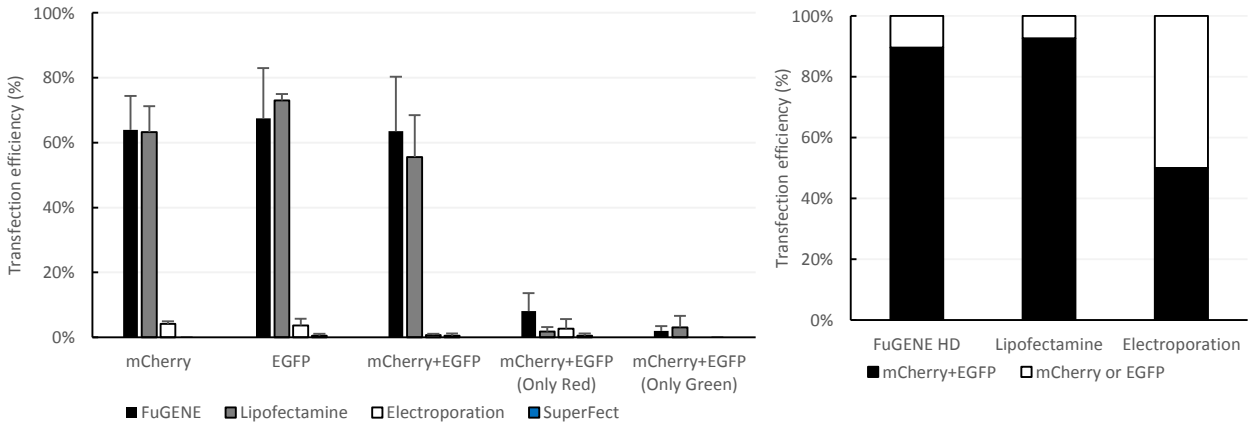

Figure 17. Left: Comparative overview of the mean transfection and co-transfection efficiency achieved with different transfection approaches (FuGENE HD, Lipofectamine, SuperFect and electroporation). ***Right****: Mean percentage of expression of both fluorescent proteins (black) and single protein uptake (white) of the transfected cells in the presence of the mCherry and EGFP plasmids in the transfection mix for FuGENE HD transfection, Lipofectamine transfection and electroporation.*

4.6. FACS analysis

In addition to the transfection efficiency analysis performed by counting colonies transiently expressing mCherry and EGFP fluorescent proteins, another type of specialized flow cytometry analysis was carried out: Fluorescence-Activated Cell Sorting (FACS). This method sorts a mixture of cells in suspension into various containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. The cell suspension is entrained in the centre of a narrow, rapidly flowing stream of liquid, which passes through a fluorescence scanning station where the fluorescent character of each cell is measured (Fluorescence Activated Cell Sorting, 2015); for these experiments, red and green fluorescence.

For this test the four transfection strategies were repeated (FuGENE® HD, Lipofectamine® 2000, SuperFect® and electroporation) following the same protocols as in the previous experiments. For each method, transfected mES cells were plated in two 6-well plates, one with a cover slip in each well (for later analysis at the fluorescence microscope) and another without cover slip, so that the cells could be trypsinized and brought freshly to the flow cytometer. In this way, these FACS results are comparable with the transfection efficiencies observed at the fluorescence microscope (*Fig. 18*).

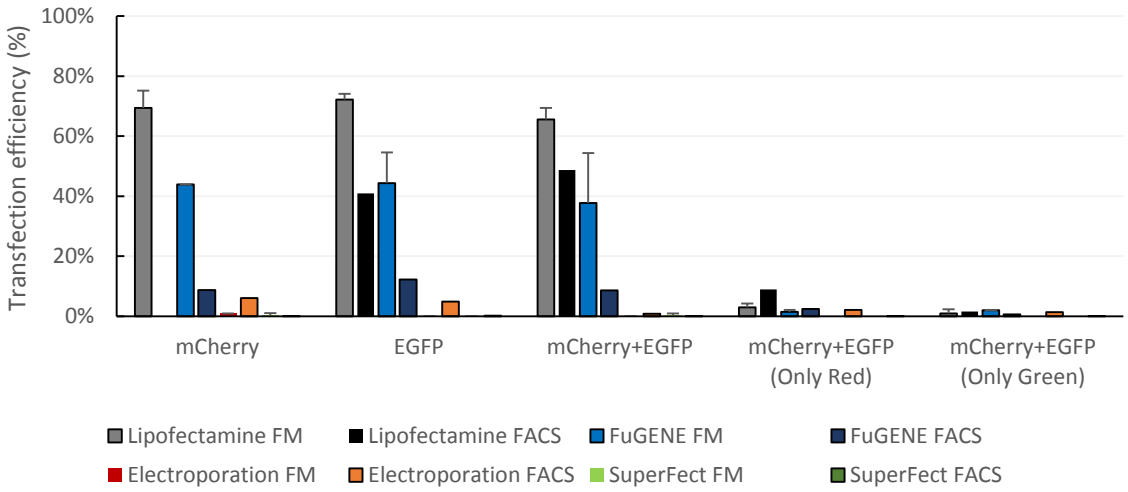

*Figure 18. Comparison of the single transfection (mCherry or EGFP) and co-transfection (mCherry+EGFP) efficiencies for the different strategies (Electroporation and FuGENE HD, Lipofectamine and SuperFect transfection) analysed at the fluorescence microscope (**FM**) or at the flow cytometer (**FACS**). The mean efficiency and standard deviation of the FM results is represented because duplicates of each transfection event were plated, while a final mean efficiency is obtained after FACS analysis. There is a blank for the mCherry Lipofectamine FACS results due to contamination of the plated wells.*

FACS analysis shows lower transfection efficiency results in all cases when compared to the results obtained at the fluorescence microscope. This is because in FACS analysis, the attached mES cell colonies are trypsinized and 50.000 individual cells are tested for mCherry and/or EGFP fluorescence, while the whole embedded colony is considered as positively transfected if only a few cells express the fluorescent proteins. Thus, in the FACS analysis individual cell transfection efficiency is tested, and on the contrary, colony transfection efficiency is studied at the fluorescence microscope (FM).

In this experiment, transfection efficiencies using FuGENE® HD were below 50% for single transfection and co-transfection at FM analysis, and between 9-12% for FACS analysis. In all of the previous transfection events, such a low efficiency was never obtained, which opens the possibility of a previous negative event like a procedure mistake or a weak contamination. For Lipofectamine® 2000 transfection FACS results, efficiencies of 41% and 49% were obtained for EGFP transfection and co-transfection, respectively. mCherry single transfection could not be tested due to the contamination of both duplicates wells were the mES cells were plated.

On the other hand, electroporation results from FACS analysis are especially valuable because of the problems to identify electroporated mES cells at the fluorescence microscope. Single mCherry and EGFP plasmid transfection showed an approximate efficiency of 5% and 6%, respectively, slightly higher than the efficiency previously detected even though considering the entire colonies instead of single cells. When both plasmids were added into the electroporation mix, 1% showed red and green fluorescence, 2% were only red and 1% only green. Finally, for SuperFect® transfection, efficiencies in the range of 0-0.02% were reached at FACS analysis, demonstrating the ineffectiveness of this strategy.

4.7. RMCE test in an RMCE-in mES cell line

After a reliable and efficient transfection technique was established in mES cells, other experiments related with the group's research where I could apply my transfection and cell culture experience were performed. The first one consisted in testing a new RMCE-in system in an RMCE-in mES cell line designed by J.E. Jakobsen (Department of Biomedicine, Aarhus University).

An RMCE-in mES cell line containing a red fluorescence gene and a puromycin resistance gene at the RMCE docking site was generated to test the cassette exchange. Four different plasmids (*Table 7*) were co-transfected into the RMCE-in mES cell line: an RMCE-in donor, containing a GFP gene as a GEI and a hygromycin resistance gene; an Flp-in donor, also containing a green fluorescence gene; a plasmid encoding for the FlpO recombinase and an empty vector (pUC19) used as a negative control. After the recombinase-mediated exchange, the mES cells that integrated the new cassette could be selected by adding hygromycin to the medium culture, and the integration of the GEI in the RMCE-in cell line could be followed by loss of red fluorescence signal and gain of GFP expression.

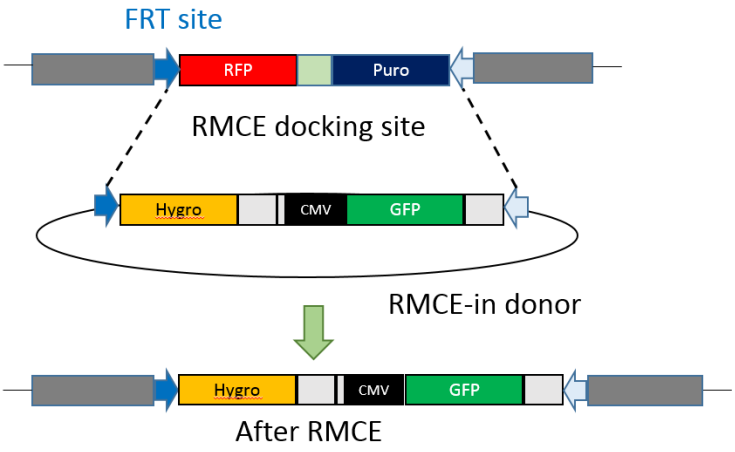

Figure 19. Simplified representation of the tested recombination mediated cassette exchange (RMCE). The RMCE-in mES cell line contains a docking site with red fluorescence and puromycin resistance genes, flanked by different FRT sites. The donor plasmid contains a GFP gene as a genetic element of interest, and a hygromycin resistance gene.

Table 7. DNA combinations (A-D) that were used to co-transfect the RMCE-in mES cell line using FuGENE HD transfection reagent.

DNA combinations			
A	RMCE-in donor + FlpO recombinase	**C**	Flp-in donor + FlpO recombinase
B	RMCE-in donor + pUC19 (neg. control)	**D**	Flp-in donor + pUC19 (neg. control)

After co-transfection of the different plasmids, part the mES cells were cultured for 5 days without selection and fixed and embedded at days 1, 2 and 5 (transient transfection). The remaining cells were cultured under Hygromycin B® (Invitrogen) selection (125 µg/ml) for 3 days in 6-well wells with cover slip (to be embedded after being cultured for 48 hours without selection), or in 60 mm dishes. The surviving colonies of the 60 mm dishes were picked and transferred into 96-well plates and cultured for 48 hours in mES cell medium to finally be either cryopreserved or sent to FACS analysis. The fluorescence signal of the embedded cells was analysed at the fluorescence microscope.

No clear green fluorescent colony was detected at days 1, 2 or 5 (transient co-transfection), and only in the Flp-in donor + FlpO recombinase (combination C) of day 2 and 5 post-transfection, some cells seemed to show more green fluorescence signal than the autofluorescence background, but any conclusion could be drawn. Surviving colonies under 3 days of hygromycin selection were analysed at the confocal microscope (*Fig.20*), and the same results were obtained. Only background signal seems to be present, and no clear GFP-expressing colony was found.

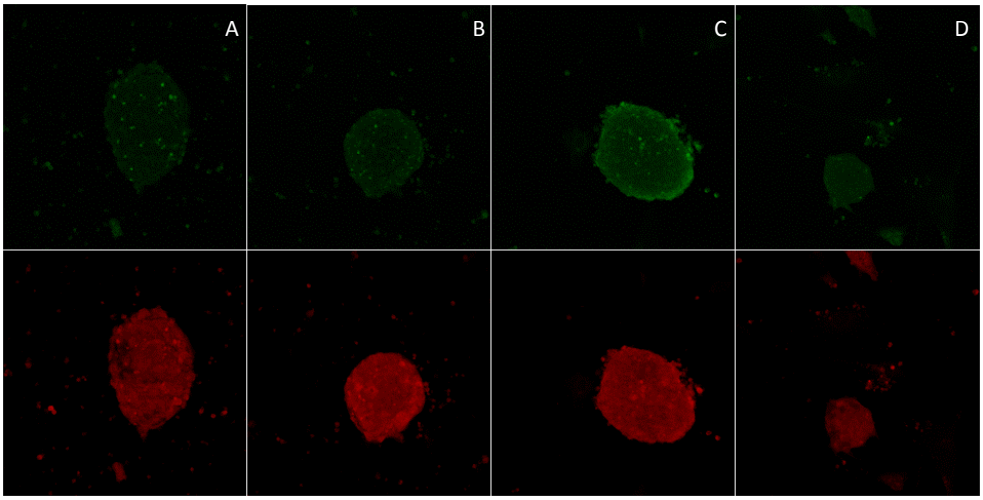

*Figure 20. Images captured with the confocal microscope (40X magnification) of the best colony that could be found in the whole cover slip for the four different co-transfections that were performed in the RMCE-in mES cell line: **A**:RMCE-in donor + FlpO recombinase; **B**: RMCE-in donor + pUC19 (neg. control); **C**: Flp-in donor + FlpO recombinase; **D**: Flp-in donor + pUC19 (neg. control).*

The surviving cells after hygromycin selection should have exchanged the cassette at the docking site and express the green fluorescent protein. The most probable explanation for this result is that not enough hygromycin concentration and culture time was applied to the mES cells. The problem with hygromycin is that it is very aggressive with ES cells, and a higher concentration would have dropped the survival rate. In addition, the GFP gene in the donor plasmid was under the control of the CMV promoter. Transient expression of protein-encoding cDNA under the control of the CMV promoter is often decreased within several population doublings in mES cells, to be finally reduced to background levels after only 3 passages. Therefore, CMV promoter is generally not used to generate stable ES cell lines (Barrow, et al., 2006). The plasmid design included this promoter because HeLa and HEK293 cell lines were first considered. After the experiments, the authors of the RMCE-in system decided to change the hygromycin resistance gene for a geneticin resistance gene, and the CMV promoter for another more robust in undifferentiated ES cells, which is still being discussed.

The surviving RMCE-in mES cell colonies were later sent to the authors of this RMCE-in system (J.E. Jakobsen). PCR analysis from these colonies showed that five out of seven picked colonies presented a band which when sequenced confirmed correct RMCE. The mES cells were targeted but the colonies were mosaic for GFP expression.

4.8. TALEN-mediated knock-in test

The second experiment were I could apply the optimized FuGENE® HD transfection and co-transfection protocol consisted in trying to knock-in a DNA sequence containing a neomycin resistance gene through transcription activator-like effector nuclease (TALEN) in the same mES cell line. Three plasmids encoding the sequence of interest (linearized), the left and the right TALEN were co-transfected together, and four 6cm dishes were plated. After 7 days under G-418® (Roche) (1 ml/100 ml mES cell medium) selection, just one colony survived (named A1). This was transferred into a 96-well well and splitted after 24 hours into two 24-well wells for then either cryopreserve the cells or for making a DNA preparation to analyse whether the neomycin resistance gene was inserted or not. After PCR with primers ROSAcaagDEST_61 and ROSA-DEST-CAAG_1355 (view point 3.7.7.), the product was analysed by electrophoresis in 1% (w/v) agarose gel.

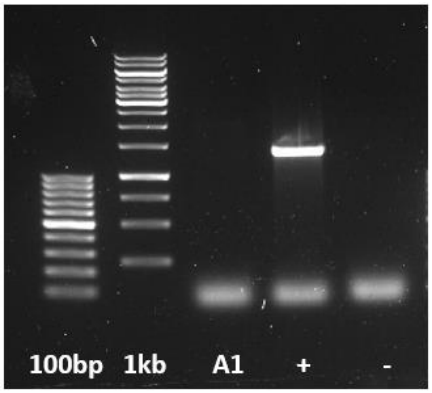

Figure 21. Agarose gel electrophoresis of the PCR product of the surviving colony after 7 days G-418 selection of a TALEN-based knock-in co-transfection, a positive and a negative control. The tested colony (A1) resulted negative.

The result of the PCR was negative for A1, which was already expected since only one colony survived the selection treatment. Another PCR could have been run for a housekeeping gene to be sure that the extracted DNA was added into the vial, but it was considered as not necessary since only one surviving colony already indicates that the TALEN-based knock-in did not success.

Prior to the beginning of the experiment, the DNA concentration was tested with NanoDrop Lite Spectrophotometer. Both plasmids encoding right and left TALEN were at a concentration of 1100-1500 ng/µl, but the linearized plasmid with the sequence of interest was at a concentration of 211 ng/µl. This may be the cause why the knock-in did not work, but no conclusions can be extracted yet. The knock-in test could not be repeated because of lack of time, and further experiments with an appropriate DNA concentration should be done to find out if this was the reason.

4.9. Cost of each transfection strategy

Finally, the cost of each transfection strategy that was tested is taken into consideration. In *Fig. 22*, the price for each µl of the different transfection reagents and the total cost for a 6-well plate experiment is represented. The optimal volumes for each reagent extracted from the concentration optimization experiments were considered: 3 µl of FuGENE HD®, 5 µl of Lipofectamine® 2000 and 8 µl of SuperFect® per 6-well well. All data were taken from the manufacturing companies, Promega, Life Technologies and Qiagen. For electroporation, the represented cost (14.4€) belongs to three Gene Pulser® electroporation cuvettes (Bio-Rad), which is the minimum number of cuvettes for each test since single mCherry, single EGFP and co-transfection are being conducted. FuGENE® HD has shown to be the most efficient and economic transfection reagent among the different gene delivery strategies that were tested in mES cells.

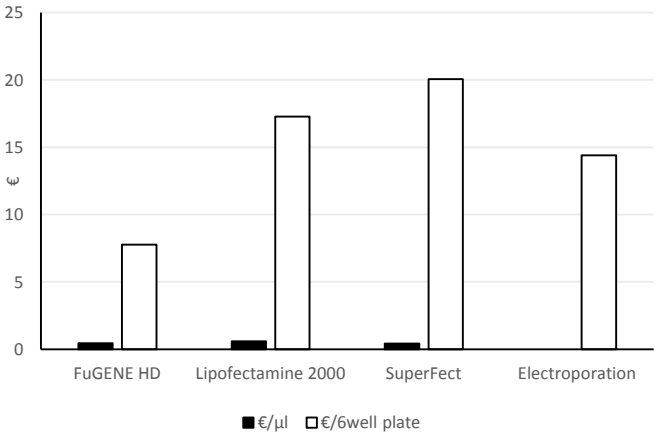

Figure 22. Cost of a transfection+co-transfection experiment using 3, 5 and 8 µl of FuGENE HD, Lipofectamine 2000 and SuperFect (respectively) per well, for 6 wells of a 6-well plate (white). Also the cost of three electroporation cuvettes is represented. The price of the transfection reagents per each µl of product is shown in black.

5. Conclusions

After the transfection and co-transfection experiments and the application of an optimized FuGENE® HD transfection procedure to test an RMCE-in system and a TALEN-based knock-in in mES cells, some conclusions can be extracted:

- Mouse embryonic stem cells, which are usually hard to transfect, were successfully transfected with two commercial reagents: FuGENE® HD (Promega) and Lipofectamine® 2000 (Invitrogen). In addition, co-transfection was concurrently tested in order to be later applied for new directed mutagenesis methods.
- FuGENE® HD was shown to be the most efficient, reliable and economic transfection reagent for the used mES cell line (52-83% transfection efficiency). Using Lipofectamine® 2000 transfection efficiencies of 40-74% were achieved.
- SuperFect® (Qiagen) and electroporation are extremely low-efficient transfection methods, (0-0.5% and 0-6.1% gene deliver efficiency, respectively) in mES cells. However, when achieving a high transfection efficiency is not the objective, electroporation is a well working strategy for stably transfecting a low number of plasmid copies.
- When two plasmids encoding different fluorescent proteins were added into the transfection mix with FuGENE® HD or Lipofectamine® 2000, in 90-93% of the transfected colonies both proteins were expressed. For the rest, only one of the two plasmids was delivered into a cell of the colony.
- FACS analysis confirmed the transfection efficiency tendencies found by fluorescence microscopy, except for FuGENE® HD transfection, were it is suspected that a single negative event dropped the percentage to levels not found before.
- PCR analysis showed that RMCE is a working system for the exchange of a desired DNA sequence in RMCE-in mES cells. The bad fluorescence microscope results helped the authors to redesign the RMCE-in donor plasmid to be more suitable for ES cells.
- The different transfection reagents have a similar price by ml of product, but FuGENE® HD showed to be approximately 1/2 and 1/3 more economic than Lipofectamine® 2000 and SuperFect®, respectively, when the cost for a complete transfection experiment is taken into account, because less reagent volume is needed.

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