ELECTROPORATION OF HUMAN MESENCHYMAL STEM CELLS FOR THE SECRETION OF FACTOR IX

ELECTROPORATION OF HUMAN MESENCHYMAL STEM CELLS FOR THE SECRETION OF FACTOR IX

By AZRA MARKAR, B.SC.

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AUTHOR: Azra Markar, B.Sc. (McMaster University)

SUPERVISOR: Associate Professor Gonzalo Hortelano

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Abstract

Mesenchymal stem cells have shown potential for success in gene therapy due to their ability to differentiate and their immunomodulatory properties *in vivo*. Although they have many inherent characteristics that are suitable for use within gene therapy, genetic modification of these cells is more difficult. Since MSCs are available in limited quantities and cannot be expanded indefinitely, the modification technique must ensure efficient expression of the transgene, a high cell survival rate and an intact ability to differentiate to various cell lineages. We optimized electroporation conditions for the genetic engineering of bone marrow-derived and umbilical cord blood-derived mesenchymal stem cells. MSCs engineered using electroporation conditions produced more transgene expression than cells engineered with cationic lipids in bone marrow-derived mesenchymal stem cells, but produced similar amounts in umbilical cord blood-derived mesenchymal stem cells. Optimal electroporation conditions also expressed more transgene than polymer based transfection reagent in umbilical cord blood-derived mesenchymal stem cells. Cell survival after optimal electroporation conditions was 67% in umbilical cord blood-derived mesenchymal stem cells. Most importantly, cells maintained their ability to differentiate into osteogenic, chondrogenic and adipogenic cell lineages. Electroporating umbilical cord blood-derived mesenchymal stem cells with a Factor IX containing plasmid lead to the FIX protein being expressed for over 12 days *in vitro*. This optimized electroporation protocol has created a fast, easy, economic and efficient method for genetically modifying mesenchymal stem cells without altering their ability to differentiate.

Acknowledgements

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List of Abbreviations and Symbols

°C – degrees Celcius

µF – microfarads

µL – microliter

µs – microsecond

BDNF – brain-derived neurotrophic factor

BMP12 – bone morphogenic protein 12

DNA – deoxyribonucleic acid

FBS – fetal bovine serum

FIX – factor IX

g – relative centrifugal force

GFP – green fluorescent protein

kb – kilobase

min – minute

ml – millileter

MSC – mesenchymal stem cell

msec – millisecond

MTT – (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

nm – nanometers

P/S – penicillin/streptomycin

PEI – polyethylenimine

RLU – relative light units

siRNA – small interfering ribonucleic acid

SOX – Sry-related HMG box

V – voltage

Ω – Ohms

Declaration of Academic Achievement

The following is a declaration that the content of the research in this document was completed by Azra Markar and recognizes the contributions of Dr. Gonzalo Hortelano, Megan Dodd and Jianping Wen in the research process and completion of this thesis.

Background

Mesenchymal Stem Cells

Stem cells have an inherent capability to differentiate into a variety of cell types, allowing them to assimilate when inserted into a biological niche. They are able to self-renew making it possible to expand populations of cells *in vitro*. This makes them suitable for *ex vivo* gene therapy. Mesenchymal stem cells (MSCs) in particular are becoming increasingly popular due to being derived from human adult tissue. This allows them to avoid the ethical concerns associated with embryonic stem cells. Mesenchymal stem cells are also well suited to be used as a form of cellular therapy due to their ability to modulate the activity of immune cells, predisposition to home to bone marrow and injury sites, and are relatively easily isolated and expanded *in vitro* [1].

Identification

In the 1970s Friedenstein *et al.* discovered colony-forming unit fibroblast-like cells from rodent bone marrow, which are now known as mesenchymal stem cells [2]. More than 40 years later specific cell surface markers exclusive to MSCs have not been identified, making true identification of these cells difficult. However MSCs are known to be adherent and able to differentiate into osteoblasts, adipocytes and chondroblasts. The consensus is that MSCs express cell surface antigens CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II markers. MSCs are from a heterogenic population

and their differentiation potential may vary depending on the source and donor [3]. The difficulty to identify MSCs, along with their heterogeneous populations makes comparing studies using MSCs difficult. Therefore it is important to keep in mind the source and the isolation techniques used when comparing MSC literature.

MSCs are a non-hematopoietic lineage of stem cells; each lineage possesses the ability to differentiate into defined cell types and subtypes. MSCs originate from mesodermal tissue and have been shown to differentiate into a variety of cells *in vitro*. This includes; bone, cartilage, adipose, tendon, ligament, muscle, stroma and non-mesodermal lineages such as neurons and Schwann cells [4-9]. The most common differentiation of MSCs shown *in vitro* is to osteogenic, chondrogenic and adipogenic cell lines. It is generally accepted that as long as the cell shows multipotent abilities, ability to adhere to tissue culture plastic, and the ability to self renew they are identified as MSCs [10].

Homing and Immunomodulatory Properties

When systemically infused, MSCs have been shown to home non-specifically to tissues including bone marrow, lung, liver, kidney and spleen. They have also been known to preferentially target tumors, inflamed tissues and sites of injury, such as genetic defects, myeloablative therapy, irradiation and myocardial infarction [11-14]. MSCs express many chemokine receptors that allow them to migrate along a chemokine gradient leading them to injured or inflamed sites [15-16]. Research into chemokines responsible for directing MSCs is ongoing but MSCs have been shown to express chemokine receptors CCR1, CCR7, CXCR4,

CXCR6 and CX3CR1. They have also been shown to migrate towards chemokines CXCL12, CX3CL1, CXCL6, CCL3 and CCL19 [17]. In particular the CXCR4 receptor has been implicated in MSC homing due to its strong attraction to the chemokine CXCL12 [6, 18].

MSCs directly injected into the tissue of interest have also shown engraftment and differentiation into the local cell type [19]. This type of delivery is particularly useful if injury repair is desired, allowing the cells to be directly implanted to the desired site.

MSCs are known to have immunomodulatory effects through a variety of mechanisms. They are able to block T cell activation [20]. They can also block and then reverse the maturation of mature dendritic cells back to immature dendritic cells, thereby helping to deplete activated T cell populations [21]. They decrease pro-inflammatory cytokines, while increasing anti-inflammatory cytokines such as IL-10 [22]. These immunomodulatory effects make MSCs particularly interesting for cell therapy applications. This ability allows the cells to survive longer within the host without eliciting an immune response. This prevents the implanted cells from being rejected and allows them to reside alongside host cells.

Sources

MSCs have been isolated from a huge variety of sources including bone marrow, umbilical cord blood, peripheral blood, muscle, vasculature, skin and adipose tissue [23-30]. Although an extensive variety of sources exist, certainly the most popular for therapeutic research today is bone marrow. Bone marrow-derived MSCs possess the potential to proliferate and differentiate, but the extraction procedure is painful and the frequency of MSCs in the bone

marrow is low, at approximately 0.00003% [3, 31]. This is resulting in umbilical cord blood and adipose tissue sources gaining popularity.

In a comparison of bone marrow, umbilical cord blood and adipose tissue-derived MSCs, differentiation into chondroblasts and osteoblasts seemed similar in MSCs from all sources. It was also found that umbilical cord blood-derived MSCs showed little to no adipogenic differentiation. Smaller lipid vacuoles were formed from umbilical cord sources when compared to bone marrow and adipose tissue sources [32-33].

Although umbilical cord blood-derived MSCs show minimal differentiation to adipogenic lineages, they still may play a useful role in gene therapy where specific differentiation to adipocytes is not necessary. They are abundantly available and are harvested at no risk to the donor [34]. They are found in low frequency, but have the highest expansion potential compared to other sources [33]. Therefore, depending on the application, umbilical cord blood-derived MSCs may show tremendous potential for cellular therapies.

MSCs are generally isolated by collecting cells from the source and centrifuging to separate cells by density. The cells of the appropriate density are grown on tissue culture plates. MSCs adhere to tissue culture plates therefore non-adherent cells can be washed away, isolating the appropriate cells [6].

Gene Therapy

Gene therapy is a strategy to treat a disease by the transplantation of genetically engineered cells (*ex vivo* gene therapy), or by the direct administration of a therapeutic gene (*in vivo* gene therapy) [35]. *In vivo* gene delivery is when DNA is delivered directly to the cells of the target tissue, without removing the tissue from the host. If this type of gene delivery is used, the target cells must be accessible for injection and the vector must infect, integrate and be expressed within the cell [36]. Recent clinical trials have shown success delivering FIX using adeno-associated virus as a vector [37].

On the other hand *ex vivo* gene delivery transfers the therapeutic genes into cells *in vitro*, and these cells are then transplanted into the target tissue. For this delivery method the cells must be readily available, able to engraft in the host and survive for a required length of time *in vivo*. They must also express the transgene at high levels for an extended period without eliciting an immune response. This makes MSCs an ideal target for *ex vivo* gene therapy. An advantage of *ex vivo* gene delivery is that the cell population can be characterized before transplantation. This increases safety by ensuring that the transgene has caused no ill effects to the cell. Another advantage is that cells can be sub-cloned and monoclonal populations that secrete high levels of transgene can be isolated. Disadvantages are that harvesting and maintaining the therapeutic cells in culture can be difficult, along with ensuring that the cells are expressing the transgene, resulting in a typically higher expense. Another disadvantage is that when the cells are transferred to the non-host tissue, they may interfere with the activity of the implantation site. For example, fibroblasts that are transplanted into the central nervous system sometimes

produce collagen, this collagen interferes with the function of the hosts central nervous system [36]. This problem can be solved by using stem cells for delivering the therapeutic gene due to their potential to engraft and repopulate host tissues.

Stem cells, including MSCs, are particularly interesting for *ex vivo* gene therapy due to their interaction with the immune system. Successful *ex vivo* gene therapy requires cells that will not be rejected by the host immune system. MSCs immunosuppressive properties make them extremely attractive for gene therapy [38]. This interaction has been demonstrated *in vitro* and *in vivo* in both animal and human models [39-42].

Gene therapy can be used to treat infectious diseases, cancers, inherited diseases and autoimmune disorders [35]. More research in this area may provide many novel and more effective ways of treating various disorders.

Challenges of using MSCs for Gene Therapy

The main challenge with using MSCs for gene therapy is that they have a low receptivity for foreign DNA, which is common of all primary cells, therefore genetic modification techniques must be carefully selected and modified to maximize DNA uptake [43].

Although MSCs are known to be easily enriched and expanded *in vitro*, they are usually recovered in low numbers [43]. After a certain number of cell divisions the cells reach senescence, limiting the amount any population can be expanded [44]. Cells have been shown to grow up to passage 12 with no reduction in telomere length, but between passage 12-16

cells undergo growth arrest [45]. It has also been shown that bone marrow-derived MSCs ability to proliferate and differentiate declines with age [46]. This is attributed to telomere shortening, which is accompanied by a slight enlargement or irregular shape of cells, resulting in cells that eventually stop dividing [44, 47]. Due to limited recovery and expansion of MSCs it is important that if transfection or transduction is desired that it be as efficient as possible to maximize usage of available cells.

MSCs have been isolated from a number of species including mouse, rat, avian, porcine, simian etc. Different abilities to modify MSCs of different species origin can pose additional challenges to *in vitro* modification and gene therapy.

Viral Transduction

Two main types of vector systems exist for gene therapy; the non-viral vectors and the viral vectors [48]. Advantages and disadvantages of each should be considered carefully when choosing vectors for gene therapy.

Although viral vectors are proven to be very efficient, the immune responses to vectors and transgenes have lead to safety concerns. Viruses gain access to host cells and exploit the hosts' cellular machinery to facilitate their own replication. Viral vectors use this infection pathway, but have been engineered to not express the viral genes that lead to replication and toxicity [48]. Popular viruses that have been used to transduce MSCs include retroviruses, lentivirus, adenovirus, adeno-associated virus and herpes virus [49].

Different viruses are more specialized for certain applications. For example, herpes simplex has a natural tropism for central nervous system making it ideal for applications in gene therapy to the brain [50]. It is also able to transfect rather large genes; up to 150kb [51].

Some viruses are able to integrate into the host genome making them ideal for long term expression of transgene. Retroviruses integrate themselves into the host genome, and provide high long-lasting transgene expression [52]. Lentivirus, a type of retrovirus, is able to infect dividing and non-dividing cells [53]. The downside is that if the integration is not targeted it can cause undesired mutation of the host genome.

Non-Viral Transfection

Non-viral vectors are advantageous over other vectors because they avoid many safety concerns presented by viral vectors. Although the immune response is low, there is some mild inflammation that may be associated with these vectors [54].

Unmethylated CpG dinucleotides have shown to induce an inflammatory response upon administration of non-viral vectors [54-55]. CpG dinucleotides cause B-cells to proliferate and secrete immunoglobulin. B-cell activation requires an unmethylated CpG dinucleotide flanked by two purines at the 5' end and two pyrimidines at the 3' end. The unmethylated CpG dinucleotide is found frequently in bacterial genomes, and less often in vertebrate genomes. Vertebrate genomes contain around one unmethylated CpG dinucleotide every 160 base pairs, compared to their bacterial counterparts, where unmethylated CpG dinucleotides are found

once every 16 base pairs [56]. This difference in frequency has resulted in an evolutionary link, where the immune system recognizes microbial DNA for attack [55]. This can cause problems in gene therapy where plasmids derived from bacterial genes are routinely used as vectors. One option is to use a non-viral system with DNA that is free of CpG dinucleotides. By altering DNA codons one can remove CpG dinucleotides, while maintaining the same amino acid sequence. By eliminating the unmethylated CpG dinucleotides the immune response to the vector is lowered extending the transgene expression [57].

Non-viral vectors can be administered repeatedly without generating an immune response, are also known for being more cost effective and able to transfect larger genes into cells [58-59]. Although viruses offer a fast and efficient way to deliver genes into cells, non-viral vectors must be seriously considered for their safety if gene therapy is to continue to clinical trials.

A variety of options for non-viral transfection exist, and are generally broken down into two categories; physical and chemical methods. Physical methods work by creating openings in the cell membrane to allow DNA entry, for example microinjections and electroporation. Chemical methods use a substance as a carrier to deliver DNA through the bilipid membrane, for example cationic lipids and polymers [60].

Cationic Lipids

Due to their positive charge cationic lipids form lipoplexes when mixed with DNA due to electrostatic interactions [61]. When applied to cells these lipoplexes are believed to be taken

up by endocytosis [62]. Once inside the cell the endosomal membrane is broken down resulting in the DNA being released into the cytoplasm [63]. Although cationic lipids are widely used for cell transfection, the mechanism that liposomes use to transport the gene into the nucleus is not clear [64]. Translocation through nuclear pores as well as access to the plasmid during cell division has been suggested [65].

Optimization for specific cell types can be achieved by altering particle size, charge and zeta potential of the lipoplexes [61]. Cationic lipids have been successfully used to transfect up to 35% of mesenchymal stem cells at an early passage without affecting the differentiation potential and viability [64]. Higher passages show different levels of gene expression. The downfall of this technique is the cytotoxicity associated with cationic lipids and limited efficiency for gene delivery and expression [66]. Another suggested challenge is that the serum-free conditions required prior to cationic lipid transfection may lead to unwanted differentiation of MSCs [67]. Although this is mentioned in the literature no substantial evidence was found to support these claims.

Polymers

Cationic polymers are generally used for gene delivery. Their chemical composition, structure, size, branching and side chain lengths can easily be altered to produce optimal polymers for various applications. Polymers used for transfection usually have a high density of positively charged groups; these interact with negatively charged phosphate groups on DNA forming polyplexes. Once applied to cells the polyplexes enter through endocytosis [68].

Polymers can be divided into two groups, synthetic and natural polymers. Polyethylenimine (PEI) is a synthetic polymer commonly used for transfection [68]. Once endocytosed it acts like a proton sponge and pumps protons into the endosome [69]. This results in an influx of chloride anions which ruptures the endosome [68]. PEI complexed with SOX-5, SOX-6 and SOX-9 genes coated onto PLGA nanoparticles and applied to MSCs have shown increased chondrogenesis and up to 80% of cells expressing the transgene after two days [70].

Polymers can be added to scaffold material to transfect MSCs in a 3D environment. This allows the cells to be transfected once they are seeded onto the scaffold eliminating the step of transfection in a 2D environment before seeding. A study by Hosseinkhani *et al.* used an acetylated form of PEI, embedded in collagen and reinforced with poly(glycolic acid) as a transfection agent [71]. Acetylation has been shown to decrease cytotoxicity of the polymers while increasing transfection efficiency [72]. This method of transfection resulted in higher transgene expression from a 3D environment than the same polymer in a 2D environment [71].

Naturally derived polymers such as chitosan and pullupan are less efficient but generally less toxic than synthetic polymers [73-75]. Pimpha et al formed a nanoparticle that combined naturally derived chitosan with PEI to benefit from the transfection ability of PEI and the lowered toxicity of chitosan. Their polymer was tested on rat MSCs and demonstrated trasfection rates higher than lipofectamine and chitosan as well as lower toxicity than PEI [75].

Electroporation

Electroporation is a non-viral transfection technique that uses a short high voltage pulse to electropermeabilize the cell membrane. There are two types of pores created during electroporation, transient and long-lived, and both play a role in transport [76]. The permeable state can last up to minutes after the application of the electric pulse, and by overcoming this barrier a variety of molecules can diffuse or be electrophoretically driven through the destabilized membrane [76-77]. The permeabilization of the membrane is transient and the membrane reseals itself after the voltage is removed [77]. If electric fields are too high, the cell membrane is disrupted beyond repair and results in cell death.

Negatively charged DNA is electrophoretically driven through the destabilized membrane while the pulse is applied [77-78]. If long-lived pores remain open after the pulse is removed DNA can continue to diffuse through the permeable membrane.

Higher electric fields lead to larger permeabilized area of cell membrane, due to higher energy for pore formation, resulting in more pores being formed. Transient pores also increase with increasing number of pulses, suggesting each pulse contributes to more/larger stable long-lived transport pores [76].

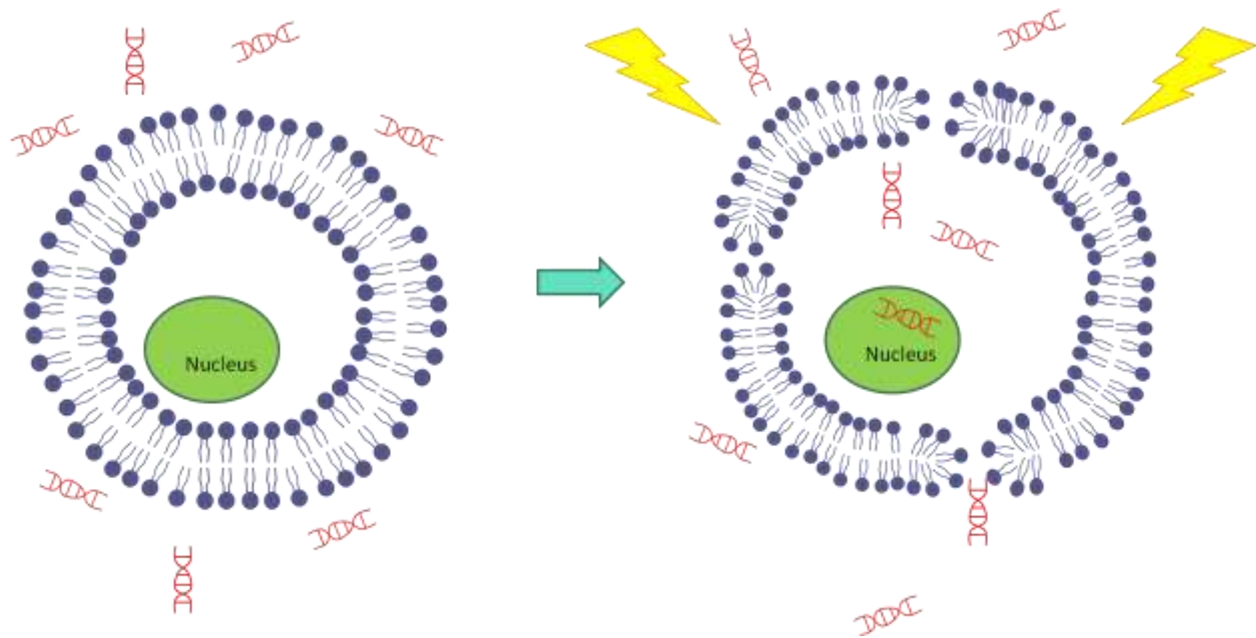


Figure 1: Electroporation. The addition of an electric pulse disrupts the cell membrane creating pores. DNA is able to enter the cell membrane through these pores. The pores are resealed once the voltage is removed and DNA taken to the nucleus will be expressed. Electroporation does not provide a mechanism for DNA to be integrated into the cell's genomic DNA.

Generally there are two waveforms available when electroporating cells; exponential decay and square. Exponential decay allows an initial chosen voltage to be applied which decays exponentially depending on the set capacitance and the resistance of the sample. Electroporation buffers with high ionic strength such as phosphate buffered saline or serum-free growth media generally require a high capacitance when electroporating mammalian cells. A square waveform allows a chosen voltage to be applied to the cells for a preset amount of time. The amount of time the voltage is applied is referred to as the pulse length. The square

waveform is generally known for being more gentle on cells and appropriate for primary cell types, and also allows for multiple pulses to be delivered [79].

All these factors can be altered to change the conditions of the electroporation. By changing one factor at a time, within a specified range, and testing for transgene production the optimal electroporation conditions can be determined. Other factors that can be modified to optimize electroporation is the amount of DNA and cell density [80].

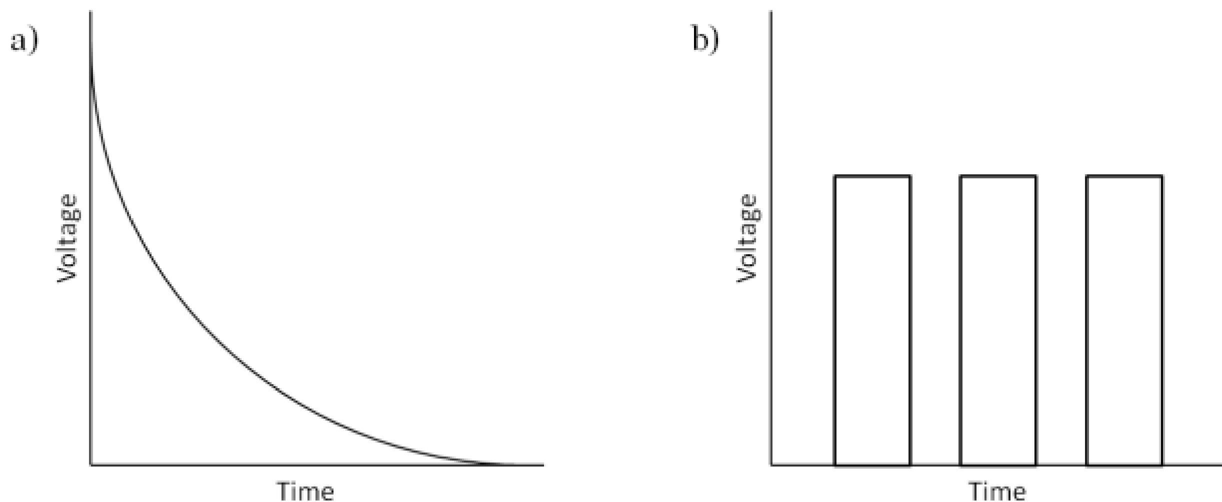


Figure 2: Exponential waveform and Square waveform. When using an exponential waveform the voltage drops exponentially over time (a). Square waveforms create a sudden burst of high voltage (b). This voltage is maintained for a certain time, or pulse length, after which the voltage drops down to zero. A square waveform allows multiple pulses to be applied to cells, depicted above.

Traditional electroporation, when compared to other non-viral transfection techniques, can lead to high transfection efficiencies but also high cell mortality [81]. Many authors publish optimization, transfection efficiency and cell viabilities, but fail to include cell recovery or yield of transfection after electroporation [81-83].

High cell mortality has been suggested to be due to high electrode surface area [84] leading to the development of a microporation technique. In microporation, cells are contained within a capillary style chamber as opposed to the traditional electroporation cuvette. This helps to decrease the electrode surface area and reduces the effects of pH variation, temperature variation and metal ion release; all attributed to poor cell survival in traditional electroporation [85]. Microporation has been shown to produce transfection efficiency of 40% with a cell survival rate of 85% in bone marrow MSCs [81] while others report transfection rates up to 83% in umbilical cord blood-derived MSCs with no mention of cell survival [85].

Nucleofection is another commonly used technique based on electroporation. This technique allows the DNA to be electroporated directly into the nucleus, which is ideal for non-dividing or difficult to transfect cell lines [86]. Although the technique uses the same basis as electroporation, little is known about the electrical parameters and details of the technique. Therefore, how the DNA is directed to the nucleus is unknown. Nucleofection has been successful in human marrow stromal cells, human and murine embryonic stem cells and bone marrow-derived MSCs [82, 86-88]. Nucleofection of bone marrow-derived MSCs had a transfection efficiency of up to 27% with cell survival of approximately 40% [82].

Optimization studies have shown traditional electroporation to be a useful technique for transfecting MSCs. Optimized electroporation conditions for rat bone marrow-derived MSCs were found to be 1500V/cm using Spinner's minimum essential medium as electroporation buffer at 22°C. These conditions achieved 29% transgene production after 48hrs and produced transgene for at least 10 days. This protocol did not affect the MSCs ability to differentiate into osteoblasts, adipocyte and chondrocyte lineages [83].

Human marrow stromal cells were also used to optimize electroporation conditions. Optimal conditions were found to be a square wave pulse with 600V and a pulse length of 100µs. Using an EGFP plasmid, 1 million cells were electroporated with 10µg linearized plasmid DNA which resulted in stable transfection. While under antibiotic selection, the cells were expanded over 2 weeks to produce 98% expression of EGFP. The cells were differentiated into adipocytes and osteoblasts successfully, demonstrating their differentiation abilities were not affected by the transfection or culturing [89].

In a comparison study of electroporation and lipofectamine transfection agents, electroporation produced higher transfection efficiencies than lipofectamine transfection. Lipofectamine agents were found to produce high levels of cell death (no data published) and altered cell morphology in human bone marrow MSCs. Electroporation was found to be harsh on cell survival as well with 35-40% of cells surviving electroporation. MSCs were found to maintain their ability to differentiate into adipogenic and osteogenic cells after electroporation. Whereas transfection with Lipofectamine™2000 was shown to reduce the cells adipogenic differentiation ability [43].

The most common application of electroporation for MSCs is to introduce genes to promote differentiation to a chosen lineage. Human umbilical cord blood MSCs electroporated using microporation with BDNF expressed transgene for two weeks, resulting in differentiation to neural cells [85]. SOX-5, SOX-6 and SOX-9 genes co-transfected into MSCs resulted in chondrogenesis [90], and rhesus bone marrow MSCs electroporated with BMP12 genes resulted in tenocyte differentiation [91]. Electroporation has also been used to create erythropoieitin secreting human bone marrow MSCs demonstrating the variety of applications for this technique [92].

Although the electroporation technique is well established for bacterial cultures its application to mammalian cells is relatively new. There is little information available about the effects of electroporation on MSCs, therefore there is no conclusive opinion on how effective it is and how it affects cell viability. From preliminary studies it appears that electroporation shows promise as a non-viral transfection method for MSCs.

Hemophilia

When a blood vessel or capillary is damaged the body's response is to form a clot comprised of platelets held together with fibrin [93]. When bleeding occurs the sub-endothelial layers of a blood vessel is exposed leading to hemostasis. Platelets aggregate at the site of injury and initiate the coagulation cascade, resulting in fibrin being formed increasing the strength of the platelet plug [94]. The multiple components involved makes the coagulation cascade a complex system, and one improperly functioning factor can lead to the inability to form blood clots.

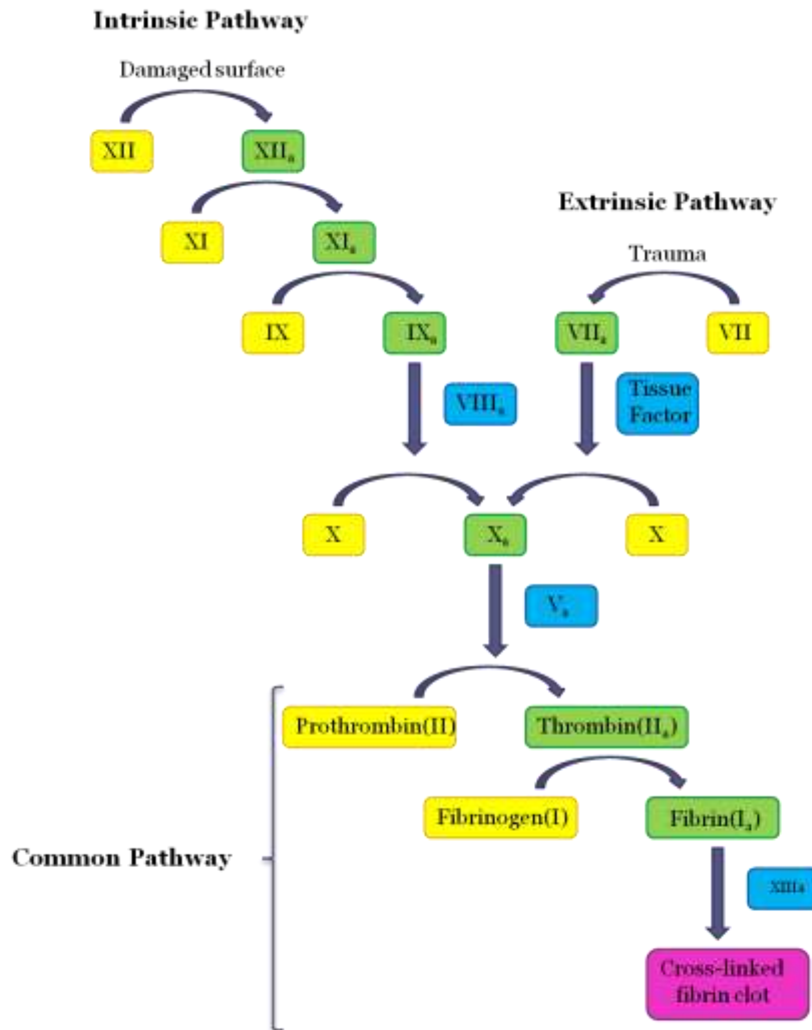


Figure 3: Inducers of the Coagulation Cascade. A blood clot is formed when cross-linked fibrin holds together platelets at the site of injury. Creating cross-linked fibrin can occur through two pathways; intrinsic or extrinsic. The intrinsic pathway is activated when the collagen in a blood vessel wall contacts blood as it is damaged. The extrinsic pathway is activated when a blood vessel is damaged. The two pathways are combined into a final common pathway that results in cross-linked fibrin.

Hemophilia B is an X-linked bleeding disorder caused by lacking or a defective coagulation factor IX (FIX) [95-96]. Severe hemophiliacs may suffer from spontaneous bleeding episodes that can be life threatening. Repetitive bleeding can also lead to chronic bleeding in joints resulting in arthropathy [96]. Hemophilia B is found in 1 in 30,000 males and is less common in females. Females are often carriers of mutated FIX genes and are usually asymptomatic but can be identified with molecular analysis [95].

Hemophilia is diagnosed from prothrombin time, activated partial thromboplastin time, and levels of factor IX [95]. Severe hemophiliacs have 1% of normal human FIX levels in their blood, whereas moderate to mild cases have 1-4% and 5-25% of normal levels respectively [97].

Current treatment involves regular infusions of either plasma derived factor IX or recombinant factor IX. Plasma derived factor IX can potentially transmit blood-borne pathogens, which caused several HIV and hepatitis infections in hemophiliacs in the 1980s. Recombinant factor IX on the other hand is much safer, but a very expensive alternative [96]. Although infection risks associated with plasma derived factor IX have been greatly minimized, the high cost of recombinant factor IX emphasizes the need for improved therapy for hemophilia patients.

Hemophilia is a suitable model for developing gene therapy strategies because expression of factor IX is not tightly regulated, therefore even a 1% increase of factor IX antigen can improve the quality of life for hemophilia B patients. Another advantage of using hemophilia to study gene therapy is that excellent murine and canine animal models exist which closely resemble the human condition [96].

Thesis Rationale

The results of electroporation studies have shown promise for a successful non-viral gene delivery to MSCs [43, 81-83, 85, 89]. MSCs are an ideal cell type for gene therapy due to their immunomodulatory effects, ease of collection, and reduced ethical concerns. Bone marrow-derived and umbilical cord blood-derived mesenchymal stem cells have been acquired from the Janowska lab in Edmonton, Alberta. The current work will focus on using the BioRad Gene Pulse Xcell Electroporation System to determine optimal electroporation conditions for bone marrow-derived and umbilical cord blood-derived MSCs.

These conditions will be compared with other non viral transfection methods such as cationic lipids and polymers, to compare the protein production and cell viability post-transfection. To ensure that electroporation has no negative effect on MSCs is vital to maintaining their unique cell characteristics, therefore their capacity to differentiate will be tested post-electroporation.

This ability to differentiate along with other characteristics makes MSCs ideal for gene therapy. Our electroporation technique will be applied to hemophilia B gene therapy. Electroporating MSCs with a CpG-free human factor IX containing plasmid can create FIX secreting cells, potentially leading to a future cell therapy for hemophilia B.

In summary, this study aims to optimize electroporation conditions for human mesenchymal stem cells. It will apply this electroporation technique to human mesenchymal stem cells with CpG-free plasmids containing the factor IX gene, to engineer mesenchymal stem cells to secrete factor IX.

Objectives

The goals of this project are to:

- Create optimized electroporation conditions for human mesenchymal stem cells
- Determine if electroporated mesenchymal stem cells retain the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages
- Compare optimized electroporation protocol with a commercially available cationic lipid and polymer transfection formulation
- Genetically engineer human mesenchymal stem cells to express factor IX

Materials and Methods

Cell Lines and Culture

Human mesenchymal stem cells were derived from either bone marrow or cord blood supplied by Janowska lab in Edmonton, Alberta. Cells were obtained with donor consent and guidelines approved by the University of Alberta Ethics Committee. Cells used for all experiments were between passage 5 and passage 9 to ensure that cells were healthy, did not reach senescence or differentiate. The cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum (FBS) and 1% penicillin (100μg/ml) and streptomycin (100μg/ml) (P/S) antibiotic. Cells were grown on uncoated polystyrene tissue culture plates at 37°C with 5% CO_2. Once cells reached 75-80% confluence they were expanded from 1 to 2 new

tissue culture plates. Ensuring cells do not grow past 80% confluency helps to prevent differentiation or senescence due to high cell concentrations [98].

To detach cells for expansion or other purposes growth medium was aspirated from the cell culture plate, and the cells were washed with phosphate buffered saline (PBS) to remove excess media. TrypLE (GIBCO) was then added to cover the bottom of the plate and the cells incubated at 37°C with 5% CO_2 for 3-5 minutes. Once removed from incubator the trypLE was deactivated by adding growth media.

To count cells, a 100µL sample of detached cells is diluted 1:100 with isoton II diluent (Beckman Coulter). Cell concentration is determined by measuring this sample using a particle counter (Beckman Coulter).

Transfection

Electroporation

Electroporation employs electricity to create transient pores in the cell membrane allowing entry of DNA or other molecules. The cells were grown until they reach 70%-80% confluence and detached in preparation for electroporation. Detached MSCs were collected into a 50ml falcon tube and centrifuged at 1000 g for 10 min at 4°C (Mendel Scientific Co. Ltd). The supernatant was discarded and the cell pellet was resuspended in Opti-MEM reduced serum medium (GIBCO). Washing with Opti-MEM is essential to remove traces of growth media which causes cell death during electroporation. 100µL of this mixture was used for counting and the

remaining was centrifuged at 1000 g for 10 min at 4°C (Mendel Scientific Co. Ltd). The supernatant was discarded and the cells were centrifuged in the appropriate amount of Opti-MEM to produce a final cell concentration of either 1×10^{6}cells/ml or 5×10^{6}cells/ml. The appropriate amount of the chosen plasmid was added to a final concentration of 50µg/ml. The cell/plasmid mixture was then aliquoted into electroporation cuvettes and electroporated using a Gene Pulser Xcell System (BioRad). After electroporation the cells are allowed to rest approximately 3 minutes at room temperature, allowing the cells to recover from electroporation and pores in the cell membrane to close. Cells were then removed using a micropipette and added to tissue culture plates containing growth medium supplemented with 20% FBS and 1% P/S.

Lipofectamine™2000

Lipofectamine™2000 is a commercially available transfection agent that employs cationic lipids to introduce DNA or siRNA into cells. Cells were grown until 80% confluence is achieved in antibiotic free media. DNA and Lipofectamine™2000 are prepared separately by diluting in Opti-MEM according to amounts in the manufacturers protocol (Invitrogen). The Lipofectamine™2000 mixture is incubated 5 minutes at room temperature. After incubation the DNA and Lipofectamine™2000 mixtures are combined, mixed gently and incubated for 20 minutes at room temperature. The solution is added to the cell culture plates containing cells and growth medium and mixed gently by rocking the plate back and forth. The cells are incubated at 37°C with 5% CO_2 for 4 hours. This time allows for the liposomes to settle to the

bottom of the plate and transfect the cells. The media is then aspirated and replaced with fresh growth medium to minimize cytotoxic effects of the liposomes.

Xfect™Stem

Xfect™Stem is a system that uses polymers to transfect cells and has been designed to meet requirements of mouse embryonic stem cell transfection. The transfection system is designed to be used for difficult to transfect cells and has low toxicity. To transfect with Xfect™Stem cells were grown until 60-65% confluence is achieved in antibiotic free media. DNA and Xfect™Stem Polymer are prepared separately by diluting each in Xfect™ Reaction Buffer according to amounts in Xfect™Stem protocol (Clontech). Each tube is vortexed to mix then the polymer solution is added to the DNA solution and vortexed well at medium speed for 10 seconds. The mixture is incubated for 10 minutes at room temperature, allowing polyplexes to form. The mixture is then added to the cell culture plates containing cells and growth medium and mixed gently by rocking the plate back and forth. The cells are incubated at 37°C with 5% CO_2 for 4 hours allowing polyplexes to transfect cells. The media is then aspirated and replaced with fresh growth medium.

Optimization of Electroporation Parameters

Luciferase Assay

A luciferase assay is an accurate way to measure the amount of transgene a cell is expressing. The luciferase gene can be introduced using the desired transfection method and conditions. After transfection with luciferase plasmid (Appendix Figure 20) if the cell is expressing the luciferase gene, luciferase protein will be produced. This protein is intracellular and by lysing the cells this protein can be measured. Cells were lysed using Cell Culture Lysis 5X Reagent (Promega) diluted to 1X concentration with double-distilled H_2O. Media was removed from cell culture plates and cells were washed 3X with PBS. The diluted lysis reagent was added to cell culture plates and a micropipette tip was used to scrape the bottom of the plate, helping to manually lyse cells. The cell lysate was collected into eppendorf tubes and centrifuged at 13000g for 5 minutes at 4°C. The supernatant was removed and placed in new tubes and the pellet was discarded.

Cell lysate with a volume of 20µl was placed in each well of a 96-well plate. The luminometer was set to add 100µl of Luciferase Assay Reagent (Promega) per well and read with a delay time of 2 seconds and a read time of 10 seconds. Light is created when the luciferase protein is oxidized by addition of the Luciferase Assay Reagent (Promega), and this light was measured by the luminometer. The amount of light produced (arbitrary light units) directly correlates to the amount of luciferase protein in the sample allowing samples to be compared in a quantitative manner.

Bradford Assay

A Bradford Assay measures the total amount of protein in a sample. This was used to normalize luciferase expression to the total amount of protein in the sample. Extra cell lysate after performing the luciferase assay is used to determine protein concentration of the sample. Dilution of standards is done according to Pierce protocol using bovine serum albumin (Pierce) and double-distilled H_2O. Standards and samples were mixed with Coomassie Reagent (Pierce) and incubated at room temperature for 10 minutes. The Coomassie Reagent in the cationic unbound form is red, once it is added to protein the protein bonds to the dye creating an anionic blue form. This blue form was measured with a spectrophotometer at 595nm which was set to zero using a cuvette containing only water before reading each sample. The OD of the sample indicates the amount of blue dye present. If samples did not fall within the range of the standard they were diluted with double-distilled H_2O and re-measured.

Green Fluorescent Protein

A marker transgene, green fluorescent protein (GFP) allows us to visualize reporter gene activities in live cells. After transfection with the pEGFP plasmid (Appendix Figure 21) GFP was visualized using a widefield deconvolution microscope (Leica DMI 6000 B). The same field of view can be visualized with a bright field view and overlaying these images allows us to compare the number of cell present with the ones expressing GFP.

Cell Recovery

Determining cell recovery allows us to assess the yield of cells that survive particular electroporation conditions. Cell recovery after electroporation was measured by comparing adherent cells after electroporation with adherent cells that were not subjected to electroporation. The control cells that were not electroporated were prepared from the same batch of cells that was electroporated. They were centrifuged, diluted and mixed with DNA as described earlier. They were then placed in the electroporation cuvette, but no electroporation occurred. These cells remained in the cuvette while their counterparts were electroporated and then plated onto cell culture plates containing growth medium. All tests were done in triplicate to ensure accuracy.

16 hours after plating each plate of cells was detached and counted using a particle counter (Beckman Coulter). By comparing the number of cells retrieved from electroporated plates to those retrieved from non-electroporated plates a percentage of cell survival was determined.

Cell Viability

An MTT Assay was used to determine cell viability after transfection. The optimal number of cells was determined according to ATCC protocol to be 5×10^5cells/well in a 96-well tissue culture plate for cord blood-derived human mesenchymal stem cells.

After transfection either by electroporation, Lipofectamine™2000 or Xfect™Stem the appropriate cell number was plated onto a 96-well tissue culture plate; cells that did not

undergo transfection were used as a control. The cells were grown 18 hours with 100µl of growth medium per well to allow adherence. Once adhered to plate growth medium was removed and replaced with 100µl of Opti-MEM with 10% FBS. 10µl of MTT Reagent was added to each well and incubated at 37°C with 5% CO_2 for 2 hours. The MTT Reagent is reduced in the presence of living cells due to mitochondrial activity to an insoluble purple form. After incubation the media was removed and discarded and 100µl dimethyl sulfoxide was added; this causes the purple dye to dissolve allowing it to be measured. The plate was covered and incubated for 2 hours at room temperature to dissolve all purple precipitate. The absorbance was read at 562nm.

Differentiation

Differentiation of MSCs into a certain cell lineage can be induced by growing them with appropriate cell densities in media specially created for differentiation. Differentiation media contains nutrition to support differentiation into the desired cell type. Growing MSCs in differentiation media allows us to determine if they are able to differentiate into that particular lineage.

Adipogenesis

By growing cells in adipogenic differentiation media adipogenesis can be induced in MSCs. Cells were detached and plated 19000 cells/well in a 12-well plate with growth media. Cells were incubated at 37°C with 5% CO_2 for 4 hours, allowing them to attach to the bottom of the plate, then media was replaced with Adipogenesis Differentiation Medium (Gibco) with Adipogenesis supplement (Gibco). Media was replaced every 2-3 days for 14 days.

After 14 days cells were stained with Oil Red O (Sigma). Oil Red O stains neutral triglycerides and lipids indicative of adipogenic cells. Oil Red O stain was prepared by mixing 3 parts Oil Red O stock with 2 parts double-distilled H_2O; incubate at room temperature 10 minutes. The stain was filtered through Whatman paper in funnel into a 15ml falcon tube. Media was discarded and cells were washed 3X with PBS. Paraformaldehyde (4%) was added to cover the surface of the plate and left to fix cells at room temperature for 30 minutes. Formaldehyde solution was discarded, and cells rinsed 2X with 2ml ddH_2O. One milliliter of 60% isopropanol was added to cells and let sit at room temperature for 5 minutes. Isopropanol was discarded and the surface covered with Oil Red O solution and allowed to sit at room temperature for 5 minutes. Oil Red O was discarded and the plate was rinsed with 2ml ddH_2O 4-6X or until wash ran clear. The surface was covered with hematoxylin (Sigma) and incubated at room temperature for 2 minutes. Hematoxylin was removed and the plate was rinsed with 2ml ddH_2O 4-6X or until wash ran clear. One milliliter of ddH_2O was added and pictures (Nikon Coolpix995) were taken through a microscope (Leica DM IL inverted microscope).

Chondrogenesis

Chondrogenic differentiation was induced by growing cells in chondrogenic differentiation media. Cells were detached and plated as pellets in each well of a 12-well plate. Each pellet consisted of 80 000 cells in 5µl of growth medium. The pellets were allowed to adhere to plates for two hours at 37°C with 5% CO_2. After the first hour 5µl of growth media was added to each pellet to maintain moisture. After the second hour Osteocyte/Chondrocyte Differentiation Medium (Gibco) with Chondrogenesis supplement (Gibco) was added to each well. Media was replaced every 2-3 days for 14 days.

After 14 days cells were stained with Alcian Blue (Sigma). Alcian Blue stains proteoglycans which is an indicator of chondrogenesis. Media was discarded and cells were washed 3X with PBS. 4% paraformaldehyde was added to cover the surface of the plate and left to fix cells at room temperature for 30 minutes. Formaldehyde solution was discarded, wells were rinsed with 1ml PBS and 1 ml ddH_2O. Alcian Blue was added to cover each well and incubated at room temperature 30 minutes. Stain was removed and rinsed 4-6X with 2ml ddH_2O or until wash ran clear. Wells were rinsed 3X with 1ml 0.1N HCl. HCl was removed and 1ml ddH_2O added. Pictures (Nikon Coolpix995) were taken through a microscope (Leica DM IL inverted microscope).

Osteogenesis

MSCs were grown in osteogenic media to induce osteogenesis. Cells were detached and plated 9500 cells/well in a 12-well plate with growth media. Cells were incubated at 37°C with 5% CO_2 for 4 hours then media was replaced with Osteocyte/Chondrocyte Differentiation Medium (Gibco) with Osteogenesis supplement (Gibco). Media was replaced every 2-3 days for 21 days.

After 21 days cells were stained with Alizarin Red S (Sigma). Alizarin Red S stains for calcium deposits produced by osteogenic cells. Media was discarded and cells were washed 3X with PBS. Paraformaldehyde (4%) was added to cover the surface of the plate and left to fix cells at room temperature for 30 minutes. Formaldehyde solution was discarded, wells were rinsed 2X with 2ml ddH_2O. Surface was covered with Alizarin Red S stain and allowed to sit at room temperature 2-3 minutes. Stain was removed and discarded. Wells were washed 4X with 2ml ddH_2O or until wash ran clear. One milliliter was added ddH_2O to cells and pictures (Nikon Coolpix995) were taken through a microscope (Leica DM IL inverted microscope).

Differentiation Control

Control cells were plated in the same dilutions as adipogenic, chondrogenic and osteogenic cells. Cells used as differentiation control were at the same passage as differentiation test cells. Cells were grown in IMDM with 10% FBS and 1%P/S and media was changed at the same time as differentiating cells. Control cells were stained at the same point and in the same way as differentiation cells described above.

All cells were viewed with bright field microscopy (Leica DM IL inverted microscope) and photographed (Nikon Coolpix995).

FIX Expression

Human Factor IX ELISA

FIX is a secretable protein; thus, transgene expression can be quantified from the supernatant of cultured cells. To measure this secretion media samples were taken from cells transfected with pCpGNFIX (Appendix Figure 22). Normal pooled human plasma (George King Biomedical) was used as a standard. Plates were coated with capture antibody diluted 1:100 in coating buffer (Affinity Biologicals Inc.), 100µl was added to each well and the plate was incubated overnight at 4°C. Wells were washed 3X with PBS-Tween (PBS with 0.1%v/v Tween-20(Sigma)) to remove unbound antibody. Standards and cell media samples were added to each well and allowed to incubate at room temperature for 90 minutes. During this time FIX in the well will attach to the capture antibody. Standards and samples were discarded and wells were washed 3X with PBS-Tween to remove unbound sample. Detection antibody was diluted 1:100 in dilution buffer, 100µl was added to each well and allowed to incubate at room temperature for 90 minutes to allow attachment to FIX. 1 o-phenylenediamine tablet (5mg) (Sigma) was diluted in 12ml substrate buffer with 12µl 30% H_2O_2 in a polystyrene tube covered with foil. The cells were washed 4X with PBS-Tween and 100µl of the mixture was added and incubated 5-10 minutes at room temperature until colour developed. To stop colour reaction 50µl of 2.5M H_2SO_4 was added. The absorbance was read at 490nm. The higher the absorbance reading

indicates more FIX in the sample. By creating a standard curve with known concentration of standards and their OD values can be compared to unknown sample OD values and determine FIX concentration in these samples.

Statistical Analysis

All measurements were done at least in triplicates, the standard deviation of the data was used to plot error bars. A 2-tailed Student's t-test was used to determine the P-value between two groups and analysis of variance was used to compare more than two groups; P-values below 0.05 were deemed significant.

Results

One of the main objectives of this investigation was to determine optimal electroporation conditions for bone marrow-derived and umbilical cord blood-derived mesenchymal stem cells. This objective was achieved using a plasmid containing the luciferase DNA (Figure 20).

Electroporation of Bone Marrow-Derived MSCs

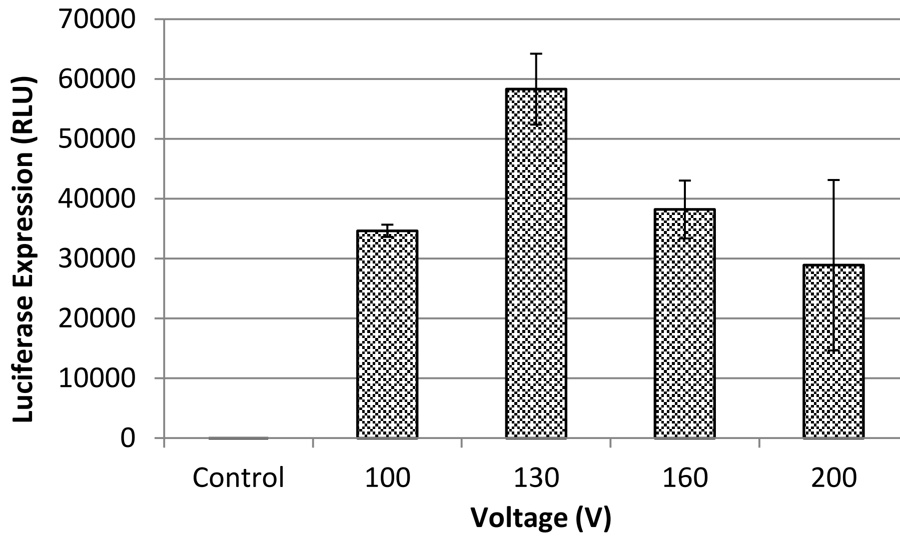
Optimization of Electroporation

Electroporation conditions can be optimized by changing different electroporation variables to determine which produces the most transgene. In order to achieve this, voltage, pulse length, and number of pulses were optimized using a square wave pulse. For the exponential wave pulse the voltage, capacitance and resistance were tested and optimized. Electroporation was done with a luciferase plasmid. If the luciferase plasmid successfully enters the cell, the cell will likely produce luciferase protein. This protein remains intracellular and can be measured by using a luciferase assay.

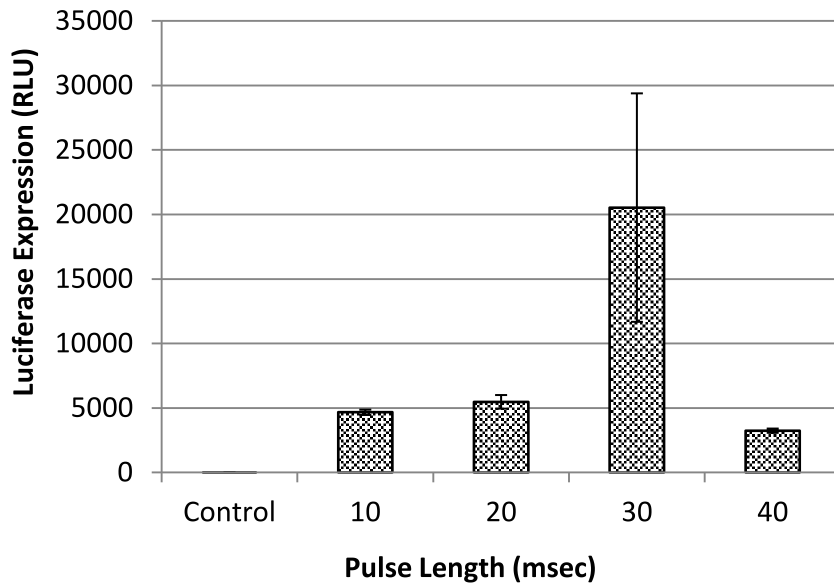
A square wave was first used to electroporate bone marrow-derived MSCs. The voltage was varied between 100V and 200V, while the other conditions remained constant. The pulse length was maintained at 25msec and one pulse was delivered. The maximum luciferase was produced when using 130V (Figure 4a). While maintaining the optimal voltage, the optimal pulse length was determined. The pulse length was varied between 10msec and 40msec and one pulse was delivered. A pulse length of 30msec produced the most luciferase (Figure 4b). Finally the number of pulses was varied between 1 and 5 pulses to determine optimal pulse number. Maximum luciferase production occurred when 1 or 4 pulses were used, with no significant difference in relative light units between the two conditions (Figure 4c).

Combining these conditions provides us with optimal electroporation conditions using a square wave for bone marrow-derived MSCs to be 130V, applied for 30msec, with either 1 or 4 pulses.

a) Optimizing Voltage



b) Optimizing Pulse Length



c) Optimizing Number of Pulses

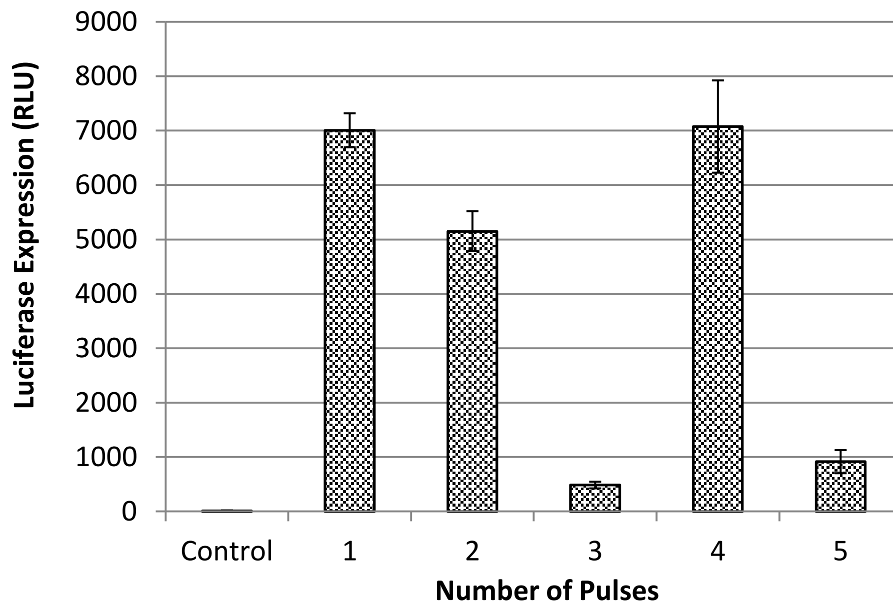


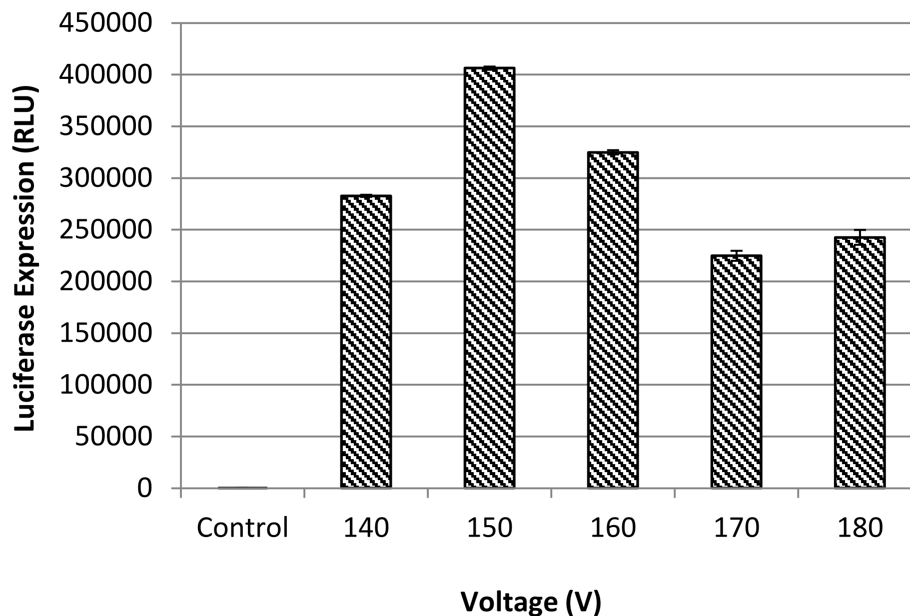
Figure 4: Optimization of bone marrow hMSCs using square waveform. Luciferase expression was compared while varying conditions to determine optimal settings. Luciferase expression was normalized against cell number by conducting a protein assay. Control represents luciferase expression of cells that were not electroporated. Error bars show standard deviation and significance was determined using single factor analysis of variance (p-values < 0.05). The data shown is representative of three replicates.

After determining optimal square waveform conditions, optimal conditions were determined using an exponential waveform. The voltage was varied between 140V and 180V using an exponential wave type, a capacitance of 950µF and resistance of 200Ω. The highest luciferase was produced when using 150V (Figure 5a). The optimal capacitance was determined by

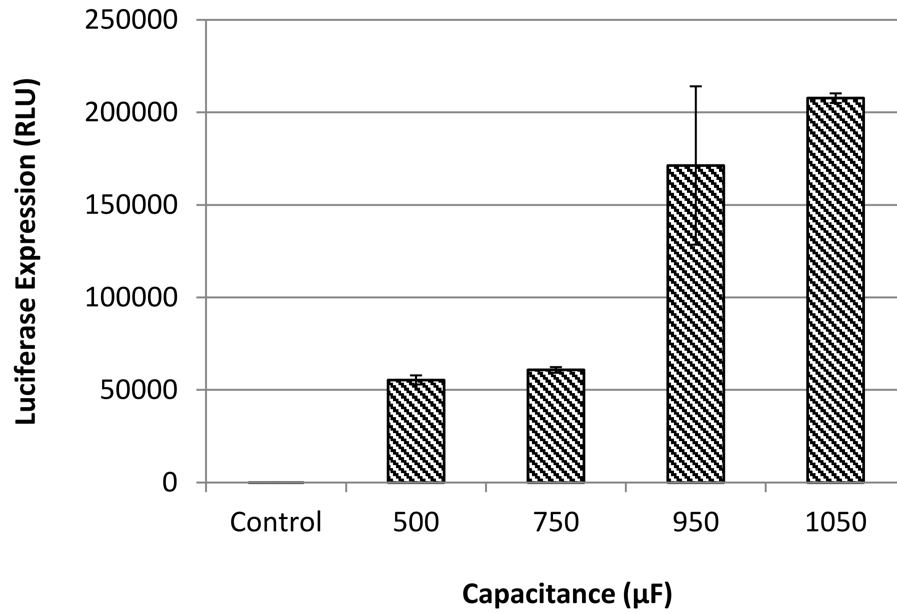
maintaining the optimal voltage as the capacitance was varied between 500µF and 1050µF. The maximum luciferase was produced using 950µF or 1050µF, with no significant difference in relative light units between the two conditions (Figure 5b). Finally the optimal resistance was determined, by maintaining the optimal voltage and capacitance and varying the resistance between 100Ω to 250Ω. A resistance of 200Ω produced the highest luciferase expression (Figure 5c).

By determining which condition produced the most luciferase protein for each variable we were able to combine the optimal conditions together providing us with optimized electroporation conditions for bone marrow-derived MSCs. These conditions are 150V with a capacitance of either 950µF or 1050µF and a resistance of 200Ω.

a) Optimizing Voltage



b) Optimizing Capacitance



c) Optimizing Resistance

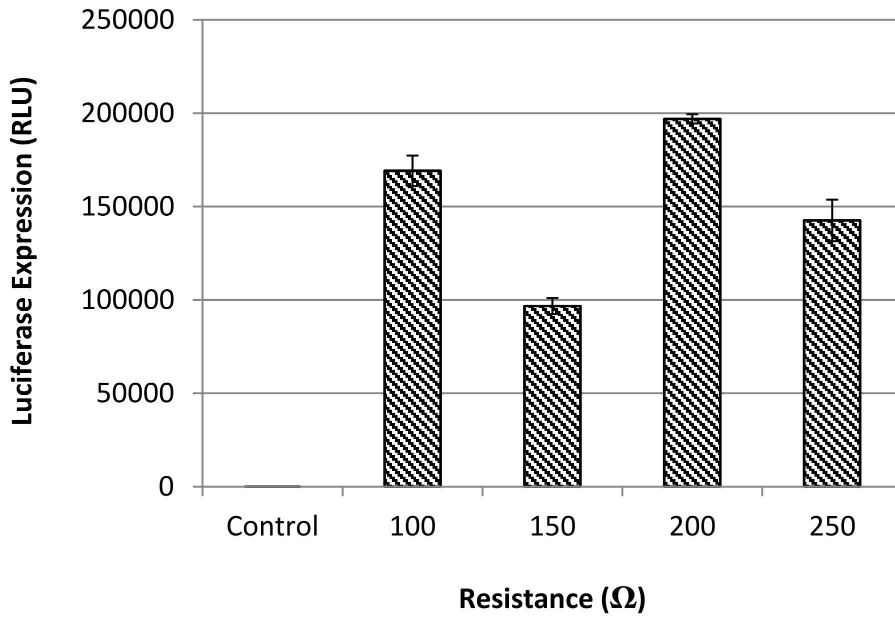


Figure 5: Optimization of bone marrow hMSCs using exponential waveform. Luciferase expression was compared while varying conditions to determine optimal settings. Luciferase expression was normalized against cell number by conducting a protein assay. The control represents luciferase expression of cells that were not electroporated. Error bars show standard deviation and significance was determined using single factor analysis of variance (p-values < 0.05). The data shown is representative of three replicates.

Electroporation versus Lipofectamine Transfection

A comparison of our optimized electroporation conditions with other transfection methods was used to determine if the new conditions produce comparable results. This was achieved by transfecting cells with a plasmid containing luciferase DNA (Figure 20). The optimal electroporation conditions for plasmid delivery into bone marrow MSCs were used to compare electroporation to transfection using Lipofectamine™2000 (Figure 6). Lipofectamine™2000 employs cationic lipids which are combined with the users chosen plasmid to transfect cells. Luciferase expression was found to be 175273RLU when using optimized electroporation conditions. The transfection protocol supplied by the manufacturer was followed and produced a luciferase expression of 111832RLU. Electroporation produced significantly increased luciferase expression than Lipofectamine™2000 transfection, and these differences in luciferase expression are a direct result of the transfection method.

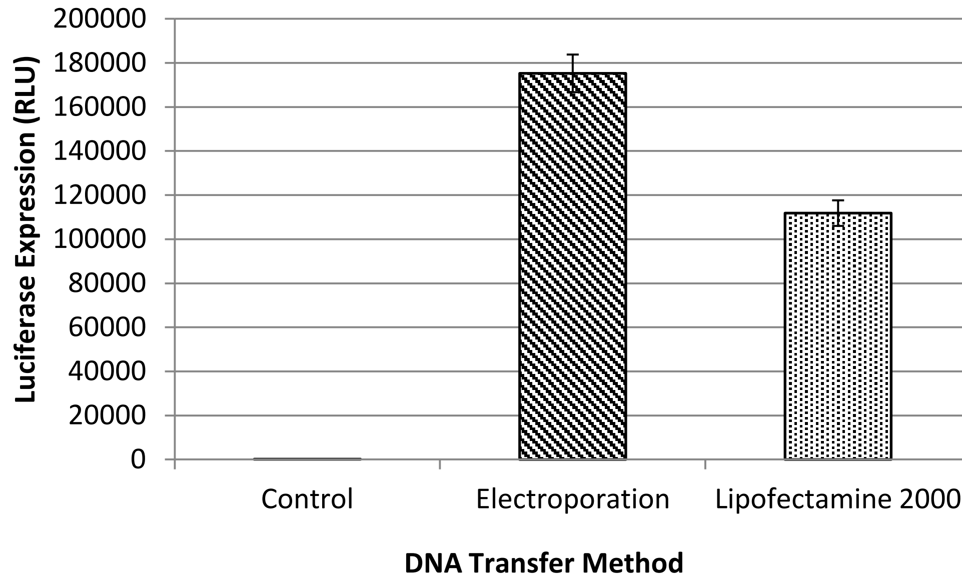


Figure 6: Optimal electroporation versus transfection. Control refers to a negative control; luciferase expression of cells that were not electroporated or transfected with Lipofectamine™2000. Optimal exponential electroporation has significantly greater luciferase expression compared to Lipofectamine™2000 determined by a 2-tailed Student's t-test (p-value 0.0007). Error bars show standard deviation. The data shown is representative of three replicates.

Due to a short supply of bone marrow-derived MSCs from healthy donors, no additional tests could be completed. Therefore there is no further data on the bone marrow-derived MSCs. As a comparison, electroporation conditions were optimized for cord blood-derived MSCs.

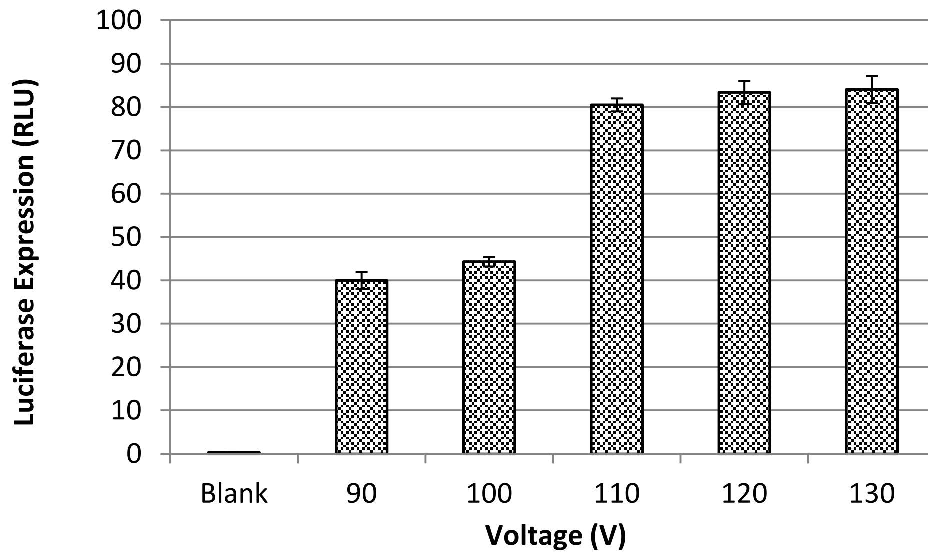
Electroporation of Cord Blood-Derived MSCs

Optimization of Electroporation

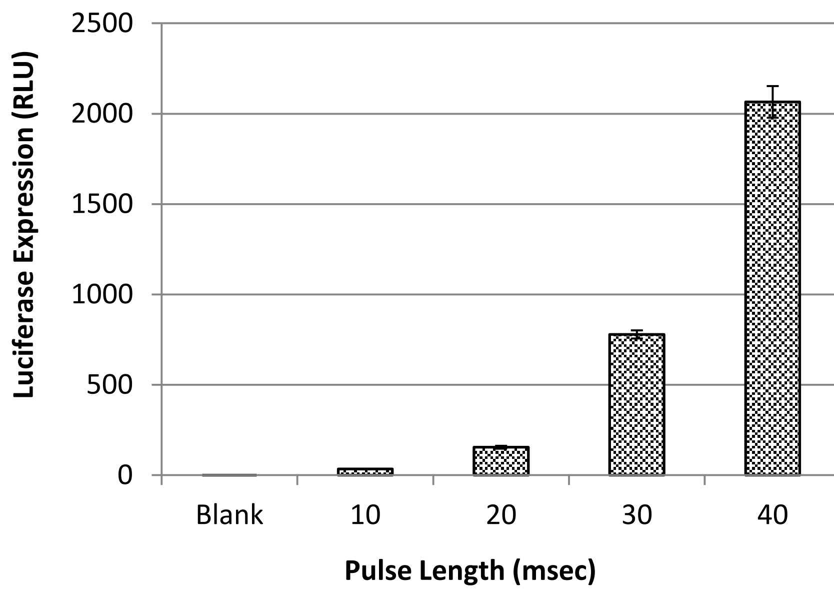
To create optimal electroporation conditions for cord blood-derived MSCs a similar method to determining electroporation conditions for bone marrow-derived MSCs was used. A square wave was first used to electroporate a plasmid containing luciferase gene into umbilical cord blood-derived MSCs. The voltage was varied between 90V and 130V, while the other conditions remained constant. The pulse length was maintained at 30msec and one pulse was delivered. The maximum relative light units was found when 120V and 130V was applied to cells, with no significant difference between the two settings (Figure 7a). While maintaining the optimal voltage the optimal pulse length was determined. The pulse length was varied between 10msec and 40msec and one pulse was delivered. The maximum luciferase production occurred with a pulse length of 40msec (Figure 7b). Finally the number of pulses was varied between 1 and 4 pulses to determine optimal pulse number. One pulse produced maximum luciferase expression (Figure 7c).

The optimal conditions for square wave electroporation cord blood-derived MSCs were found to be a single pulse with a voltage between 120V and 130V, with a pulse length of 40msec.

a) Optimizing Voltage



b) Optimizing Pulse Length



c) Optimizing Number of Pulses

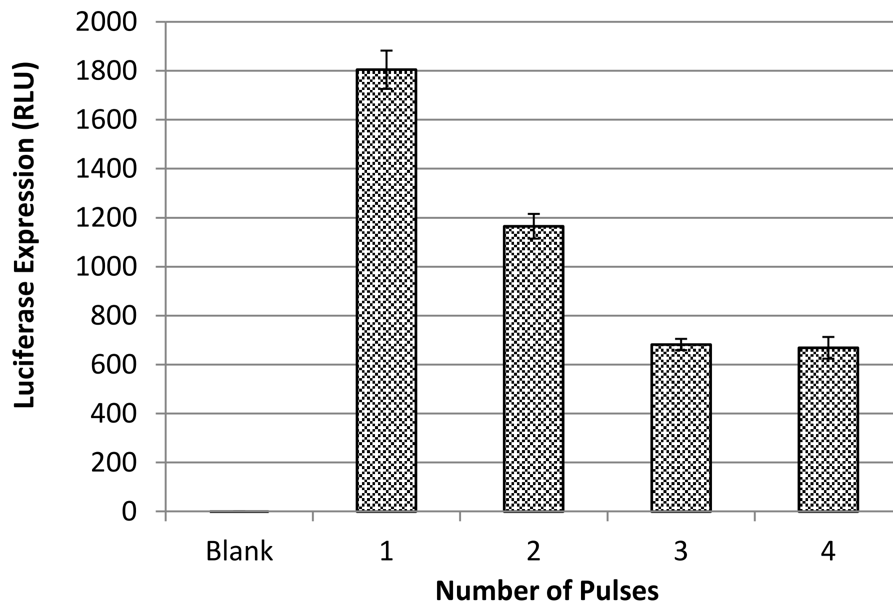


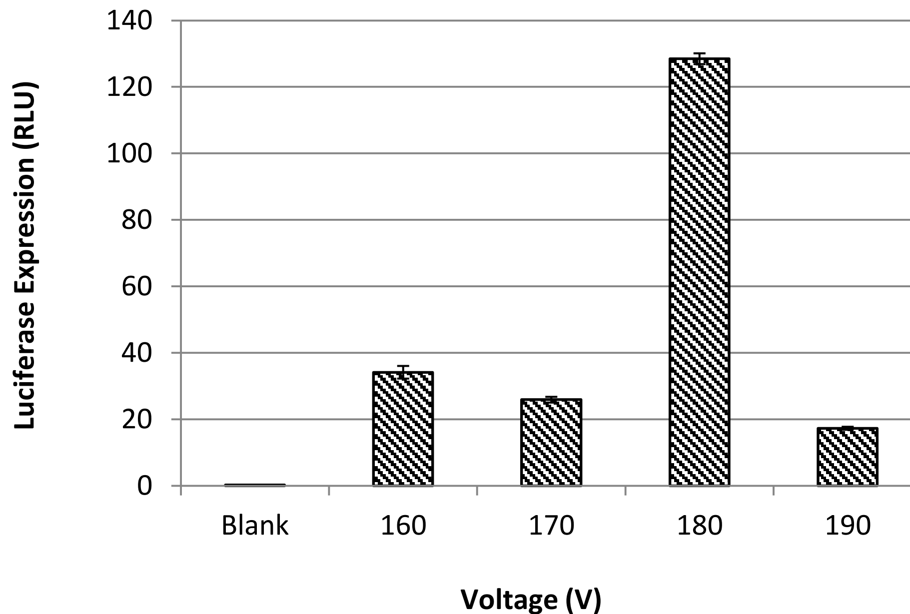
Figure 7: Optimization of umbilical cord blood hMSCs using square waveform. Luciferase expression was compared while varying conditions to determine optimal settings. Luciferase expression was normalized against cell number by conducting a protein assay. Blank refers to negative controls; luciferase expression of cells that were not electroporated. Error bars show standard deviation and significance was determined using single factor analysis of variance (p-values < 0.05). The data shown is representative of three replicates.

Optimal electroporation conditions were determined using an exponential waveform in the same manner. The voltage was varied between 160V and 190V using an exponential wave type, a capacitance of 1050µF and resistance of 200Ω. The maximum luciferase expression occurred with 180V (Figure 8a). The optimal capacitance was determined by maintaining the optimal

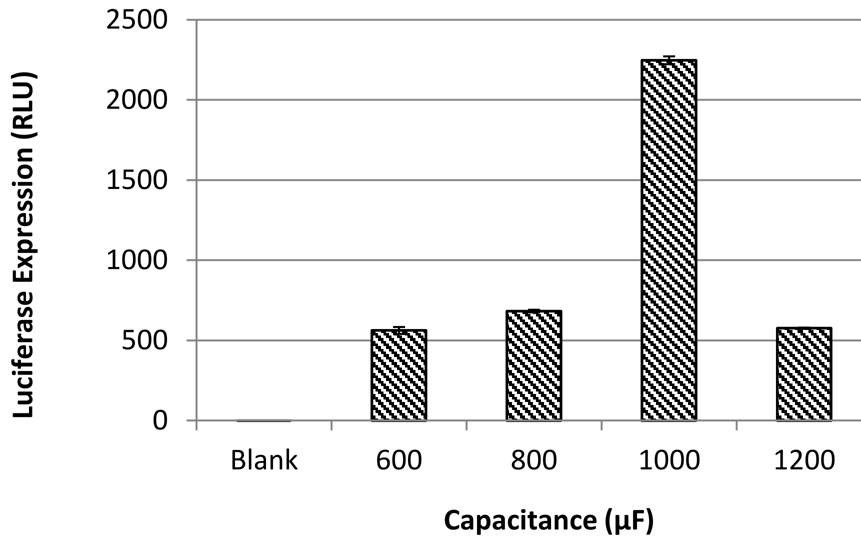
voltage as the capacitance was varied between 600μF and 1200μF. The maximum production of luciferase occurred with a capacitance of 1000μF (Figure 8b). Finally, the optimal voltage and capacitance was maintained, and the resistance was varied between 100Ω and infinity to determine optimal resistance. Infinite resistance produced the maximum luciferase expression (Figure 8c).

By combining the conditions that produced the highest luciferase expression we were able to determine optimal conditions using each waveform. These conditions were found to be a voltage of 180V, with a capacitance of 1000μF, and infinite resistance.

a) Optimizing Voltage



b) Optimizing Capacitance



c) Optimizing Resistance

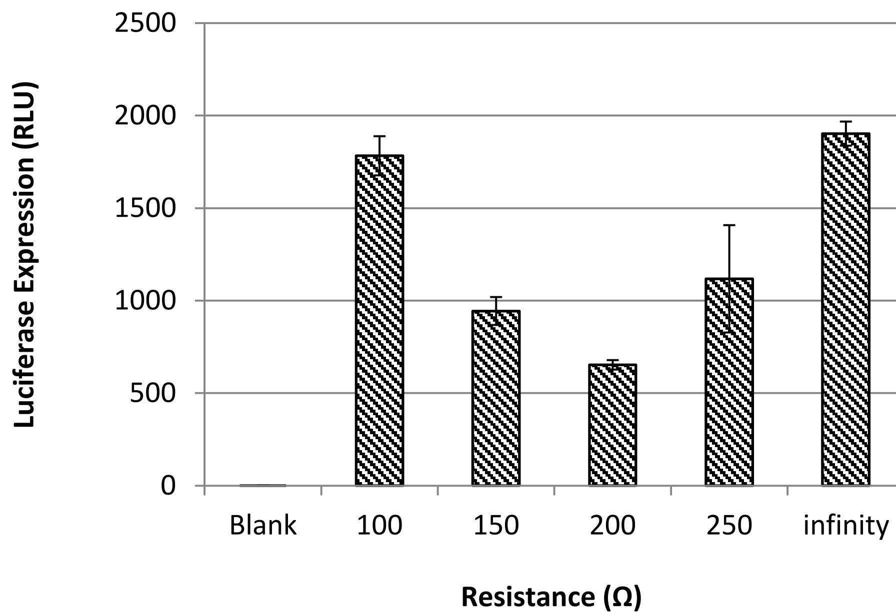


Figure 8: Optimization of umbilical cord blood hMSCs using exponential waveform. Luciferase expression was compared while varying conditions to determine optimal settings. Luciferase

expression was normalized against cell number by conducting a protein assay. Blank refers to negative controls; luciferase expression of cells that were not electroporated. Error bars show standard deviation and significance was determined using single factor analysis of variance (p-values < 0.05). The data shown is representative of three replicates.

Since electroporation conditions were optimized using a cell concentration of 1 million cells/ml, it was necessary to determine if increasing the cell concentration would alter electroporation efficiency. By using higher cell concentrations it will be easier to scale up the technique to transfect more cells in one round. Cells were electroporated with the luciferase plasmid at cell concentrations of 1 million cells/ml and 5 million cells/ml and luciferase expression was measured (Figure 9). No significant difference was found in luciferase expression using either technique (p-value 0.28).

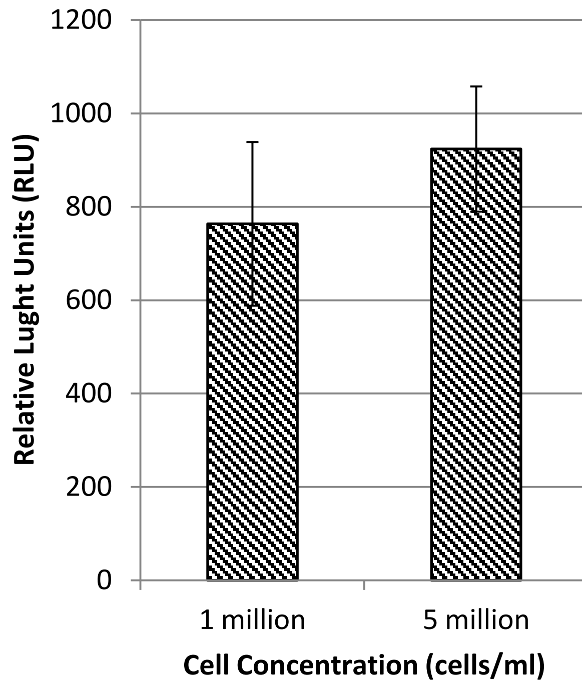


Figure 9: Varying cell concentration. Optimized exponential electroporation conditions showed luciferase expression did not change significantly when cell concentration was increased from 1 million cells/ml to 5 million cells/ml. Statistical analysis done with 2-tailed Student's t-test (p-value 0.28) and error bars represent standard deviation. The data shown is representative of three replicates.

Although some optimized electroporation conditions for similar cell types have been published, many do not include the yield of cells that will survive their electroporation conditions. Since it is well known that MSCs cannot be expanded indefinitely it is important that a realistic number of cells survive the procedure.

MSCs are adherent cells and require only a few hours to attach to tissue culture plates, allowing dead cells to be washed away. The surviving cells can be counted and compared to cells that were not electroporated. This provides a percentage of cells that survive the procedure (Figure 10). Square wave conditions maintain an impressive cell survival at 82%, where exponential wave conditions are more harsh, resulting in 67% cell survival.

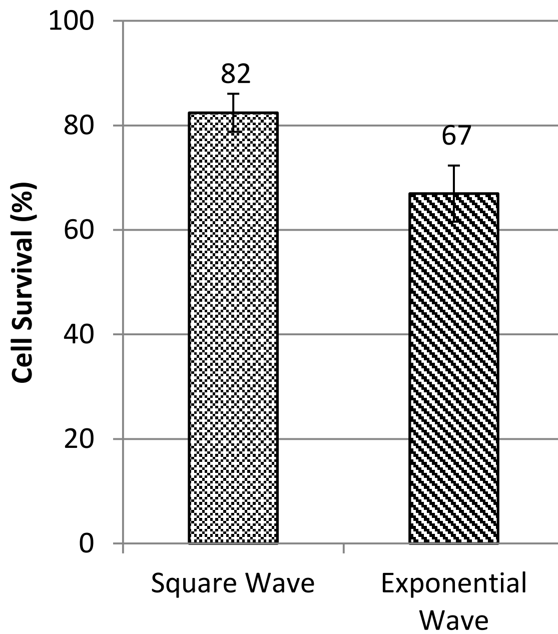


Figure 10: Cell Survival Post-Electroporation. Percentage of cells recovered after electroporation with optimized conditions. Statistical analysis done with 2-tailed Student's t-test (p-value 0.01) and error bars represent standard deviation. The data shown is representative of three replicates.

Comparison with other Techniques

Comparability with commercially available techniques was achieved by comparing our electroporation technique with other non-viral methods of gene therapy. Our technique was compared to Lipofectamine^{TM}2000 and Xfect^{TM}Stem using an EGFP plasmid in Figure 11. This provides a visual comparison of transgene production. All transfection methods show GFP production. Variations are found in the number of transfected cells and in the intensity of GFP production.

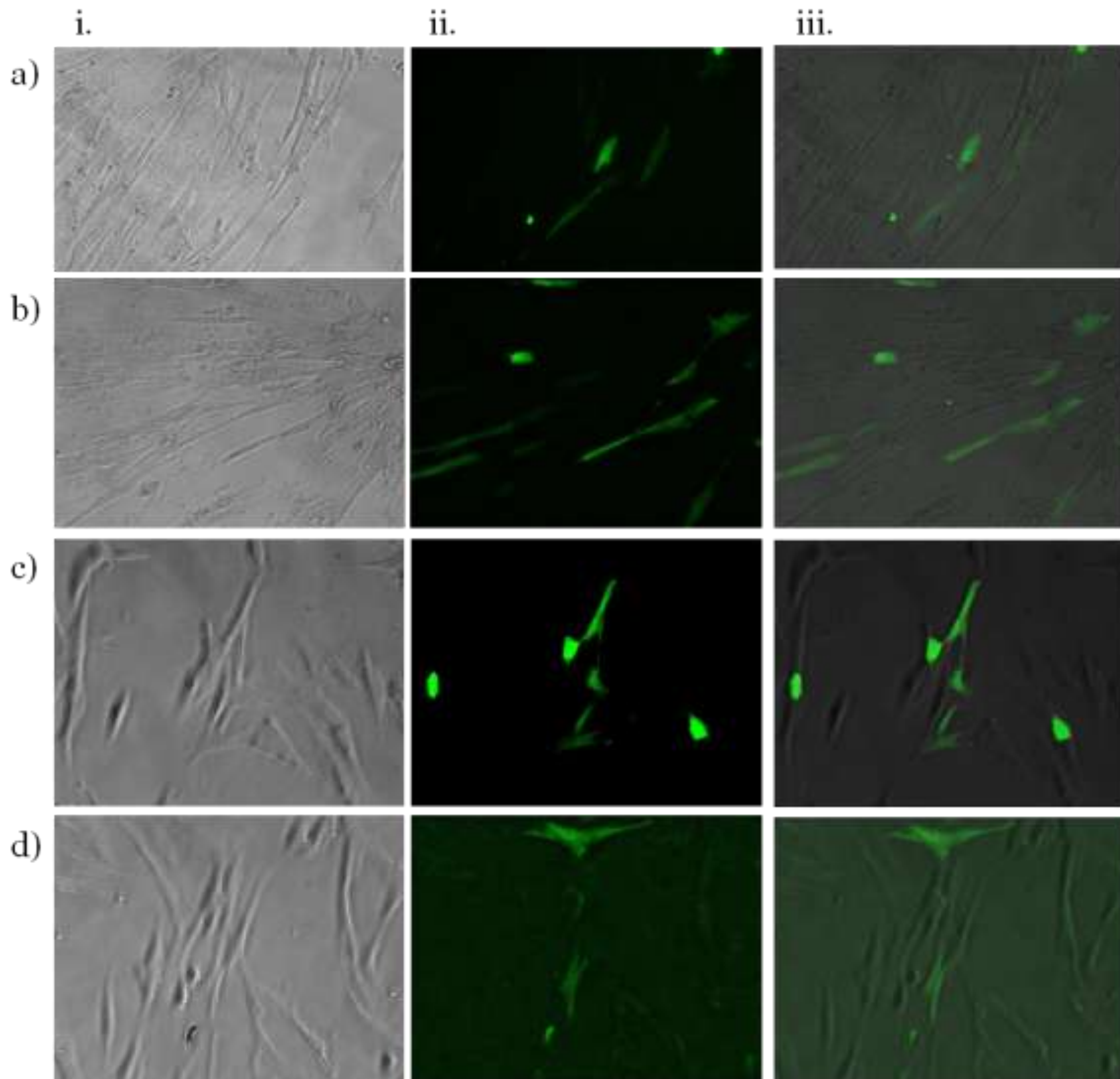


Figure 11: Compare electroporation to other non-viral transfection techniques using GFP. Comparison of green fluorescence protein expression from Lipofectamine^{TM}2000(a), Xfect^{TM}Stem(b) and electroporated (optimal exponential(c) and square(d) waves). Bright field images of cells are shown in the column i, column ii shows the same field of view but only green fluorescence protein, and column iii shows and overlay of the two images.

By conducting a luciferase assay of transfected cells we were able to determine a measureable quantity of transgene. The luciferase expression of electroporated, Lipofectamine™2000 and Xfect™Stem transfected cells were compared to determine which technique produces cells expressing the most transgene (Figure 12). All transfection techniques produced relevant amounts of luciferase, but it was evident that electroporation and Lipofectamine™2000 produced far more than Xfect™Stem.

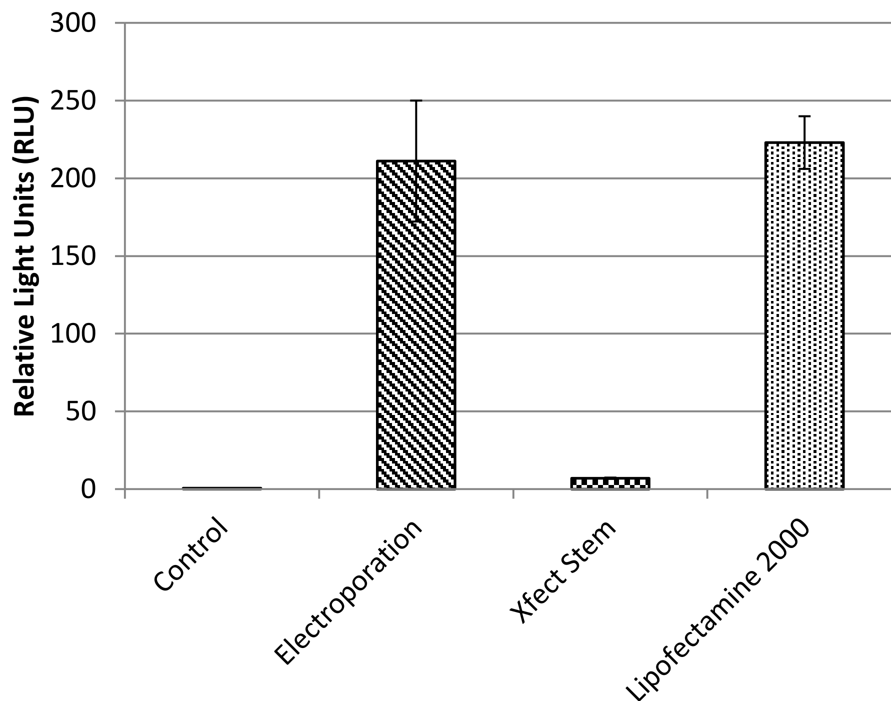


Figure 12: Compare electroporation to other non-viral transfection techniques using luciferase. Comparison of luciferase expression from electroporation (optimal exponential wave), Xfect™Stem and Lipofectamine™2000. Control represents untransfected cells. All transfections produced significantly more relative light units when compared to control; single factor analysis of variance (p-values < 0.01). Error bars represent standard deviation. The data shown is representative of three replicates.

Although transfection techniques may be effective at producing the required transgene we must ensure that the viability of the cells is not affected. This is particularly important since an unhealthy cell is not ideal for any type of therapeutic use. An MTT assay was used to determine the viability of control cells (no transfection), cells electroporated with the optimized protocol, cells transfected with Xfect^{TM}Stem and cells transfected with Lipofectamine^{TM}2000 (Figure 13). No significant change was found in viability after any type of transfection, indicating no measurable decline in cell viability.

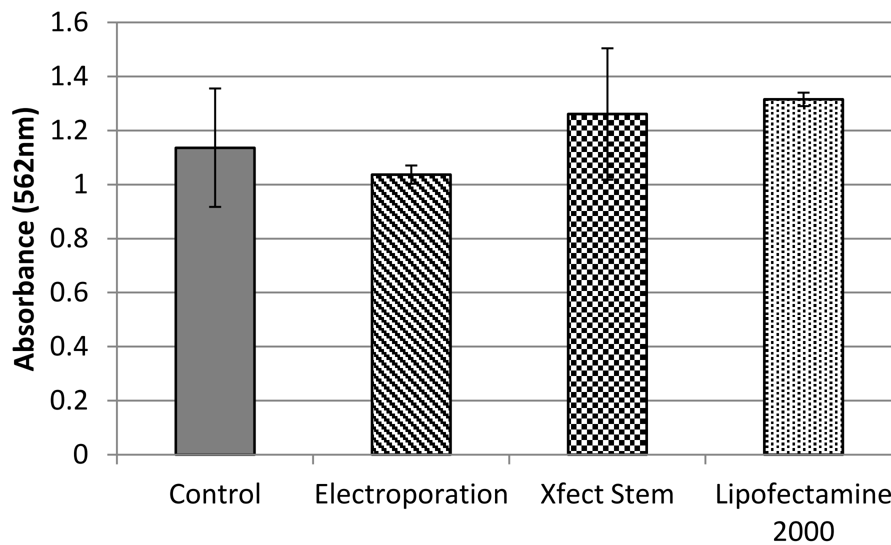


Figure 13: Viability Post-Transfection. Comparison of viability using an MTT assay. There was no significant difference found between viability of untransfected control cells with any transfection methods; single factor analysis of variance (p-value 0.07). Error bars represent standard deviation. The data shown is representative of five replicates.

Although the MTT assay determined that the viability of all transfected cells appeared to be similar to untransfected cells, not all cells appeared as healthy under the microscope. Proliferation of Lipofectamine™2000 and Xfect™Stem modified cells slowed in comparison to electroporated and untransfected cells. Bright field images show some differences in morphology and presence of cell debris (Figure 14). The most pronounced change in cell morphology was seen in Lipofectamine™2000 transfected cells, along with more cell debris. Some cell debris was also present in Xfect™Stem modified cells.

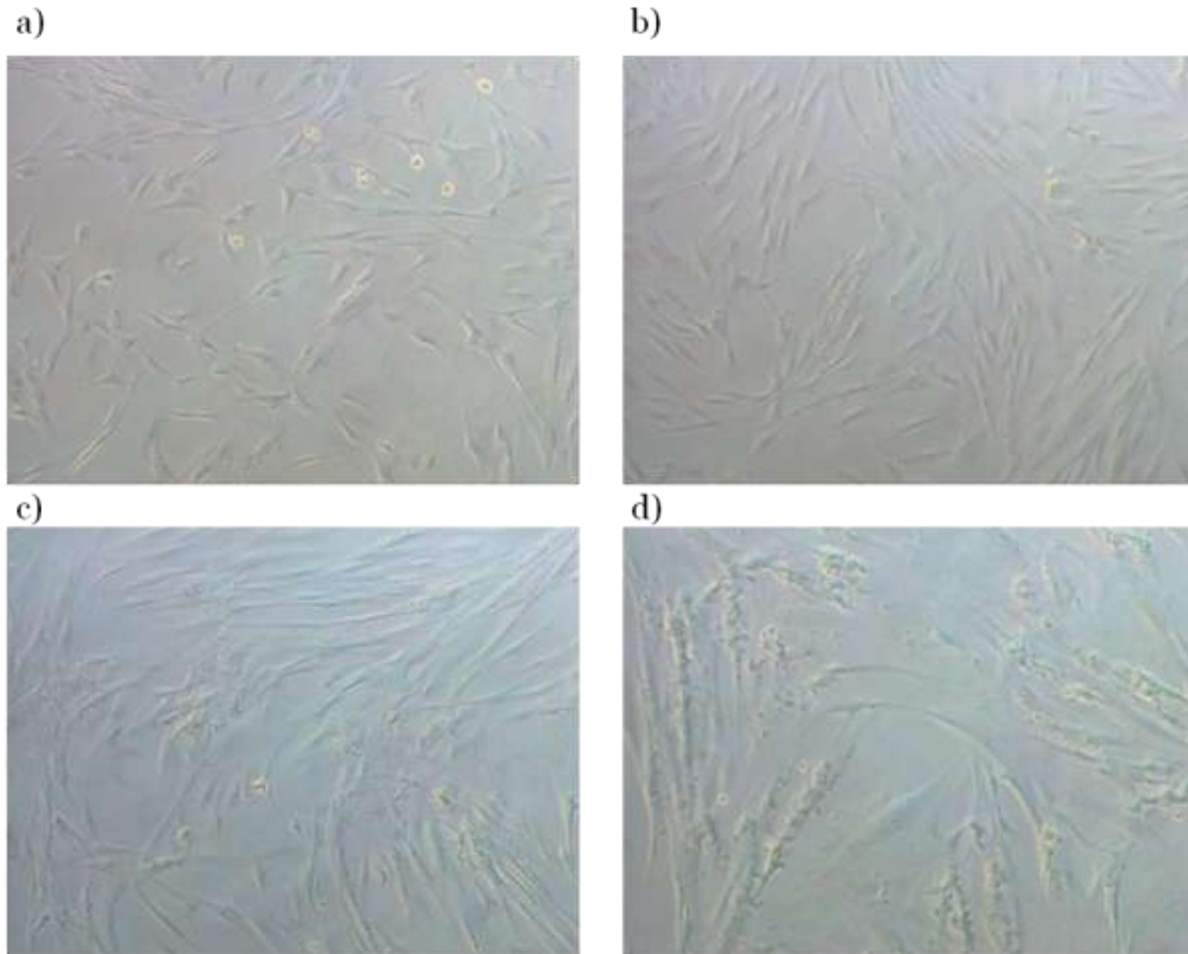


Figure 14: Cell Morphology Post-Transfection. Bright field images of untransfected control and transfected cells indicating change in morphology and cell debris (100X magnification). Untransfected control(a), electroporated(b), Xfect^{TM}Stem(c), Lipofectamine^{TM}2000(d).

It was hypothesized that perhaps the change in morphology may be due to some type of differentiation caused by transfection agents. To determine if this was the case the cells were stained for calcium deposits, proteoglycans and lipids using Alizarin Red S, Alcian Blue and Oil red O respectively. No staining occurred indicating no osteogenesis, chondrogenesis or

adipogenesis (Figure 15). Although no staining occurred, the cells stained with Oil red O showed some type of fibrous cell debris in Xfect^{TM}Stem and Lipofectamine^{TM}2000 transfected cells (Figure 15 c and d). This fibrous debris is not found in untransfected differentiated positive control cells stained with Oil red O (Figure 15 a).

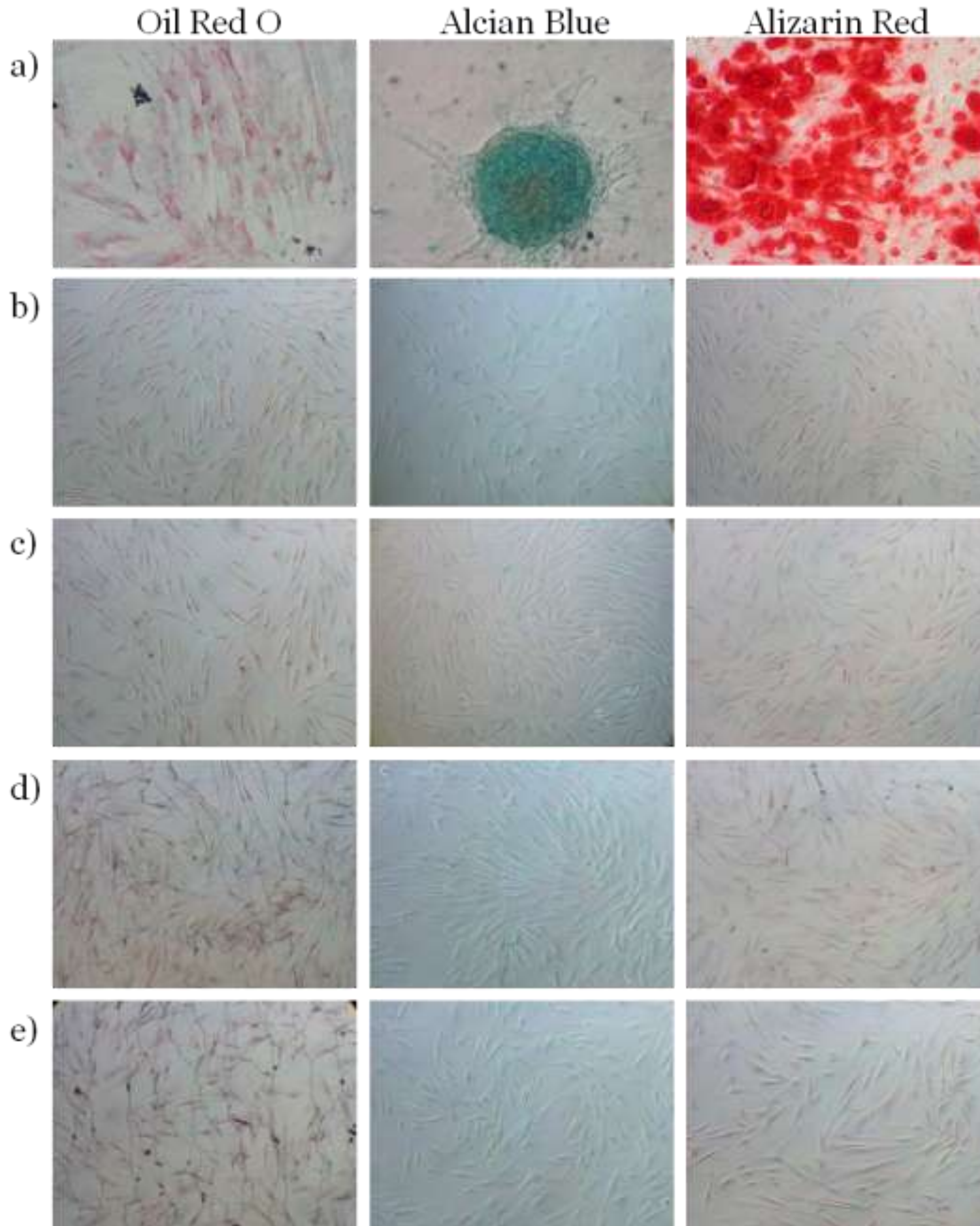


Figure 15: Stained Cell Morphology Post-Transfection. Bright field images of untransfected control and transfected cells stained with Oil red O, Alcian Blue and Alizarin Red S (100X magnification). Untransfected differentiated positive control(a), Untransfected negative control(b), electroporated(c), Xfect™Stem(d), Lipofectamine™2000(e).

Capacity of MSCs to Differentiate

For the electroporation technique to be successful in transfecting MSCs, the technique should not induce or hinder differentiation into a particular cell lineage. To determine if this is happening non-electroporated cells (no transfection) and electroporated cells were differentiated. This was done using media designed for specific differentiation into either osteogenic, chondrogenic or adipogenic cell lineages. After differentiation cells were stained with Alizarin Red S (osteogenic), Alcian Blue (chondrogenic) and Oil red O (adipogenic). To determine if similar degrees of differentiation occurred in both control and electroporated cells were viewed under a microscope.

Cells induced into osteogenesis showed strong Alizarin Red S staining of calcium deposits in both non-electroporated and electroporated differentiated cells (Figure 16 c and d). Undifferentiated cells show no Alizarin Red S staining (Figure 16 a and b).

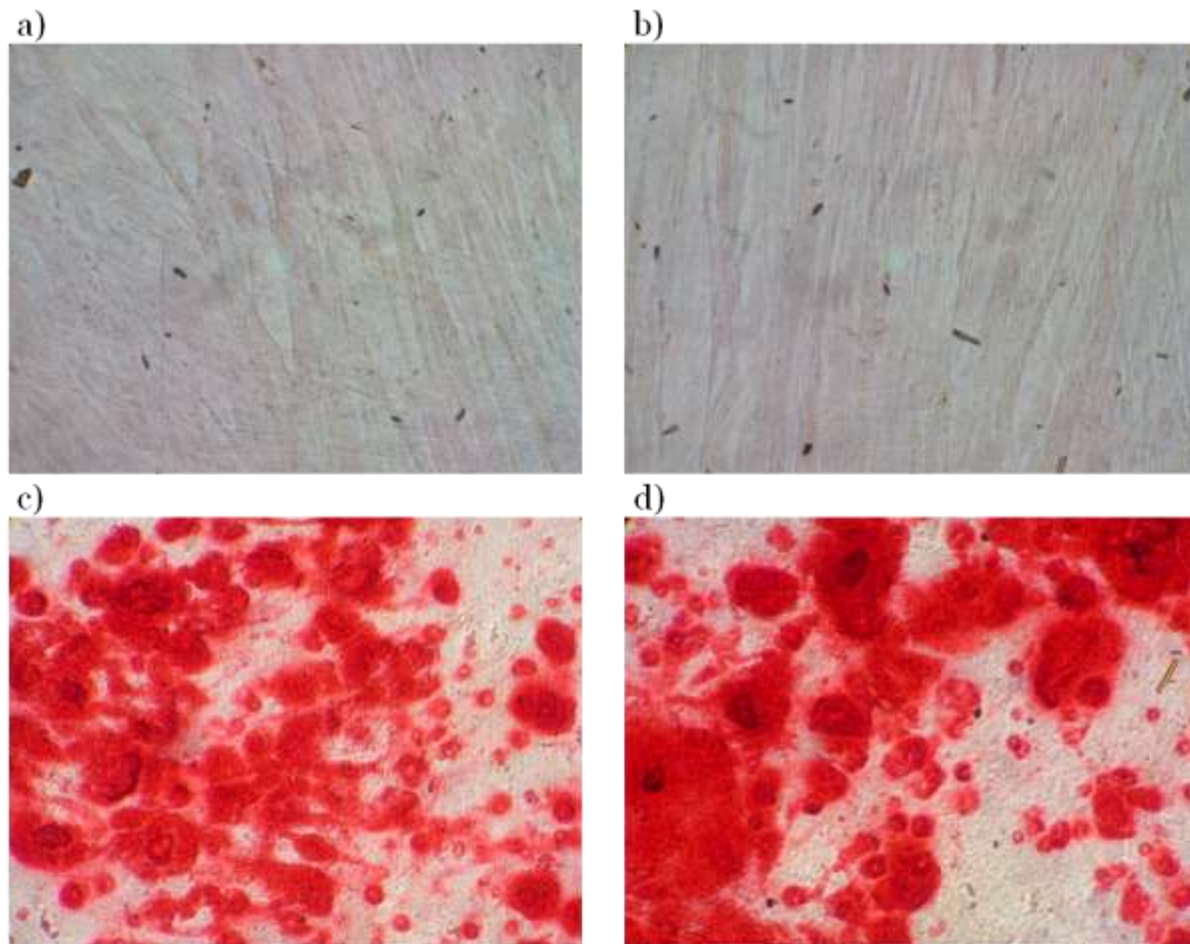


Figure 16: Osteogenesis of electroporated and non-electroporated cells. Bright field images of osteogenic differentiation (400X magnification). Undifferentiated non-electroporated CBMSCs(a), undifferentiated electroporated CBMSCs(b), differentiated non-electroporated CBMSCs(c), differentiated electroporated CBMSCs(d). All cells stained with Alizarin Red S.

Cells grown in chondrogenesis differentiation media show non-electroporated and electroporated cells stained in a similar way. Cells were plated in pellets of highly concentrated cells. Cells grown in control growth medium show no blue staining; the cells from the pellets

continued to divide and eventually covered the bottom of the plate (Figure 17 a and b). Cells in pellets with chondrogenesis differentiation media came together into a dense ball of cells, and show strong blue staining of proteoglycans (Figure 17 c and d).

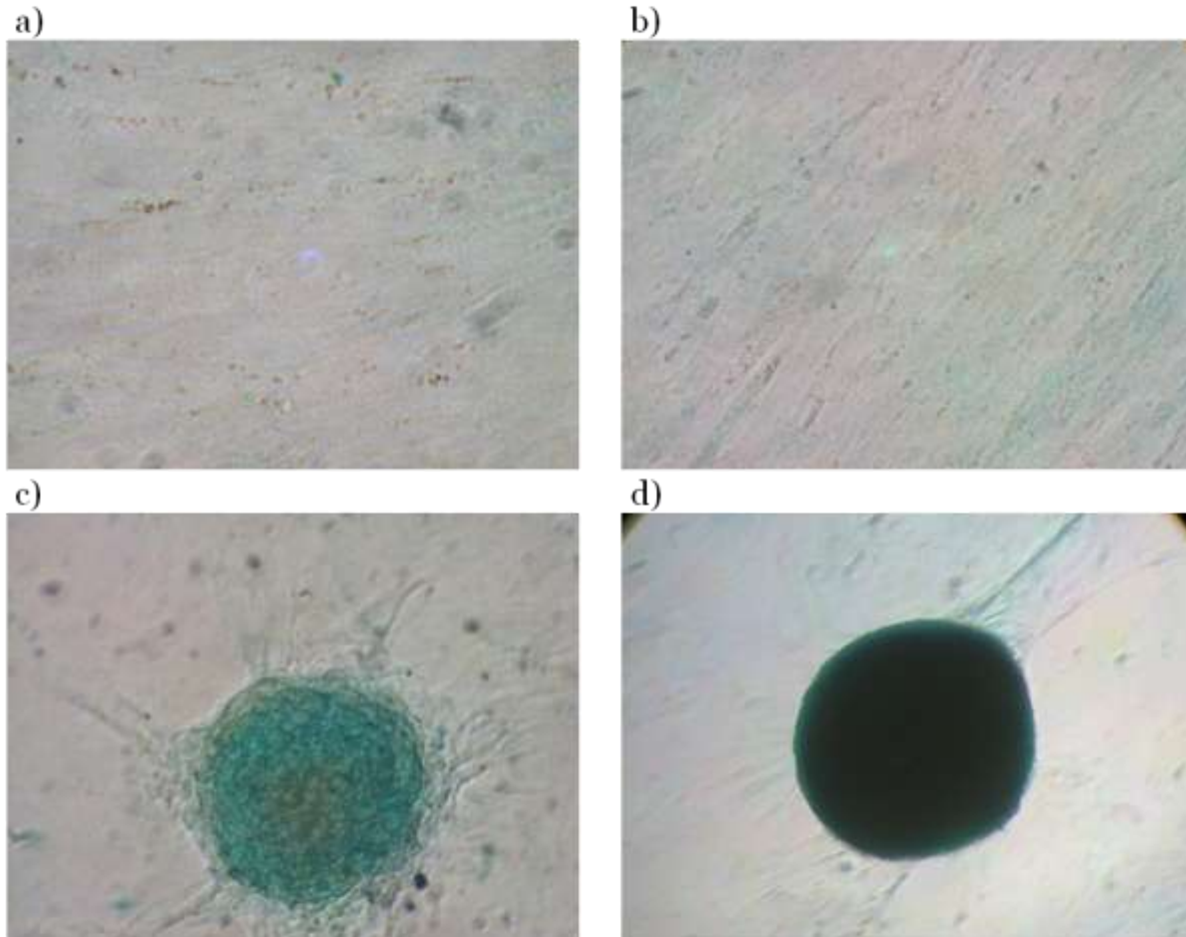


Figure 17: Chondrogenesis of electroporated and non-electroporated cells. Bright field images of chondrogenic differentiation (100X magnification). Undifferentiated non-electroporated CBMSCs(a), undifferentiated electroporated CBMSCs(b), differentiated non-electroporated CBMSCs(c), differentiated electroporated CBMSCs(d). All cells stained with alician blue.

Umbilical cord blood-derived MSCs are known to not differentiate well into adipocytes [32-33]. Regardless, growing these cells with adipogenesis differentiation media showed a small amount of differentiation in non-electroporated and electroporated cells (Figure 18 c and d). Oil red O stains for neutral triglycerides and lipids within the cell; both non-electroporated and electroporated cells with differentiation media have a small amount of red/purple staining, representing fat vacuoles, and cell appear to be larger and more rounded. Non-electroporated and electroporated cells without differentiation media, divided and covered the plate and showed no red/purple staining (Figure 18a and b).

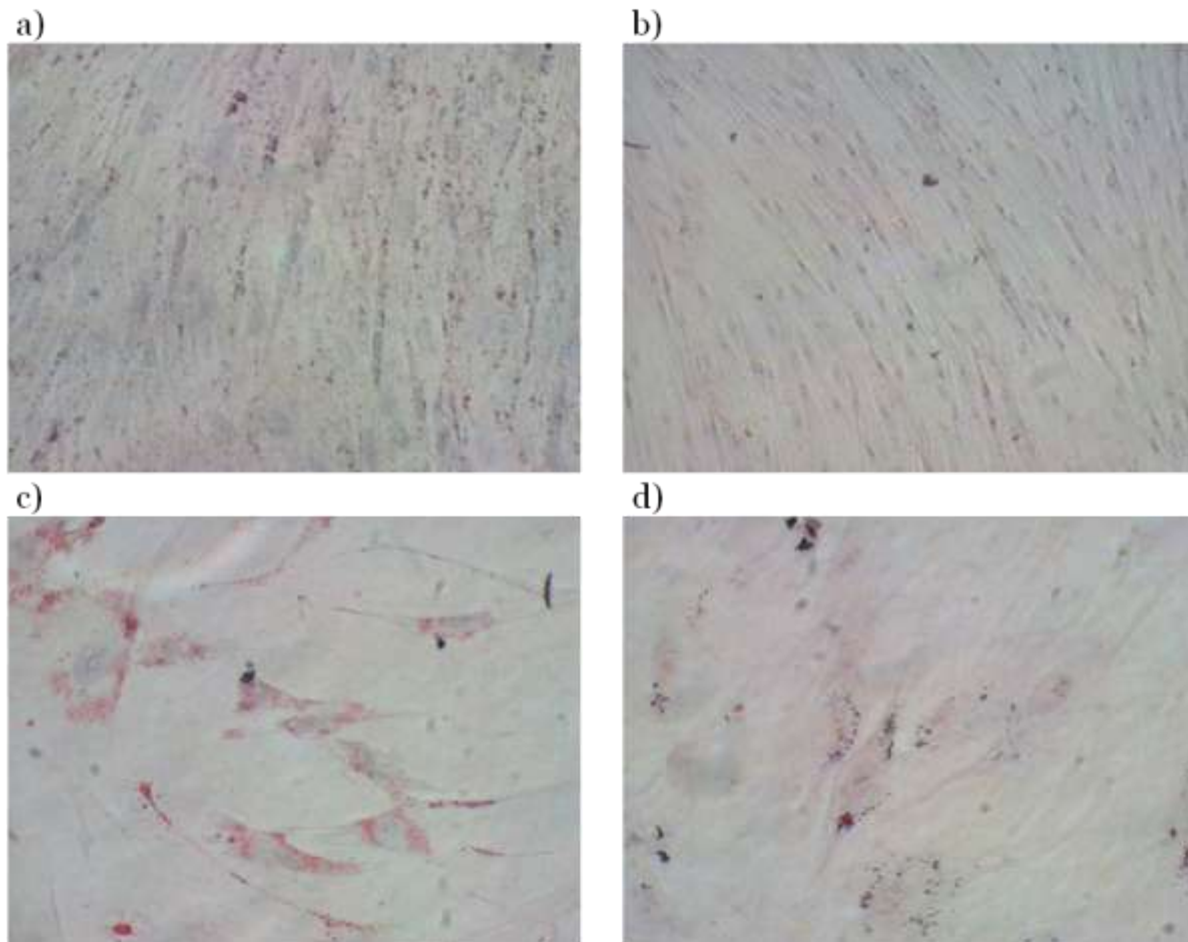


Figure 18: Adipogenesis of electroporated and non-electroporated cells. Bright field images of adopogenic differentiation (400X magnification). Undifferentiated non-electroporated CBMSCs(a), undifferentiated electroporated CBMSCs(b), differentiated non-electroporated CBMSCs(c), differentiated electroporated CBMSCs(d). All cells stained with Oil red O and hematoxalin.

Factor IX Expression

Creating FIX secreting MSCs was attempted by electroporation cord blood-derived MSCs with a Factor IX (FIX) containing plasmid. If successful this could lead to a possible method to deliver FIX to patients with hemophilia B. This will also be able to give us an idea of how long the transgene is secreted. The cells were electroporated using optimal exponential and square wave pulses with CpGNFIX plasmid, cell media samples were then used to measure the amount of FIX secreted by the cells. The cells were subjected to antibiotic selection which began 48 hours after transfection.

Exponential wave electroporation conditions produced a maximum of 185ng/ml produced on day 2 which declined thereafter but remained significant producing 35ng/ml by day 12. Square wave electroporation conditions produced 3ng/ml on day 1 and reduced to insignificant levels thereafter (Figure 19).

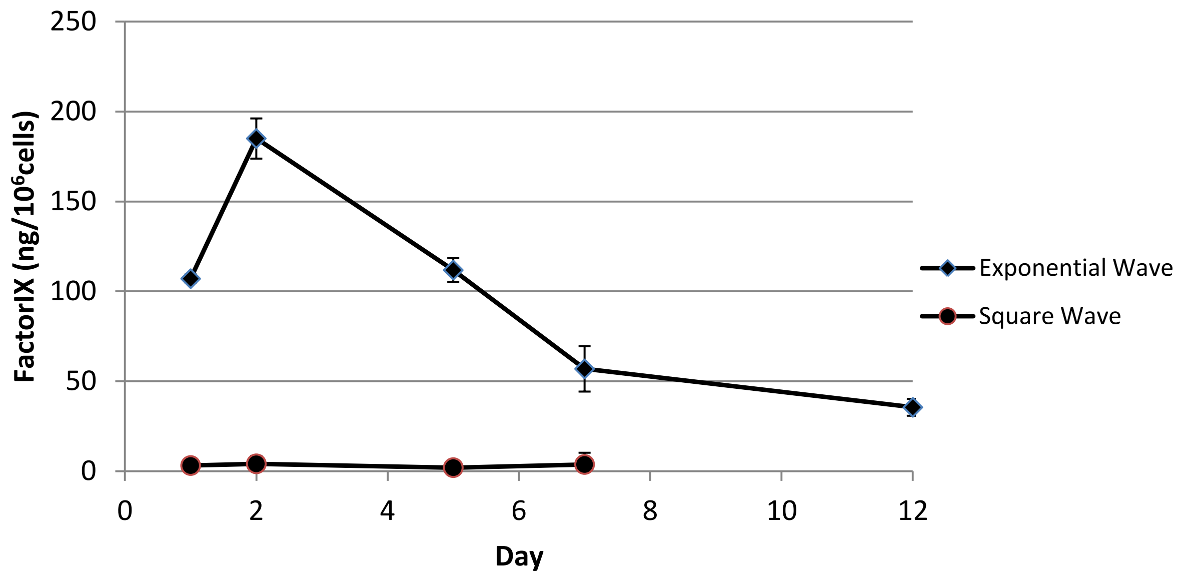


Figure 19: Factor IX secretion from electroporated cells. Factor IX expression from CBMSCs electroporated with CpGNFIX plasmid. Error bars represent standard deviation. The data shown is representative of three replicates.

Discussion

Optimization of Electroporation

Bone Marrow-Derived MSCs

To determine optimal electroporation conditions MSCs were electroporated while varying electroporation conditions, with a luciferase plasmid.

While varying the voltage, with a square waveform, the optimal luciferase expression was found when electroporating bone marrow-derived cells at 130V (Figure 4a). Voltages below 130V were not strong enough to efficiently destabilize the membrane and drive the plasmid DNA into the cell. Voltages over 130V were too harsh, and resulted in cell death. The optimal pulse length was determined to be 30msec (Figure 4b), and the optimal number of pulses was found to be 1 (Figure 4c). Once again low pulse lengths did not provide enough time to destabilize the membrane and drive the plasmid into the cell whereas higher pulse length was lethal to cells. As the number of pulses increases the number of transient pores increased, allowing more DNA to enter the cells [76]. Although more pulses allow more DNA to enter the cell, fewer cells survived the procedure. A high level of luciferase expression was found when four square waveform pulses were used (Figure 4c). This expression may be due to the few surviving cells expressing luciferase at very high levels. Although four pulses produced similar levels of transgene to a single pulse, decreasing cell survival due to more pulses is not necessary. Therefore, one pulse was chosen as optimal.

Exponential waveforms on the other hand were able to generate much higher luciferase expression in bone marrow-derived MSCs. The optimal voltage was determined to be 150V (Figure 5a). Voltages below 150V were unable to create pores in the membrane to introduce DNA into the cell, whereas voltages above 150V were too harsh for cells and lead to higher rates of cell death. The optimal capacitance was found to be 1050μF (Figure 5b). There was no significant difference in luciferase expression produced using 950 μF or 1050μF, but 1050μF was chosen as the optimal conditions due to reduced error. The increase of capacitance was able to increase the duration of the voltage decay [79]. Therefore, increasing capacitance was

able to increase the amount of time the voltage is applied to the cells, thus increasing entry of DNA into the cells. A further increase of capacitance is possible, but is likely to create conditions which may be too harsh for cells. Also serum free Opti-MEM was used as the electroporation buffer, which has a high ionic strength, and requires high capacitance for electroporation [79]. Therefore the high capacitance found to be optimal was expected due to the chosen electroporation buffer. The optimal resistance was found to be 200Ω. Using these conditions the optimal luciferase expression produced was 207719RLU, almost one third higher than luciferase expression with optimal square waveform conditions (58321RLU).

Therefore the optimal conditions for electroporating bone marrow-derived MSC are using an exponential waveform with 150V, 1050μF and 200Ω. These conditions allow for high transgene expression while ensuring a fair number of cells survive the procedure.

The most obvious difference between these conditions and others published is the voltage. Studies using rat and human bone marrow-derived MSCs demonstrated optimal electroporation at voltages closer to 300-600V [43, 83, 89]. The cells used in these studies were unable to survive electroporation at such high voltages. As discussed earlier MSCs from varying sources can differ considerably, therefore differences in collection, culture, passage and confluency may attribute to these inconsistencies.

Cord Blood-Derived MSCs

Optimal electroporation conditions for cord blood-derived MSCs was determined by electroporating with a plasmid containing a luciferase reporter gene. This was achieved in the same way optimal conditions were determined for bone marrow-derived MSCs. Square wave conditions were found to be one pulse at 120V-130V with a pulse length of 40msec producing 1805RLU (Figure 7). No significant difference in luciferase expression was found between 120V and 130 V; 130V was arbitrarily chosen for future experiments. Pulse lengths longer than 40msec may produce higher luciferase expression, but also contribute to high rates of cell death, therefore 40msec was the longest pulse length tested. Exponential wave conditions were found to be 180V, 1050µF and infinite resistance producing 1902RLU (Figure 8). A Student's t-test shows that exponential wave conditions produce significantly more luciferase than square wave conditions and therefore exponential wave conditions were used as optimal conditions for following experiments.

The optimized electroporation conditions for umbilical cord blood-derived MSCs has higher voltage and resistance than bone marrow-derived MSC optimized conditions while optimal capacitance remained the same. Optimized square wave protocols found umbilical cord blood-derived MSCs required a slightly higher pulse length for optimal electroporation compared to bone marrow-derived MSCs. The number of pulses and voltage remained constant for both MSC sources. From experience umbilical cord-derived MSCs seem to grow faster and in general seem less fragile than their bone marrow counterparts. This allows them to survive harsher electroporation conditions including higher voltage and pulse lengths.

Increasing the cord blood-derived MSC concentration from 1 million cells/ml to 5 million cells/ml was compared using the optimal exponential wave conditions. This was to ensure that scaling up experiments is possible by increasing cell concentration, rather than having to electroporate many cuvettes. This indicated no significant change in luciferase production between the two cell concentrations (Figure 9). Meaning cell concentration can be increased, if necessary, with no change in transgene expression.

Although our optimal electroporation conditions showed that transgene is being produced, determining cell survival is important. Since electroporation is known for being harsh, with sometimes only a few surviving cells, determining the number of cells that survive the procedure is important. A percent survival for square wave and exponential wave conditions was determined by comparing the number of cord blood-derived MSCs recovered from a cuvette without electroporation to the number recovered after electroporation. Square wave conditions resulted in 82% survival and exponential wave conditions in 67% survival (Figure 10). Square wave forms are known for being gentler than exponential wave forms on cells. Along with the fact that square wave optimized conditions were set at a lower voltage, this difference was expected.

The survival rates of square wave and exponential wave electroporation conditions were surprisingly high, since most literature refers to the major downfall of electroporation in mammalian cells to be low survival rates [43, 81]. High survival rates are attributed to very careful handling of the cells before and after electroporation. In particular, ensuring all growth media was removed from cells before electroporation, allowing the cells to rest after

electroporation, and growing in medium supplemented with 20% FBS for 24 hours post-electroporation.

Although survival is lower for exponential wave conditions, transgene expression is significantly higher. When harsher electroporation conditions are used there is more breakdown of the cell membrane. This allows for more plasmid DNA to enter the cells, but also results in lower cell survival. Therefore exponential wave conditions of 180V, 1000μF and infinite resistance were chosen as our optimal conditions for further experiments. These conditions allow for significantly higher transgene expression, but lower cell survival than their square wave counterparts.

Comparison with Lipofectamine Transfection and Cationic Polymers

Bone Marrow-Derived MSCs

Comparison studies were conducted to establish how well our electroporation conditions compare with those of other commercially available non-viral transfection agents. Bone marrow-derived MSCs were either electroporated at optimal conditions with a luciferase plasmid, or transfected (Lipofectamine™2000) with a luciferase plasmid (Figure 6). Lipofectamine™2000 is a cationic lipid based transfection reagent commonly used to transfect a variety of cell types. Transfection yielded a luciferase expression of 111832RLU, on the other hand the optimized electroporation conditions produced a luciferase expression of 175273RLU; nearly one third greater than the luciferase expression when cells were transfected. These

results clearly demonstrate that electroporation is superior to transfection for DNA transfer in bone marrow-derived MSC.

Although electroporation conditions were different, these results agree with previously published data that indicate that electroporation with an exponential wave is superior to transfection with cationic lipids in bone marrow-derived MSCs [43].

Cord Blood-Derived MSCs

Using cord blood-derived MSCs electroporation conditions were compared with Lipofectamine^{TM}2000 and Xfect^{TM}Stem using a luciferase plasmid and an EGFP plasmid. Xfect^{TM}Stem is a polymer based transfection reagent designed to transfect stem cells.

The microscope images show GFP expression from all transfection techniques (Figure 11). This gives us a visual indication of transgene expression to compare transfection techniques. Although this method shows that all techniques produce transgene, it is not ideal to compare the techniques. Some cells show strong GFP expression, and some are weaker. Regardless all techniques show some degree of GFP production.

A more quantifiable comparison of transgene expression was done using luciferase (Figure 12). Electroporation conditions and Lipofectamine^{TM}2000 produced significantly more relative light units than Xfect^{TM}Stem. There was no significant difference between electroporation conditions and Lipofectamine^{TM}2000.

When comparing luciferase expression to the GFP expression from Xfect^{TM}Stem modified cells, there are some clear differences. That is to say that Xfect^{TM}Stem modified cells appeared to express similar levels of GFP to cells modified with electroporation or Lipofectamine^{TM}2000 (Figure 11), but significantly less luciferase (7RLU) compared to cells modified with electroporation or Lipofectamine^{TM}2000 (211RLU and 223RLU, respectively) (Figure 12). It is likely that there were a proportion of modified cells producing low amounts of GFP in Xfect^{TM}Stem cells, whereas a similar proportion of cells were modified by electroporation and Lipofectamine^{TM}2000, but express higher amounts of GFP. This can explain the apparent difference in expression levels, because the luciferase assay measures total protein expression, rather than a visual of if the cell is expressing or not.

Xfect^{TM}Stem was not used as a transfection agent for bone marrow-derived MSCs, but a similar comparison was done between Lipofectamine^{TM}2000 and optimized electroporation.

Electroporation was superior to lipofectamine transfection when bone marrow-derived cells were tested, but no significant difference was found in umbilical cord blood-derived cells. There only explanation for these differences is that the cells are from varying sources. These results underline the differences in MSCs from varying sources, and the importance of optimizing transfection methods for each cell source.

To ensure cells maintained their viability after transfection an MTT assay was conducted (Figure 13). Comparing the MTT results of un-transfected cells to cells transfected with electroporation, Lipofectamine^{TM}2000, or Xfect^{TM}Stem, it was shown that there was no significant difference in viability after any transfection. This indicates that all three types of transfection do not

negatively or positively affect the viability of MSCs. It is important that the viability of the cells remains high for use in *ex vivo* gene therapy.

Although MTT results indicate that the transfection methods attempted does not compromise cell viability, some changes in cell morphology were evident and are important to note. Most prominent changes were in Lipofectamine™2000 cells where cells seemed enlarged, and there is an increase in cellular debris. Some increased cell debris was also found in Xfect™Stem modified cells (Figure 14).

To determine if this change in morphology was due to differentiation cells were stained with Alizarin Red S, Alcian Blue and Oil red O for osteogenesis, chondrogenesis and adipogenesis respectively (Figure 15). There was no differentiation shown to osteogenic, chondrogenic or adipogenic cell lines from any of the control or transfected MSCs. This indicated that transfection did not cause differentiation into these three lineages. An interesting finding occurred in cells stained for adipogenesis. Lipofectamine™2000 and Xfect™Stem transfected cells showed some type of net-like staining over the cells. This is likely excess cell debris that has taken up the Oil red O or hematoxylin stain.

Helledie *et al.* also reported altered cell morphology and cell death in human bone marrow-derived MSCs after Lipofectamine™2000 transfection [43]. Although from different sources the similar characteristics of the cells is an obvious parallel.

Although the MTT assay results that show no alteration in cell viability, it is important to note the change in morphology after certain types of transfection. Differentiation is not confirmed as the cause of this change, but it is possible that the cells are entering senescence or cell health is beginning to deteriorate.

Capacity of MSCs to Differentiate

A potential concern is that electroporation would alter the ability of MSCs to differentiate. Cord blood-derived cells subjected to electroporation were compared with those that were not electroporated. This allowed us to determine if the both groups of cells differentiated in similar ways when grown in media designed to stimulate differentiation. Cells in differentiation media were also compared to cells grown in control media (IMDM) to determine if any differentiation occurs in control media cells due to electroporation. If control media electroporated cells exhibit differentiation this may indicate that electroporation induces differentiation into a particular lineage.

Figure 16 shows cells stained for osteogenesis. Figure 16a shows non-electroporated MSCs grown in control media and no red staining is visible. This can be compared to Figure 16b which shows electroporated MSCs grown in control media, once again no red staining is visible indicating that no osteogenic differentiation occurred in either case. Figure 16c and d show non-electroporated and electroporated MSCs grown in osteogenic differentiation media. Both show strong red staining of calcium deposits, indicating osteogenic differentiation. This also

indicates a similar degree of differentiation, and that electroporation did not promote or hinder MSCs ability to differentiate into osteogenic cell lineages.

Figure 17 shows MSCs stained for chondrogenesis. Figure 17a and b show non-electroporated and electroporated MSCs grown in control media. Although cells were plated in pellets they eventually grew to cover the tissue culture plate. None of the cells show blue staining, indicative of chondrogenic differentiation. The cells grown in chondrogenesis differentiation media show the cells in a densely packed ball, with obvious blue staining (Figure 17c and d). This indicates chondrogenesis of cells. Both electroporated and non-electroporated cells formed these cell masses. The darkness of the cell mass is a result of density. Since the balls are quite dense they do not allow light from the microscope through, resulting in dark shadows. Overall chondrogenic potential of MSCs does not seem to be affected by electroporation.

Adipogenesis staining is shown in Figure 18. Figure 18a and b show non-electroporated and electroporated MSCs grown in control media. Both show cells that have divided to cover the tissue culture plate, and show blue hematoxylin staining of the nucleus. Figure 18c and d show non-electroporated and electroporated MSCs in adipogenic differentiation media. There seem to be fewer cells compared to the control, indicating that they slowed dividing possibly due to differentiation. The cells seem larger and have purplish/pink and red staining within the cell, indicating Oil red O staining of fat vacuoles. Although some staining occurred, it was minimal, which may be attributed to the limited ability of umbilical cord blood-derived MSCs ability to differentiate into adipocytes. Regardless the amount of differentiation seen in both

electroporated and non-electroporated cells are similar indicating that adipogenic potential was not affected by electroporation.

Overall electroporated umbilical cord blood-derived MSCs were able to differentiate into osteogenic, chondrogenic and adipogenic cell lineages in a similar manner to un-transfected cells. This indicates that electroporation did not alter their differentiation ability, an integral characteristic of MSCs.

Factor IX Expression

The newly determined optimal electroporation conditions were applied to a potential hemophilia B therapy by developing a FIX-secreting cord blood-derived MSC line.

Optimal square wave and exponential wave conditions were used to electroporate MSCs with FIX containing plasmids (Figure 19). Square wave conditions showed poor transgene production, with only 2ng/ml of FIX produced on day one and insignificant amounts thereafter. Exponential wave conditions on the other hand show some potential. Maximum transgene expression is shown on day 2 at 135ng/ml, with declining expression thereafter. Although declined, there still remained 35ng/ml on day 12 indicating some form of continued expression. Although low levels of FIX were found, measurements were concluded on day 12. At which point very few cells survived the antibiotic selection and the experiment was terminated.

This decline in FIX expression is expected in transiently transfected cells. This indicates that the gene did not integrate and was likely lost during cell division. Antibiotic selection during the 12

day period would result in any cells not expressing the FIX plasmid to die. The remaining amount of FIX being produced on day 12 indicates that the FIX gene may have been integrated into host DNA in a few cells, resulting in permanent transfection. This would explain the detectable, albeit low, FIX secretion by day 12.

These results indicate that exponential wave conditions are able to produce cells with transgene expression lasting up to 12 days.

Conclusions and Future Directions

Optimized electroporation conditions for bone marrow-derived MSCs were found to be an exponential waveform with 150V, 1050µF and 200Ω. These conditions were found to produce higher transgene expression than Lipofectamine™2000 transfected cells after 24 hours. Future studies may increase the testing range to compare our electroporation method with polymer based transfection reagents, determine differentiation potential after transfection, and engineer FIX secreting bone marrow-derived cells.

Optimized electroporation conditions for umbilical cord blood-derived MSCs were also found. They were an exponential waveform with 180V, 1000µF and infinite resistance. These conditions produced the maximum amount of luciferase 24 hours after electroporation when compared to other conditions. Scaling up these conditions to electroporate more cells is easily possible and shown when cell concentration was increased from 1million cells/ml to 5 million

cells/ml and the luciferase expression using the optimized conditions did not change significantly. When the optimized square and exponential waveform conditions were used to determine cell survival it was found that 82% and 67% survived respectively. This is a tremendous increase than what is expected from the literature [43, 81].

When comparing optimized electroporation conditions of umbilical cord blood MSCs with other transfection agents, it was found that GFP expression was detected in all transfection types. A comparison using a luciferase reporter gene gives a more clear representation of quantitative amounts of protein produced. The luciferase assay showed that electroporation and Lipofectamine™2000 produced significantly more transgene than Xfect™Stem. There was no decrease in viability compared to untransfected cells after all types of transfection. Although MTT assays showed no decrease in viability it is important to note elongated cell morphology and an increase of cell debris, most prominent in Lipofectamine™2000 transfected cells and also seen in Xfect™Stem cells. These changes were not attributed to differentiation to osteogenic, chondrogenic or adipogenic cell lineages.

The capacity of electroporated umbilical cord blood MSCs to differentiate was not affected by electroporation. Electroporated cells were successfully differentiated into osteogenic, chondrogenic and adipogenic cell lineages showing that the cells maintained their multipotent ability after electroporation.

Finally optimized electroporation with CpGNFIX plasmid was able to produce factor IX. Maximum transgene production was on day 2, but expression was sustained over the 12 day trial period.

Overall, optimized electroporation conditions produced for umbilical cord blood-derived MSCs were successful at modifying cells to express transgene. They maintained their ability to differentiate into the three cell lineages indicative of MSC identity. Electroporation conditions were able to provide sustained transgene expression at low levels.

A common technique when genetically engineering cells is to isolate individual clones. These clones can be screened for FIX secretion. This allows transfected cells secreting the highest levels of FIX to be isolated for therapeutic use. Unfortunately the nature of MSCs do not allow them to be passaged indefinitely. This makes this technique impractical as cells would need to be passaged many times to create clones.

The transient expression created by electroporation may be useful when short term gene expression is needed. For example, expressing a homing factor such as CXCR4 may be desired to promote homing when implanting cells. Expression would be necessary for a short time after implantation until cells settle into their environment, making electroporation ideal. Another possibility would be to induce differentiation of MSCs. This has been done using particular genes to induce differentiation into a desired cell line [90-91]. Short-lived expression would provide sufficient amounts of transgene to induce differentiation.

The low levels of FIX transgene expression are likely too low to result in any sort of therapeutic effect if the electroporated umbilical cord blood MSCs were to be used as *ex vivo* gene therapy for hemophilia B. Although the technique may not be applicable to hemophilia gene therapy it still holds promise. The optimized procedure shows a much higher rate of survival compared to

what is expected from literature. Additionally, despite the fact that transgene expression is low it is sustained over 12 days.

As a simple and economical transfection method electroporation has proved successful in modifying MSCs. It is effective in producing transgene while maintaining MSC viability. Future applications of these electroporation techniques may be to modify human MSCs for use in low dose transient gene therapy.

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Appendix

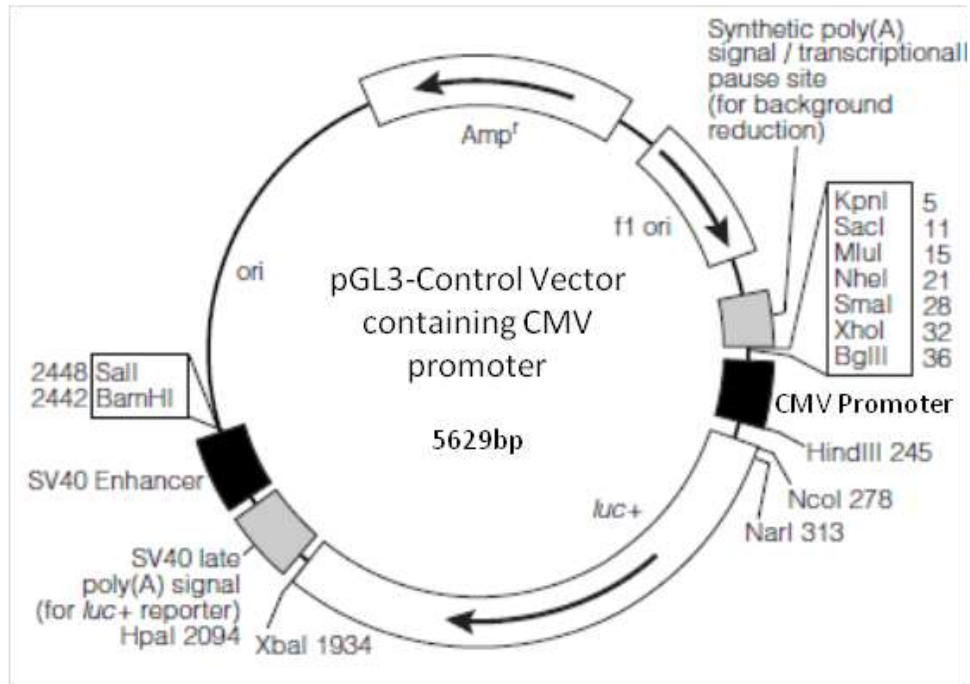

Figure 20: Luciferase Vector. Adapted from Promega pGL3 control vector. The SV40 promoter of the promega promoter was removed and a CMV promoter was added. By replacing the promoter the plasmid increased by 374 bp to a total size of approximately 5629bp. This vector was the luciferase plasmid used to determine optimal electroporation conditions and compare electroporation to transfection.

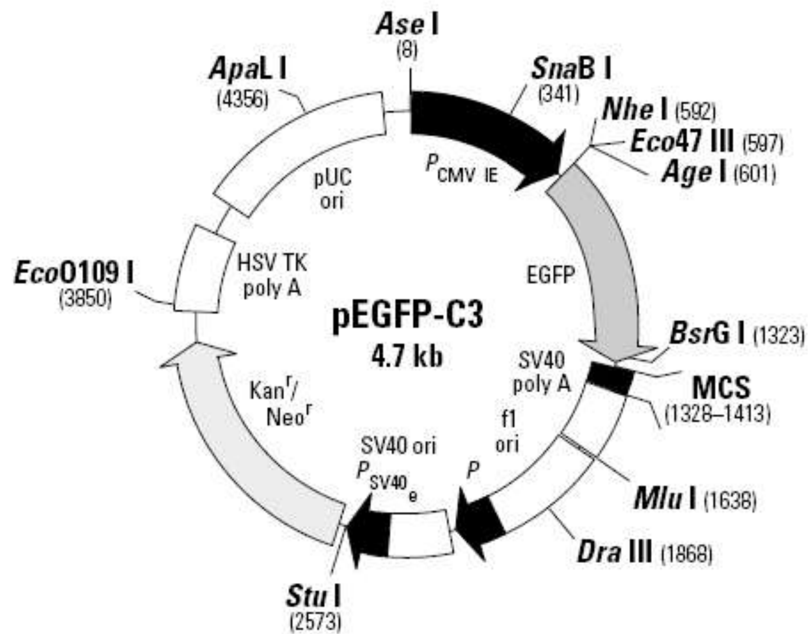

Figure 21: Green Fluorescent Protein Vector. The pEGFP-C3 plasmid has been optimized for higher expression in mammalian cells and was used for transfection comparison studies (Clontech).

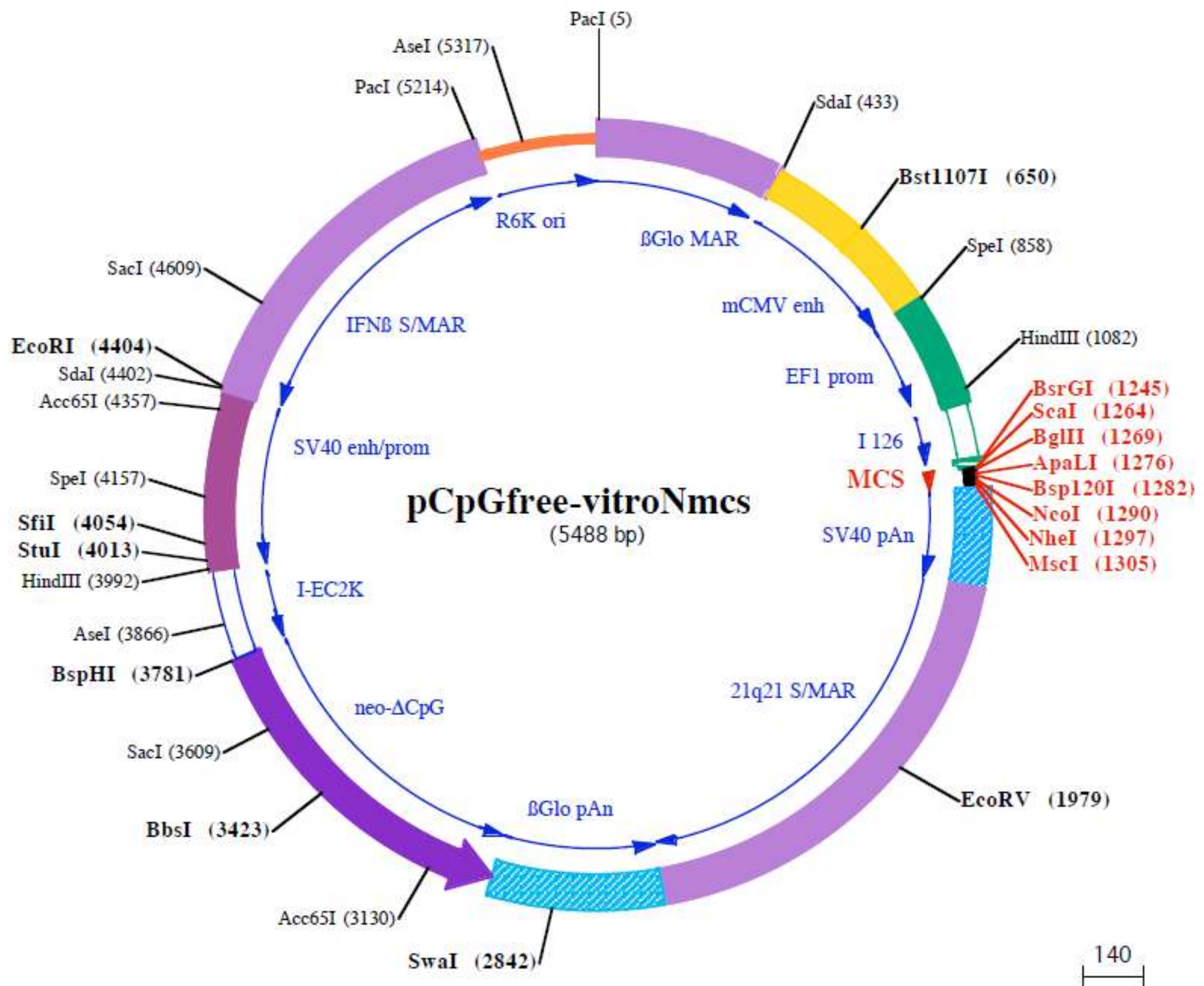

Figure 22: Factor IX Vector. This pCpGfree-vitroNmcs plasmid with a multiple cloning site was used to create the CpG-free hFIX plasmid vector. The hFIX DNA was cloned into the multiple cloning site of this plasmid (InvivoGen).